

Thèse de Doctorat

Laurène LECLAIR-VISONNEAU

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Etude physiopathologique de la diffusion de la maladie de Parkinson au système nerveux autonome

JURY

Président du jury	Isabelle ARNULF, Professeur des universités–Praticien Hospitalier, Université Pierre et Marie Curie, Paris 6
Rapporteurs :	Isabelle ARNULF, Professeur des universités–Praticien Hospitalier, Université Pierre et Marie Curie, Paris 6 Pierre BURBAUD, Professeur des universités–Praticien Hospitalier, Université de Bordeaux
Directeur de Thèse :	Pascal DERKINDEREN, Professeur des universités–Praticien Hospitalier, Université de Nantes
Co-directeur de Thèse :	Yann PEREON, Professeur des universités–Praticien Hospitalier, Université de Nantes

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Abréviations

ATP	adénosine triphosphate
BEI	barrière épithéliale intestinale
BGSA	biopsie des glandes salivaires accessoires
CGRP	calcitonin gene-related peptide
Da	dalton
DFNIE	densité des fibres nerveuses intra-épidermiques
DN4	questionnaire de la douleur neuropathique en 4 points
GFAP	glial fibrillary acidic protein
IRLS	international restless legs scale
MIBG	méta-iodobenzylguanidine
MMSE	mini-mental state examination
MoCA	Montreal cognitive assessment
MP	maladie de Parkinson
NMS-Quest	non-motor symptoms questionnaire
NO	monoxyde d'azote
OR	odds-ratio
PDQ-39	39-item Parkinson's disease questionnaire
PDSS	Parkinson's disease sleep scale
PSQI	Pittsburgh sleep quality index
QCD	questionnaire concis sur les douleurs
SCOPA-Aut	SCales for Outcomes in PArkinson's disease-autonomic symptoms
SNA	système nerveux autonome
SNC	système nerveux central
SNE	système nerveux entérique
SNpc	substance noire <i>pars compacta</i>
TCSP	trouble du comportement en sommeil paradoxal
UKPDSBB	United Kingdom Parkinson's disease survey brain bank
UPDRS-III	unified Parkinson's disease rating scale- partie III
VIP	vasoactive intestinal peptide
ZO	zona occludens

Contexte du travail de thèse

Les symptômes de la maladie de Parkinson (MP) sont classiquement représentés par la triade motrice : tremblement, raideur et lenteur. Si le handicap résulte en partie de ces symptômes, les signes non moteurs de la MP influencent fortement son cours évolutif et son pronostic. Le retentissement sur la qualité de vie des symptômes non moteurs est majeur au cours de la MP. Parmi eux, l'incontinence urinaire, la constipation et les douleurs neurogènes sont des symptômes dysautonomiques qui, seuls ou associés, altèrent de manière significative le vécu de la MP. Les études histopathologiques, autopsiques ou *in vivo*, montrent que le système nerveux autonome (SNA) serait affecté tant dans son contingent central (centres autonomes du tronc cérébral et de la moelle épinière), que périphérique, jusqu'aux organes cibles. Des inclusions d'alpha-synucléine phosphorylée ou une perte neuronale y sont observées et l'atteinte histologique précéderait même la triade symptomatique motrice.

Introduction

I- Caractérisation clinique et fonctionnelle de la MP

Affectant environ 150 000 personnes en France, la MP est la deuxième maladie neurodégénérative en fréquence (1). Sa prévalence augmente avec l'avancée en âge, ainsi alors qu'elle est autour de 1 ‰ dans la 6^{ème} décennie, elle atteint plus de 1% chez les personnes de 70 à 79 ans (2). La MP est caractérisée par une hétérogénéité significative dans sa variabilité clinique (3,4), mais aussi dans ses mécanismes physiopathologiques complexes faisant intervenir des facteurs génétiques (formes monogéniques notamment) et environnementaux (toxicité vs protection) (3,5).

1. La triade motrice

Nommée *paralysis agitans (shaking palsy)* par Sir James Parkinson en 1817 (6), la MP est classiquement décrite sous le prisme de l'atteinte motrice, caractérisée par la triade : akinésie, hypertonie plastique et tremblement de repos, résultant de la perte des neurones dopaminergiques de la substance noire (7,8). On peut distinguer les formes akinéto-rigides de celles où le tremblement prédomine (3). Contrairement au tremblement qui se modifie peu au cours du temps (9), l'akinésie et l'hypertonie s'aggravent avec l'évolution de la maladie, de manière plus rapide dans les 5 premières années (10,11). L'atteinte motrice axiale se manifeste par une posture et des réponses posturales anormales, une altération de la variabilité et de la vitesse de marche, des phénomènes de freezing, une amimie, une hypophonie et une dysarthrie (11,12). Les signes axiaux peuvent être présents en début de maladie, discrets et peu invalidants, ils sont alors sensibles au traitement dopaminergique ; puis ils progressent avec la durée d'évolution de la maladie, en lien avec des lésions extra-nigrales, et indiquent un pronostic défavorable (9).

2. La MP au-delà du mouvement : les troubles neuropsychiatriques

Décrits dès le XIXème siècle (6), les symptômes non moteurs font partie intégrante de la MP, et en grèvent également la qualité de vie et le pronostic (13,14). Si certains signes peuvent précéder la triade du syndrome extra-pyramidal, les signes non moteurs apparaissent le plus souvent au cours de l'évolution de la maladie.

Les troubles neuropsychiatriques sont fréquents, parmi eux l'altération cognitive concerne 20 à 57% des parkinsoniens dans les 3 à 5 ans suivant le diagnostic et peut atteindre jusqu'à 80% des patients ayant une MP évoluée (15,16). Elle se traduit par des perturbations pouvant toucher les fonctions exécutives, visuo-spatiales, attentionnelles, la mémoire ou encore le langage. Les profils d'altération cognitive peuvent être très variables d'un patient à l'autre, impliquant à divers degrés des voies cholinergiques, dopaminergiques ou noradrénergiques (15), ces données sont illustrées par la Figure 1. Un âge avancé et une atteinte motrice axiale sont des facteurs de risque de démence parkinsonienne (16,17).

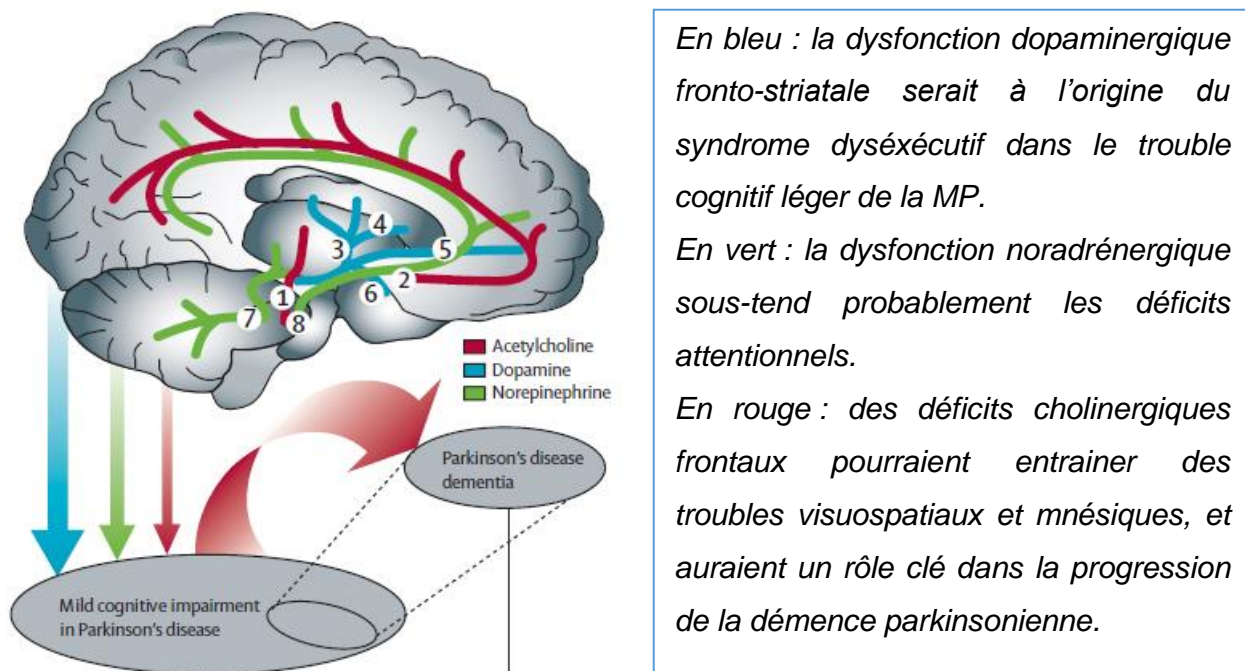


Figure 1 : Voies neuronales impliquées dans la physiopathologie des troubles cognitifs dans la MP, du trouble cognitif léger à la démence parkinsonienne (tiré de 15).

Des hallucinations sont présentes chez 16 à 42% des patients, sous forme d'hallucinations visuelles et d'illusions principalement, les hallucinations olfactives, auditives et tactiles étant plus rares (18,19). Leur survenue est associée à la raréfaction de l'innervation dopaminergique rétinienne, à des dysfonctionnements des aires visuelles primaires et associatives ou encore à des troubles cognitifs (18).

Alors qu'une dépression est estimée chez 10 à 15% des patients au stade initial de MP, suggérant son aspect prodromal, environ 35 % des parkinsoniens souffrent de symptômes dépressifs cliniques à la phase d'état (16,20). Le sexe féminin, les troubles cognitifs et la gravité de la maladie sont des facteurs de risque de dépression au cours de la MP. Ses mécanismes physiopathologiques impliquent des facteurs psychologiques et neurobiologiques (20). L'apathie, l'anxiété et les troubles du contrôle des impulsions peuvent également survenir au cours de la MP et complexifier son vécu et sa prise en charge (16).

Les troubles du sommeil concernent 60 à 98% des patients parkinsoniens (21). Les patients souffrent, de manière isolée ou associée, d'insomnie d'endormissement ou de maintien, d'un sommeil fragmenté, d'un syndrome des jambes sans repos, de somnolence diurne excessive ou d'un trouble du comportement en sommeil paradoxal (21,22). Une mauvaise qualité de sommeil est rapportée dans 20 à 80% des cas, favorisée par les fluctuations motrices, les douleurs, l'état psychique, la nycturie ou les médicaments (22). La somnolence diurne excessive concerne 16 à 50% des patients, prenant rarement la forme d'attaques de sommeil (21,23). Si, comme dans la population générale, la somnolence peut être secondaire à un syndrome d'apnées du sommeil ou une dépression, elle pourrait résulter d'une altération des systèmes d'éveil centraux liée à la MP et serait majorée par les traitements dopaminergiques (8,22–24). Elle s'aggrave avec la sévérité de la maladie et sa durée d'évolution (25).

Le trouble du comportement en sommeil paradoxal (TCSP) correspond à l'extériorisation des rêves sous forme de comportements complexes et souvent violents en sommeil paradoxal, se traduisant en polysomnographie par une abolition imparfaite du tonus musculaire axial ou

des membres, normalement observé dans ce stade (26,27). Décrit pour la première fois chez l'homme en 1986, le TCSP avait précédemment été observé chez le chat dès 1965, lorsque Michel Jouvet et son équipe étaient parvenu à supprimer l'atonie musculaire physiologique du sommeil paradoxal en coagulant la partie médio-ventrale des noyaux du locus coeruleus alpha ou la voie descendante qui en est issue, donnant naissance à des comportements oniriques (26,28). Les mécanismes du TCSP chez l'homme supposent une perte de l'atonie musculaire, par désinhibition des motoneurons spinaux, du fait d'une lésion ou d'une dégénérescence de noyaux pontiques ou bulbaires (notamment locus subcoeruleus, équivalent du locus coeruleus alpha, chez l'homme), probablement associée à une activation de générateurs locomoteurs (sous l'influence du diencephale ou du télencéphale) (Figure 2, 27).

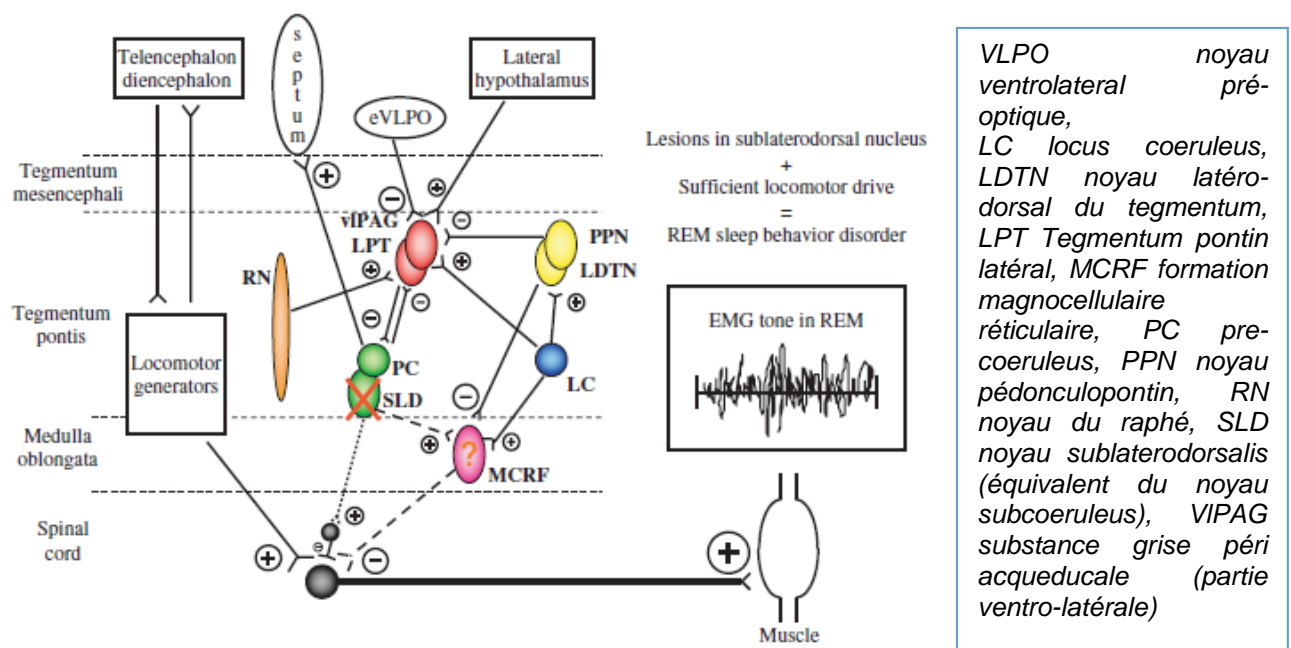


Figure 2 : Modèle physiopathologique du TCSP chez l'homme (tiré de 27)

S'il est fréquemment observé dans la MP, en moyenne dans 42% des cas (29), le TCSP peut exister en l'absence de toute pathologie neurodégénérative apparente, nommé alors TCSP idiopathique. Cependant, des signes infracliniques habituellement détectés dans la MP ou dans des pathologies apparentées (démence à corps de Lewy et atrophie

multisystématisée) sont mis en évidence dans le TCSP idiopathique. On observe chez ces patients de discrètes anomalies motrices (30,31), des déficits olfactifs plus fréquents, des altérations de la vision des couleurs (31), un dysfonctionnement mnésique, exécutif et de construction visuo-spatiale (32,33) et une diminution de l'innervation dopaminergique striatale (34,35). Jusqu'à 81 % des sujets avec TCSP idiopathique évoluent vers un syndrome parkinsonien ou démentiel dans un délai moyen de 14 ans (36). Le TCSP constitue ainsi un symptôme pré moteur cardinal de MP. Au cours de la MP, le TCSP constitue un des signes de gravité de la maladie, comme nous le détaillerons plus loin (4).

3. La MP au-delà de l'encéphale : la dysautonomie

Les manifestations cliniques liées à l'atteinte du système nerveux autonome (SNA) peuvent être précoces au cours de la MP et peuvent même précéder l'apparition de la triade motrice. Ainsi, un ralentissement du transit intestinal (<1 selle quotidienne) serait un signe précurseur ou un facteur de vulnérabilité, puisqu'associé à un risque relatif de 2,7 de développer une MP dans une population d'âge supérieur à 51 ans (37). Une dénervation cardiaque a été mise en évidence 4 ans avant l'apparition des signes moteurs chez un patient (38). Chez les patients présentant un TCSP idiopathique, symptôme pré moteur de MP, il a été observé une prévalence plus importante de dysautonomie cardiovasculaire et urinaire (31,39,40).

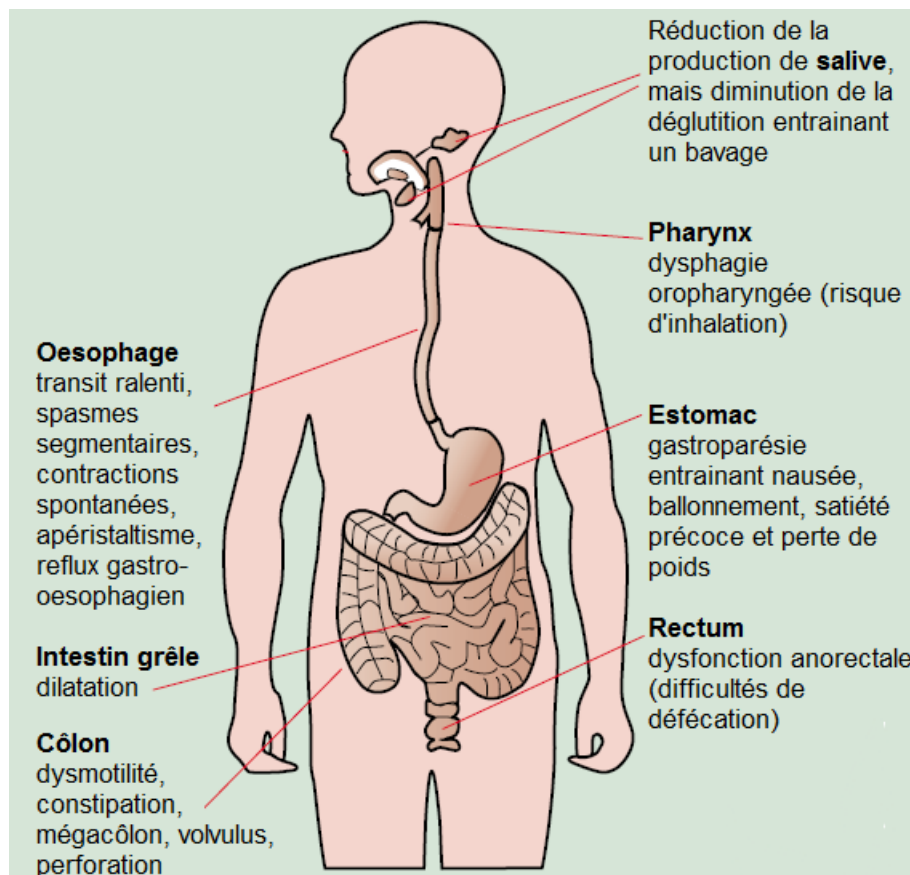
L'atteinte du SNA, notamment urinaire et digestif, est le signe non moteur le plus fréquemment rapporté par les patients et est retrouvée dès les cinq premières années d'évolution (41). Cette dysautonomie peut être diffuse et toucher les systèmes pupillaires, glandulaires (lacrymal, salivaire, sudoripare), cardiovasculaires, digestifs, génito-urinaires ou cutanés (42,43).

Parmi d'autres symptômes neuro-ophtalmologiques, la dysfonction pupillaire est à l'origine d'une vision trouble et d'une photophobie chez les patients parkinsoniens. La pupillométrie montre des latences de contraction pupillaire augmentées et un diamètre au repos supérieur aux sujets contrôle (44,45). Une sécheresse oculaire existe chez 88% des patients

parkinsoniens (contre 21% chez des sujets contrôle) et des anomalies au test de Schirmer sont retrouvées plus fréquemment chez les patients parkinsoniens (46). Une hypersialorrhée ou une tendance au bavage sont notées respectivement chez 70% et 54 % des patients et peuvent entraîner une gêne sociale. Des tests fonctionnels suggèrent que la production de salive serait réduite et que l'hypersialorrhée résulterait d'une diminution de la déglutition (47–49).

La dysautonomie cardiovasculaire se manifeste principalement par une hypotension orthostatique, concernant 20 à 40% des patients parkinsoniens et pouvant entraîner de nombreuses chutes (50,51). L'hypotension post-prandiale, une labilité tensionnelle, une hypertension et possiblement une intolérance à l'effort pourraient également être des conséquences de l'atteinte du SNA cardiovasculaire (52).

Si la musculature oropharyngée et le sphincter anal externe sont contrôlés par le système nerveux somatique, la majeure partie du tube digestif est innervée par le SNA (53, figure 3).



[Figure 3](#) : Manifestations cliniques de la dysautonomie digestive (adapté de 53)

La déglutition fait intervenir différentes efférences somatiques et viscérales. Son altération chez 30 à 82% des patients peut être une expression clinique de la dysautonomie, elle peut se compliquer d'une inhalation (53,54). L'atteinte digestive haute peut également se manifester par une perturbation de la motricité œsophagienne (55) ou une gastroparésie entraînant des nausées, un ballonnement abdominal postprandial ou une satiété précoce (53,54).

La constipation, parfois rebelle, définie par une fréquence des selles inférieure à 3 par semaine, atteint 20 à 52% des patients (53,54,56). Elle semble liée à un ralentissement du transit colique et peut, dans les cas les plus sévères, entraîner une pseudo-obstruction intestinale et ses complications (53). Sur des données d'interrogatoire, la sévérité de la constipation semble corrélée avec la gravité de la maladie (stades de Hoehn et Yahr) et avec un âge plus avancé au diagnostic (57,58). La dysfonction anorectale dans la MP se caractérise par des efforts excessifs avec douleurs et sensation d'évacuation incomplète, atteignant jusqu'à 67% des patients (54), elle pourrait être liée à une dyssynergie abdominopelvienne (59).

Les troubles urinaires dans la MP peuvent trouver leur origine dans l'altération du SNA ou des voies de régulation centrale, ils concernent 27 à 64% des patients (14,57,60). L'impériosité mictionnelle est rencontrée chez 34 à 54 % des parkinsoniens, l'incontinence urinaire est tardive, concernant un quart d'entre eux, et semble associée à l'incontinence fécale. Plus de 60% des patients parkinsoniens rapportent une nycturie, une dysurie est observés chez 44 à 70% des hommes (14,57). Vingt à 79 % des patients parkinsoniens rapportent des troubles sexuels, notamment sous forme de troubles de l'érection (pour les hommes) ou de l'orgasme (14,57).

Une dysrégulation de la sudation est rapportée par deux tiers des patients, l'hyperhidrose étant plus fréquente qu'une hypohidrose, notamment lors des périodes off de traitement (61). Des anomalies de la réponse cutanée sympathique sont observées chez les patients ayant une plainte d'hyperhidrose, suggérant une hypersudation de la face et du tronc contrastant

avec une hypohidrose des extrémités (62). Des douleurs sont rapportées dans 30 à 83% des cas selon les études, les seuils de douleurs et de sensibilité au chaud et au froid semblent abaissés, leur variation sous l'effet du traitement dopaminergique reste controversée (63). Les douleurs neurogènes, non expliquées par les fluctuations motrices, pourraient avoir une origine à la fois centrale (locus cœruleus, striatum, cortex préfrontal et cingulaire) et périphérique, liées à l'atteinte du SNA cutané (63,64).

II- Physiologie du SNA

1. Organisation générale du SNA

En opposition avec le système nerveux somatique qui permet à l'organisme d'interagir avec son environnement, le SNA régit les activités automatiques du corps humain, indépendamment du contrôle volontaire. Il régule ainsi les fonctions vitales, en innervant les glandes endocrines et exocrines, la musculature lisse et le myocarde. On distingue une division parasympathique, principalement mise en jeu dans les situations basales de « repos et digestion » et une division orthosympathique sollicitée dans les situations d'alerte : « fuir ou combattre » (65,66). La Figure 4 résume les structures anatomiques du SNA.

Les voies nerveuses du SNA sont constituées d'un premier neurone émergeant du système nerveux central (SNC), neurone pré-ganglionnaire, et dont l'axone fait synapse avec un deuxième neurone, post-ganglionnaire, au sein d'un ganglion viscéral. L'axone du neurone post-ganglionnaire fait synapse avec l'organe effecteur.

Les corps cellulaires des neurones pré-ganglionnaires du SNA parasympathique sont localisés dans le tronc cérébral, associés aux noyaux des nerfs crâniens (III noyau d'Edinger-Westphal, VII noyau salivaire supérieur et lacrymal, IX noyau salivaire inférieur et X noyau dorsal du vague) et dans la moelle spinale sacrée (substance grise intermédio-latérale de S2 à S4). Le nerf vague assure l'innervation de l'essentiel des organes : cœur, bronches, œsophage, estomac, intestin grêle, partie proximale du colon, foie, vésicule biliaire, pancréas, partie supérieure des uretères (67). Les ganglions viscéraux, sites de la synapse, se situent à proximité des organes cibles, fréquemment à leur surface ou dans leur paroi. L'acétylcholine est le neuromédiateur des deux synapses de la division parasympathique, se fixant sur des récepteurs nicotiques et muscariniques au sein du ganglion et sur des récepteurs muscariniques dans l'organe cible.

Les centres orthosympathiques sont localisés dans la colonne intermédio-latérale de la moelle spinale (de T1 à L3), les axones émergent par la racine ventrale de la moelle puis

empruntent le rameau communicant blanc jusqu'aux ganglions de la chaîne paravertébrale du même segment, où se situe le plus souvent la synapse avec le neurone post-ganglionnaire.

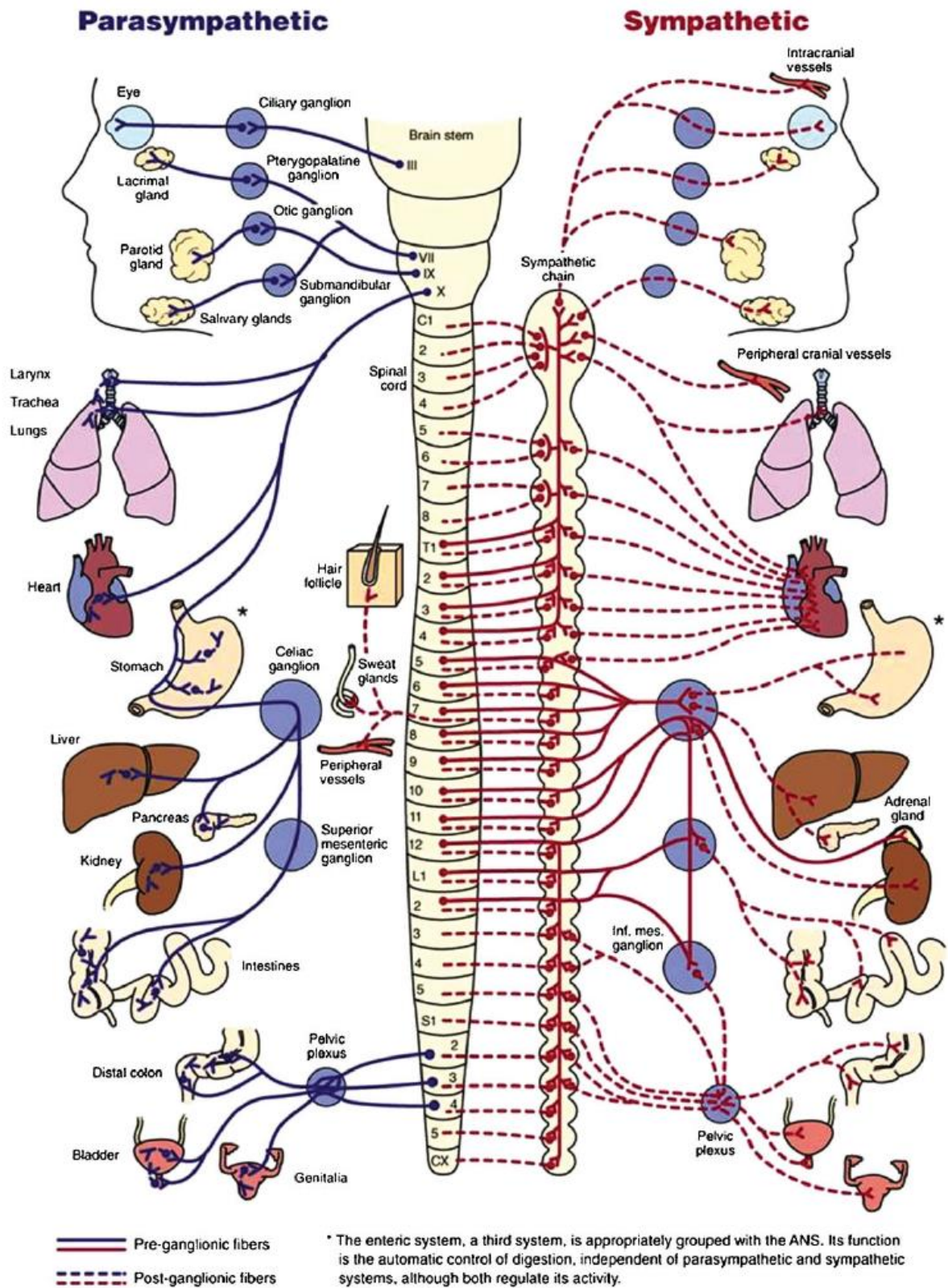


Figure 4 : Organisation anatomique du SNA (tiré de 68)

Certains relais ont lieu à l'extérieur de la chaîne paravertébrale (ganglions mésentériques supérieur et inférieur, coélique) et la glande médullo-surrénale reçoit une innervation des neurones pré-ganglionnaires. Le neurone pré-ganglionnaire libère de l'acétylcholine se fixant principalement sur des récepteurs nicotiques, les neurones post-ganglionnaires libèrent majoritairement de la noradrénaline et dans certaines synapses l'acétylcholine (dans les glandes sudoripares notamment), des neuropeptides et de l'ATP (65).

2. Le système nerveux entérique et la barrière épithéliale intestinale

Le système nerveux entérique (SNE) peut être considéré comme une troisième division du SNA, appartenant à son contingent périphérique. Les divisions orthosympathiques et parasymphathiques y communiquent au sein du vaste réseau de neurones qui s'étend depuis le tiers inférieur de l'œsophage jusqu'au rectum. Les 400 à 600 millions de neurones du SNE, organisés en 2 plexus nerveux, assurent l'innervation du tractus digestif (69).

La paroi du tube digestif s'organise en 5 tuniques successives, comprenant depuis la lumière vers l'extérieur : la muqueuse, la musculaire muqueuse, la sous-muqueuse, la musculuse et la séreuse (Figure 5).

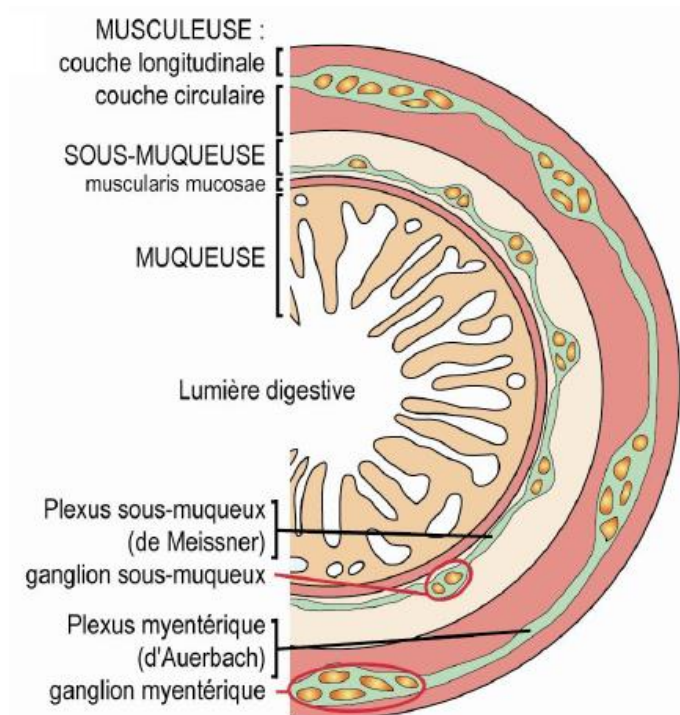
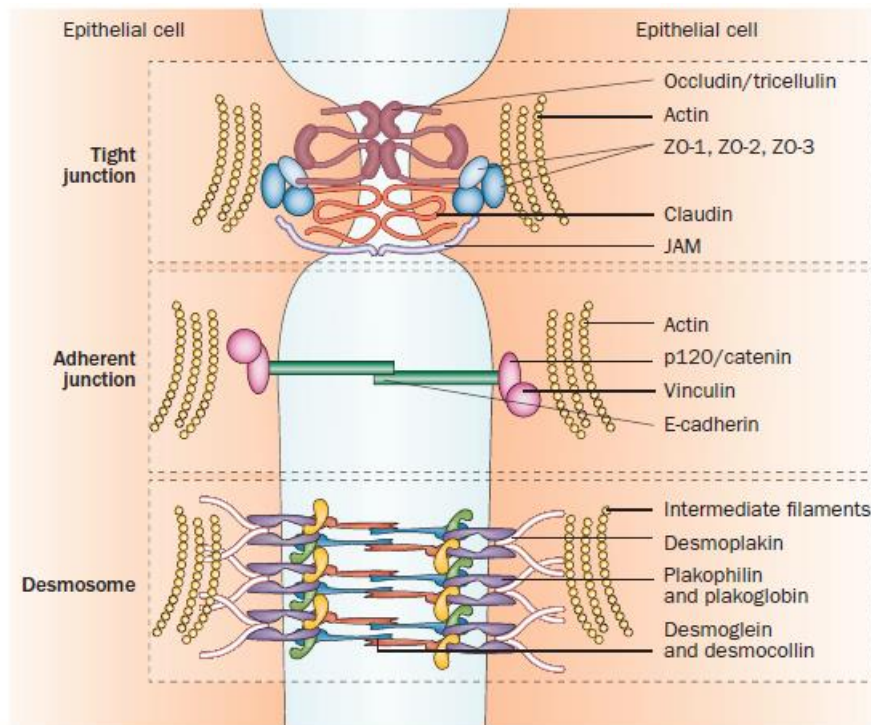


Figure 5: Schéma d'une coupe transversale d'intestin grêle.

La muqueuse se compose d'une monocouche de cellules épithéliales et d'un tissu conjonctif (*lamina propria*) comprenant des follicules lymphoïdes et riche en vaisseaux sanguins, ayant une fonction dans l'absorption des nutriments. La musculaire-muqueuse est formée d'une mince couche de tissu musculaire lisse. La sous-muqueuse est constituée de tissu conjonctif et contient le plexus sous-muqueux (ou plexus nerveux de Meissner) ainsi que des vaisseaux sanguins et lymphatiques destinés à la muqueuse. La musculature est disposée en 2 couches de cellules musculaires lisses : circulaire interne et longitudinale externe ; entre ces deux couches se situe le plexus myentérique ou nerveux d'Auerbach. La tunique externe est le plus souvent une séreuse (tissu conjonctif tapissé sur son versant externe par un épithélium simple).

La barrière épithéliale intestinale (BEI) est constituée de l'épithélium intestinal, de la lamina propria et de la musculaire muqueuse. Son rôle est de favoriser le passage de l'eau, des électrolytes et des nutriments tout en protégeant l'organisme des agressions extérieures (70). Les cellules épithéliales intestinales permettent cette perméabilité sélective par 2 passages : la voie transcellulaire et la voie paracellulaire. La voie transcellulaire est impliquée dans l'absorption et le transport des nutriments comme les sucres, les acides aminés, les vitamines, les acides gras, les minéraux et les peptides. La membrane cellulaire étant imperméable, ce passage se fait via l'utilisation de transporteurs et de canaux localisés sur les membranes apicales et basolatérales des cellules épithéliales intestinales. La voie paracellulaire est associée au transport des molécules dans l'espace intercellulaire, entre les cellules épithéliales adjacentes, organisé par trois complexes jonctionnels (les jonctions serrées, les jonctions adhérentes et le desmosome). Le complexe jonctionnel apical, composé des jonctions serrées et des jonctions adhérentes, régule principalement la perméabilité paracellulaire (70,71). L'organisation de l'espace intercellulaire est détaillée dans la figure 6.



[Figure 6](#) : Complexes jonctionnels régulant l'espace intercellulaire (tiré de 70)

Les chambres d'Ussing permettent d'étudier la perméabilité *ex vivo* en mesurant la quantité de fluorescence passant d'une chambre à l'autre, à travers la muqueuse. L'utilisation de molécules de différentes tailles couplées à une molécule fluorescente permet de mesurer sélectivement les différents types de perméabilité (acide sulfonique pour la perméabilité paracellulaire et peroxydase de raifort pour la perméabilité transcellulaire) (72).

Les plexus du SNE sont constitués de ganglions nerveux, comprenant des neurones et cellules gliales entériques (considérés comme des équivalents digestifs des astrocytes du SNC), interconnectés entre eux par des prolongements axonaux et dendritiques (69,70). On distingue une vingtaine de sous-types de neurones entériques, répartis en trois catégories fonctionnelles : les neurones sensitifs intrinsèques (ou neurones afférents primaires intrinsèques), les interneurons et les neurones moteurs (73). Les neurones sensitifs intrinsèques participent à la détection des modifications chimiques intra-luminales et des

distorsions mécaniques de l'épithélium et du tissu musculaire externe. Leurs neuromédiateurs sont des peptides tels que la substance P et le CGRP (calcitonine gene-related peptide). Les afférences des neurones sensitifs intrinsèques sont intégrées par les interneurones, ascendants et descendants, et les neurones moteurs (excitateurs, inhibiteurs, sécrétomoteurs, sécrétomoteurs-vasodilatateurs et ceux innervant les cellules endocrines). Une quarantaine de neurotransmetteurs a été identifiée dans le SNE, les neurones moteurs excitateurs libèrent principalement l'acétylcholine (ainsi que la tachykinine et l'enképhaline) et les neurones inhibiteurs libèrent majoritairement le NO (monoxyde d'azote) et le VIP (vasoactive intestinal peptide) (69,74). Le SNE comprend également 14 à 20% de neurones cathécolaminergiques, majoritairement dopaminergiques, répartis selon un gradient oral-aboral, ils ne représentent que 1 à 6% des neurones innervant le côlon (75,76).

Le plexus sous-muqueux assure l'innervation de l'épithélium intestinal, participant ainsi au rôle de barrière du tube digestif (77). Les axones innervant la muqueuse libèrent majoritairement le VIP, mais aussi l'acétylcholine, la substance P et le neuropeptide Y (70). Les neurones du SNE sont en interactions étroites et réciproques avec la glie entérique et l'épithélium. L'association fonctionnelle des cellules épithéliales intestinales, des neurones entériques et des cellules gliales entériques peut être considérée comme une unité neuro-glio-épithéliale entérique, évoquant l'organisation de la barrière hémato-encéphalique (70). La perméabilité de la BEI est modifiée selon l'activation des neurones du plexus sous-muqueux, que ce soit par une stimulation électrique, sur des modèles *in vitro* ou *in vivo* (77,78), ou par des neuromédiateurs chimiques habituellement présents dans le SNE (70).

Les neurones du plexus sous-muqueux ont également des effets sur la prolifération et la différenciation des cellules de la muqueuse intestinale, et sur les processus de réparation, en produisant notamment le VIP, l'acétylcholine, la substance P, la sérotonine ou des endocannabinoïdes (70). De manière réciproque, des modifications expérimentales de la glie influent sur l'épithélium intestinal et les neurones entériques (79) et les cellules épithéliales peuvent sécréter des facteurs activant les neurones du SNE (70,80).

Le plexus sous-muqueux présente l'intérêt de pouvoir être prélevé et analysé en utilisant des biopsies digestives obtenues en routine lors d'une endoscopie digestive (81,82). Notre équipe a mis au point une technique de microdissection de biopsie digestive qui permet d'isoler le plexus sous-muqueux et de l'analyser, entre autres, par immunohistochimie ou par Western Blot (83, Figure 7). Cette approche permet une analyse morphologique et quantitative des neurones du plexus sous muqueux (83–85), ainsi que la détection d'inclusions d'alpha-synucléine chez les patients parkinsoniens (voir chapitre III).

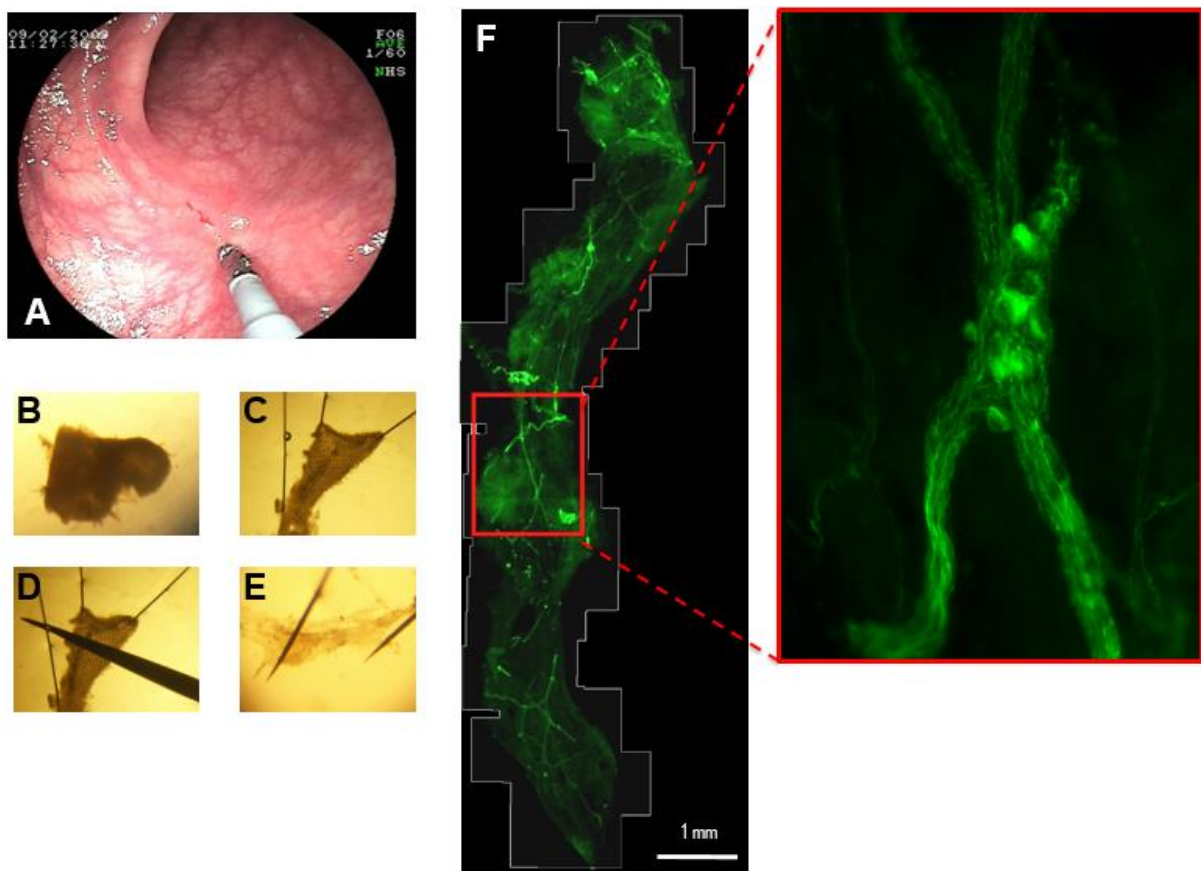


Figure 7 : Etude du plexus sous-muqueux *in vivo* (A : prélèvement d'une biopsie colique au cours d'une endoscopie, B à E : microdissection de la biopsie permettant de séparer la muqueuse de la sous-muqueuse, F : ganglions et prolongements axonaux et dendritiques du plexus sous-muqueux marqués par la PGP 9.5 en immunohistochimie).

Le contrôle de la motricité digestive est assuré par le plexus myentérique : péristaltisme, segmentation, complexes moteurs migrants et rétro propulsion (lors de vomissements). Si la motricité œsophagienne est principalement assurée par le système nerveux somatique via des générateurs centraux (*central pattern generator* dans le bulbe), le SNE est déterminant dans la motricité intestinale et colique. De ce fait, l'absence de plexus myentérique dans la maladie de Hirschsprung a des conséquences cliniques majeures (69).

3. Régulation du SNA

Les systèmes parasympathiques et orthosympathiques sont continuellement activés à basse fréquence. Selon les situations, la sollicitation de l'un ou de l'autre de ces systèmes modifie l'équilibre de base, augmentant ou diminuant l'activité des structures cibles (66). Les effets de chacune des divisions sur les organes cibles sont résumés dans le Tableau 1.

Le SNA est régulé via les afférences viscéro-sensitives provenant des organes cibles, stimulées par des modifications mécaniques ou chimiques, véhiculées par des fibres peu ou non myélinisées (A δ et C), principalement au sein des afférences parasympathiques (nerf vague notamment). Les fibres afférentes orthosympathiques, de type C, véhiculent principalement les informations nociceptives des organes cibles. Les afférences viscéro-sensibles sont intégrées au niveau des ganglions périphériques, de la moelle spinale, du tronc cérébral (noyau du tractus solitaire dans le bulbe notamment pour les afférences parasympathiques) puis dans des structures supérieures comme l'hypothalamus, les corps mamillaires, le thalamus ou le cortex. Dans le cadre des réflexes autonomes, l'intégration des afférences d'un organe au sein des ganglions viscéraux peut déclencher la modulation de l'innervation efférente de ce même organe ou d'une autre organe viscéral (65). La modulation de l'activité autonome par le biais de voies réflexes périphériques est particulièrement développée au sein du système nerveux entérique, avec des réflexes entéro-entériques (86). Ainsi lorsqu'il est expérimentalement isolé du SNC, le SNE peut

contrôler le fonctionnement du tube digestif, en particulier la motricité de l'intestin grêle et du côlon (67,69).

Tableau 1 : Effets de l'activation des divisions orthosympathiques et parasympathiques sur les organes cibles (65,66)

Stimulation orthosympathique	Organe cible	Stimulation parasympathique
mydriase	Pupille (iris)	myosis
	Glandes lacrymales	sécrétion
vasoconstriction, réduction de sécrétion	Glandes salivaires	vasodilatation et sécrétion
	Cœur	
augmentation	Contractilité du myocarde	diminution
accélération	Nœud sinusal	ralentissement
relâchement	Muscles bronchiolaires	contraction
	Vaisseaux périphériques	
vasoconstriction	peau	
vasoconstriction	viscères	vasodilatation
vasodilatation	muscles squelettiques	
	Tractus gastro-intestinal	
néoglucogénèse, glycogénolyse	Glandes hépatiques	
inhibition de la sécrétion	Glandes gastriques	sécrétion
relâchement	Vésicule biliaire	contraction
relâchement	Paroi du tube digestif	contraction
contraction	Sphincters	relâchement
libération de rénine	Rein	
	Vessie	
relâchement	Détrusor	contraction
contraction	Sphincters	relâchement
	Peau	
sécrétion	Glandes sudorales	
contraction	Muscles piloérecteurs	

L'hypothalamus assure l'essentiel du contrôle central descendant du SNA, intégrant les afférences provenant de diverses structures telles que le cortex entorhinal, le cortex insulaire, l'hippocampe, les ganglions de la base ou le cervelet (67). Il émet des efférences vers les centres bulbaires et spinaux du SNA. Parallèlement il active la libération

d'hormones, via le système endocrinien, agissant directement ou indirectement sur des cibles périphériques (65,67).

III-Neuropathologie de la MP

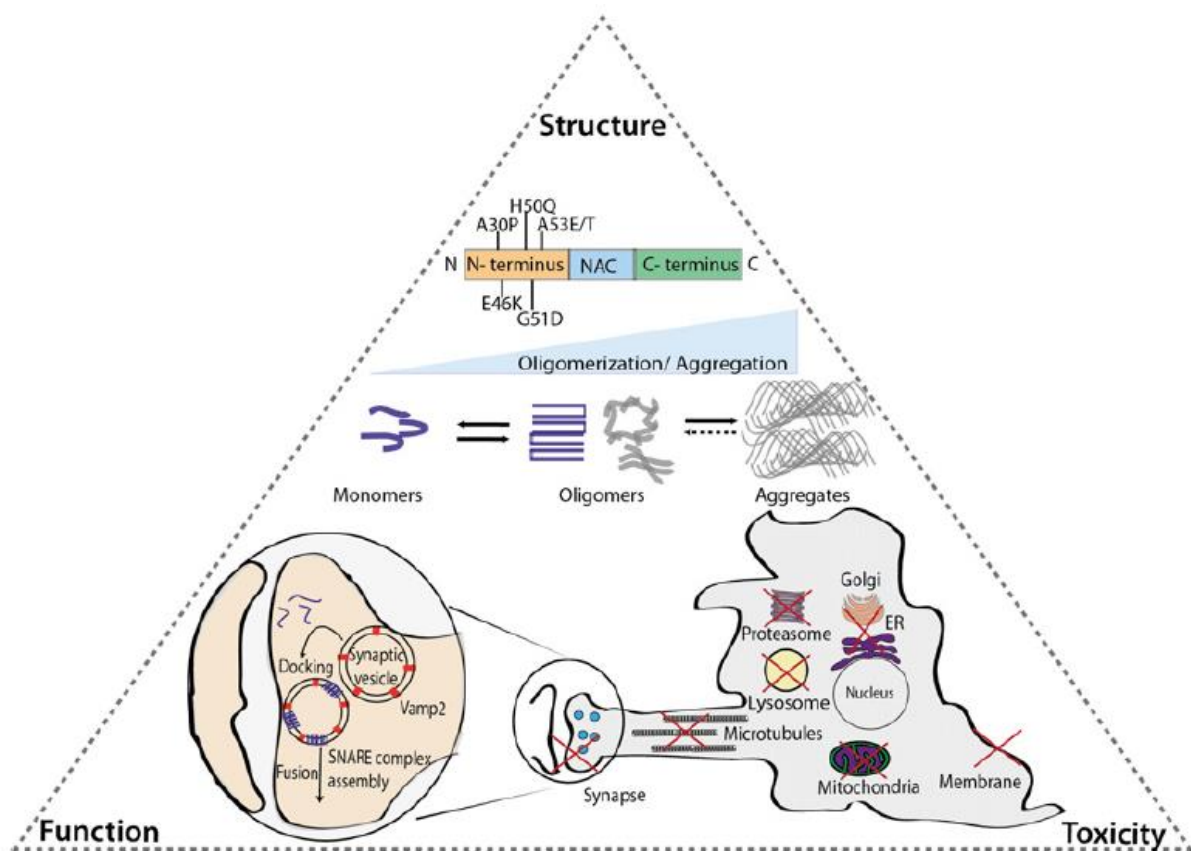
1. Voie nigro-striée

La signature histopathologique de la maladie de Parkinson est la perte des neurones dopaminergiques de la *pars compacta* de la substance noire (SNpc), localisée dans le mésencéphale, prédominant dans ses régions caudales et ventrolatérales (8,87). Ces neurones dopaminergiques projettent leurs afférences sur le striatum : putamen et noyau caudé. La dégénérescence des neurones dopaminergique est progressive, ainsi les premiers signes moteurs deviennent apparents lorsque, selon les études, 30 à 60% des neurones ont dégénéré (88,89). La perte neuronale dans la SNpc est associée à la formation de corps de Lewy intra-cytoplasmiques et de neurites de Lewy dystrophiques. Les corps de Lewy sont des inclusions neuronales sphériques éosinophiles, mesurant 8 à 30 µm de diamètre, constitués de multiples protéines, parmi lesquelles l'alpha-synucléine est majoritaire, principalement sous forme phosphorylée et agrégée (90,8,91).

L'alpha-synucléine est abondamment exprimée dans le cerveau, mais aussi dans des tissus périphériques et des cellules circulantes comme les globules rouges (92). Ses fonctions sont connues de manière incomplète. Elle serait active dans les terminaisons pré-synaptiques et régulerait la transmission synaptique. Son rôle serait important dans la dynamique des vésicules synaptiques, leur mobilisation et leur endocytose (93).

Des études génétiques ont montré l'implication causale de l'alpha-synucléine dans la pathogenèse de la MP, les mutations ponctuelles de cette protéine entraînant des formes autosomiques dominantes de MP (92,93). L'alpha-synucléine est une petite protéine de 140 acides aminés, elle peut être divisée en 3 domaines majeurs : le domaine N-terminal (1-60) structuré en hélice α , le domaine central (61-95) impliqué dans l'agrégation de l'alpha-synucléine et le domaine C-terminal (96-140) qui donne sa flexibilité au polypeptide. Elle peut subir de nombreuses modifications post-traductionnelles qui modifient son fonctionnement physiologique ou pathologique (92). La phosphorylation principalement sur

son résidu sérine 129 (Ser-129) est observée dans les corps et neurites de Lewy (94). Le stress oxydatif majore cette phosphorylation Ser-129 et la formation d'inclusions (95) ; inversement la diminution de la phosphorylation Ser-129 entraîne une réduction de l'agrégation de l'alpha-synucléine (96). Les monomères d'alpha-synucléine peuvent subir une multimérisation, qui pourrait avoir un rôle physiologique (sous forme de tétramères (97)), mais conduisant également à un repliement anormal (*misfolding*) avec la formation de protofibrilles, qui s'associent pour former des fibrilles amyloïdes matures, s'accroissant progressivement avec l'agrégation de nouveaux monomères (92, Figure 8).



[Figure 8](#) : Fonction physiologique et toxicité de l'alpha-synucléine, en lien avec sa structure (tiré de 92)

Le rôle toxique de l'alpha-synucléine et des corps de Lewy sur les neurones reste imprécis. La toxicité pourrait s'exercer sur les membranes, les mitochondries, le cytosquelette et les voies de clairance protéique, via une altération des organites intracellulaires (protéasome,

lysosome, microtubules ou encore réticulum endoplasmique) (92,98). Par ailleurs, alors qu'il existe une corrélation clinico-pathologique entre la mort neuronale dans la SNpc et les symptômes moteurs, la densité de neurones porteurs de corps de Lewy reste remarquablement constante (3,6% en moyenne) quels que soient la durée d'évolution et les symptômes (99). Les formes oligomériques d'alpha-synucléine seraient toxiques (98,100) et le rôle protecteur ou toxique des corps de Lewy reste discuté (99,101). L'agrégation de l'alpha-synucléine pourrait débuter dans la partie distale de l'axone au niveau des terminaisons synaptiques, entraînant sa dégénérescence, puis remonter vers le soma où se forment préférentiellement les corps de Lewy (89).

L'alpha-synucléine semble avoir des propriétés de diffusion, *in vitro* sous forme de fibrilles ou de fraction insoluble (102,103) ou *in vivo* comme l'a montré sa capacité à se propager d'un neurone à l'autre, sur le mode des protéines prions, atteignant notamment des neurones dopaminergiques fœtaux greffés chez des patients parkinsoniens (104,105).

2. Diffusion encéphalique

Au-delà de la substance noire, des accumulations d'alpha-synucléine associées à une perte neuronale ont été constatées dans certains noyaux du tronc cérébral : les noyaux sérotoninergiques du raphé, le noyau dorsal du vague dans le bulbe, le complexe cœruleus-subcœruleus et le noyau pédonculo-pontin dans le pont, le noyau d'Edinger-Westphal dans le mésencéphale (87,106). À l'étage sus-tentorial, elles ont également été observées dans le bulbe olfactif, le noyau basal de Meynert, l'amygdale temporale, les noyaux limbiques du thalamus, l'hypothalamus et l'épiphyse postérieure. La synucléinopathie de type Lewy (corps et neurites de Lewy) peut être présente dans une région limitée de l'hippocampe et le gyrus parahippocampique, le cortex entorhinal et transentorhinal, le cortex insulaire, le gyrus cingulaire, et les cortex frontaux et pariétaux (87).

L'atteinte diffuse des structures encéphaliques serait à l'origine de l'expression clinique complexe de la MP (107). La densité de corps de Lewy observés dans le cortex entorhinal et

le gyrus cingulaire antérieur prédirait l'altération cognitive dans la MP (108). Le déficit cognitif léger et le syndrome dyséxécutif fronto-striatal seraient liés à une extension de la synucléinopathie de type Lewy aux voies dopaminergiques fronto-striatales mais également aux noyaux cholinergiques, à leurs voies ascendantes et au locus cœruleus adrénérique (15,16). La démence parkinsonienne serait le reflet d'une extension du processus pathologique au lobe temporal et au cortex pariéto-occipital (15). Enfin, dans la démence parkinsonienne, les hallucinations visuelles apparaissent fortement liées à une synucléinopathie touchant l'amygdale (109).

3. Diffusion au SNA

La présence de corps et neurites de Lewy à l'extérieur du système nerveux central est connue depuis les années 1930, en particulier au niveau des voies et organes cibles du SNA (42,110,111). L'atteinte des centres autonomes concerne des structures encéphaliques (noyaux parasympathiques du tronc cérébral : noyau d'Edinger-Westphal et noyau dorsal du vague) mais aussi extra-encéphaliques telles que les centres orthosympathiques de la colonne intermédiolatérale de la moelle spinale et les neurones parasympathiques de la moelle spinale sacrée (112,113). Des accumulations d'alpha-synucléine et une perte neuronale sont également mises en évidence à tous les niveaux du réseau autonome : dans les voies pré-ganglionnaires, les ganglions autonomes, les voies post-ganglionnaires et les organes cibles, comme l'illustre la Figure 9.

Des analyses autopsiques ont mis en évidence des corps et neurites de Lewy dans les glandes sous-maxillaires, le ganglion cervical supérieur et les troncs sympathiques et le nerf vague (au niveau de la bifurcation carotidienne) de patients parkinsoniens (114). *In vivo*, des biopsies des glandes salivaires accessoires ont également montré la présence d'alpha-synucléine agrégée dans les espaces périacineux où est localisée l'innervation autonome

dans une étude pilote (3 patients) (115), mais ces résultats n'ont pas été confirmés dans une population plus importante, avec un manque de spécificité (116).

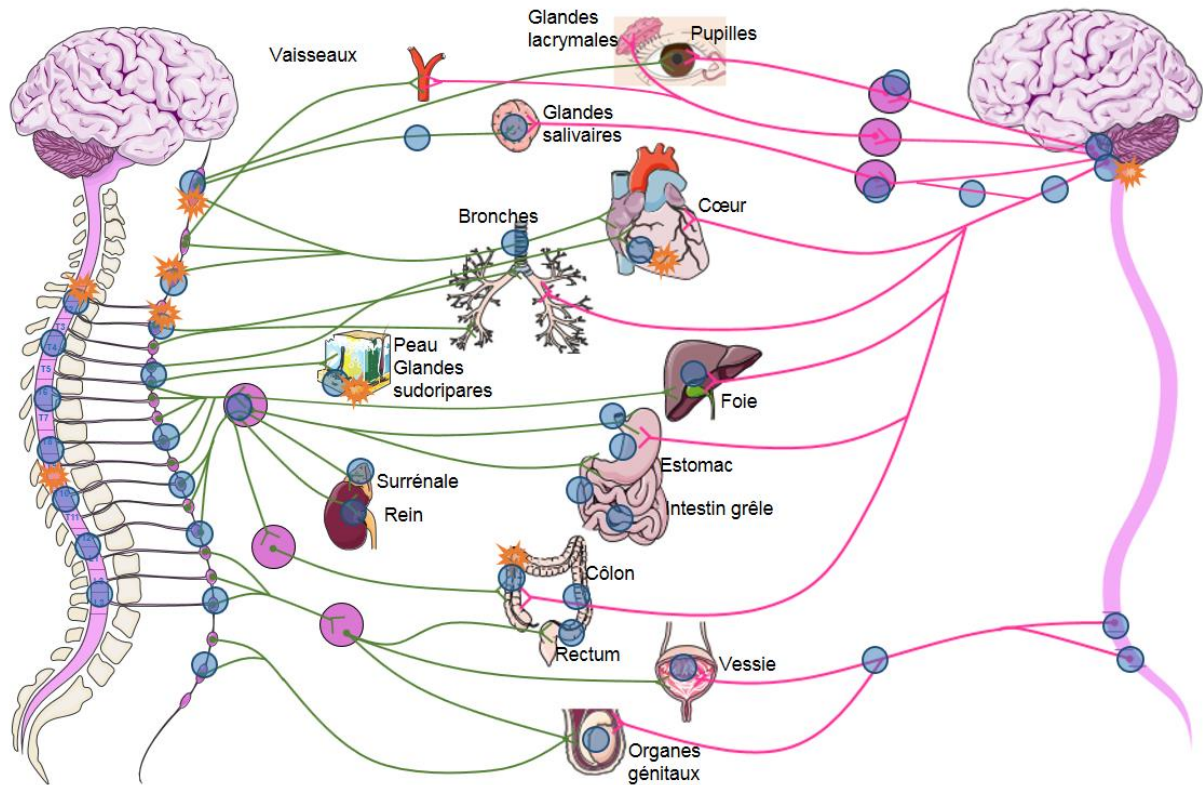


Figure 9 : Représentation schématique de l'atteinte du SNA (les ronds bleus correspondent aux corps et neurites de Lewy, les étoiles oranges à la perte neuronale).

La scintigraphie myocardique au ^{123}I -MIBG (metaiodobenzylguanidine), explorant l'innervation cardiaque sympathique post-ganglionnaire, a mis en évidence une dénervation autonome cardiaque plus importante dans la MP par rapport aux syndromes parkinsoniens atypiques, elle apparaissait quasi-constante lorsque les patients avaient une dysautonomie clinique (117,118). La dénervation autonome atteint les ganglions sympathiques, les voies pré et post-ganglionnaires, jusqu'aux tissu nerveux cardiaque et au myocarde (119). Des études anatomo-pathologiques suggèrent que la synucléinopathie pourrait atteindre initialement les axones distaux des fibres catécholaminergiques cardiaques, entraînant leur

dégénérescence, puis suivre une progression centripète jusqu'aux corps cellulaires dans les ganglions paravertébraux orthosympathiques (120,121). L'expression clinique de la dysautonomie cardiaque est principalement une hypotension orthostatique. Goldstein *et al.* évoquent une atteinte triple dans la physiopathologie de l'hypotension orthostatique : post-ganglionnaire sympathique cardiaque, pré-ganglionnaire parasympathiques et orthosympathiques, altérant le baroréflexe (50,52). La dénervation cardiaque s'aggrave avec la durée d'évolution (52).

De la même manière, les structures autonomes contrôlant le tube digestif sont atteintes de manière diffuse dans la MP (113,122). Des inclusions d'alpha-synucléine sont rapportées au sein du SNE, dans la muqueuse, les plexus sous-muqueux et myentérique, suivant un gradient rostro-caudal (85,123–125). La dysautonomie digestive haute est caractérisée par une dysphagie et une gastroparésie. La déglutition fait intervenir des structures somatiques et autonomes. Même si les structures autonomes pourraient participer à la physiopathologie de la dysphagie, elle semble surtout liée à sa composante somatique motrice dans la MP et pourrait s'expliquer par l'altération des programmes moteurs (*central pattern generator*) liées à l'atteinte du noyau pédonculopontin (122,126,127). La gastroparésie pourrait être liée à une atteinte du noyau moteur dorsal du vague mais également du SNE (122,128). La constipation serait liée à un ralentissement du temps de transit colique, possiblement par altération des circuits intrinsèques du SNE (53,122). La question de la mort neuronale dans le SNE colique en lien avec la synucléinopathie reste débattue, les résultats des études pouvant être contradictoires (76,123,129,130). Le nombre de neurones apparaissait réduit de 15% dans les ganglions du plexus sous-muqueux porteurs de neurites de Lewy sur des biopsies coliques *in vivo* (83). Une diminution des neurones dopaminergiques du plexus myentérique avait été observée chez 9 patients parkinsoniens sur 11, en comparaison avec des sujets contrôles (129) ; mais une étude autopsique plus récente n'a pas confirmé ces résultats (130). Dans le plexus sous-muqueux, il n'a pas été observé de perte neuronale dopaminergique, et plus largement catécholaminergique (76). Par ailleurs, une étude

autopsique ancienne chez 3 sujets avait montré que les neurones porteurs de corps de Lewy du SNE étaient VIPergiques et non dopaminergiques (131), des données non confirmées par la suite (130). La physiopathologie de la constipation dans la MP reste imprécise, en raison des corrélations anatomo-cliniques avec l'atteinte du SNE contradictoires, une atteinte de l'innervation préganglionnaire pourrait également être impliquée (76,113). L'atteinte du SNE colique pourrait être très précoce dans la MP, dès la phase prémotrice, comme cela a été montré dans le TCSP idiopathique (132).

Plusieurs analyses autopsiques ont montré l'atteinte des structures autonomes centrales à visée urinaire dans la MP, notamment dans les neurones orthosympathiques de la colonne intermédiolatérale de la moelle spinale et les centres parasympathiques sacrés (113,133). Une synucléinopathie de type Lewy a également été observée dans les plexus autonomes vésicoprostatiques (134).

Une dénervation autonome périphérique est fréquemment mise en évidence chez les patients parkinsoniens sur des biopsies cutanées, avec une réduction des fibres nerveuses intra-épidermiques chez 44% d'entre eux (en comparaison avec les normes d'une population contrôle) et une diminution de l'innervation des glandes sudoripares chez 76% des patients contre 42% des témoins (135). La dénervation n'étant pas corrélée aux scores cliniques, les lésions histologiques pourraient précéder l'apparition de symptômes dysautonomiques cutanés (64). Cependant, la sévérité des symptômes sensitifs était associée à une dénervation plus marquée, suggérant un lien entre les douleurs neurogènes diffuses dans la MP et l'atteinte autonome périphérique (64). Plusieurs études autopsiques et *in vivo* sur des biopsies cutanées ont montré la présence de pathologie de Lewy dans les réseaux autonomes cutanés, en particulier dans les nerfs pilomoteurs et sudomoteurs, associée à une dysfonction autonome adrénergique et cholinergique cutanée, s'aggravant avec la sévérité de la MP (136).

Les corrélations anatomo-cliniques entre la charge lésionnelle en corps et neurites de Lewy et la dysautonomie restent imprécises, car les symptômes seraient mieux expliqués par la perte neuronale que par la synucléinopathie (99).

4. Dynamique de l'atteinte histopathologique

La multiplicité des localisations lésionnelles de la MP pose la question de leur organisation temporelle et des mécanismes la sous-tendant. L'équipe d'Heiko Braak a proposé en 2003 une gradation chronologique de la MP, nommée classification de Braak (Figure 10). Le processus pathologique débiterait dans les organes périphériques, progressant le long des voies nerveuses jusqu'au noyau moteur dorsal (nerfs vague et glosso-pharyngien) et aux noyaux olfactifs antérieurs (stade 1). Puis la synucléinopathie progresserait de manière ascendante via le complexe coeruleus-subcoeruleus dans le pont (stade 2), atteignant ensuite le mésencéphale, le noyau tubéromammillaire de l'hypothalamus et les noyaux magnocellulaires de la base du cerveau (stade 3), le néocortex temporal interne (stade 4), les néocortex associatifs pariétaux polymodaux (stade 5) et les cortex prémoteurs, primaires ou associatifs unimodaux (stade 6) (137).

Elle permettrait d'expliquer l'existence des symptômes prémoteurs de la MP, tels que le TCSP idiopathique, la constipation et l'hyposmie (138) (Figure 11). Cette progression pourrait provenir de la transmission par voie nerveuse et synaptique d'un agent pathogène de nature indéterminée (139,140). Cette hypothèse de progression de la MP demeure controversée (141), notamment car elle s'applique peu aux cas génétiques par mutation de l'alpha-synucléine et des exceptions sont observées à cette gradation temporelle (91,142,143). La dynamique de progression pourrait s'expliquer par une vulnérabilité plus importante de certaines structures à l'agrégation pathologique de l'alpha-synucléine (91).

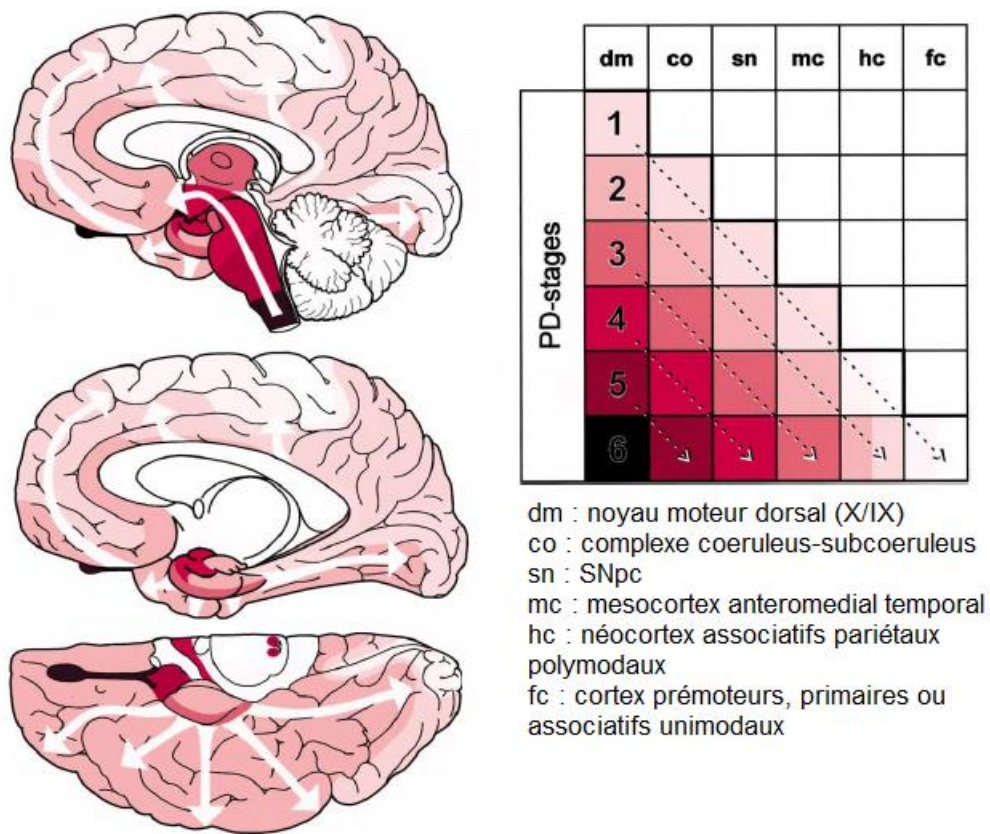


Figure 10 : Représentation schématique de la classification de Braak (137)

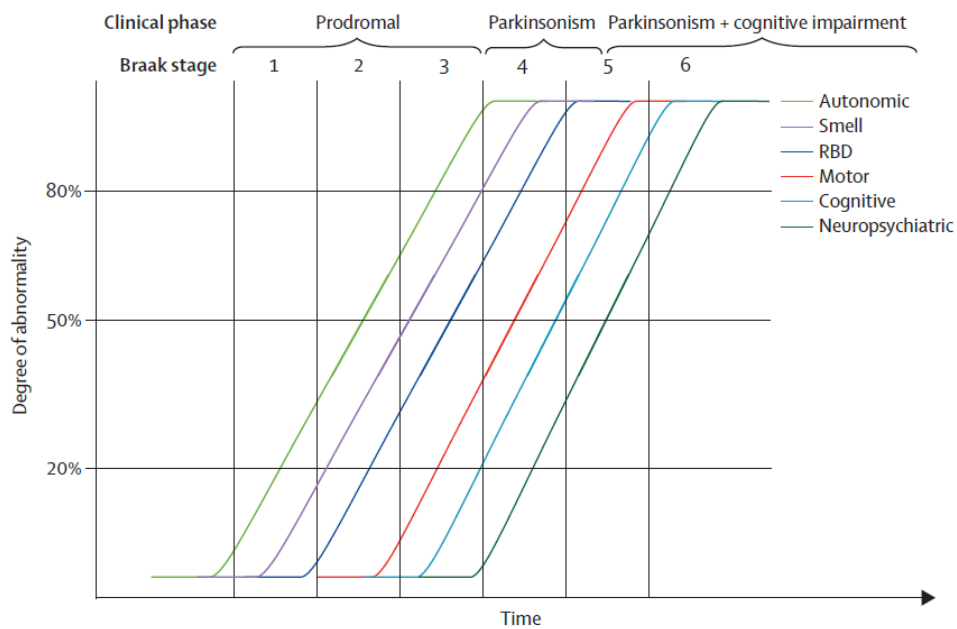
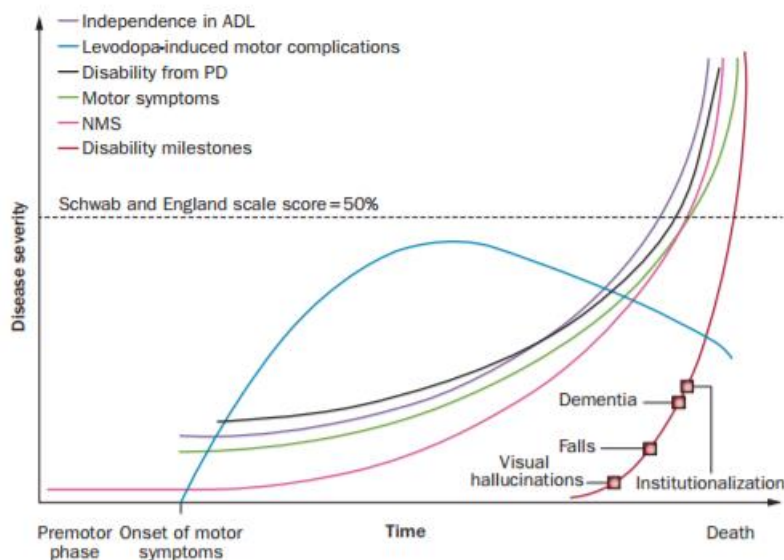


Figure 11 : Hypothèse sur la place des troubles non moteurs (dysautonomie, troubles de l'odorat, TCSP, cognition, troubles neuropsychiatriques) dans l'évolution de la MP, corrélée à la classification de Braak (144).

IV-Gravité de la maladie

La MP entraînerait une surmortalité d'un facteur 1,6 à 3. La mortalité ajustée de la MP serait supérieure à celle d'autres pathologies telles que la cardiopathie ischémique, l'accident vasculaire cérébral ou la bronchopneumopathie chronique obstructive (145,146).

L'évolution clinique de la MP a été considérablement modifiée par l'avènement des traitements médicamenteux et la stimulation cérébrale profonde. Les phases avancées de la MP étaient définies auparavant par des critères essentiellement moteurs : la restriction de mobilité, telle que l'illustre l'échelle de Hoehn et Yahr, et les complications motrices de la dopathérapie. L'augmentation de l'espérance de vie et les modifications du phénotype de la maladie sous traitement font entrevoir une contribution croissante des symptômes non moteurs au pronostic à long terme (147). Le handicap à un stade avancé de la MP peut être défini par la dépendance à un tiers pour les activités de la vie quotidienne. Il est principalement associé aux symptômes moteurs dopa-résistants, comme la dysarthrie et les troubles posturaux, et aux symptômes non moteurs tels que la démence, les hallucinations ou la dysautonomie (147) (Figure 12).



[Figure 12](#) : Représentation schématique de la progression de la MP au cours du temps (étapes décisives : hallucinations visuelles, chutes, démence, institutionnalisation) (tiré de 147).

Certaines étapes de la MP apparaissent décisives dans son pronostic, ainsi les hallucinations visuelles, les chutes, la démence et l'institutionnalisation sont proposées comme des événements marquant négativement le cours évolutif de la maladie dans ses cinq dernières années (147–149).

Parmi les facteurs pronostiques de la MP, l'âge influence le taux de dépendance, probablement par son effet sur les troubles posturaux et la démence, qui sont plus sévères chez des sujets plus âgés, à durée d'évolution équivalente (150,17,151–153). Cet effet de l'âge est également observé sur le plan de la charge lésionnelle de type synucléinopathie (149).

Afin d'étudier la diffusion de la MP au SNA dans notre travail, nous proposons de retenir trois critères de gravité : les signes moteurs axiaux, les troubles cognitifs et le trouble du comportement en sommeil paradoxal.

1-Les signes moteurs axiaux

Parmi les signes moteurs axiaux, les troubles posturaux apparaissent en moyenne après 10 à 15 ans d'évolution de la maladie, chez 20 à 80 % des patients (154). A 20 ans d'évolution, 87% des patients présentent des chutes, entraînant des fractures chez 35% d'entre eux (146). La survenue des troubles de la posture, de l'équilibre et de la marche constitue un tournant évolutif dans la maladie et un facteur pronostic péjoratif important (147,149,154). Ils sont associés à une vitesse d'aggravation plus rapide des symptômes, en particulier les troubles cognitifs (17,155). Le score moteur axial augmente de manière indépendante le taux de dépendance (152) et les troubles de la marche sont un facteur de risque de mortalité (156).

2- Les troubles cognitifs

Entre 20 et 57% des patients parkinsoniens présentent des troubles cognitifs légers dans les 3 à 5 ans suivant le diagnostic de MP (157). Ces troubles cognitifs légers prédisent le risque

de développer une démence (158), observée chez 10% des patients dans les 3 à 5 premières années et dans 83% des cas à 20 ans d'évolution (146,157). Un faible score au mini-mental state examination (MMSE) est corrélé de manière indépendante au taux de dépendance (152) et l'altération cognitive est un facteur de risque de mortalité dans la MP (145).

3- Le trouble du comportement en sommeil paradoxal

L'existence d'un trouble comportemental en sommeil paradoxal au cours d'une maladie de Parkinson est également associée à une plus grande sévérité de la maladie (4). Les troubles cognitifs et la démence sont plus fréquents (159–163), il est observé plus d'hallucinations (164–166) et les patients présentent plus de chutes, de fluctuations motrices et un score moteur plus élevé (167). Une étude a montré l'association du TCSP avec une hypotension orthostatique ou une dysfonction urinaire, suggérant un lien entre le TCSP et l'atteinte du système nerveux autonome (31). Le TCSP serait également un des déterminants négatifs de la qualité de vie (168). Non seulement lié à une pathologie plus sévère, marquée par des hallucinations et des troubles cognitifs augmentant la mortalité, le TCSP pourrait également être un facteur de risque indépendant de mortalité (169,170).

Hypothèses et objectifs de travail

De nombreux travaux histopathologiques ont montré l'atteinte du SNE au cours de la MP, dans les plexus sous-muqueux et myentérique, suivant un gradient rostro-caudal. Le plexus sous-muqueux colique innerve principalement la muqueuse colique, renforçant son rôle de BEI. Les données concernant la perméabilité au cours de la MP sont contradictoires, et l'implication de la BEI dans la pathogenèse de la MP est indéterminée. Nous avons étudié la BEI tant dans son aspect morphologique (aspect des cryptes) que fonctionnel (perméabilité para et transcellulaire) chez des patients parkinsoniens en comparaison avec des sujets contrôle. Nous avons également regardé la corrélation de la perméabilité de la BEI avec la charge lésionnelle en alpha-synucléine phosphorylée et agrégée (Article 1).

Fréquemment rapporté au cours de la MP, le TCSP est un signe non moteur pouvant avoir des conséquences cliniques notables, mais il est également un marqueur de sévérité de la maladie et témoigne d'une diffusion du processus pathologique au tronc cérébral. Selon la théorie de Braak, les atteintes du SNA périphérique et du complexe cœruleus-subcœruleus pourrait être successives dans le temps. Nous avons étudié l'atteinte du SNE colique chez des patients parkinsoniens avec et sans TCSP, d'un point de vue clinique, fonctionnel et histologique (Article 2).

Les données de la littérature sur la dysautonomie au cours de la MP étant partielles, éparses et incomplètes, nous avons analysé de manière systématique l'atteinte du SNA au cours de la MP en la caractérisant sur le plan clinique et fonctionnel. Nous avons effectué des corrélations anatomo-cliniques entre l'ensemble des symptômes dysautonomiques et l'histologie du SNA périphérique. Nous avons étudié une éventuelle association entre la dysautonomie et des marqueurs de sévérité de la MP tels que les signes moteurs axiaux, les troubles cognitifs et la présence d'un TCSP (Article 3).

Méthodes

Les études de cette thèse ont été réalisées dans le cadre du protocole SYNAPark (numéro d'enregistrement NTC01748409), financé par l'appel d'offre interne du CHU de Nantes 2012 et l'Association France Parkinson.

Résumé du protocole (version après amendement du 4/11/2014) :

Titre de l'étude	Etude physiopathologique monocentrique prospective de la diffusion de la maladie de Parkinson au système nerveux autonome.
Mots clés	Dysautonomie, maladie de Parkinson, corrélation anatomo-clinique
Promoteur de l'étude	CHU DE NANTES
Investigateur principal (si étude monocentrique)	Dr Laurène LECLAIR-VISONNEAU
Nombre de centres prévus	Monocentrique – CHU de Nantes
Type d'étude	Etude pilote observationnelle et physiopathologique de type Hors Produits de Santé.
Planning de l'étude	<ul style="list-style-type: none"> ❖ Durée totale : 41 mois ❖ Période de recrutement : 39 mois ❖ Durée de suivi par patient : 3 mois
Design de l'étude	<ul style="list-style-type: none"> ❖ Monocentrique ❖ Etude pilote ❖ Contrôlée : classification selon l'atteinte du système nerveux autonome (SNA) périphérique et selon l'existence d'une plainte dysautonomique ❖ Non randomisée
Objectifs de l'étude	<p><u>Objectif principal</u> :</p> <ul style="list-style-type: none"> - Rechercher l'existence d'associations entre la présence d'une atteinte du système nerveux autonome (SNA) périphérique chez les patients Parkinsoniens en anatomo-pathologie et une atteinte clinique, d'une ou plusieurs modalités du SNA, mise en évidence par des symptômes cliniques ou des tests fonctionnels <p><u>Objectif(s) secondaire(s)</u> :</p> <ul style="list-style-type: none"> - Rechercher une association entre une atteinte du SNA périphérique en anatomo-pathologie et une atteinte clinique de la même modalité, mise en évidence par des symptômes ou des tests fonctionnels, pour 4 modalités : salivation, digestion, sensibilité cutanée et sudation. - Etudier une association entre l'atteinte du SNA périphérique et les facteurs de gravité de la maladie de Parkinson (MP) : âge avancé, troubles cognitifs, trouble comportemental en sommeil paradoxal (TCSP) et signes moteurs axiaux. - Evaluer les conséquences de la dysautonomie (selon la plainte et selon l'atteinte du SNA périphérique) sur la qualité de vie et sur le sommeil des patients inclus dans cette étude.

<p>Nombre de cas prévisionnel</p>	<p>45 patients Parkinsoniens recrutés selon 3 classes de durée d'évolution de la maladie afin d'avoir une représentativité de la population des patients atteints : de 1 à 5 ans (15 patients), de 5 à 10 ans (15 patients) et d'évolution de plus de 10 ans (15 patients). <u>NB</u> : Ces classes de durée d'évolution de la maladie n'impacteront pas les analyses des critères principal et secondaires.</p>
<p>Calendrier des différentes visites et des différents examens</p>	<p>Présentation de l'étude lors d'une visite de suivi du patient</p> <p><u>Visite V1 (J0 : Inclusion)</u> : recueil du consentement, examen physique général et neurologique (vérification des critères diagnostiques, score UPDRS moteur, MMSE), prise de sang (critères de non inclusion), bilan neuropsychologique (MoCA, échelle de Mattis).</p> <p><u>Visite V2 (3 semaines +/- 7 jours après la V1)</u> : Rectosigmoïdoscopie avec 12 biopsies</p> <p><u>Visite V3 (3 semaines +/- 7 jours après la V2)</u> : - Entretien clinique et explorations fonctionnelles du système nerveux autonome - Biopsie cutanée - Biopsie des glandes salivaires accessoires (BGSA)</p> <p><u>Visite V4 (3 semaines +/- 7 jours après la V3)</u> : - Questionnaires de sommeil, de qualité de vie PDQ39, dépression, anxiété et fatigue - Enregistrement polysomnographique</p>
<p>Critères principaux de sélection, d'inclusion, de non-inclusion et d'exclusion</p>	<p><u>Critères d'inclusion</u> :</p> <ul style="list-style-type: none"> -Patients atteints de maladie de Parkinson selon les critères de la UKPDSBB (United Kingdom Parkinson's disease survey brain bank) - Agés de 45 à 80 ans - ayant donné leur consentement pour la participation à cette étude <p><u>Critères de non inclusion</u></p> <ul style="list-style-type: none"> -Ethyilisme chronique (prise d'alcool > 40 g/j) -Traitement anticholinergique, cholinomimétique, sympathomimétique, alphabloquant ou bêtabloquant -Cécité -Neuropathie connue préexistante -Atteinte préexistante des glandes salivaires : amylose, sarcoïdose, syndrome de Gougerot-Sjögren, lymphome -Antécédents de maladie colique authentifiée (maladie inflammatoire, adénocarcinome) -Colopathie fonctionnelle ayant précédé les premiers signes de la MP depuis plus de 5 ans -Coagulopathie ou traitement anticoagulant -Insuffisance rénale (Clairance de la créatinine < 30 ml/min) -Diabète (glycémie à jeun > 1,26 g/l ou glycémie non à jeun > 2g/l). -Hypovitaminose B12 (vitamine B12 < 200 pg/ml) -Présence d'une démence avec MMSE < 24 - Femmes enceintes ou, si femmes en âge de procréer : femme ne bénéficiant pas d'une contraception efficace - Majeurs sous tutelle <p><u>Critères de sortie prématurée d'étude</u> :</p> <ul style="list-style-type: none"> -Mise en évidence d'anomalies coliques significatives lors de la

	rectosigmoïdoscopie (inflammation, néoplasie colique)
Indication cible	Patients atteints d'une maladie de Parkinson et suivis au CHU de Nantes par un spécialiste des mouvements anormaux.
Critère de jugement principal	Le critère d'atteinte clinique d'une ou plusieurs modalités du SNA est : présence d'une plainte d'un ou plusieurs symptômes dysautonomiques ou présence d'une altération d'un des tests fonctionnels
Critère(s) de jugement secondaire(s)	<p><u>Les critères d'atteinte clinique des 4 modalités</u> pour lesquelles une analyse anatomo-pathologique est réalisée sont :</p> <ul style="list-style-type: none"> - Salivation : plainte (SCOPA-Aut Q2, NMS-Quest Q1), anomalie fonctionnelle (test de Saxon, test au sucre et mesure du pH) - Digestion : plainte : SCOPA-Aut (Q1, 3, 4, 5, 6, 7, 26a), NMS-Quest (Q3, 4, 5, 6, 7), constipation et GSRS - Sensibilité cutanée : plainte [SCOPA-Aut (Q20, 21), NMS-Quest (Q10), DN4, QCD], élévation du seuil de sensibilité thermique - Sudation : plainte [SCOPA-Aut (Q17, 18), NMS-Quest (Q28)], altération de la réponse cutanée sympathique <p><u>Les critères de gravité de la MP</u> sont :</p> <ul style="list-style-type: none"> - Age - Présence d'un TCSP (présence ou absence et sévérité) - Troubles cognitifs (échelle de Mattis et MoCA) - Signes moteurs axiaux (sous-score axial UPDRS) <p><u>Les critères d'altération du sommeil</u> sont :</p> <ul style="list-style-type: none"> - Altération de la qualité du sommeil : plainte du patient (PDSS, PSQI) et paramètres fonctionnels (polysomnographie) - Somnolence diurne excessive : échelle de somnolence d'Epworth - Syndrome des jambes sans repos : critères diagnostiques et échelle de sévérité IRLS, paramètres fonctionnels (polysomnographie) - <u>Les critères d'altération de la qualité de vie</u> reposent sur le questionnaire de qualité de vie PDQ39
<p>En cas d'étude ancillaire :</p> <p><input type="checkbox"/> Pharmacogénétique</p> <p><input type="checkbox"/> Pharmacocinétique</p> <p><input type="checkbox"/> Pharmacodynamique</p> <p><input type="checkbox"/> Pharmaco-économique</p> <p><input checked="" type="checkbox"/> Autres analyses</p>	<p>Le but de l'étude ancillaire est la mise au point d'une technique de recherche qui pourra éventuellement être utilisée en routine.</p> <p>L'étude ancillaire pourra préciser l'intérêt de la biopsie des glandes salivaires accessoires (technique très peu invasive mais encore en développement) par rapport aux biopsies coliques (l'intérêt majeur de cette analyse est bien connu mais la technique de prélèvement est plus invasive).</p>
Analyse statistiques	<p><u>Critère principal</u> : La présence des symptômes dysautonomiques et la présence d'altération des tests fonctionnels seront comparées entre les patients avec atteinte du SNA périphérique et les patients sans atteinte du SNA périphérique au moyen de tests non paramétriques de Wilcoxon, de tests du Chi-2 et de tests de Fisher.</p> <p><u>Critères Secondaires</u> :</p> <ul style="list-style-type: none"> - Pour la salivation, la sensibilité cutanée et la sudation : recherche d'associations entre la présence de symptômes, le résultat des tests fonctionnels et le résultat du test anatomo-pathologique (dénervation oui/non), pour la fonction digestive : recherche d'associations entre les symptômes cliniques et le test anatomo-pathologique (biopsie colique: dénervation oui / non), grâce à des tests de Wilcoxon, des tests du Chi-2 et de Fisher. - L'existence de signes de gravité de la MP : âge, TCSP, troubles

	<p>cognitifs, et signes moteurs axiaux sera comparé selon la présence ou l'absence d'atteinte du SNA périphérique grâce des modèles logistiques univarié et multivariés (si les effectifs sont suffisants).</p> <p>- Les paramètres du sommeil (cliniques et fonctionnels) et la qualité de vie (échelle PDQ39) seront comparés entre les patients avec atteinte du SNA périphérique et les patients sans atteinte du SNA périphérique par des régressions logistiques univarié. Les paramètres du sommeil et de qualité de vie seront comparés entre les patients avec une plainte dysautonomique significative et les patients sans plainte, par des régressions logistiques univarié. Les analyses seront ajustées sur la dépression, l'anxiété et la fatigue au moyen de régression logistique multivarié (si les effectifs sont suffisants).</p> <p><u>Analyse de l'étude ancillaire :</u></p> <p>L'objectif est de comparer le résultat des biopsies coliques aux résultats des biopsies salivaires. Le gold standard est le résultat de la biopsie colique. Les performances diagnostiques de la biopsie salivaire seront évaluées par les valeurs prédictives positives et négatives, la sensibilité et la spécificité.</p>
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Résultats

I- Article 1 : Altérations structurales de la barrière épithéliale intestinale dans la maladie de Parkinson

Clairembault T*, Leclair-Visonneau L*, Coron E, Bourreille A, Le Dily S, Vavasseur F, Heymann MF, Neunlist M, Derkinderen P. Structural alterations of the intestinal epithelial barrier in Parkinson's disease. Acta Neuropathologica Communications. 2015 Mar 10;3:12.

*co-auteurs

Résumé

Des modifications morphologiques et fonctionnelles de la BEI ont été régulièrement rapportées dans les maladies digestives telles que le syndrome du côlon irritable et les maladies inflammatoires chroniques de l'intestin. De nombreuses données montrent que la MP n'est pas seulement une maladie du cerveau, mais également un trouble digestif. L'atteinte gastro-intestinale est un événement fréquent et précoce au cours de la MP qui pourrait jouer un rôle crucial dans le développement initial de la maladie. Nous avons mené cette étude afin de déterminer si des modifications dans la fonction et/ou la morphologie de la BEI se produisaient au cours de la MP.

Des biopsies coliques ont été prélevées chez 31 patients parkinsoniens et 11 témoins sains appariés en âge. Les perméabilités para et transcellulaire ont été évaluées en mesurant, respectivement, les flux d'acide sulfonique et de peroxydase de raifort dans des biopsies de muqueuse colique montées dans des chambres d'Ussing. L'expression et la localisation des protéines de jonctions serrées ZO-1 et occludine ont été analysées respectivement par Western blot et immunofluorescence.

Les perméabilités para et transcellulaire n'étaient pas différentes entre les patients parkinsoniens et les sujets témoins. L'expression de l'occludine, mais pas celle de ZO-1, était significativement diminuée dans les biopsies coliques de patients parkinsoniens par rapport

aux témoins et la distribution cellulaire des deux protéines était altérée dans les muqueuses coliques des patients parkinsoniens.

Nos résultats montrent une altération morphologique de la BEI au cours de la MP, renforçant ainsi le rôle potentiel du tractus gastro-intestinal dans l'initiation et/ou la progression de la maladie.

RESEARCH

Open Access

Structural alterations of the intestinal epithelial barrier in Parkinson's disease

Thomas Clairembault^{1,2,3†}, Laurene Leclair-Visonneau^{1,2,4†}, Emmanuel Coron^{1,2,3,4}, Arnaud Bourreille^{1,3,4}, Séverine Le Dily⁴, Fabienne Vavasseur^{3,4}, Marie-Françoise Heymann^{2,5,6}, Michel Neunlist^{1,2,3} and Pascal Derkinderen^{1,2,4,7*}

Abstract

Functional and morphological alterations of the intestinal epithelial barrier (IEB) have been consistently reported in digestive disorders such as irritable bowel syndrome and inflammatory bowel disease. There is mounting evidence that Parkinson's disease (PD) is not only a brain disease but also a digestive disorder. Gastrointestinal involvement is a frequent and early event in the course of PD, and it may be critically involved in the early development of the disease. We therefore undertook the present survey to investigate whether changes in the IEB function and/or morphology occur in PD. Colonic biopsies were performed in 31 PD patients and 11 age-matched healthy controls. The para- and transcellular permeability were evaluated by measuring sulfonic acid and horseradish peroxidase flux respectively, in colonic biopsies mounted in Ussing chambers. The expression and localization of the two tight junctions proteins ZO-1 and occludin were analyzed by Western blot and immunofluorescence, respectively. The para- and transcellular permeability were not different between PD patients and controls. The expression of occludin, but not ZO-1, was significantly lower in colonic samples from PD patients as compared to controls and the cellular distribution of both proteins was altered in colonic mucosal specimens from PD patients. Our findings provide evidence that the IEB is morphologically altered in PD and further reinforce the potential role of the gastrointestinal tract in the initiation and/or the progression of the disease.

Keywords: Parkinson's disease, Intestinal epithelial barrier, Enteric nervous system, Tight junctions, Occludin, ZO-1

Introduction

The intestinal epithelium forms a regulated barrier, known as intestinal epithelial barrier (IEB), between the blood circulation and the contents of the intestinal lumen [1]. It prevents the passage of noxious contents while allowing the absorption and secretion of nutrients [1]. Penetration of this barrier occurs via two routes, either between epithelial cells via the paracellular pathway, or through epithelial cell via the transcellular pathway [1]. Among the most important structures of the intestinal barrier are the epithelial tight junctions (TJs) that connect adjacent enterocytes together to determine paracellular permeability through the lateral intercellular space [2]. They are formed by transmembrane proteins such as claudins and occludins connected to the actin cytoskeleton via high

molecular weight proteins called zona occludens (ZO-1, ZO-2 and 3) [2]. Increased permeability of the IEB along with changes in the expression levels of TJs proteins have been consistently reported in several digestive disorders such as inflammatory bowel disease [3,4] and irritable bowel syndrome [5,6].

It has become evident over the last 20 years that PD is a gut disorder (reviewed in [7]). Gastrointestinal symptoms occur in almost every PD patient at some point and are among the most debilitating non-motor features of the disease [8]. These clinical data have been supported by *post mortem* studies that demonstrated the presence of Lewy bodies and neurites in the enteric neurons in nearly every case examined pathologically [9,10]. The German pathologist Heiko Braak suggested that the appearance of Lewy pathology in enteric neurons develop early in the course of disease, prior to the involvement of the central nervous system [11]. This led him to suggest that the gastrointestinal tract might be a portal of entry for a putative pathogen that would breach the IEB to induce the

* Correspondence: derkinderen@yahoo.fr

†Equal contributors

¹Insem U913, 1 rue Gaston Veil, Nantes F-44035, France

²University Nantes, Nantes F-44093, France

Full list of author information is available at the end of the article



formation of Lewy bodies and neurites in the enteric neurons [11].

The high prevalence of gastrointestinal symptoms and pathology in PD and the possible derangement of gastrointestinal permeability in the pathogenesis of the disease prompted several groups to investigate IEB permeability in parkinsonian patients. The three studies, which have been carried out to date have all used absorption of sugar probes as a means to investigate non-invasively the paracellular permeability [12]. These studies, which have included a small number of patients, led to conflicting results. Two studies found a pattern of sugar absorption reminiscent of small intestine hyperpermeability in a subset of patients [13,14] while the third one showed an increase in sucralose excretion without changes in the lactulose/mannitol ratio, a pattern consistent with increased colonic permeability [15]. We therefore undertook the present research to analyze in more details the IEB in PD. To this end, a functional and structural characterization of the IEB was performed in colonic biopsies from PD patients.

Materials and methods

Subjects

A total of 42 subjects participated in this study, 31 PD patients and 11 healthy controls. PD patients aged 43–74 years were recruited from the movement disorder clinic at Nantes University Hospital, France. Diagnosis of PD was made according to criteria provided by the United Kingdom Parkinson's Disease Survey Brain Bank [16]. Collected demographic data included gender, age at onset and disease duration, as well as age at colonoscopy. Complete drug history was obtained, and an approximation of the cumulative dose of L-dopa was made based on the equation developed by Parkkinen and collaborators [17]. Control subjects were healthy subjects who had a normal colonoscopy performed for colorectal cancer screening. All controls subjects underwent a detailed neurological examination to rule out PD symptoms and cognitive deficiency. Controls and PD patients were excluded if they suffered from irritable bowel syndrome and/or anorectal dysfunction. 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 NCT01748409). Written informed consent was obtained from each patient and from each normal volunteer according to the principles of Helsinki.

Endoscopic procedure and colonic biopsies

For each subject, nine biopsies were taken in the sigmoid/descending colon during the course of a rectosigmoidoscopy for PD patients and during a colonoscopy for control subjects. Five biopsies were immersed in 4°C Hank's Balanced Salt Solution (Life Technologies, Saint Aubin, France): three of these biopsies were immediately processed

for the assessment of para- and transcellular permeability in Ussing chambers while the two other biopsies were used for immunohistochemistry experiments. Two biopsies were stored at -80°C in lysis buffer RA1 (Macherey-Nagel, Hoerd, France) with 1% (v/v) β -mercaptoethanol (Sigma, Saint Quentin Fallavier, France) for further analysis by immunoblotting. The two remaining biopsies were snap frozen in liquid nitrogen at the time of collection and kept at -80°C .

Para- and transcellular permeability of colonic biopsies in Ussing chambers

Three biopsies were mounted in Ussing chambers (World Precision Instruments; WPI, Hertfordshire, UK) exposing a surface of 0.011 cm^2 . Tissues were bathed on each side with 3 ml of F12 supplemented Dulbecco's Modified Eagle medium (Invitrogen, France) containing 0.1% (v/v) fetal bovine serum, 200 mM Glutamine and 45 g/L of NaHCO_3 . The medium was continuously oxygenated and maintained at 37°C by a gas flow (95% O_2 /5% CO_2). After a 30 min baseline period, 275 μL of apical medium was replaced with 200 μL of media containing 1 mg/mL of fluorescein-5,6-sulfonic acid (molecular weight: 400 Da) (Life Technologies) for a final concentration of 0.1 mg/mL to assess paracellular permeability. Seventy-five microliters of media with 10 mg/mL of Horse Radish Peroxydase (HRP) (Sigma) were also added to the basolateral chamber for a final concentration of 0.375 mg/mL to measure transcellular permeability in a subset of PD patients and control subjects. The fluorescence level of basolateral aliquots of 150 μL , reflecting paracellular transit from the luminal surface was measured every 30 min over a 3-hour period using a fluorimeter (Varioskan[®], ThermoFisher Scientific, Cillebon sur Yvette, France). HRP quantities in the basolateral chamber, reflecting transcellular transit from the apical surface, was measured using an enzymatic activity assay with 3,3',5,5'-tetramethylbenzidine reagent (BD Bioscience, Le Pont de Claix, France). Paracellular and transcellular permeabilities were determined by averaging the gradient of change in fluorescence intensity over time in the three biopsies that were analyzed per patient, using a linear regression fit model (GraphPad Prism 5, La Jolla, USA).

Western blot

For the analysis of ZO-1 expression, total proteins from the 2 biopsies stored in RA1 buffer were precipitated and prepared for Polyacrylamide Gel Electrophoresis (PAGE) using protein precipitator and resuspension buffer (Protein solving buffer and tris(2-carboxyethyl) phosphine) TCEP reducing agent, PSB/TCEP from NucleoSpin Triprep Kit (Macherey-Nagel, Hoerd, France) according to the manufacturer's instructions. For experiments on the transmembrane protein occludin, the two

dry frozen biopsies stored at -80°C were lysed in UTC buffer (7 M Urea, 2 M Thiourea and 4% CHAPS) containing a protease inhibitor cocktail (Complete[®], Roche, Meylan, France) using the "Precellys 24" tissue homogenizer (Bertin technologies, Saint Quentin-en-Yvelines, France) and followed by sonication with "vibracell 75 186" device (Sonics, Newton CT, USA). Equal amounts of lysate were separated using the Invitrogen NuPage Novex 3-8% Tris-Acetate Midi Protein Gels[™] for ZO-1 or NuPage Novex 4-12% Bis-Tris MidiGels[™] for occludin before electrophoretic transfer to nitrocellulose membranes with the iBlot[™] Dry Blotting System also from Invitrogen. Membranes were processed for immunoblotting using rabbit polyclonal anti-ZO-1 (1:500, Life Technologies) and rabbit anti-occludin (1:250, Abcam, Paris, France) antibodies and the relevant immunoreactive bands were quantified as previously described [18].

Microdissection and immunohistochemistry

Microdissection was performed as previously described [19] in two out of the nine biopsies taken per patient. Each whole-mount preparation of submucosa obtained from a single biopsy was permeabilized for 3 hours in phosphate buffered saline (PBS)/NaN₃ containing 1% (v/v) Triton X-100 and 10% (v/v) horse serum and then incubated with antibodies against phosphorylated alpha-synuclein (1:5000, WAKO, Osaka, Japan) and PGP9.5 (1:10,000; Ultracode Limited, UK). Each whole-mount preparation of mucosa was treated for 24 h with Scale A2 solution composed of 4 M urea, 10% (w/v) glycerol and 0.1% (v/v) Triton X100 [20] then incubated with rabbit polyclonal antibodies to ZO-1 (1:100, Life Technologies) and occludin (1:100, Abcam). Suitable secondary antibodies conjugated to Alexa Fluor 488 and 594 were used (Invitrogen, Cergy-Pontoise, France). Following incubation with the secondary antibodies, the mucosa samples were treated for 10 minutes with a solution of 0.3% (w/v) of Sudan Black B powder (Sigma) dissolved in 70% (v/v) ethanol, then washed extensively with PBS. Whole specimen of submucosa and mucosa were viewed under an Axio Zoom.V16 stereomicroscope (Zeiss, Marly Le Roi, France). All samples were deidentified and studied in a blinded manner. For the analysis of ZO-1 and occludin immunofluorescence, the percentage of morphologically normal crypts per biopsy was calculated and the following classification was used: 'normal', more than 2/3 of morphologically normal crypts; 'mild disruption': between 1/3 and 2/3 of morphologically normal crypts; 'disrupted': less than 1/3 of morphologically normal crypts.

Statistics

All data are given as the mean \pm standard error of the mean (SEM). For comparisons of means between groups, a Mann-Whitney test was performed. Differences were deemed statistically significant if $p < 0.05$.

Results

A total of 31 PD patients and 11 controls were included. Table 1 shows the main clinical and demographic features of all patients. Age and sex did not differ significantly between PD patients and control subjects (mean age was 64.2 ± 2.1 for PD and 60.6 ± 1.4 for controls, $p = 0.25$; 22/31 male in PD group and 6/11 male in control group, $p = 0.13$).

Para- and transcellular permeability are unaffected in PD

In a first set of experiments, we evaluated whether IEB is functionally altered in PD patients. The para- and

Table 1 Main clinical characteristics of PD patients

	Age	Sex	Disease duration (years)	Cumulative lifetime dose of L-dopa (mg)
1	70	F	2	0
2	64	M	NK	NK
3	70	M	NK	NK
4	66	M	23	66111000
5	47	F	2	0
6	62	M	4	3823375
7	74	M	13	1505625
8	53	M	4	2372500
9	69	M	2.5	273750
10	43	F	11	508050
11	67	M	2.3	0
12	58	M	7	2135250
13	58	M	9	1533000
14	72	F	14	1442662.5
15	64	M	4	492750
16	64	M	10	355875
17	56	M	NK	NK
18	66	M	4.6	0
19	62	M	26	1560375
20	70	F	2.2	219000
21	57	M	1.8	0
22	58	M	13	2007500
23	50	M	8	1286625
24	69	F	28	3759500
25	62	M	8	511000
26	56	F	8	1241000
27	46	M	10	1213625
28	56	F	8.4	866875
29	58	F	4.8	447125
30	54	M	24	3085423
31	58	M	10	3374130

NK not known.

transcellular permeability of colonic biopsies were measured in Ussing chambers in both PD patients and control subjects using sulfonic acid and HRP, respectively. No difference in the sulfonic acid flux was observed between PD patients and control subjects ($n = 31$ and 11 , respectively; $p = 0.65$) (Figure 1A). HRP flux was also comparable between PD subjects and healthy controls ($n = 21$ and 9 , respectively; $p = 0.39$) (Figure 1B). Although not statistically different from controls, the sulfonic acid and HRP flux values were heterogeneous between PD patients (Figure 1A and B). We thus investigated if the main clinical features of the disease had any influence on IEB

permeability. We did not observe any correlation between age, disease duration or lifetime cumulative dose of L-DOPA and the values of sulfonic acid or HRP flux (Table 2).

There is a growing body of evidence supporting a key role for submucosal enteric neurons in the regulation of IEB functions [21-23]. This prompted us to study if the flux values of sulfonic acid and HRP were related to the presence of Lewy bodies and Lewy neurites in the submucosa. To this end, two biopsies per patient were immunohistochemically assessed for the presence of Lewy pathology using antibodies against phosphorylated alpha-synuclein and PGP9.5 (Figure 2A and B). A biopsy was deemed positive when containing at least one inclusion immunoreactive for both phosphorylated alpha-synuclein and PGP9.5 (Figure 2C). A patient was noted as positive when at least one of the two biopsies displayed inclusion(s). In accordance with our previous reports [24,25], the intraneuronal inclusions found in the submucosal plexus were chiefly observed in the neuronal processes and thus reminiscent of Lewy neurites (Figure 2A-C). Twenty-three out of 31 PD patients were positive for phosphorylated alpha-synuclein inclusions. All control subjects were devoid of inclusions. The values of sulfonic acid and HRP flux were not different between PD patients with or without inclusions (Figure 2D and E).

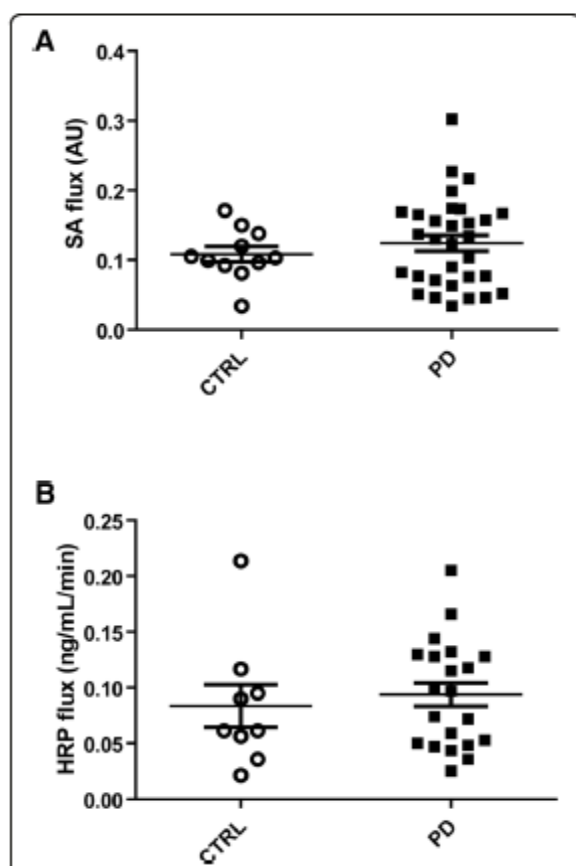


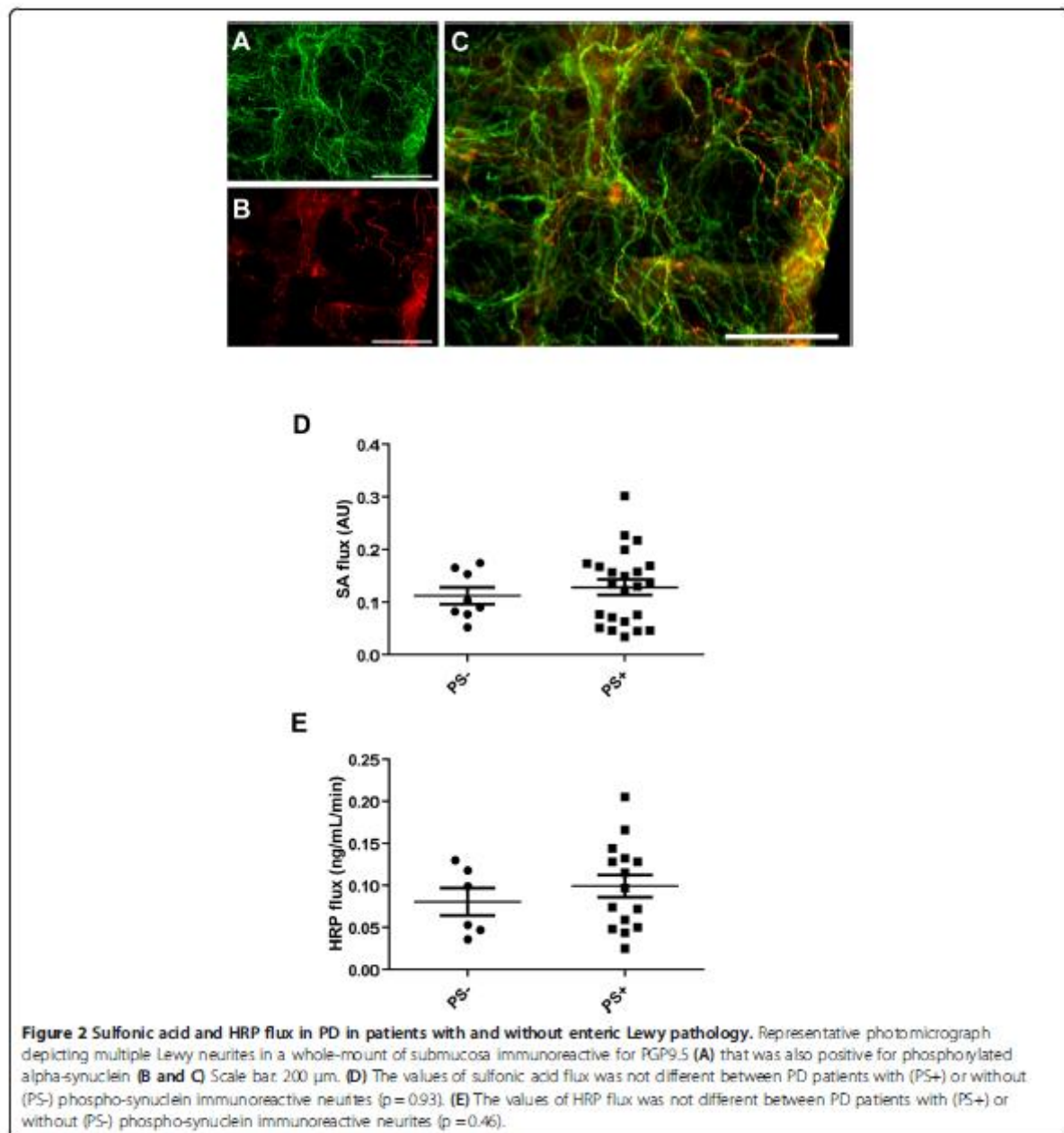
Figure 1 Comparison of para- and transcellular permeability in PD patients and healthy controls. **(A)** For the evaluation of paracellular permeability, the flux of sulfonic acid (SA flux) was measured in colonic biopsies mounted in Ussing chambers, expressed in arbitrary units (AU), in PD patients ($n = 31$) and controls (CTRL, $n = 11$). No significant changes were observed between the two groups ($p = 0.65$). **(B)** For the evaluation of paracellular permeability, the flux of horseradish peroxidase (HRP flux) was measured in colonic biopsies mounted in Ussing chambers, expressed in ng/mL/min, in PD patients ($n = 21$) and controls (CTRL, $n = 9$). No significant changes were observed between the two groups ($p = 0.39$).

Expression of the tight junction protein occludin is decreased in PD

We next investigated whether structural changes in the IEB occur in PD. The expression levels of the TJs proteins ZO-1 and occludin were analyzed by Western blot in colonic biopsies from PD subjects and healthy controls. A significant decrease in the expression of occludin was observed in colonic samples of PD patients as compared to controls (Figure 3A and B). A doublet band of approximately 220 kDa was observed on Western blot with antibodies against ZO-1 (Figure 3A). As previously shown, these two bands most likely represent the two ZO-1 isoforms [26,27]. By contrast to occludin, no change in ZO-1 expression levels was observed in PD whether the two bands were quantified together (Figure 3C) or separately (data not shown).

Table 2 Spearman's correlation with sulfonic acid (SA) and HRP permeability in PD patients

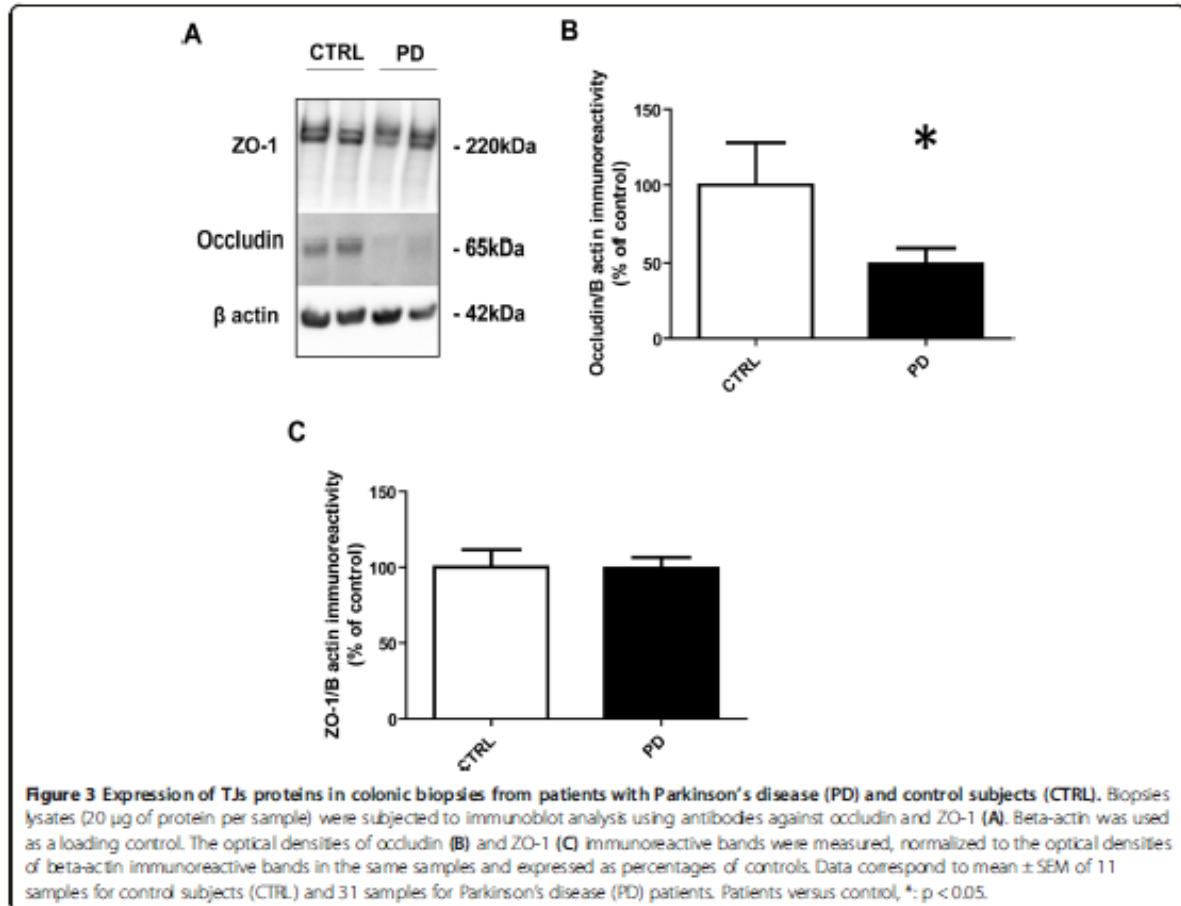
	Age	Disease duration	Cumulative lifetime dose of L-dopa
SA	$p = 0.090$ $r = 0.303$	$p = 0.788$ $r = 0.052$	$p = 0.830$ $r = -0.046$
HRP	$p = 0.165$ $r = 0.323$	$p = 0.469$ $r = 0.172$	$p = 0.754$ $r = 0.082$



Cellular distribution of the TJs proteins is altered in PD

The cellular distribution of occludin and ZO-1 was further investigated by immunofluorescence in 8 controls and 31 PD subjects. Samples from 3 controls were excluded because the mucosa was too small and/or too damaged to allow a reliable analysis of the TJs morphology. A mean of 96.4 crypts per biopsy were analyzed. We observed differences in the cellular distribution of both ZO-1 and occludin between PD patients and

controls (Figure 4). A normal and typical reticular pattern of occludin and ZO-1 staining was observed in the colonic samples of 6 out of 8 controls (Figure 4A and C, Additional file 1) and in only 9/31 PD patients (Figure 4B and D, Additional file 1). TJs morphology was disrupted and irregularly distributed in the mucosa of 1 out of 8 controls (Figure 4A and C, Additional file 1) and in 14/31 PD patients (Figure 4B and D, Additional file 1). An occasional and mild disruption of TJs morphology was

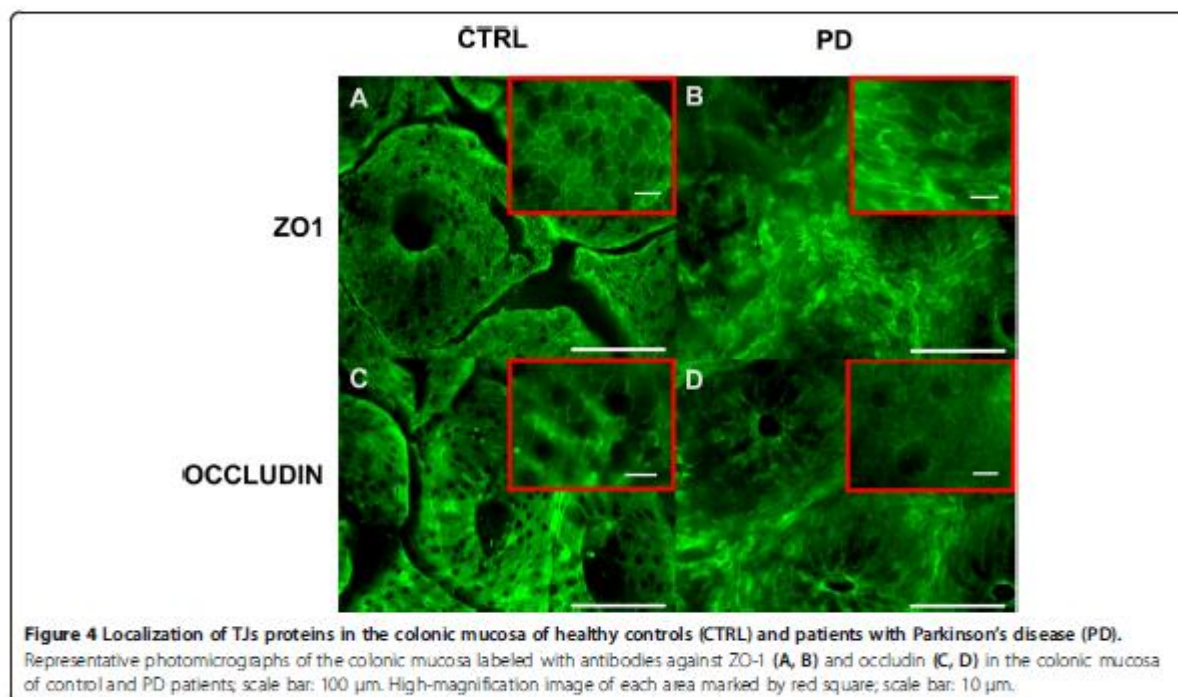


observed in the remaining control subject and in 8/31 PD samples (Figure 4 and Additional file 1). An increased staining of occludin in the cytoplasm of colonic enterocytes, suggestive of protein internalization, was observed in PD samples as opposed to the healthy group where occludin was mostly located in the TJs (Figure 4C and D). Worthy of note was the presence of moderate to severe TJs disorganization in the 5 patients who had never received levodopa, suggesting that the altered TJs morphology was not related to chronic levodopa intake (Additional file 2 and Table 1).

Discussion

The TJs are intercellular protein complex located at the apical portions of the lateral membranes of epithelial cells which play a key role in the regulation of IEB paracellular permeability. They are composed of transmembrane proteins, such as claudins and occludin and a wide spectrum of cytosolic proteins among which is ZO-1 [1]. By showing a decrease in occludin expression along with TJs disorganization, our study is the first to

provide evidence that the IEB is structurally altered in PD. Previous studies have shown intestinal tissue expression and distribution of occludin to be markedly decreased in patients with intestinal permeability disorders, including inflammatory bowel disease [28] and irritable bowel syndrome [6]. Recent data from genetic and epidemiological studies provided support for an association between diseases of the gastrointestinal tract and the susceptibility to developing PD. The CARD15 gene known to be associated with Crohn's disease is over-represented in patients with PD [29]; *vice versa*, the Leucine-rich repeat kinase 2 (LRRK2) gene, a causative PD mutation, was recently identified as a major susceptibility gene for Crohn's disease by genome-wide association studies [30]. Moreover, patients with irritable bowel syndrome are almost 50% more likely to develop PD than people who are free of this gastrointestinal disorder [31]. Our results show that the two disorders also share similarities at the molecular levels further supporting the assumption that irritable bowel syndrome may actually belong to early signs of gastrointestinal involvement in PD [32]. They also support



the hypothesis that the brain gut-axis might be critically involved in the pathophysiology of both disorders [7,33].

Studies of intestinal permeability in humans have mainly been carried out with *in vivo* techniques, usually with oral ingestion of various sugar probes and measurement of urinary excretion [12]. To date, the three studies that attempted to evaluate intestinal permeability in PD using this technique have provided only preliminary and conflicting results. Two of these studies focused on the lactulose/mannitol ratio, which evaluate small intestinal permeability. As a group, the 15 PD patients studied by Davies and collaborators had a significant increase in the lactulose/mannitol ratio when compared to age and sex matched controls, but individual results in both groups were highly overlapping [14]. Salat-Foix *et al.* showed that the lactulose/mannitol ratio was only marginally higher in 3 out of 12 PD patients [13]. In addition to lactulose/mannitol ratio, Forsyth *et al.* also used sucralose absorption for the assessment of colon permeability in 9 PD patients and 10 controls. They did not observe any difference in the lactulose/mannitol ratio between the two groups but found a significantly greater permeability to sucralose in PD subjects [15]. The inconsistent results on intestinal permeability in PD obtained with sugar probes prompted us to measure IEB permeability by another technique, namely Ussing chambers, in a larger sample size. Although less commonly used than sugar absorption, the Ussing chambers has proven to be a reliable and effective tool to measure IEB permeability of

gastrointestinal biopsies either paracellularly or transcellularly over a 3 hour period [34]. Using this approach, we showed that there were no significant differences in para- and transcellular permeabilities between PD subjects and controls. Nevertheless, the values of paracellular permeability as assessed by the sulfonic acid flux in Ussing chamber were highly heterogeneous between PD patients, some displaying a level comparable to controls while others had a more than a 2.5 fold increase in sulfonic acid flux. These data suggest that increased colonic permeability may be a feature for a subset of PD patients, as already reported when sugar probes were used [15]. The factors responsible for this heterogeneity still remain to be determined, as we did not observe any correlation between age, cumulative dose of L-Dopa disease duration and the severity of altered permeability.

On the surface, our results may seem contradictory, as the decreased expression of occludin observed in PD patients was not accompanied by changes in paracellular permeability. Occludin is a tetraspan protein with two extracellular loops, which homophilically interact with the adjacent cells [2]. Its role on IEB has been debated since its initial discovery in 1993 [35]. Initial studies strongly suggested that occludin was not required for the TJs formation or the maintenance of barrier function as occludin knockout mice lacked any noticeable defect in intestinal TJs morphology or barrier function [36]. This has been recently challenged in an elegant study

published by Al-Sadi *et al.* [37]. The purpose of their research was to better delineate the involvement of occludin in IEB by studying the transepithelial flux of various-sized probes after knocking down occludin both *in vitro* and *in vivo*. They showed that the occludin knock down caused a marked increase in the flux rates of macromolecules above 5 kDa such as inulin and dextran but had only modest effect on flux of smaller-sized probes under 200 Da such as mannitol and urea [36]. Fluorescein-5,6-sulfonic acid, which was used for the assessment of paracellular permeability in our study has a molecular weight of 400 Da, likely to be too small for detecting defects in IEB permeability induced by a mere down regulation of occludin. This may explain the lack of significant changes in IEB permeability observed in PD patients in our study in spite of the occurrence of structural changes.

The question arises as to what might be the clinical relevance of our experimental findings. A current theory, the so-called Braak's theory, assumes that PD originates in the gastrointestinal tract [11]. Braak and co-workers suggested that the appearance of Lewy pathology occurs in the earliest stage of PD in both the enteric nervous system and the dorsal motor nucleus of the vagus [11,38]. This led Braak to postulate that a pathogen may breach the IEB to trigger Lewy pathology in the terminal axons of the enteric neurons, further spreading to the central nervous system via the vagal preganglionic innervation of the gut [11,39]. In light of these considerations, our results demonstrating altered intestinal TJ's structure in PD gain in importance as the down regulation of occludin may favor the entry of a putative pathogen. This must be however balanced, as the stomach in contrast to the colon appears to be the most suitable target for the pathologic insult to occur in Braak's scenario. Several studies have indeed described that Lewy pathology is distributed following a rostro-caudal gradient in PD, with the lower esophagus and stomach having the greatest involvement and the colon and rectum the lowest [9,10], a distribution that parallels the vagal innervation of the gastrointestinal tract [40]. Further studies are therefore warranted to analyze the mucosal barrier permeability and morphology in gastric and duodenal samples from PD patients.

Conclusions

In conclusion, we provide evidence for the first time that morphological changes in the IEB occur in PD patients. Our results further reinforce the possible role of the gastrointestinal tract in the pathophysiology of PD. Further work is needed to determine if occludin down regulation in the gut might facilitate the spreading of PD pathology in the enteric nervous system and in the brain.

Additional files

Additional file 1: Percentage of morphologically normal crypts in the colonic mucosa of healthy controls (CTRL) and patients with Parkinson's disease (PD).

Additional file 2: Localization of TJ's proteins in the colonic mucosa of the 5 with Parkinson's disease (PD) who had never received levodopa. Scale bar: 100 μ m.

Competing interests

The authors declare that they have no competing interests.

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Author details

¹Inserm U913, 1 rue Gaston Veil, Nantes F-44035, France. ²University Nantes, Nantes F-44093, France. ³CHU Nantes, Institut des Maladies de l'Appareil Digestif, Nantes F-44093, France. ⁴Inserm, CIC-04, Nantes F-44093, France. ⁵CHU Nantes, Service d'Anatomie Pathologique, Nantes F-44093, France. ⁶Inserm, UMR957, Nantes F-44093, France. ⁷CHU Nantes, Department of Neurology, Nantes F-44093, France.

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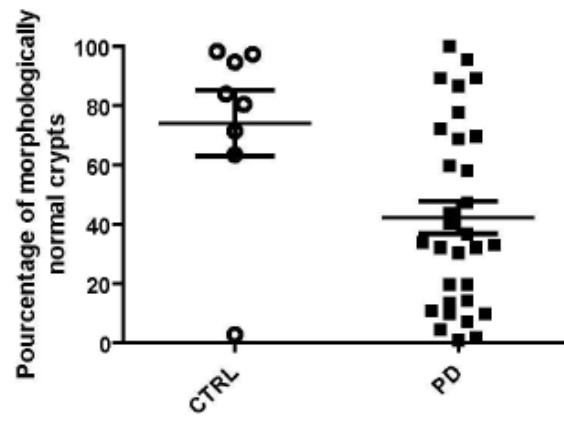
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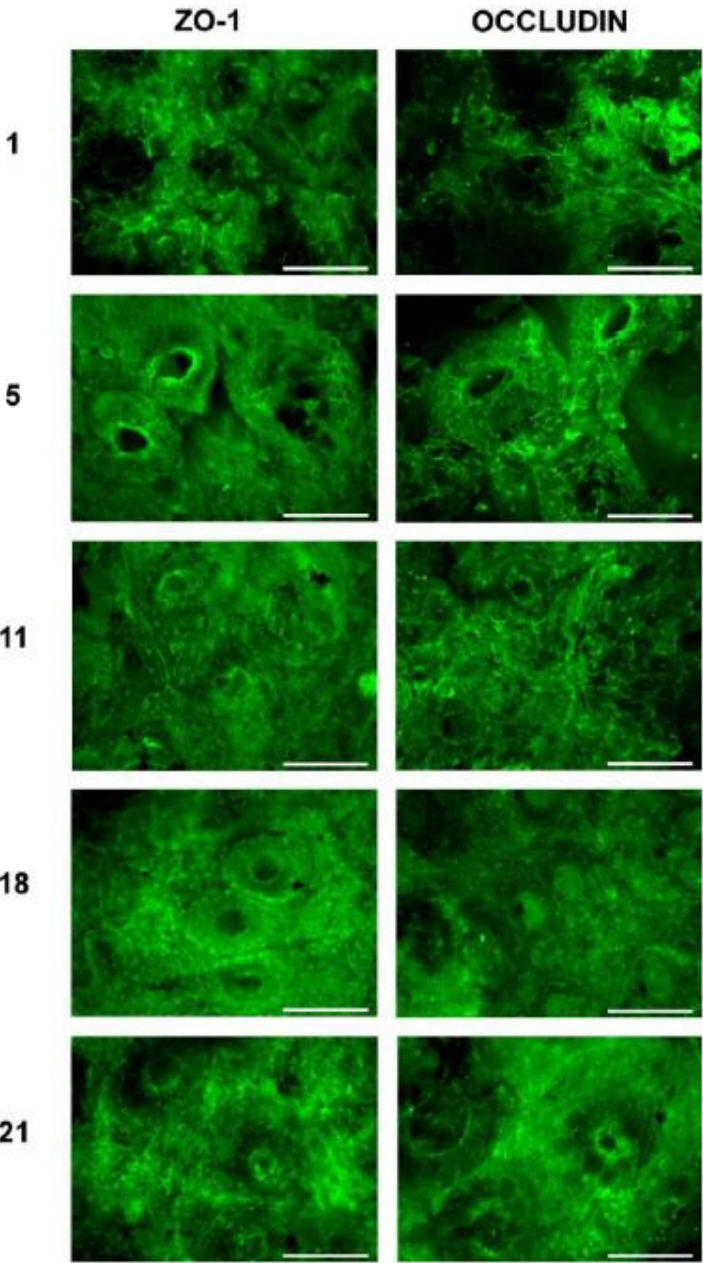
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Supplementary figure 1



Supplementary figure 2



II- Article 2 : Le trouble du comportement en sommeil paradoxal est associé à l'atteinte histologique du système nerveux entérique dans la maladie de Parkinson

Leclair-Visonneau L*, Clairembault T*, Coron E, Le Dily S, Vavasseur F, Dalichampt M, Péréon Y, Neunlist M, Derkinderen P. REM sleep behavior disorder is related to enteric neuropathology in Parkinson's disease. Neurology. In press.

*co-auteurs

Résumé

L'objectif de l'étude était de déterminer si le TCSP dans la MP est associé avec des lésions et dysfonctions du SNA, et en particulier du SNE, en évaluant les lésions histopathologiques d'alpha-synucléine phosphorylée et la perméabilité entérique.

Quarante-cinq patients ont été inclus dans cette étude transversale. Le TCSP était diagnostiqué par un entretien clinique standardisé et confirmé par vidéo-polysomnographie. Chez chacun des 45 patients, cinq biopsies ont été prélevées à la jonction entre le côlon sigmoïde et descendant au cours d'une rectosigmoïdoscopie. Afin de détecter les lésions d'alpha-synucléine phosphorylée, deux biopsies coliques étaient analysées en immunohistochimie avec des anticorps dirigés contre d'alpha-synucléine phosphorylée et la PGP 9.5 chez 43 patients (2 patients ont été exclus en raison d'un nombre insuffisant de biopsie disponible). La perméabilité para et transcellulaire était évaluée en mesurant, respectivement, les flux d'acide sulfonique et de peroxydase de raifort sur les trois biopsies restantes, montées dans des chambres d'Ussing.

La synucléinopathie entérique était plus fréquente chez les patients parkinsoniens avec TCSP en comparaison avec les patients parkinsoniens sans TCSP (18/28, 64,3% vs 2/15, 13,3%, respectivement, $p < 0.01$). Aucune différence de perméabilité n'était observée entre les patients parkinsoniens avec et sans TCSP.

En conclusion, les lésions d'alpha-synucléine phosphorylée dans le SNE sont plus fréquentes chez les patients parkinsoniens avec TCSP que sans TCSP, suggérant que le TCSP au cours de la MP est associé à une neuropathologie de l'alpha-synucléine plus diffuse.

REM sleep behavior disorder is related to enteric neuropathology in Parkinson disease

Laurène Leclair-Visonneau, MD*
Thomas Clairembault, PhD*
Emmanuel Coron, MD, PhD
Séverine Le Dily, MS
Fabienne Vavasseur, PhD
Marie Dalichamp, MSc
Yann Péréon, MD, PhD
Michel Neunlist, PhD
Pascal Derkinderen, MD, PhD

Correspondence to
Dr. Leclaire-Visonneau:
laurene.leclair@chu-nantes.fr

ABSTRACT

Objective: To determine whether REM sleep behavior disorder (RBD) in Parkinson disease (PD) is associated with lesions and dysfunctions of the autonomic nervous system by evaluating enteric phosphorylated α -synuclein histopathology (PASH) and permeability.

Methods: A total of 45 patients with PD were included in this cross-sectional study. RBD was diagnosed on the basis of a standardized clinical interview and confirmed by polysomnography. For each patient, 5 biopsies were taken at the junction between the sigmoid and descending colon during the course of a rectosigmoidoscopy. For the detection of enteric PASH, 2 colonic biopsies were analyzed by immunohistochemistry with antibodies against phosphorylated α -synuclein and PGP9.5 in 43 patients (2 patients were excluded because only 1 biopsy was available). The paracellular permeability and transcellular permeability were evaluated by measuring sulfonic acid and horseradish peroxidase flux, respectively, in the 3 remaining biopsies mounted in Ussing chambers.

Results: Enteric PASH was more frequent in the subgroup of patients with PD with RBD compared to patients without RBD (18 of 28, 64.3%, vs 2 of 15, 13.3%, respectively, $p < 0.01$). No differences were observed in intestinal permeability between patients with PD with and without RBD.

Conclusions: Patients with PD and RBD have a greater frequency of synuclein pathology in the enteric nervous system, suggesting that RBD is associated with widespread synuclein neuropathology. *Neurology*® 2017;89:1612–1618

GLOSSARY

FFPE = formalin-fixed, paraffin-embedded; PASH = phosphorylated α -synuclein histopathology; PD = Parkinson disease; RBD = REM sleep behavior disorder; UPDRS-III = Unified Parkinson's Disease Rating Scale part III.

Since its description in 1986, REM sleep behavior disorder (RBD) has generated considerable interest in the field of neurodegenerative disorders, especially Parkinson disease (PD).¹ RBD is reported in up to 50% to 60% of patients with PD,² and a number of studies have analyzed the association between RBD and disease phenotype. This suggests that patients with PD and RBD are more likely to have a nontremor phenotype, more gait dysfunction, increased cognitive impairment, and more autonomic dysfunction.^{3,4} It has been proposed that such an aggressive clinical course was explained by a more widespread distribution of PD pathology, i.e., α -synuclein inclusions, throughout the CNS. This hypothesis has been supported by a recent autopsy study that showed an increased density of α -synuclein inclusions in several brainstem and cortical structures in patients with PD and RBD compared to patients without RBD.⁵

α -Synuclein inclusions are not limited to the CNS but are also found in the peripheral autonomic neuronal circuits.⁶ Some of these peripheral tissues, among which is the enteric nervous system, by being assessable through routine biopsies, could represent a window to assess PD neuropathology in living patients.⁷ We have indeed shown that whole mounts of

Supplemental data
at Neurology.org

*These authors contributed equally to this work.

From Inserm (L.L.-V., T.C., E.C., M.N., P.D.), U1235, Nantes; University Nantes (L.L.-V., T.C., E.C., Y.P., M.N., P.D.); Inserm (L.L.-V., E.C., S.L.D., F.V., P.D.), CIC-04; CHU Nantes (L.L.-V., Y.P.), Department of Clinical Neurophysiology; CHU Nantes (T.C., E.C., F.V., M.N.), Institut des Maladies de l'Appareil Digestif; CHU Nantes (M.D.), Plateforme de Biométrie, Département Promotion DRG; and CHU Nantes (P.D.), Department of Neurology, France.

Go to Neurology.org for full disclosures. Funding information and disclosures deemed relevant by the authors, if any, are provided at the end of the article.

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submucosa from routine colonic biopsies allow a morphologic and quantitative analysis of enteric phosphorylated α -synuclein histopathology (PASH) in PD.⁷⁻⁹ In addition, gastrointestinal biopsies can be used to evaluate the functional consequences of enteric nervous system dysfunction on intestinal permeability.^{9,10} In the current study, we therefore have used colonic biopsies to analyze whether patients with PD and RBD have differences in enteric neuropathology and permeability compared to patients without RBD.

METHODS **Participants.** In this cross-sectional study, a total of 45 patients 45 to 80 years of age with idiopathic PD were prospectively and consecutively recruited from the movement disorder clinic at Nantes University Hospital, France, from February 2013 to February 2016. To limit recruitment bias and to span the entire course of PD, the 45 patients were recruited regarding their disease duration (duration 1-5 years, $n = 15$ patients; 5-10 years, $n = 16$; >10 years, $n = 14$). PD was diagnosed according to criteria provided by the UK Parkinson's Disease Survey Brain Bank. Patients were excluded if they had irritable bowel syndrome, anorectal dysfunction (fecal incontinence or chronic proctalgia),¹¹ or dementia (Mini-Mental State Examination score ≤ 23).

Standard protocol approvals, registrations, and patient consents. The study protocol was approved by the local Committee on Ethics and Human Research (Comité de Protection des Personnes Ouest IV, registration IdRCB 2012-A00917-36) and registered on ClinicalTrials.gov (identifier NCT01748409). Written informed consent was obtained from each patient after a full explanation of the purpose and a detailed description of the study procedures according to the principles of Helsinki.

Patients' evaluation. Collected demographic data included sex, age at onset, disease duration, and L-dopa equivalent daily dose. Complete drug history was obtained, and the lifetime cumulative dose of L-dopa was approximated.¹² Motor symptoms were evaluated with the Unified Parkinson's Disease Rating Scale part III (UPDRS-III) and its axial subscore (sum of items 18 [speech], 19 [facial expression], 22 [neck rigidity], 27 [arising from a chair], 28 [posture], 29 [gait], and 30 [postural stability]). Constipation was diagnosed as defined by Rome III Diagnostic Criteria for Functional Constipation; stool frequency and the Bristol stool form scale were collected.¹³ Orthostatic hypotension was defined as a decrease in systolic blood pressure ≥ 20 mm Hg and/or in diastolic blood pressure ≥ 10 mm Hg from the supine to upright position at 1, 3, 5, and 10 minutes. Structured interview and overnight polysomnography were carried out in all 45 patients and interpreted according to the recommendations of American Academy of Sleep Medicine.¹⁴ Sleep clinical measures included Parkinson Disease Sleep Scale, Pittsburgh Sleep Quality Index, Epworth Sleepiness Scale, and Fatigue Severity Scale. RBD was diagnosed according to the International Classification of Sleep Disorders-II diagnostic criteria.¹⁵ When patients had absent or infrequent ($\leq 1\%$) REM sleep in polysomnographic recording, diagnosis of probable RBD was considered according to a clinical interview performed by a trained sleep specialist (L.L.-V.).

Endoscopic procedure and colonic biopsies. For each participant, 5 biopsies were taken at the junction between sigmoid and descending colon during the course of a rectosigmoidoscopy

(short colonoscopy). In contrast to a full colonoscopy, this painless and safe procedure can be performed in 5 to 10 minutes without the need for sedation or bowel preparation.¹⁶ All biopsies were immersed in 4°C Hank balanced salt solution (Life Technologies, Saint-Aubin, France); 3 of them were immediately processed for the assessment of paracellular and transcellular permeability in Ussing chambers, while the 2 other biopsies were used for immunohistochemistry experiments (see below).

Microdissection and immunohistochemistry. Submucosa samples were processed for whole-mount immunostaining as previously described.^{7,9} The primary antibodies used were those directed against phosphorylated α -synuclein (1:5,000, WAKO, Osaka, Japan) and PGP9.5 (1:10,000; Ultraclone Limited, Cambridge, UK). Whole specimens of submucosa were viewed under an Axio Zoom V16 stereomicroscope (Zeiss, Marly Le Roi, France). All samples were deidentified and analyzed by 2 experienced raters (T.C. and P.D.) who were blinded to the patients' RBD status.

Paracellular and transcellular permeability of colonic biopsies in Ussing chambers. Three biopsies were mounted in Ussing chambers (World Precision Instruments, Hertfordshire, UK) exposing a surface of 0.011 cm² and analyzed for paracellular and transcellular permeability as previously described with sulfonic acid and horseradish peroxidase, respectively.⁹

Statistics. Continuous variables are expressed as the mean \pm SE or median and interquartile range, and categorical data were expressed as numbers and percentage. Continuous variables were compared between groups (patients with and without RBD) with the 2-sample Student test or the nonparametric Wilcoxon test (when normality assumption could not be assumed). The χ^2 test or Fisher tests were used for categorical variables (SAS software, version 9.4 SAS Institute Inc, Cary, NC). A logistic model adjusted for age was used to compare clinical measures between groups. Correlation coefficients between paracellular and transcellular permeability and continuous variables (disease duration, L-dopa cumulative dose, and age) were calculated with the Spearman test (GraphPad Prism 7 software, La Jolla, CA). For all statistical tests, a value of $p < 0.05$ was deemed significant.

RESULTS Clinical features and sleep characteristics of the study population are shown in table 1 and table e-1 at [Neurology.org](#), respectively. There were 30 patients with PD and RBD (23 with polysomnography-confirmed RBD and 7 with probable RBD) and 15 without RBD (1 patient showed infrequent REM sleep on polysomnography and negative structured interview for RBD). In the RBD group, RBD preceded the onset of parkinsonism or was simultaneous (early RBD) in 9 patients (30%). Patients with RBD were older and had higher UPDRS-III scores than patients without RBD (table 1). After adjustment for age, total UPDRS-III score, but not the axial subscore, remained different between the 2 groups ($p = 0.04$ and $p = 0.08$, respectively). Disease duration, L-dopa lifetime cumulative dose, L-dopa equivalent daily dose, Mini-Mental State Examination score, orthostatic hypotension, and constipation did not differ between the 2 groups (table 1). Clinical and polysomnographic sleep

Table 1 Main clinical characteristics and colonic PASH of patients with PD with and without RBD

	PD + RBD	PD - RBD	p Value
No. (%)	30 (67)	15 (33)	
Male, n (%)	22 (73.3)	8 (53.3)	0.18
Age, y	62.1 (6.2)	57.1 (9.0)	0.03*
Disease duration, y	9.7 (6.6)	6.6 (6.0)	0.14
L-Dopa lifetime cumulative dose, kg	1.3 (1.2)	0.9 (1.4)	0.12
L-Dopa equivalent daily dose, g	0.8 (0.5)	0.8 (0.6)	0.54
UPDRS-III "on"			
Total (0-108)	24.7 (12.1)	15.8 (9.2)	0.02*
			0.04 ^{ab}
Axial (0-32)	7.9 (4.4)	5.1 (3.2)	0.04*
			0.08 ^b
MMSE (0-30), n (%)	28.5 (1.6)	27.6 (2.1)	0.15
Constipation Rome III criteria, n (%)	20 (66.7)	7 (46.7)	0.20
Stools per week, n	6.9 (5.7)	8.7 (7.4)	0.60
Predominant Bristol stool forms 1 and 2, n (%)	16 (53.3)	4 (26.7)	0.12
Colonic PASH, n (%)	18/28 (64.3)	2/15 (13.3)	<0.01*
Orthostatic hypotension, n (%)	23 (76.7)	12 (80.0)	1.0

Abbreviations: MMSE = Mini-Mental State Examination; PASH = phosphorylated α -synuclein histopathology; PD = Parkinson disease; RBD = REM sleep behavior disorder; UPDRS-III = Unified Parkinson's Disease Rating Scale part III.

* Significant.

^b After age adjustment.

measures were similar in patients with and without RBD (table e-1).

Two colonic biopsies per patient were immunohistochemically assessed for the presence of enteric PASH. Of the 45 patients with PD, 2 were excluded because only 1 biopsy was available for immunohistochemistry. A biopsy was considered positive when containing at least 1 structure immunoreactive for both phosphorylated α -synuclein and PCP9.5 (figure 1). A patient was noted as positive (PASH+) when at least 1 of the 2 biopsies contained PASH (figure 1). In accordance with our earlier findings,^{7,8} PASH was observed mainly in the neuronal processes and thus was morphologically reminiscent of Lewy neurites (figure 1). Among the 43 patients with PD who were analyzed, 20 (46.5%) were PASH+. PASH+ patients were more frequent in the subgroup with RBD compared to patients without RBD (18 of 28, 64.3%, vs 2 of 15, 13.3%, respectively, $p < 0.01$) (table 1). This difference was still observed when the analysis was limited to patients with polysomnography-confirmed RBD status (14 of 21, 66.7%, for patients with RBD vs 2 of 14, 14.3%, for patients without RBD, $p < 0.01$). In the RBD group, clinical characteristics were not different between PASH+ and PASH- patients (table e-2). The proportion of PASH+ patients did not differ according to RBD onset (6 of 8, 75%, for

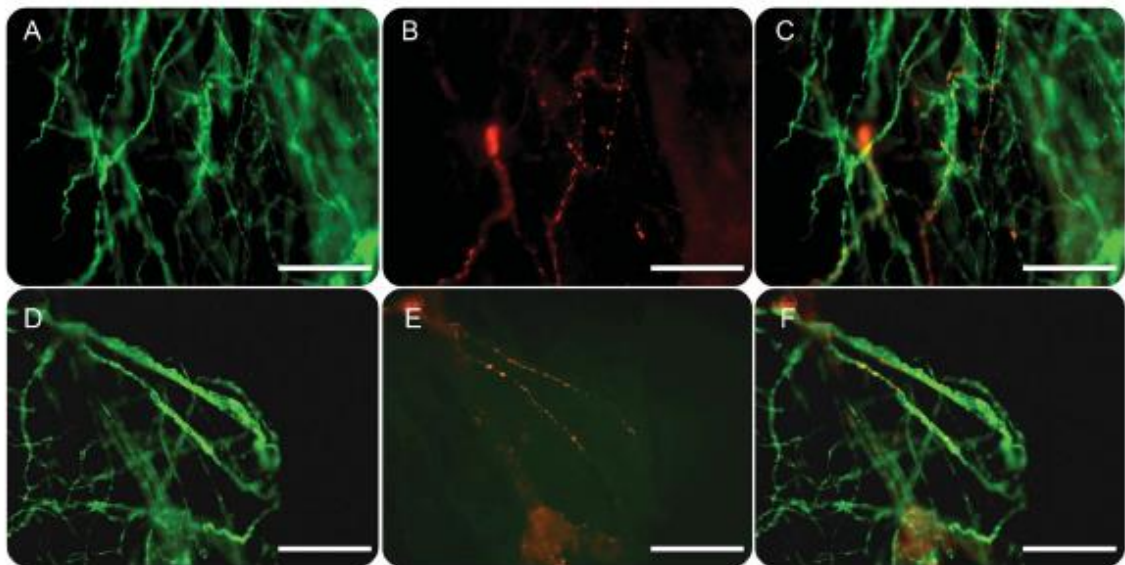
patients with early RBD vs 12 of 20, 60%, for patients with RBD after parkinsonism, $p = 0.67$).

The paracellular permeability and transcellular permeability of colonic biopsies were measured in using chambers with sulfonic acid and horseradish peroxidase, respectively. There were no correlations between paracellular or transcellular permeability and disease duration ($p = 0.69$ and $p = 0.63$, respectively) or L-dopa cumulative dose ($p = 0.89$ and $p = 0.41$, respectively). Paracellular permeability but not transcellular permeability was correlated with age ($r = 0.35$, $p = 0.02$ and $p = 0.95$, respectively). No differences were observed for paracellular or transcellular permeability between patients with and without RBD ($n = 28$ for patients with RBD and 15 for patients without RBD, $p = 0.45$, and $n = 22$ for patients with RBD and 9 for patients without RBD, $p = 0.06$, respectively) (figure 2).

DISCUSSION In this prospective study, we found that RBD among patients with PD was closely associated with a greater frequency of enteric PASH. In contrast, we did not observe any differences in intestinal permeability, either paracellular or transcellular, between patients with PD with and those without RBD. Sixty-seven percent of our patients with PD had RBD, which is toward the upper end of the range reported in previous series.⁷ Consistent with earlier findings, patients with RBD were significantly older^{3,17} and had higher UPDRS-III scores than patients without RBD.¹⁸ Regarding dysautonomic manifestations, we did not find evidence of an association between RBD and orthostatic hypotension or constipation. Even if these findings are in concordance with some of the previously published series (no difference in orthostatic hypotension in the report by Yonitaka et al.¹⁷; no difference in constipation in the report by Postuma et al.¹⁹), most of the existing studies reported more orthostatic hypotension^{3,13,20} and constipation^{17,21} in PD with RBD.

An increasing number of studies have addressed the feasibility of PASH detection in peripheral tissues suitable to biopsy taking. The skin, salivary glands, and gastrointestinal tract have been regarded as the most promising tissue targets because they are affected by PASH early and specifically in a large proportion of patients.²² This has logically led several groups to investigate whether PASH could be detected in peripheral tissues biopsies from patients with idiopathic RBD to identify a subgroup of patients at high short-term risk of developing PD. A Catalan consortium performed transcutaneous core needle biopsy of the submandibular gland in 21 patients with idiopathic RBD but obtained material containing glandular parenchyma suitable for histologic analysis in only 9 participants.²³ PASH was observed in

Figure 1 PASH in the submucosa of patients with PD



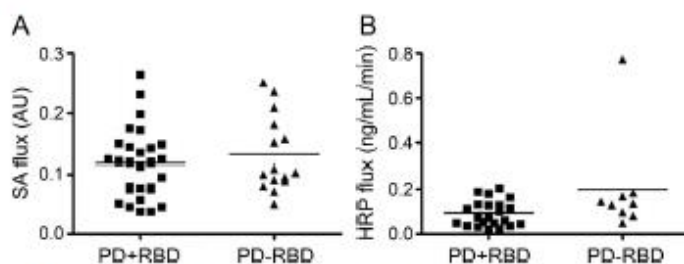
Colonic biopsies of 2 different patients with PD and RBD (top and bottom) were microdissected and analyzed by immunohistochemistry. Representative photomicrographs show multiple PASH in whole mounts of submucosa immunoreactive for PGP9.5 (A, D) that were also positive for phosphorylated α -synuclein (B, E; merged in C, F). Scale bar: 100 μ m. PASH = phosphorylated α -synuclein histopathology; PD = Parkinson disease; RBD = REM sleep behavior disorder.

nerve fibers in 8 of these 9 patients. When skin and colonic biopsies were analyzed, PASH was visualized in 10 of 18 and in 4 of 17 patients with idiopathic RBD, respectively.^{24,25} In addition to idiopathic RBD, all of these studies enrolled patients with symptomatic PD but without specifying whether these patients had concomitant RBD. Thus, our study attempted to fill this void by analyzing the enteric

neuropathology in a cohort of patients with PD with or without RBD.

In recent years, there has been considerable debate about the immunohistochemical method that should be used for the detection of pathologic α -synuclein in gastrointestinal biopsies. Most existing studies have been performed on formalin-fixed, paraffin-embedded (FFPE) tissue, which has the main advantages of being readily accessible to most hospital-based laboratories and allowing retrospective analyses. A number of single-center studies have had conflicting results regarding the sensitivity and specificity of FFPE colonic biopsies for the detection of PASH.²⁶⁻³⁰ This was confirmed by a recent multicenter blinded study that showed that adequate diagnostic accuracy of PD was not achieved by any of the 4 staining methods evaluated.³¹ This overall lack of specificity of FFPE biopsies prompted us to use another approach for the detection of enteric PASH in the current study. Combining routine colonic biopsies and microdissection techniques, we have previously shown that collecting whole mounts of submucosa enables a comprehensive assessment of the submucosal neural network, along with the detection of PASH in patients with PD.⁷⁻⁹ With this method and in accordance with our previous results,⁸ we showed in the current survey that 20 of 43 patients with PD had enteric PASH when 2 descending/sigmoid colon

Figure 2 Comparison of paracellular and transcellular permeability in patients with PD with or without RBD



(A) For the evaluation of paracellular permeability, the flux of sulfonic acid (SA) was measured in colonic biopsies mounted in Ussing chambers, expressed in arbitrary units (AU), in patients with PD with (n = 28) and without (n = 15) RBD. No significant changes were observed between the 2 groups (p = 0.45). (B) For the evaluation of paracellular permeability, the flux of horseradish peroxidase (HRP) was measured in colonic biopsies mounted in Ussing chambers, expressed in nanograms per milliliter per minute, in patients with PD with (n = 22) and without (n = 9) RBD. No significant changes were observed between the 2 groups (p = 0.06). PD = Parkinson disease; RBD = REM sleep behavior disorder.

biopsies were analyzed. When patients were divided into 2 groups, with or without RBD, PASH was more frequent in the subgroup with RBD compared to patients without RBD.

Numerous recent studies have suggested that the presence of RBD in PD is associated with a more aggressive course of the disease. Patients with PD with RBD are indeed older; sleepier; more likely to have cognitive deficits, hallucinations, and dysautonomia; and less likely to have a tremor-predominant motor phenotype than patients with PD without RBD.^{3,20,21,32} One possible explanation proposed for this finding is that α -synuclein pathology is different and more diffuse in RBD-associated PD than in disease without RBD, not only in the CNS but also in peripheral autonomic networks. Regarding the CNS, this hypothesis is supported by the only existing autopsy study that compared the distribution and density of PASH between 40 patients with PD with probable RBD and 41 patients without RBD.⁵ This study showed that the RBD group had a greater density in PASH in cortical (basal transentorhinal and basal cingulate) and brainstem (dorsal motor nucleus of the vagus and locus ceruleus) structures.⁵ Our study, by demonstrating a close relationship between enteric PASH and RBD, provides additional evidence that patients with PD and RBD have a more diffuse α -synuclein neuropathology.

Accumulating evidence indicates that cholinergic dysfunction is critically involved in RBD. The cholinergic neurons of the pedunculopontine/laterodorsal tegmental nucleus are active during REM sleep,³³ and limited pontine lesions encompassing this nucleus are sufficient to cause RBD.³⁴ Furthermore, these neurons are prone to degenerate and are particularly vulnerable to α -synuclein pathology in PD.³⁵ Cholinergic signaling is also critically involved in gastrointestinal physiology. Cholinergic neurons, the main physiologic role of which is to increase gastrointestinal peristalsis, are by far the most abundant neurons in the human adult enteric nervous system.³⁶ With regard to PD, a recent anatomic report showed that α -synuclein expression in the human colon is restricted to cholinergic neurons, suggesting that these neurons may be vulnerable to PD pathology.³⁷ As a whole, these observations suggest that cholinergic neurons, from either the CNS or peripheral nervous system, may be particularly susceptible to α -synuclein pathology, thereby providing a possible explanation for the greater frequency of enteric synuclein pathology in patients with PD with RBD.

Most of the existing studies on intestinal permeability in PD, which have been performed with oral ingestion of sugar probes, have provided conflicting results.^{38–40} This explains why we have chosen another approach, namely *ex vivo* measurement with

Ussing chambers, to evaluate intestinal permeability. Although less commonly used than sugar absorption, the Ussing chamber has proved to be a reliable and effective tool to measure intestinal permeability of gastrointestinal biopsies either paracellularly or transcellularly over a 3-hour period.¹⁰ Using this approach, we previously showed that there were no differences in paracellular and transcellular permeability between a sample of patients with PD and healthy controls.⁹ We show in the current study that, in contrast to enteric neuropathology, intestinal permeability is not linked to RBD.

There are some limitations of this study. The first is the absence of polysomnographic confirmation of RBD status in a subset of cases. Eight patients had absent or infrequent REM sleep, possibly due to a first-night effect or related to PD insomnia. This issue can be overcome in future studies by recording polysomnography on 2 consecutive nights. The second limitation is the relatively small sample size. That said, our study has also several strengths. First, colonic biopsies were analyzed with a whole-mount method that has been optimized for the detection of PASH.⁷ Second, PASH raters were blinded to the RBD status of the patients. Finally, our study is the first to compare intestinal permeability between patients with PD with and without RBD.

Our results, together with previous findings, provide evidence that the presence of RBD in PD predicts the development of widespread neuropathologic changes not only in the brain but also in the peripheral autonomic nervous system. They also suggest that patients with PD without RBD and with no identifiable PASH in colonic biopsies may have a more benign disease course. Future studies focusing on the presence of PASH in other peripheral nervous tissues accessible to biopsies such as skin and salivary glands²² are needed to fully demonstrate that RBD in PD is associated with a diffuse pathologic involvement of peripheral autonomic neuronal circuits.

AUTHOR CONTRIBUTIONS

Laurène Leclaire-Vionneux: study concept and design, acquisition and interpretation of data, statistical analysis. Thomas Clément: study concept and design, acquisition and interpretation of data. Emmanuel Corot: acquisition of data, critical revision of manuscript for intellectual content. Séverine Le Dily and Fabienne Vassesseur: acquisition of data, study supervision. Marie Dalchamps: interpretation of data, statistical analysis, study supervision. Yann Péron and Michel Neurilic: study concept and design, critical revision of manuscript for intellectual content. Pascal Derkinderen: study concept and design, study supervision, interpretation of data.

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DISCLOSURE

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Supplemental Material

	PD+RBD	PD-RBD	p-value
no., %	30 (67%)	15 (33%)	
Clinical measures			
PDSS (0-150)	91.1 (20.0)	95.3 (16.8)	0.49
PSQI (0-21)	8.2 (3.6)	9.9 (5.1)	0.32
ESS (0-24)	9.8 (5.2)	11.7 (4.7)	0.21
FSS (0-63)	36.5 (13.4)	30.1 (16.5)	0.17
Polysomnographic measures			
Total sleep time (min)	344.9 (103.6)	328.1 (123.5)	0.63
Sleep efficiency (%)	71.4 (20.4)	74.4 (20.2)	0.64
Sleep latency (min)	17.2 (28.6)	19.1 (37.8)	0.85
N1 stage (%)	16.4 (13.2)	9.7 (8.9)	0.08
N2 stage (%)	50.8 (11.4)	50.5 (8.4)	0.93
N3 stage (%)	21.6 (10.3)	26.9 (7.6)	0.09
REM stage (%)	11.1 (9.0)	12.7 (8.7)	0.56
Arousal index	14.9 (6.9)	13.5 (6.5)	0.50

Table e-1: Clinical and polysomnographic sleep measures of PD patients with (PD+RBD) and without RBD (PD-RBD)

	PD+RBD	PD+RBD	p-value
	PASH+	PASH-	
no., %	18	10	
Sex, % male	15 (83%)	5 (50%)	0.09
Age, y	62.6 (6.1)	61.8 (7.2)	0.56
Disease duration, y	10.2 (7.3)	9.4 (6.6)	0.90
L-dopa lifetime cumulative dose, kg	1.5 (1.15)	0.9 (1.1)	0.17
L-dopa equivalent daily dose, g	1.0 (0.5)	0.6 (0.5)	0.06
UPDRS part III 'on'			
total (0-108)	26.2 (12.4)	22.9 (12.5)	0.56
axial (0-32)	8.8 (4.7)	6.7 (3.9)	0.30
MMSE (0-30)	27.3 (2.5)	28.0 (2.0)	0.42
Constipation Rome III criteria (no., %)	10 (56%)	8 (80%)	0.25
Stools per week	5.3 (3.0)	9.4 (8.5)	0.26
Predominant Bristol stool form 1 and 2 (no., %)	8 (44%)	7 (78%)	0.25
Paracellular permeability (SA flux, AU)	0.12 (0.07)	0.11 (0.04)	0.79
Transcellular permeability (HRP flux, ng/mL/min)	0.09 (0.06)	0.08 (0.05)	0.72

Table e-2: Clinical characteristics and permeability measures in patients with RBD (PD+RBD) with (PASH+) and without (PASH-) colonic phosphorylated alpha-synuclein histopathology.

III-Article 3 : La distribution de la dysfonction autonome est hétérogène dans la maladie de Parkinson

Leclair-Visonneau L, Magy L, Volteau C, Clairembault T, Le Dily S, Preterre C, Peyre A, Damier P, Neunlist M, Péréon Y, Derkinderen P. Heterogeneous pattern of autonomic dysfunction in Parkinson's disease. Soumis

Résumé :

La plainte dysautonomique est un des symptômes non moteurs les plus fréquents au cours de la MP. De nombreuses études neuropathologiques ont montré une large distribution des corps et neurites de Lewy, les marqueurs histopathologiques de la MP, du SNA périphérique jusqu'aux organes cibles. Néanmoins, peu d'études ont exploré de manière globale les symptômes et la physiologie de la dysautonomie dans la MP.

Dans une étude transversale prospective, nous avons réalisé une évaluation complète du SNA dans un groupe de 45 patients parkinsoniens. Les modalités du SNA (fonctions pupillaire, lacrymale, salivaire, cardiovasculaire, digestive, urinaire, sexuelle, sudorale et la sensibilité cutanées) étaient évaluées à l'aide de questionnaires et des tests fonctionnels. Une quantification de la densité des fibres nerveuses intra-épidermiques (DFNIE) était effectuée sur une biopsie cutanée. Par ailleurs, un enregistrement vidéopolysomnographique et une évaluation neurologique et neuropsychologique était réalisée chez chacun des 45 patients.

L'analyse des différentes modalités du SNA montrait que les symptômes et signes dysautonomiques étaient distribués de manière hétérogène dans la population de patients parkinsoniens. La dénervation cutanée (évaluée par la DFNIE) était corrélée avec le seuil de sensibilité thermique (OR=12,0, p=0,02) et uniquement associée avec la constipation (OR=5,5, p=0,01) et une plainte de sécheresse oculaire (OR=8,29, p=0,04). L'altération cognitive était associée avec des symptômes cardiovasculaires (OR=4.33, p=0.03), une hypotension orthostatique (OR=5.83, p=0.02) et une constipation (OR=5.38, p=0.02).

Néanmoins, l'atteinte motrice axiale et le TCSP n'étaient liés à aucun symptôme ou dysfonction autonome.

Nos résultats montrent que les différentes modalités du SNA sont atteintes de manière hétérogène, suggérant ainsi que la dysfonction autonome ne suivrait pas une progression par paliers mais une évolution erratique et éparse.

Heterogeneous pattern of autonomic dysfunction in Parkinson's disease

Laurène Leclair-Visonneau, MD^{1,2,3,4}, Laurent Magy, MD, PhD⁵, Christelle Volteau, MS⁶, Thomas Clairembault, PhD^{1,2,7}, Séverine Le Dily, MS³, Cécile Preterre, MD^{1,2,3,8}, Arnaud Peyre, MD^{1,2,3,4}, Philippe Damier, MD, PhD^{2,3,8}, Michel Neunlist, PhD^{1,2,7}, Yann Péréon, MD, PhD^{2,4}, and Pascal Derkinderen, MD, PhD^{1,2,3,8}

¹Inserm, U1235, Nantes, F-44035, France

²University Nantes, Nantes, F-44093, France

³Inserm, CIC-04, Nantes, F-44093, France

⁴CHU Nantes, Department of Clinical Neurophysiology, Nantes, F-44093, France

⁵CHU Limoges, Department of Neurology, Centre de Référence “Neuropathies Périphériques Rares”, Limoges, F-87042, France

⁶CHU Nantes, Plateforme de Biométrie, département Promotion DRCI, F-44093, France

⁷CHU Nantes, Institut des Maladies de l'Appareil Digestif, Nantes, F-44093, France

⁸CHU Nantes, Department of Neurology, Nantes, F-44093, France

Corresponding author:

Laurène Leclair-Visonneau

Laboratoire d'Explorations fonctionnelles - CHU de Nantes - Bd Jacques Monod

44093 Nantes CEDEX 01 – France

Telephone: 0033 2 40 16 58 89

laurene.leclair@chu-nantes.fr

Running title: Dysautonomia in Parkinson's disease

Abstract

Autonomic symptoms are frequent non-motor complaints in patients with Parkinson's disease. Numerous neuropathological studies have shown that Lewy bodies and neurites, the pathological hallmarks of Parkinson's disease, are widely distributed throughout the peripheral autonomic nervous systems and across end organs. However, few investigations extensively explored the symptoms and physiology of dysautonomia in Parkinson's disease. We therefore performed a comprehensive evaluation of the autonomic function in a prospective group of 45 patients with idiopathic Parkinson's disease. Autonomic components (pupillomotor, tear, salivary, cardiovascular, digestive, urinary, sexual, sudomotor functions and skin sensitivity) were evaluated using questionnaires and functional tests. Skin biopsy was performed for intraepidermal nerve fiber density quantification. In addition, all patients underwent polysomnography and a complete neuropsychological and neurological assessment. The analysis of autonomic components showed that dysautonomic signs and symptoms were heterogeneously distributed among patients. Skin denervation as assessed by intraepidermal nerve fiber density quantification was correlated with quantitative thermal sensory testing (OR=12.0, p=0.02) and only associated with constipation (OR=5.5, p=0.01) and ocular dryness symptoms (OR=8.29, p=0.04). Cognitive alteration was associated with cardiovascular symptoms (OR=4.33, p=0.03) and orthostatic hypotension (OR=5.83, p=0.02) as well as with constipation (OR=5.38, p=0.02). Axial motor impairment and rapid eye movement sleep behaviour disorder were not related to any of the autonomic complaint or dysfunction. Our results show that autonomic functions are affected in a heterogeneous pattern in Parkinson's disease, thereby suggesting that the progression of autonomic dysfunction follows an erratic rather than a stepwise progression.

Keywords:

dysautonomia, electrophysiology, intraepidermal nerve fibre density, Parkinson's disease, cognitive alteration

Abbreviations:

ANS= Autonomic Nervous System; IENF= intraepidermal nerve fibre; MDRS= Mattis dementia rating scale; MMSE= Mini-Mental State Examination; MoCA= Montreal cognitive assessment; NCV= nerve conduction velocity; NMS-Quest= Non-motor symptoms questionnaire; QST= Quantitative thermal sensory testing; RBD= rapid eye movement sleep behaviour disorder; SCOPA-Aut= Scales for Outcomes in Parkinson's disease-autonomic symptoms; SSR= sympathetic skin response; UPDRS-III= Unified Parkinson's Disease Rating Scale part III

Introduction

Autonomic involvement in Parkinson's disease is critical because it underlies several frequent and debilitating symptoms of autonomic failure, such as orthostatic hypotension, constipation, urinary problems, heat or cold intolerance, drooling, sweating and swallowing problems (Jain, 2011; Gallagher *et al.*, 2010). This wide variety in dysautonomic symptoms is likely to result from a dysregulation of more than one subdivision of the autonomic nervous system (ANS) (McCorry, 2007). For instance, constipation may result from combined parasympathetic cholinergic failure and enteric nervous system dysfunction, while decreased sweating and orthostatic hypotension are more likely explained by sympathetic cholinergic and noradrenergic dysfunctions, respectively (Kaufmann and Goldstein, 2013; Goldstein, 2014).

These clinical observations are supported by neuropathological studies, which found Lewy bodies and neurites, the pathological hallmarks of Parkinson's disease, in various peripheral autonomic networks, including preganglionic efferent parasympathetic projection neurons in the dorsal motor nucleus of the vagus as well as pre-ganglionic and post-ganglionic sympathetic projection neurons (Wakabayashi *et al.*, 2010). Lewy pathology has also been found in several end organs including the submandibular gland, enteric nervous system, epicardium, skin and adrenal medulla (Wakabayashi *et al.*, 2010). In addition, neuronal loss and/or denervation have been observed in the dorsal motor nucleus of the vagus (Eadie, 1963; Halliday *et al.*, 1990; Benarroch *et al.*, 2006), in sympathetic ganglia (Orimo *et al.*, 2005), in the epicardium (Orimo *et al.*, 2008) and the skin (Dabby *et al.*, 2006).

The main limitation of most of previous reports on autonomic involvement in Parkinson's disease lies in the fact that they have focused on a single symptom and/or on a specific subdivision of the ANS. The purpose of the current study was therefore to perform a comprehensive assessment of the autonomic and non-motor features in a prospective group of

idiopathic parkinsonians in order to explore autonomic dysfunction across Parkinson's disease natural history, its distribution and its relationship with disease progression and severity.

Materials and methods

Study population

From February 2013 to February 2016, 50 patients aged 45-80 years with idiopathic Parkinson's disease as per the UK Brain Bank Criteria were prospectively screened for this cross-sectional study. Exclusion criteria were (i) confounding factors for autonomic failure or neuropathy (autonomic active treatment, diagnosed peripheral neuropathy, renal failure, diabetes, alcoholic abuse and low levels of vitamin B12) (ii) dementia (Mini-Mental State Examination (MMSE) < 24). Of the 47 patients who initially met inclusion criteria, two patients were subsequently excluded (one patient withdrew consent before ANS analysis and the diagnosis of Parkinson's disease was not further confirmed in another patient). This study was carried out in accordance with the Declaration of Helsinki, conducted with the approval of the local Ethical Committee (*Comité de protection des personnes Ouest VI, France*) and registered on ClinicalTrials.gov (identifier NCT01748409). Each participant gave written informed consent. This population was part of a previously published report (Leclair-Visonneau *et al.*, in press).

Clinical-demographic data set

Collected demographic data included sex, age at onset and disease duration, treatments with L-dopa equivalent daily dosage (Tomlinson *et al.*, 2010) and an approximation of lifetime cumulative dose of L-dopa (Parkkinen *et al.*, 2011).

Motor and cognitive function evaluation

All 45 patients were evaluated using the Unified Parkinson's Disease Rating Scale part III (UPDRS-III) in ON state. 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 (Bejjani *et al.*, 2000). Neuropsychological evaluation was carried out using the Mini-mental state examination (MMSE), Montreal cognitive assessment (MoCA) and Mattis dementia rating scale (MDRS).

Autonomic nervous system evaluation

ANS components (pupillomotor, tear, salivary, cardiovascular, digestive, urinary, sexual, sudomotor functions and skin sensitivity) were evaluated in 3 dimensions: symptoms, function and histology.

Dysautonomic symptoms were evaluated with the following questionnaires: SCOPA-Aut (Scales for Outcomes in Parkinson's disease-autonomic symptoms) (Visser *et al.*, 2004), NMS-Quest (Non-motor symptoms questionnaire) (Chaudhuri *et al.*, 2006) and Rome III constipation criteria (Rome Foundation, 2006). A question on dry eye was added, the proposed options followed the SCOPA-Aut model (from never to often).

ANS functional testing was carried out between 9:30 am and 12:30 am (total visit duration 2.5 hours), in a temperature-controlled room set between 20 and 24°C. It was divided into the assessment of pupillomotor function (pupillometry), tear secretion (Schirmer's test), saliva production (Saxon test), cardiovascular function (heart rate variability and orthostatic hypotension), skin sensitivity (Quantitative thermal sensory testing (QST) and nerve conduction velocity (NCV)) and sudomotor function (sympathetic skin response (SSR)). Pupillometry was performed after 2 min adaptation in scotopic condition, binocular pupils were measured by infra-red illumination and a high resolution camera (940 nm) at 30 images

per second (Metrovision® Mon2012H, Perenchies, France), an average of at least ten validated recordings was registered for maximal and minimal diameters, constriction's amplitude, latency and velocity, dilation's latency and velocity. All data were compared to laboratory normative data for age-matched healthy controls and the test was considered abnormal when at least one abnormal value was observed. For Schirmer's test, strips were placed over the inferior lid margin of both eyes, eyes closed and tear level was measured at 5 minutes. Results were the mean value of both eyes (dry eyes under 5 mm, intermediate results between 5 and 10 mm). Saxon test was performed to evaluate the stimulated saliva flow volume by weighing a cotton pad before and after the participant had chewed on it for 2 min (normal value ≥ 2.75 g). For heart-rate variability, the RR interval was recorded with electrocardiogram electrodes placed on presternal region (Dantec™ Keypoint® G4, Natus, Paris, France) during 1 minute in four conditions: normal breath (mean, variation max-min/mean, standard deviation), deep breath (mean, variation max-min/mean, standard deviation), 15 seconds Valsalva's manoeuvre (max/min) and standing up from sitting position (30th/15th battement ratio). The individual results were compared to laboratory normative data for age-matched healthy controls and the test was deemed abnormal when at least one abnormal value was observed. Orthostatic hypotension was defined as a decrease in systolic blood pressure ≥ 20 mm Hg and/or in diastolic blood pressure ≥ 10 mm Hg from supine to upright position at 1, 3, 5 and 10 minutes. QST (Pathway system, Medoc, Abioz Technologies, France) was performed on hand and foot in predominant side of Parkinson's disease. The baseline temperature was 32°C and the contact area of the thermode was 9.0 cm² with ramped stimuli (1°C/s) ending when patient pressed a button. The mean threshold temperature of at least six consecutive measurements was calculated for cold and warm detection as well as for cold pain and heat pain. Range between detection and pain was calculated for cold and heat stimulation. Quartiles were compared between patients, lower

quartiles in at least one of four conditions (cold and heat on hand, cold and heat on foot) were deemed abnormal. NCV (Dantec™ Keypoint® G4) was studied in two motor nerves (left fibular and right tibial) and in two sensory nerves (left sural and right radial). Individual results were compared to laboratory normative data for age-matched healthy controls, and classified as normal, axonal and/or demyelinating neuropathy. Sudomotor function was estimated by SSR (Dantec™ Keypoint® G4). The test was performed after a 15-min baseline resting period. Surface electrodes were placed on palms and plants, with reference electrodes placed on the dorsum, recorded 10-second signal after a 15 mA stimulation on the contralateral wrist. The widest amplitude response on three measurements was used, results were compared to laboratory normative data for age-matched healthy controls, and a reduction in amplitude or absence was considered as ANS dysfunction.

A single skin biopsy was performed under local anaesthesia with 1% xylocaine, with a 4-mm punch on 7-mm depth, on the lower limb (10 cm above the lateral malleolus) on the side predominantly affected by Parkinson's disease. Immunofluorescence was performed using protein gene product 9.5 immunostaining and intraepidermal nerve fibre (IENF) density was quantified at high magnification (x400) with a light microscope by two blinded operators as previously described (Duchesne *et al.*, 2015).

Rapid eye movement sleep behaviour disorder

Sleep clinical assessment comprised rapid eye movement sleep behaviour disorder (RBD) history and severity by structured interview. Overnight polysomnography was performed and interpreted according to the recommendations of American Academy of Sleep Medicine (Iber *et al*, 2007). RBD was defined according to International Classification of Sleep Disorders–II diagnostic criteria (American Academy of Sleep Medicine, 2005). When rapid eye movement sleep was considered absent or infrequent in polysomnographic recording ($\leq 1\%$), the

diagnosis of probable RBD was considered according to a clinical interview performed by a trained sleep specialist (LLV).

Statistical analysis

Continuous data were expressed as the mean \pm standard deviation and categorical data were expressed as numbers and percentage. In order to conduct clinical-functional correlations, we defined one or two items representative for each ANS component and dimension (symptoms, function) whenever possible. We performed multiple comparisons (Kruskal-Wallis tests for ordinal variables and Fisher tests for categorical variables) between these items to explore autonomic failure associations. Clinical features of Parkinson's disease patients with and without skin denervation were compared with Student test for ordinal variables and Fisher tests for categorical variables, and a logistic regression model was done to adjust these comparisons on disease duration. Logistic regression models were also done for evaluating correlation between autonomic symptoms or dysfunction and skin denervation or severity criteria. Odds ratio and their 95% confidence interval were estimated. Correlation coefficients between SCOPA-Aut and continuous variables (age, disease duration, UPDRS III total and axial subscore, neuropsychological tests) were calculated with Spearman's test. For all statistical tests $p < 0.05$ was deemed significant.

Results

Population description

Among the 45 Parkinson's disease patients, 30 (66.7%) were men. The mean (\pm standard deviation) age was 60.4 ± 7.5 years and mean disease duration was 8.7 ± 6.5 years. Twenty-seven (60%) patients were treated with a combination of several antiparkinsonian

medications, seven (15.6%) with levodopa only, 10 (22.2%) with dopamine agonists only and one was untreated. Mean levodopa equivalent daily dose was 810.5 ± 532.9 mg. Eight (17.8%) patients were also treated by deep brain stimulation. Mean UPDRS-III score was 21.7 ± 11.9 and mean UPDRS-III axial subscore was 7 ± 4.2 . Neuropsychological assessment showed altered MMSE score (< 26) in five patients (mean 28 ± 2), altered MoCA score (< 26) in 17 patients (mean 25.5 ± 3.7) and altered MDRS score (< 135) in nine patients (mean 138.2 ± 5). There were 30 (60.7%) patients with RBD (23 with polysomnographic-confirmed RBD and 7 with probable RBD) and 15 (33.3%) without RBD (one patient showed infrequent rapid eye movement sleep on polysomnography and negative structured interview for RBD).

Correlations between dysautonomic symptoms and functional testing

As expected, intra-component analysis showed associations between symptoms and functional measures of several ANS components, including tear (dry eye complaint and Schirmer's test) and cardiovascular functions (light-headed for some time and orthostatic hypotension test) (Supplemental Table 1). By contrast, no significant association was observed between abnormal QST measurements, pain and skin sensitivity complaints. Regarding loco-regional ANS impairment, associations were observed between pupillomotor dysfunction and dry eye complaint, dysphagia and reduced salivary flow, dysuria and fecal incontinence, hyperhidrosis and skin sensitivity symptoms, and between hyperhidrosis and altered QST (Supplemental Table 1). Multiple comparisons revealed a heterogeneous pattern of inter-component associations: pupillomotor symptoms were correlated with abnormal heart-rate variability, early abdominal fullness or skin sensitivity symptoms; sialorrhea with constipation or abnormal Schirmer's test; orthostatic hypotension with constipation, abnormal QST or abnormal SSR (Supplemental Table 1). Sexual symptom and pain were not associated with any of ANS symptom or dysfunction (Supplemental Table 1).

Correlations between dysautonomia and peripheral ANS denervation

As a measure of small fibre neuropathy, 13 (29%) Parkinson's disease patients showed reduced IENF density in skin biopsy. Parkinson's disease patients with skin denervation were more likely to be male, had longer disease duration and higher L-dopa lifetime cumulative dose, showed poorer MDRS score and displayed more frequently non-motor symptoms (Table 1). After adjustment on disease duration, only poorer MDRS score remained significant. Axonal neuropathy was diagnosed on NCV study in 15 (34%) patients, without difference between patients with and without skin denervation (46.2% vs 29%, respectively, $p=0.31$). There were no demyelinating neuropathies. When autonomic components were compared between parkinsonian patients with and without skin denervation, only dry eye complaint, constipation and QST were associated with skin denervation (Table 2). Three out of four QST ranges were altered in patients with skin denervation when compared to patients without skin denervation, reflecting functional-histological correlations (heat on hand, $p=0.03$, cold on hand, $p<0.01$, cold on foot, $p=0.03$). Of note was the absence of relationship between skin denervation, thermal intolerance and pain (Table 2).

Correlations between dysautonomia and Parkinson's disease severity

The presence of axial symptoms in parkinsonian patients has been consistently associated to disease severity and poor prognosis (Post *et al.*, 2011; Kotagal *et al.*, 2014). Regarding non-motor features, cognitive impairment and RBD have been identified in a subset of parkinsonian patients in whom a rapid progression rate could be expected (Fereshtehnejad *et al.*, 2015). We therefore investigated the possible correlation between dysautonomia and these three severity criteria of Parkinson's disease, i.e. cognitive alteration (MoCA < 26), axial motor impairment (UPDRS-III axial subscore ≥ 10) and RBD. Cognitive alteration was

associated with cardiovascular symptoms (light headed when standing up) and dysfunction (orthostatic hypotension) as well as with constipation (Table 3). Conversely, axial motor impairment and RBD were not related to any of the autonomic complaint or dysfunction (Table 3). There were no correlations between total SCOPA-Aut and age ($p=0.12$), disease duration ($p=0.07$), cognitive alteration (MoCA, $p=0.58$; MDRS, $p=0.21$; MMSE, $p=0.53$) or axial sub-score ($p=0.17$), however SCOPA-Aut was correlated with total UPDRS score ($r=0.32$, $p=0.03$).

Discussion

Anatomically, the peripheral ANS can be divided into sympathetic (cholinergic and noradrenergic), parasympathetic pathways and the enteric nervous system. Dysfunction of a particular component causes characteristic signs and symptoms. Enteric nervous system dysfunction is responsible for delayed gastric emptying and constipation. Parasympathetic cholinergic failure induces constipation, hyposialorrhea, an invariable pulse rate, urinary retention, and erectile failure, while sympathetic cholinergic failure induces decreased sweating. Sympathetic noradrenergic failure presents as orthostatic intolerance and orthostatic hypotension (McCorry, 2007). Dysautonomia in Parkinson's disease encompasses a wide range of symptoms and signs, including, among the most common, constipation, orthostatic or postprandial light-headedness, orthostatic hypotension, urinary dysfunction, excessive or decreased sweating and hyposialorrhea (Goldstein, 2014). Hence, in Parkinson's disease there seems to be a dysregulation of most if not all components of the autonomic nervous networks.

The widespread autonomic dysregulation encountered in parkinsonian patients prompted us to carry out a comprehensive evaluation of autonomic symptoms and signs in a prospective sample of patients affected by Parkinson's disease. We observed several ANS inter-component associations that were heterogeneously distributed, following either a

locoregional or a remote distribution pattern. Apart from its expected correlation with skin sensitivity, skin denervation was only associated with constipation and dry eye. In line with previous studies (Doppler *et al.*, 2014), reduced IENF density cannot be ascribed to L-dopa potential toxicity, as L-dopa cumulative doses were similar after adjustment on disease duration. Cardiovascular symptoms, orthostatic hypotension and constipation were more likely to occur in parkinsonian patients with cognitive alteration; however, autonomic complaints and dysfunction were not associated with the two other criteria of disease severity, namely axial motor impairment and RBD (Post *et al.*, 2011; Fereshtehnejad *et al.*, 2015).

Most of the existing studies on ANS complaints or dysfunctions in Parkinson's disease have focused on single or dual components of dysautonomia, and especially on cardiovascular dysfunction. Cardiovascular dysfunction in Parkinson's disease, indicating predominantly parasympathetic (heart-rate variability) or sympathetic (orthostatic hypotension) dysfunction (Goldstein *et al.*, 2002), has been shown to be associated with disease severity. This includes, for example, the higher risk of developing dementia in patients with orthostatic hypotension (Anang *et al.*, 2014) and the higher Hoehn and Yahr stage in patients with cardiovascular autonomic dysfunction (Kim *et al.*, 2014). Our results, which showed that orthostatic hypotension was strongly associated with cognitive alteration in our Parkinson's disease sample, are therefore in line with these previous observations. A few studies have attempted to correlate sudomotor and skin vasomotor dysfunction with disease severity in Parkinson's disease (Asahina *et al.*, 2014) and with dementia (Akaogi *et al.*, 2009). Akaogi *et al.* showed that skin vasomotor function was more severely affected in patients with dementia than in Parkinson's disease without dementia, while Asahina *et al.* noted an inverse correlation between the amplitude of sympathetic sweat response and Hoehn and Yahr stage. Contrasting with these results, we did not observe any association between SSR and MoCA.

Numerous pathological studies have shown that every single component of the ANS may be affected by Parkinson's disease pathology. Lewy pathology has been observed in a chain of neurons forming the sympathetic autonomic pathways including the intermediolateral cell column of spinal cord (Wakabayashi and Takahashi, 1997), sympathetic paravertebral ganglia (Hartog Jager and Bethlem, 1960; Orimo *et al.*, 2005; Beach *et al.*, 2010), adrenal medulla and skin nerve fibres (Ikemura *et al.*, 2008). This pathological process also reaches several structures of the parasympathetic nervous system (Eadie, 1963; Hunter, 1985; Halliday *et al.*, 1990; Takeda *et al.*, 1993; Beach *et al.*, 2010; Braak *et al.*, 2003; Del Tredici *et al.*, 2002; Braak *et al.*, 2007) as well as the enteric nervous system (Qualman *et al.*, 1984; Wakabayashi *et al.*, 1988; Beach *et al.*, 2010). It is suggested that this widespread distribution of Lewy pathology throughout the central and peripheral autonomic networks is responsible for the disabling dysautonomic symptoms observed in parkinsonian patients (Adler and Beach, 2016). At first glance, these neuropathological observations appear to be at odds with our results, which show a patchy and heterogeneous pattern of dysautonomic signs and symptoms in Parkinson's disease. However, it should be kept in mind that the mere presence of Lewy pathology in a subset of neurons does not necessarily imply that these neurons are dysfunctional. For example, the motor symptoms of Parkinson's disease are driven primarily by neuronal loss rather than Lewy pathology in the *substantia nigra* (Greffard *et al.*, 2006). As such, an evaluation of the density of the autonomic neurons in the different components of the peripheral ANS will be a critical step toward understanding the pathophysiology of dysautonomic symptoms in Parkinson's disease.

We confirm the results of a number of studies, which have shown a reduction of distal IENF in Parkinson's disease patients and a correlation of IENF density with disease duration and disease severity (Nolano *et al.*, 2008; Kass-Iliyya *et al.*, 2015). In addition, we show in the current survey that skin denervation is poorly related to dysautonomic signs and

symptoms, as ocular dryness and constipation were the only signs to be significantly correlated with IENF density, therefore suggesting that the mere analysis of IENF density in skin biopsy cannot be used as a surrogate marker of ANS failure in Parkinson's disease.

There are some limitations of this study. First, we did not perform any functional evaluation of the lower urinary tract and digestive systems, as urodynamic testing and colonic manometry were considered too invasive. Another aspect is the relatively small sample size of Parkinson's disease patients, which hinders us from further stratification of patients into subgroups with different motor severity and/or disease duration. Finally, alpha-synuclein deposition in skin biopsies was not evaluated in our study. The principal reason for not performing this evaluation is that there is still an open debate about the optimized immunohistochemical method that should be used for the detection of alpha-synuclein in skin nerve fibers (Zange *et al.*, 2016; Doppler *et al.*, 2016). On the other hand, our study has several strengths. All patients in our study had a comprehensive assessment of the ANS, addressing a variety of non-motor outcomes. In addition, all patients had a precise evaluation of cognitive function with three different validated scales and a confirmation of RBD by polysomnography.

In summary, our results show that autonomic functions are affected in a heterogeneous pattern in Parkinson's disease. They suggest that autonomic dysfunction in Parkinson's disease progresses in an erratic rather than a stepwise fashion pointing out the complexity of the pathophysiology of disease progression. They also imply that it is not possible in a single patient with Parkinson's disease to predict the progression pattern of dysautonomia.

Supplemental table 1. Autonomic nervous system intra and inter-component multiple comparisons.

Component	Item	Pupillomotor		Tear		Salivary		Cardio-vascular		Upper digestion symptom		Lower digestion symptom		Urinary symptom		Sexual symptom		Skin sensitivity		Sudomotor		
		Symptom	Dysfunction n	Symptom	Dysfunction n	Symptom	Dysfunction n	Symptom	Dysfunction n	Symptom: orthostatic hypotension n	Dysfunction: abnormal heart-rate variability	Difficulty swallowing or choking	Early abdominal fullness	Faecal incontinence	Constipation n	Urgency	Weak stream of urine	Difficulty to have sex	Symptom: cold or heat intolerance	Symptom: unexplained pain	Dysfunction n	Symptom
Pupillomotor	Symptom: oversensitivity to bright light		0.99	0.25	0.51	0.25	0.59	0.12	0.66	0.11	0.90	0.04*	0.22	0.50	0.88	0.86	0.53	0.05	0.96	0.94	0.80	0.98
	Dysfunction: altered pupillometry	0.61		0.10	0.91	0.54	0.29	0.91	0.41	0.69	0.08	0.81	1.00	0.73	0.79	0.66	0.09	0.81	1.00	0.72	0.47	0.59
Tear	Symptom: dry eye	0.53	0.03*		<0.01†	0.38	0.31	0.13	0.36	0.60	0.54	0.80	0.19	0.54	0.73	0.81	0.85	0.69	0.61	0.07	0.71	0.29
	Dysfunction: abnormal Schirmer's test	0.99	0.84	<0.01†		0.03*	0.87	0.22	0.84	0.65	0.48	0.80	0.88	0.48	0.99	0.96	0.25	0.39	0.91	0.68	0.45	0.29
Salivary	Symptom: sialorrhea	0.27	0.59	0.28	0.16		0.45	0.54	0.28	0.25	0.16	0.75	0.27	0.04*	0.78	0.90	0.82	0.59	0.96	0.28	0.25	0.75
	Dysfunction: abnormal sialometry	0.74	0.20	0.33	0.50	0.73		0.34	0.75	0.53	0.02*	0.95	0.69	0.07	0.87	0.58	0.09	0.90	0.65	0.12	0.49	0.80
Cardio-vascular	Symptom: light-headed when standing up or for some time	0.07	0.63	0.66	0.38	0.62	0.53		0.15	0.96	0.11	0.25	0.08	0.69	0.95	0.47	0.06	0.03*	0.05	0.75	0.29	0.80
	Dysfunction: orthostatic hypotension	0.45	0.27	0.16	0.17	0.56	0.75	0.35	0.07	0.07	0.47	0.80	0.06	0.03*	0.08	0.66	0.53	0.56	0.59	0.04*	0.25	0.02*
Upper digestion symptom	Dysfunction: abnormal heart-rate variability	0.03*	0.46	0.26	0.89	0.45	0.53	0.74	0.07	0.93	0.30	0.30	0.24	0.44	0.98	0.32	0.43	0.60	0.23	0.93	0.57	0.79
	Difficulty swallowing or choking	0.86	0.08	0.85	0.56	0.15	0.02*	0.32	0.72	1.00	0.41	0.61	1.00	0.07	0.95	0.45	0.54	0.63	0.31	0.22	0.35	0.63
Lower digestion symptom	Early abdominal fullness	0.04*	0.61	0.35	0.96	0.63	0.77	0.14	0.51	0.12	0.41	0.13	0.13	0.43	0.43	0.32	0.74	0.10	0.42	0.78	0.06	0.31
	Faecal incontinence	0.29	0.40	0.11	0.99	0.51	0.69	0.18	0.06	0.24	0.81	0.30		0.15	0.83	0.04*	0.53	0.56	0.11	0.17	0.25	0.61
Urinary symptom	Constipation	0.08	0.73	0.85	0.56	0.01*	0.12	1.00	0.03*	0.51	0.07	0.36	0.26		0.39	0.67	0.76	0.16	0.50	0.54	0.76	0.63
	Urgency	0.78	0.92	0.62	0.62	0.76	0.79	0.94	0.07	0.96	0.90	0.29	0.59	0.25		0.44	0.19	0.65	0.49	0.31	0.25	0.62
Sexual symptom	Weak stream of urine	0.50	0.37	0.39	0.95	0.98	0.30	0.40	0.59	0.93	0.65	0.28	0.38	0.37	0.53		0.14	0.25	0.32	0.88	0.54	0.37
	Difficulty to have sex	0.80	0.09	0.17	0.21	0.78	0.13	0.15	0.72	0.51	0.54	0.91	0.61	0.76	0.41	0.22		0.69	0.51	0.75	0.75	0.66
Skin sensitivity	Symptom: cold or heat intolerance	0.02*	0.47	0.28	0.22	0.37	0.68	0.03*	0.33	0.44	0.68	0.42	0.42	0.22	0.95	0.42	0.69		1.00	0.41	1.00	0.22
	Symptom: unexplained pain	1.00	1.00	0.90	0.76	0.91	0.74	0.07	0.71	0.29	0.31	0.13	0.17	0.50	0.85	0.61	0.51	1.00	1.00	1.00	0.73	1.00
Sudomotor	Dysfunction: altered DST	0.58	0.72	0.18	0.69	0.51	0.14	0.86	0.06	1.00	0.22	0.77	0.28	0.54	0.08	0.92	0.75	0.63	1.00		0.02*	0.14
	Symptom: hyperhidrosis	0.62	0.47	0.58	0.79	0.60	0.54	0.36	0.29	0.73	0.35	0.19	0.28	0.76	0.20	0.78	0.75	0.02*	0.73	0.02*		0.14
	Dysfunction: abnormal SSR	0.91	0.44	0.41	0.44	0.87	0.80	0.96	0.02*	0.79	0.37	0.54	0.61	0.32	0.90	0.49	0.56	0.21	0.79	0.06	0.08	

† : intra-component association

*: inter-component association

Table 1. Main clinical features of Parkinson's disease patients with and without skin denervation.

	PD+SD	PD-SD	p-value	adjusted p-value ‡
number, %	13 (29%)	32 (71%)		
Sex, % male	12 (92.3%)	18 (56.3%)	0.03	0.05
Age, y	62.2 (7.9)	59.7 (7.4)	0.31	0.45
Disease duration, y	11.9 (5.6)	7.3 (6.5)	0.03	/
L-dopa therapy (no, %)	12 (92%)	22 (69%)	0.14	0.39
L-dopa lifetime cumulative dose, kg	1.9 (1.4)	0.8 (1.1)	<0.01	0.19
UPDRS part III 'on'				
total	22.1 (9.6)	21.6 (12.9)	0.90	0.35
axial	7.2 (3.4)	6.9 (4.5)	0.84	0.24
Neuropsychological tests				
MMSE < 26	3 (23.1%)	2 (6.3%)	0.13	0.13
MoCA < 26	6 (46.2%)	11 (34.4%)	0.51	0.39
MDRS < 135	6 (46.2%)	3 (9.4%)	0.01	0.03
Overall non motor symptoms				
SCOPA-Aut	22.9 (8.0)	19.1 (7.7)	0.14	0.33
NMS	15.3 (3.4)	12.1 (4.7)	0.04	0.09

‡ adjusted p-value on disease duration

PD+SD= Parkinson's disease patients with skin denervation, PD-SD= Parkinson's disease patients without skin denervation. Data are mean (standard deviation) or number (%).

Table 2. Correlations between autonomic symptoms and dysfunction and skin denervation.

	Component	Item	Odds ratio	p-value
Pupillo- motor	Symptom: oversensitivity to bright light	SCOPA-Aut Q19	1.99 [0.51 - 7.79]	0.33
	Dysfunction: altered pupillometry		0.49 [0.09 - 2.69]	0.41
Tear	Symptom: dry eye	"do you complain from dry eye?"	8.29 [1.65 -41.58]	0.01*
	Dysfunction: abnormal Schirmer's test		7.20 [0.83 -62.55]	0.07
Salivary	Symptom: sialorrhea	SCOPA-AUT Q2	1.88 [0.47 - 7.41]	0.37
	Dysfunction: abnormal sialometry	Saxon's test	1.71 [0.47 - 6.25]	0.42
Cardio-vascular	Symptom: light-headed when standing up	SCOPA-AUT Q14	2.25 [0.57 - 8.82]	0.24
	Symptom: light-headed when standing for some time	SCOPA-AUT Q15	1.03 [0.28 - 3.75]	0.96
	Dysfunction: orthostatic hypotension		1.93 [0.44 - 8.42]	0.38
	Dysfunction: abnormal heart-rate variability		1.67 [0.37 - 7.44]	0.50
Upper digestion	Difficulty swallowing or choking	NMS Q3	1.43 [0.39 - 5.26]	0.59

symptom	Early abdominal fullness	SCOPA-AUT Q4	3.33 [0.77 -14.42]	0.11
Lower	Faecal incontinence	SCOPA-AUT Q7	5.64 [0.46 -68.46]	0.17
digestion	Constipation	Rome III criteria	5.50 [1.05 -28.88]	0.04*
symptom				
Urinary	Urgency	SCOPA-AUT Q8	1.50 [0.41 - 5.48]	0.54
symptom	Weak stream of urine	SCOPA-AUT Q11	1.60 [0.43 - 5.96]	0.48
Sexual	Difficulty to have sex	NMS Q19	1.83 [0.48 - 6.90]	0.37
symptom				
Skin	Symptom: cold	SCOPA-AUT Q20	0.39 [0.10 - 1.46]	0.16
sensitivity	intolerance			
	Symptom: heat	SCOPA-AUT Q21	0.97 [0.27 - 3.54]	0.96
	intolerance			
	Symptom: unexplained	NMS Q10	0.77 [0.17 - 3.45]	0.73
	pain			
	Dysfunction: altered		12.0 [1.39 -	0.02*
	QST		>99.99]	
Sudo-	Symptom: hyperhidrosis	NMS Q28	0.74 [0.19 - 2.94]	0.67
motor	Dysfunction: abnormal		1.93 [0.28 -13.30]	0.50
	SSR			

Q= item number of the questionnaire

Table 3. Correlation between autonomic symptoms and dysfunction and Parkinson's disease severity criteria.

Component		Cognitive alteration		Axial motor impairment		RBD	
		Odds Ratio	p-value	Odds Ratio	p-value	Odds Ratio	p-value
Pupillo-motor	Symptom: oversensitivity to bright light	0.73 [0.22 - 2.46]	0.61	0.28 [0.06 - 1.32]	0.11	0.57 [0.16 - 2.08]	0.40
	Dysfunction: altered pupillometry	0.95 [0.23 - 3.95]	0.95	0.79 [0.14 - 4.55]	0.80	0.33 [0.08 - 1.34]	0.12
Tear	Symptom: dry eye	1.42 [0.32 - 6.22]	0.65	1.18 [0.20 - 6.98]	0.85	0.55 [0.12 - 2.45]	0.43
	Dysfunction : abnormal Schirmer's test	0.61 [0.16 - 2.27]	0.46	4.00 [0.45 - 35.79]	0.21	0.50 [0.11 - 2.19]	0.36
Salivary	Symptom: sialorrea	1.64 [0.44 - 6.08]	0.46	1.30 [0.27 - 6.22]	0.74	0.73 [0.19 - 2.79]	0.64
	Dysfunction: abnormal sialometry	0.36 [0.10 - 1.30]	0.12	0.29 [0.05 - 1.57]	0.15	0.39 [0.11 - 1.38]	0.14
Cardio-	Symptom:	4.33 [1.13 -	0.03*	1.00 [0.23 -	1.00	1.14 [0.33 -	0.83

vascular	light-headed when standing up	16.68]		4.35]		3.97]	
	Symptom: light-headed when standing for some time	3.20 [0.89 - 11.56]	0.08	2.00 [0.43 - 9.26]	0.38	1.49 [0.43 - 5.19]	0.53
	Dysfunction: orthostatic hypotension	5.83 [1.25 - 27.16]	0.02*	2.07 [0.41 - 10.39]	0.38	1.22 [0.27 - 5.58]	0.80
	Dysfunction: abnormal heart-rate variability	1.50 [0.38 - 6.00]	0.57	0.67 [0.13 - 3.33]	0.62	0.89 [0.22 - 3.61]	0.87
Upper digestion symptom	Difficulty swallowing or choking	2.37 [0.69 - 8.20]	0.17	4.00 [0.85 - 18.84]	0.08	1.53 [0.42 - 5.58]	0.52
	Early abdominal fullnes	1.07 [0.32 - 3.64]	0.91	0.28 [0.06 - 1.32]	0.11	1.97 [0.56 - 6.94]	0.29
Lower digestion symptom	Faecal incontinence	3.60 [0.30 - 43.06]	0.31		0.97		0.98
	Constipation	5.38 [1.26 - 27.16]	0.02*	1.43 [0.31 - 3.33]	0.65	2.29 [0.64 - 5.58]	0.20

		22.98]		6.64]		8.11]	
Urinary symptom	Urgency	1.03 [0.31 - 0.97 3.43]		2.80 [0.60 - 0.19 13.01]		0.67 [0.19 - 0.53 2.32]	
	Weak stream of urine	0.67 [0.20 - 0.51 2.24]		1.12 [0.26 - 0.88 4.86]		2.25 [0.63 - 0.21 7.97]	
Sexual symptom	Difficulty to have sex	0.22 [0.06 - 0.83]	0.02*	0.94 [0.20 - 0.94 4.39]		1.07 [0.30 - 0.92 3.84]	
Skin sensitivity	Symptom: cold intolerance	1.80 [0.50 - 0.37 6.50]		2.50 [0.45 - 0.29 13.76]		0.48 [0.12 - 0.28 1.84]	
	Symptom: heat intolerance	1.03 [0.31 - 0.97 3.43]		5.50 [1.00 - 0.05 30.36]		1.00 [0.29 - 1.00 3.46]	
	Symptom: unexplained pain	1.25 [0.32 - 0.75 4.82]		0.74 [0.13 - 0.74 4.20]		0.38 [0.10 - 0.16 1.47]	
	Dysfunction: altered QST	1.80 [0.50 - 0.37 6.50]		1.27 [0.27 - 0.76 5.93]		0.75 [0.20 - 0.66 2.75]	
Sudo-motor	Symptom: hyperhidrosis	0.64 [0.18 - 0.50 2.34]		0.88 [0.19 - 0.88 4.14]		0.75 [0.21 - 0.66 2.70]	
	Dysfunction: abnormal SSR	1.19 [0.18 - 0.86 8.00]		0.97 [0.09 - 0.98 9.91]		0.10 [<0.01 - 0.05* - 0.98]	

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Supplementary material

Supplemental table 1. Autonomic nervous system intra and inter-component multiple comparisons. Data on 'light-headed for some time' and 'when standing up' are combined for clarity reasons, as well as data on cold and heat intolerance. A separate analysis shows that light-headed for some time and orthostatic hypotension test were significantly associated ($p=0.02$).

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Discussion

Au cours de ce travail de thèse, nous avons montré une altération morphologique de la BEI, sans lien apparent avec la charge lésionnelle en synucléinopathie dans le plexus sous-muqueux. Par ailleurs, l'atteinte histopathologique du SNE était associée à l'atteinte probable du locus subcœruleus dans la MP, se manifestant cliniquement par un TCSP. Enfin nous avons étudié la répartition de la dysfonction du SNA dans ses différentes modalités et avons observé une distribution hétérogène et éparse.

L'altération de la BEI au cours de la MP pourrait contribuer à sa physiopathologie, car elle pourrait être le point d'entrée d'un pathogène environnemental déclenchant la phosphorylation et l'agrégation de l'alpha-synucléine (128). Les études portant sur les modifications de perméabilité intestinale dans la MP reposent principalement sur la mesure *in vivo* de l'absorption et l'excrétion de sucres non métabolisables (lactulose, mannitol, sucralose). Elles ont montré des résultats contradictoires sur la perméabilité de l'intestin grêle ou du côlon (171–173). Nous avons utilisé une technique différente, la mesure *ex vivo* sur des biopsies coliques montées dans des chambres d'Ussing (174). Bien que nous n'observions pas de différence entre les moyennes des deux groupes, les patients parkinsoniens présentaient des valeurs de perméabilité paracellulaire plus hétérogènes que les témoins, dépassant de plus de 2 fois la moyenne des témoins. Des modifications de perméabilité paracellulaire pourraient concerner un sous-groupe de patients, comme cela a été évoqué par une autre équipe (172). Nous n'avons pas mis en évidence de facteur explicatif à ces modifications, en l'absence de corrélation avec l'âge, le traitement par L-dopa ou la durée d'évolution de la maladie. Notre étude a montré une altération morphologique de la BEI, avec une diminution de l'expression de l'occludine et une désorganisation des jonctions serrées. Cette modification de l'espace intercellulaire pourrait augmenter la perméabilité à des macromolécules de plus de 5 kDa, qui ne serait alors pas détectée par la

mesure de la perméabilité à l'acide sulfonique (400 Da) (175). Une limitation de notre travail est de n'avoir pu corrélérer les anomalies morphologiques observées à des modifications fonctionnelles. Par ailleurs, nous n'avons pas observé de différence de perméabilité entre les patients parkinsoniens présentant une synucléinopathie dans le SNE et ceux dont les biopsies coliques étaient indemnes. Ainsi, la pathologie de Lewy dans le plexus sous-muqueux ne se traduirait pas physiologiquement par une dysrégulation de la BEI.

Des anomalies des protéines de jonction ont également été observées dans des tissus inflammatoires, et non dans les tissus sains, chez des patients atteints de maladies inflammatoires chroniques de l'intestin (176). Notre équipe a précédemment mis en évidence une augmentation des cytokines pro-inflammatoires (*tumor necrosis factor alpha*, interféron gamma, interleukine 6 et interleukine 1 beta) et de marqueurs d'activation gliale (GFAP (Glial fibrillary acidic protein) and Sox-10) dans des biopsies du côlon ascendant (177). Ces marqueurs étaient corrélés négativement avec la durée d'évolution de la MP et n'étaient pas associés aux lésions de synucléinopathie (177). Nous avons confirmé ces résultats par une étude montrant un niveau d'expression de la GFAP supérieur dans la MP, en comparaison avec une population contrôle et des patients atteints d'atrophie multisystématisée et de paralysie supranucléaire progressive (178). Ces données renforcent ainsi l'hypothèse d'une implication de la réactivité gliale dans l'initiation et/ou la progression du processus pathologique dans la MP (179,180). Dans un modèle animal de MP, l'injection intestinale de lipopolysaccharide induisait une augmentation de la perméabilité intestinale initiale (probablement colique), puis une normalisation de cette perméabilité parallèlement à l'accumulation d'alpha-synucléine phosphorylée dans le plexus myentérique (181).

Ces données sont en adéquation avec l'hypothèse de Braak, reposant sur une progression du processus pathologique à travers le SNE, dans le plexus sous-muqueux, se propageant au plexus myentérique puis au SNC via le nerf vague (137,140). Ainsi, chez le rat, l'alpha-synucléine (sous forme de lysat, de monomère, d'oligomère ou de fibrille) injectée dans la paroi gastrique et duodénale, à proximité du plexus myentérique, est transportée via les

fibres cholinergiques du nerf vague jusqu'au noyau moteur dorsal du vague, mettant en jeu des transports axonaux de vitesses différentes (182). Par ailleurs, une étude sur des registres nationaux danois a mis en évidence une réduction de 15% du risque de MP à long terme après vagotomie totale (183). Néanmoins, les données statistiques étant à la limite de la significativité, elles nécessitent d'être répliquées (184,185). Parallèlement à une potentielle voie d'entrée digestive, l'existence d'une hyposmie dès le stade prémoteur de la MP suggère que le bulbe olfactif pourrait également être la porte d'entrée d'un pathogène environnemental (186,187). Les propriétés de propagation d'un neurone à l'autre de l'alpha-synucléine, sur le modèle de la protéine prion, supportent également cette hypothèse de diffusion (103,105,180).

Des explorations complémentaires concernant une éventuelle porte d'entrée digestive sont nécessaires. L'atteinte histologique du SNE suivant un gradient rostro-caudal (85,123,124,188), l'étude de la BEI gastrique et duodénale permettrait de comprendre de potentiels mécanismes de l'initiation de la MP. Par ailleurs, l'altération du microbiote intestinal pourrait jouer un rôle dans la pathogénie de la MP (187,189). Les modifications du microbiote chez les patients parkinsoniens sont explorées de manière récente, montrant une augmentation des populations supposées pro-inflammatoires et une réduction des populations supposées anti-inflammatoires (190), une dysbiose modifiant la perméabilité intestinale et réciproquement (191) et une corrélation avec des phénotypes cliniques de la maladie (192). La dysbiose favorisant la biosynthèse du lipopolysaccharide (190), elle pourrait expliquer l'inflammation de bas grade et l'activation gliale observées plus volontiers dans le côlon des patients en début de maladie (177). L'étude du microbiote dans la MP n'en est qu'à ses débuts, il ouvre un large champ de recherches qui devront s'affranchir des effets du mode de vie (tabac, café) et des médicaments (193–196).

Dans notre deuxième étude, nous avons montré que le TCSP au cours de la MP était associé à la synucléinopathie dans le SNE. La coïncidence entre l'atteinte du SNE et celle

du locus subcœruleus au cours de la MP est également compatible avec le scénario de Braak (137). Cependant, les durées d'évolution ne différant pas entre les patients parkinsoniens avec et sans TCSP, notre étude n'est pas en faveur d'une gradation chronologique dans l'apparition de ces atteintes, mais plutôt de profils de MP différents (3). Nos données supportent l'existence de formes de MP plus agressives et plus diffuses, les patients avec TCSP étant plus âgés et plus sévères sur le plan moteur (UPDRS). De nombreuses études cliniques ont montré que les parkinsoniens avec TCSP présentaient plus d'éléments de gravité de la maladie, tant sur le plan des symptômes neuropsychiques (159,161,165,166), que moteurs (167) ou dysautonomiques (31). A la différence de certaines études, nous n'avons pas observé plus de constipation et d'hypotension orthostatique chez les patients parkinsoniens avec TCSP que sans (31,197). Une étude autopsique a montré que les patients parkinsoniens avec TCSP présentaient une augmentation de la charge lésionnelle en alpha-synucléine par rapport à des patients parkinsoniens sans TCSP, dans 9 régions encéphaliques d'intérêt (sur 10 analysées), comprenant le locus cœruleus, l'amygdale, les noyaux du nerf vague, le cortex entorhinal ou le cortex cingulaire (198). Enfin, le TCSP pourrait être particulièrement liée à l'accumulation anormale d'alpha-synucléine dans la MP, plutôt qu'à d'autres mécanismes délétères évoqués dans la physiopathologie de la MP tels qu'une altération mitochondriale, un stress oxydatif ou une réaction inflammatoire (198,199).

Des signes d'atteinte extra-nigrale, tels que le TCSP ou les troubles cognitifs, apparaissent liés à la gravité de la MP (4). Nous avons voulu étudier la diffusion de la MP à l'ensemble du SNA afin d'en déterminer la place au cours de l'histoire naturelle de la maladie et le lien avec sa sévérité. Notre étude systématique des modalités du SNA chez 45 patients parkinsoniens montre que la dysfonction autonome ne semble pas suivre une progression par étapes, au fil de la maladie, mais se répartit de manière éparse et asynchrone. Ainsi, si certaines atteintes peuvent suggérer une composante locorégionale (dysfonction pupillaire et

sécheresse oculaire par exemple), les corrélations intermodalités montrent des associations ponctuelles, sans lien physiopathologique apparent, ne partageant notamment pas de mécanisme orthosympathique ou parasympathique commun. Des associations locorégionales avaient auparavant été mises en évidence, notamment entre l'incontinence urinaire et l'incontinence fécale (57). Certaines associations intermodalités du SNA ont fait l'objet d'études, indiquant un lien entre la dysfonction cardio-vasculaire et la dysfonction sudorale (200,201) ou la dysfonction pupillaire (202). Notre analyse plus globale du SNA ne confirme pas ces données. Nous avons utilisé la dénervation cutanée comme *gold standard* de l'atteinte histopathologique du SNA cutané, non liée à un potentiel effet toxique de la L-dopa (203), et nous n'avons observé des corrélations intermodalités qu'avec la constipation et la plainte de sécheresse oculaire. Les conclusions de notre étude peuvent être limitées par la taille de notre échantillon ne permettant que difficilement des explorations sous forme d'analyse en *clusters*. Nous avons cependant réalisé des études statistiques exploratoires dont les résultats étaient négatifs (données non publiées).

Une étude sur questionnaire s'était attachée à explorer de manière complète le SNA en 2002, et de manière intéressante, les auteurs n'avaient pas observé de corrélation entre les symptômes dysautonomiques dans leur ensemble ou la dysfonction gastro-intestinale avec la sévérité de la maladie (âge, durée d'évolution, échelle de Hoehn et Yahr, échelle de Schwab et England et score moteur), échouant dans l'élaboration d'un modèle statistique prédisant la dysautonomie (204). Nos données sont concordantes avec ces résultats. En effet, nous n'observons pas de corrélation globale de la dysautonomie avec la gravité de la MP (corrélations de la SCOPA-Aut uniquement avec l'UPDRS total) et, seules la constipation et l'hypotension orthostatique (symptômes et test fonctionnel) apparaissent corrélés à l'atteinte cognitive. La dysautonomie dans son ensemble ne peut ainsi pas être considérée comme un marqueur de gravité de la MP. Cependant, en adéquation avec de précédentes études, la constipation et l'hypotension orthostatique apparaissent comme des facteurs prédictifs de sévérité de la MP (197,205–207).

Nous souhaitons poursuivre l'exploration de la dysautonomie dans la MP par une étude longitudinale de notre échantillon de patients parkinsoniens, portant sur l'évolution des symptômes et des dysfonctions autonomiques à 5 ans de leur première évaluation. Cette étude physiopathologique pourrait nous indiquer une éventuelle organisation temporelle des dysfonctions en lien avec une susceptibilité à la synucléinopathie des différentes fibres pré et post-ganglionnaires, orthosympathiques (cholinergiques ou catécholaminergiques) et parasympathiques. En effet, des données expérimentales chez le cochon d'inde et sur des biopsies coliques humaines suggèrent que l'alpha-synucléine est principalement localisée dans les neurones cholinergiques du SNE (208). Les fibres cholinergiques pourraient être particulièrement vulnérables à l'accumulation pathologique d'alpha-synucléine, comme cela a été montré dans des noyaux du tronc cérébral (209). Ces données pourraient expliquer la préservation des neurones catécholaminergiques des plexus sous-muqueux et myentériques au cours de la MP, puisqu'il n'a pas été mis en évidence de perte neuronale dans des études récentes autopsique et *in vivo* (76,130). Elles pourraient également expliquer certains symptômes dysautonomiques observés au cours de la MP. Ainsi, l'évolution au cours du temps de la dysfonction cardiovasculaire pourrait nous aider à comprendre ses mécanismes (par exemple : altération de la variabilité de l'intervalle RR d'origine parasympathique vs hypotension orthostatique de mécanisme adrénérique prédominant) (66). De la même manière, l'étude de la réactivité pupillaire permettrait de distinguer une dysfonction parasympathique d'une atteinte orthosympathique (66).

Enfin, la corrélation clinicopathologique entre la synucléinopathie et les symptômes dysautonomiques demeure imprécise. Les données de notre équipe concernant le lien entre la synucléinopathie dans le plexus sous-muqueux et la constipation sont contradictoires (pas de corrélation dans des données non publiées et corrélation dans 83). De plus, une synucléinopathie cutanée peut être observée précocement au cours de la MP, en l'absence de symptomatologie clinique (210). Des études complémentaires de corrélation anatomo-

clinique sont nécessaires, afin de clarifier le rôle de la pathologie de Lewy dans la genèse des symptômes.

Conclusion

Ce travail de thèse a permis d'explorer l'implication du système nerveux autonome dans l'histoire naturelle de la MP. Chez certains patients, l'altération de la BEI pourrait contribuer à l'initiation de la maladie en permettant la transmission d'un pathogène environnemental au plexus sous-muqueux, déclenchant l'agrégation anormale de l'alpha-synucléine phosphorylée au sein des terminaisons axonales des neurones du SNE, puis, suivant une progression ascendante, jusqu'au soma des neurones. L'alpha-synucléine agrégée pourrait se propager de proche en proche, à travers le plexus myentérique, le nerf vague jusqu'au noyau moteur dorsal du vague. Après avoir atteint le tronc cérébral, le processus pathologique pourrait alors gagner le complexe cœruleus-subcœruleus, se manifestant cliniquement par un TCSP, que nous avons montré lié à l'atteinte du SNE. Cette pathogénie de la MP ne pourrait concerner qu'un sous-groupe de patients, plus sévères, d'autres portes d'entrée de la maladie étant évoquées. La diffusion de la MP à l'ensemble du SNA ne suivrait pas une progression chronologique définie, mais apparaît comme éparse et erratique. Néanmoins, l'atteinte du SNA cardiovasculaire et la constipation semblent être des marqueurs de gravité de la MP, associés aux troubles cognitifs, et donc à une diffusion cortico-sous-corticale de la pathologie.

Travaux annexes et articles de revue

Article 4 : Valeur diagnostique de la biopsie des glandes salivaires accessoires dans la détection de la pathologie de type Lewy.

Folgoas E, Lebouvier T, Leclair-Visonneau L, Cersosimo MG, Barthelaix A, Derkinderen P, Letournel F. Diagnostic value of minor salivary glands biopsy for the detection of Lewy pathology. *Neurosci Lett*. 2013 Sep 13;551:62-4.

Article 5 : Expression et phosphorylation de la GFAP entérique dans la maladie de Parkinson.

Clairembault T, Kamphuis W, Leclair-Visonneau L, Rolli-Derkinderen M, Coron E, Neunlist M, Hol EM, Derkinderen P. Enteric GFAP expression and phosphorylation in Parkinson's disease. *J Neurochem*. 2014 Sep;130(6):805-15.

Article 6 : Les cellules gliales entériques : une participation nouvelle dans la maladie de Parkinson ?

Clairembault T, Leclair-Visonneau L, Neunlist M, Derkinderen P. Enteric glial cells: new players in Parkinson's disease? *Mov Disord*. 2015 Apr;30(4):494-8.

Article 7 : Que peut nous apprendre une biopsie gastro-intestinale sur la maladie de Parkinson ?

Corbillé AG, Clairembault T, Coron E, Leclair-Visonneau L, Preterre C, Neunlist M, Derkinderen P. What a gastrointestinal biopsy can tell us about Parkinson's disease? *Neurogastroenterol Motil*. 2016 Jul;28(7):966-74.

Article 4 : Valeur diagnostique de la biopsie des glandes salivaires accessoires dans la détection de la pathologie de type Lewy.

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Diagnostic value of minor salivary glands biopsy for the detection of Lewy pathology



Emmanuelle Folgoas^{a,1}, Thibaud Lebouvier^{a,b,1}, Laurène Leclair-Visonneau^{b,c}, Maria-Graciela Cersosimo^d, Annick Barthelaix^{e,f}, Pascal Derkinderen^{a,b,*}, Franck Letournel^{e,f,1}

^a CHU Nantes, Department of Neurology, Nantes, F-44093, France

^b Inserm, U913, Nantes, F-44093, France

^c CHU Nantes, Department of Physiology, Nantes, F-44093, France

^d Parkinson's Disease and Movement Disorder Unit, Hospital de Clínicas, University of Buenos Aires, Argentina

^e CHU Angers, Neurobiology and Neuropathology Laboratory, Angers F-49033, France

^f Université of Angers, UPRES EA3143, F-49033, France

HIGHLIGHTS

- Lewy pathology is present in the major salivary glands in PD.
- Lewy pathology was assessed in minor salivary glands biopsies in 16 PD patients.
- Lewy pathology was found in 3 out of 16 PD patients.
- Our results do not support the use of minor salivary glands biopsy for the detection of Lewy pathology.

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ABSTRACT

The recent demonstration of the presence of Lewy pathology in the submandibular glands of Parkinson's disease (PD) patients prompted us to evaluate the diagnostic performance of minor salivary gland biopsy for PD. Minor salivary glands were examined for Lewy pathology using phosphorylated alpha-synuclein antibody in 16 patients with clinically diagnosed PD and 11 control subjects with other neurological disorders. Abnormal accumulation of alpha-synuclein was found in 3 out of 16 PD patients. Two control subjects exhibited weak phosphorylated alpha-synuclein immunoreactivity. Our results do not support the use of minor salivary glands biopsy for the detection of Lewy pathology in living subjects.

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1. Introduction

PD is anatomically characterized by the progressive loss of dopaminergic neurons in the *substantia nigra* and the appearance of intracellular inclusions, named Lewy bodies and Lewy neurites whose main component is aggregated alpha-synuclein [8]. Comprehensive autopsy surveys have nevertheless demonstrated that Lewy pathology is much more extensive affecting brain regions

other than the *substantia nigra* and also peripheral nervous systems [2].

Some of the peripheral neuronal circuits affected by Lewy pathology are accessible to biopsies [11,12,14–16], making them an original source of markers that will directly assess the neuropathological process in living PD patients [13]. Because of the high density of Lewy pathology in the autonomic innervation of the submandibular gland [2,7], a recent report suggested that this major salivary gland might represent a promising biopsy site for PD [1]. This is however unlikely as histological analysis of the submandibular gland can only be achieved through incisional biopsy, an invasive procedure not devoid of complications [10]. Conversely, minor salivary gland biopsy is safe and routinely performed for diagnostic purposes [6]. A pilot study found alpha-synuclein aggregates in the minor salivary gland biopsy in 2 PD patients but these

* Corresponding author at: Department of Neurology, 44093 Nantes, France. Tel.: +33 0240065205; fax: +33 0240065203.

E-mail addresses: derkinderen@yahoo.fr, pascal.derkinderen@chu-nantes.fr (P. Derkinderen).

¹ These authors contributed equally to this work.

Table 1

Demographic and clinicopathological features of patients with Parkinson's disease. UPDRS: the Unified Parkinson's Disease Rating Scale; Phospho-syn: immunoreactivity for phosphorylated alpha-synuclein. NA: not assessed; ++: robust; -: negative.

PD patient number	Sex	Age	Disease duration	UPDRS III	Phospho-syn
1	M	73	21	NA	++
2	M	53	8	38	-
3	F	76	11	NA	-
4	M	73	10	26	-
5	M	88	9	37	-
6	F	76	13	51	-
7	F	65	12	14	-
8	M	69	8	38	-
9	F	66	16	51	-
10	M	63	14	28	-
11	M	71	26	34	-
12	F	51	11	49	++
13	F	61	9	30	-
14	M	49	11	13	++
15	M	62	8	22	-
16	F	70	10	34	-

preliminary results have to be confirmed [4]. We therefore undertook the present research to determine whether Lewy pathology can be detected in minor salivary gland biopsy in a larger population of PD and hence be used for a *premortem* diagnosis of the disease.

2. Patients and methods

Sixteen patients with PD who were diagnosed according to the United Kingdom PD Society Brain Bank [9] (9 male, mean age 66.6 ± 10 , Table 1) and 11 subjects with other neurological disorders (4 male, mean age 50.7 ± 16 , Table 2) participated in the study. Both PD patients and controls were excluded if they had Sicca syndrome. All controls underwent a neurological examination to rule out PD symptoms. For PD patients, motor impairment was assessed by the Unified Parkinson's Disease Rating Scale (UPDRS) Part III in off-state. No genetic screening was performed in PD patients. This study was carried out in accordance with the Declaration of Helsinki, conducted with the approval of the local Ethical Committee (*Comité de protection des personnes Ouest VI, Brest, France*) and registered on ClinicalTrials.gov (identifier NCT01748409). Written informed consent was obtained from each participant.

All minor salivary glands specimens were obtained by a unique biopsy technique that was performed through normal-appearing mucosa in the lower labial mucosa between the midline and commissure. Anesthesia was obtained with local infiltration of 2% xylocaine with vasoconstrictor. The lip was everted and a single

Table 2

Demographic and clinicopathological features of control subjects with other neurological disorders. CIDP: chronic inflammatory demyelinating polyneuropathy; Phospho-syn: immunoreactivity for phosphorylated alpha-synuclein; +: positive; -: negative; ±: weak/dubious.

Control number	Sex	Age	Diagnosis	Phospho-syn
1	F	61	CIDP	-
2	F	60	Multiple sclerosis	-
3	M	48	Myelitis	-
4	F	78	Vascular leucopathy	-
5	M	70	Paraneoplastic	-
6	F	57	Cranial multineuropathy	±
7	M	41	Cranial multineuropathy	-
8	F	23	Peripheral neuropathy	-
9	F	63	Peripheral neuropathy	-
10	F	38	Multiple sclerosis	±
11	M	40	Myelitis	-

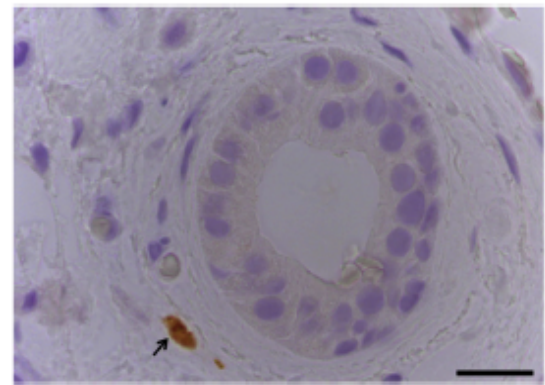


Fig. 1. Photomicrograph of immunohistochemical staining for phosphorylated alpha-synuclein in paraffin sections of a minor salivary gland showing a Lewy-like neurite around an acinus (arrow, patient 1). Scale bar: 20 μ m.

1.5–2 cm horizontal incision was made through the mucosa. The salivary glands were then released from their surrounding fascia, removed separately and immediately fixed with paraformaldehyde. Serial 4- μ m section of paraffin embedded tissue samples were cut and stained with Hematein–Eosin or incubated with anti-phospho-alpha-synuclein antibody (1:10,000, Wako, Osaka, Japan). All procedures were done using Leica-BOND III instruments. Sections were examined under a Zeiss microscope with AxioVisio 4.6 software. For each sample 3 levels of minor salivary gland were blindly examined by a neuropathologist (FL).

3. Results

Robust phosphorylated alpha-synuclein immunostaining was observed in minor salivary glands biopsy in 3 of 16 patients with PD (Fig. 1 and Table 1), while weak immunoreactivity was observed in 2 control subjects (Table 2). These phosphorylated alpha-synuclein-positive structures were mainly located in the periacinar stroma in both PD patients (Fig. 1) and controls.

No complications occurred after the biopsy procedure either in PD patients or control subjects.

4. Discussion

This study was primarily aimed at assessing whether minor salivary glands biopsy can be used as a routine diagnostic tool for the detection of Lewy pathology. Accordingly, we chose a conventional immunohistochemical method and examined 3 sections per biopsy. Definite Lewy pathology was observed in 3 of 16 PD patients.

Recently, two autopsy studies shed light on major salivary glands as a potential source of biopsiable markers for PD diagnosis [2,7]. Both surveys consistently showed that Lewy pathology was detectable at high density in the submandibular gland of almost all PD patients (9/9 and 22/23 cases, respectively). These results led Beach and collaborators to simulate needle core biopsy of submandibular glands that had been frozen at autopsy using a 18-gauge needle [1]. They demonstrated the presence of alpha-synuclein immunoreactive structures reminiscent of Lewy neurites in 17 of 19 PD patients when 3–4 tissues core per gland were analyzed [1]. Although encouraging, these results have been obtained on autopsy material and there is still a critical need for studies that will assess the feasibility and safety of submandibular gland biopsy and/or needle biopsy in living patients. Fine needle aspiration of the submandibular glands have been performed for the detection of tumoral cells in living patients [5], but is unlikely that

such a procedure that only gives access to smears of epithelial cells, will allow to detect Lewy pathology. Owing to the risk of injury of the marginal mandibular branch of the facial nerve, its analysis will probably never become a routine biomarker for PD [10].

By contrast, labial minor salivary glands biopsy is a technically simple, safe and straightforward procedure [3]. Moreover, the analysis of minor salivary glands allows the identification of autonomic fibers innervating both the acini and blood vessels, potentially making it an interesting biopsy site for the detection of Lewy pathology and hence for the diagnosis of PD. Our results, however, do not support this assumption as the sensitivity of this technique to detect Lewy pathology is only 19%, contrasting with the 100% sensitivity found when large submandibular gland sections were examined [1]. A potential explanation for these discrepancies is that the involvement of the accessory salivary gland with Lewy pathology could be too sparse to allow a high diagnostic sensitivity. One could also argue that the number of accessory salivary glands examined in our study is too small to reproducibly identify Lewy pathology. Nevertheless, performing biopsy with a larger incision in order to remove and analyze more minor salivary glands samples would go beyond the routine procedure and therefore increase the rate of complications such as lip numbness and bleeding [6].

In conclusion, due to its lack of sensitivity, our results do not support the use of routine labial biopsies for the detection of Lewy pathology in PD patients.

Author's contribution

EF, LLV and TL performed the minor salivary glands biopsies. EF, LLV, TL and PD performed the clinical assessment of patients. FL performed and analyzed the immunostaining. TL, MGC, AB, PD and FL designed the study, wrote the first draft and the final version of the manuscript.

Competing interests

The authors have no competing interests that could interfere with the present research.

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Article 5 : Expression et phosphorylation de la GFAP entérique dans la maladie de Parkinson.

ORIGINAL
ARTICLE



Enteric GFAP expression and phosphorylation in Parkinson's disease

Thomas Clairembault,^{*,†,‡} Willem Kamphuis,[§]
Laurène Leclair-Visonneau,^{*,†,¶} Malvyne Rolli-Derkinderen,^{*,†}
Emmanuel Coron,^{*,†,‡,¶} Michel Neunlist,^{*,†,‡} Elly M. Hol^{§,***,††} and
Pascal Derkinderen^{*,†,¶}

^{*}Inserm U913, Nantes, France

[†]University Nantes, Nantes, France

[‡]CHU Nantes, Institut des Maladies de l'Appareil Digestif, Nantes, France

[§]Astrocyte Biology & Neurodegeneration, Netherlands Institute for Neuroscience, an institute of the Royal Netherlands Academy of Arts and Sciences, Amsterdam, the Netherlands

[¶]Inserm CIC-04, Nantes, France

^{**}Swammerdam Institute for Life Sciences, Center for Neuroscience, University of Amsterdam, the Netherlands

^{††}Department of Translational Neuroscience, Brain Center Rudolf Magnus, University Medical Center Utrecht, Utrecht, the Netherlands

Abstract

Enteric glial cells (EGCs) are in many respects similar to astrocytes of the central nervous system and express similar proteins including glial fibrillary acidic protein (GFAP). Changes in GFAP expression and/or phosphorylation have been reported during brain damage or central nervous system degeneration. As in Parkinson's disease (PD) the enteric neurons accumulate α -synuclein, and thus are showing PD-specific pathological features, we undertook the present survey to study whether the enteric glia in PD become reactive by assessing the expression and phosphorylation levels of GFAP in colonic biopsies. Twenty-four PD, six progressive supranuclear palsy (PSP), six multiple system atrophy (MSA) patients, and 21 age-matched healthy controls were included. The expression levels and the phosphorylation state of GFAP were analyzed in colonic biopsies by western blot. Additional

experiments were performed using real-time PCR for a more precise analysis of the GFAP isoforms expressed by EGCs. We showed that GFAP κ was the main isoform expressed in EGCs. As compared to control subjects, patients with PD, but not PSP and MSA, had significant higher GFAP expression levels in their colonic biopsies. The phosphorylation level of GFAP at serine 13 was significantly lower in PD patients compared to control subjects. By contrast, no change in GFAP phosphorylation was observed between PSP, MSA and controls. Our findings provide evidence that enteric glial reaction occurs in PD and further reinforce the role of the enteric nervous system in the initiation and/or the progression of the disease.

Keywords: enteric glial cells, enteric nervous system, glial fibrillary acidic protein, multiple system atrophy, Parkinson's disease, progressive supranuclear palsy.

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Glial fibrillary acidic protein (GFAP) is a major constituent of glial intermediary filaments that form the cytoskeleton of mature astrocytes. To date, nine splice variants of GFAP have been described in the human central nervous system (Kamphuis *et al.* 2014). GFAP α is the canonical isoform and most of the immunohistochemical studies on astroglia have used antibodies that do not discriminate between GFAP isoforms. The assembly of GFAP is controlled by its

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Address correspondence and reprint requests to Pascal Derkinderen, Inserm U913, 1 place Alexis Ricordeau, 44093 Nantes, France. E-mails: derkinderenp@yahoo.fr; pascal.derkinderen@chu-nantes.fr

Abbreviations used: EGCs, enteric glial cells; ENS, enteric nervous system; GFAP, glial fibrillary acidic protein; MSA, multiple system atrophy; PAGE, polyacrylamide gel electrophoresis; PD, Parkinson's disease; PSP, progressive supranuclear palsy.

phosphorylation state, as its soluble phosphorylated pool is in dynamic equilibrium with the polymerized non-phosphorylated fraction of the protein (Inagaki *et al.* 1994). Several lines of evidence support a tight regulation of GFAP in neural development and also in the pathophysiology of several neurodegenerative disorders (Middeldorp and Hol 2011). GFAP gene mutations have indeed been associated with the fatal neurodegenerative condition Alexander disease (Yoshida and Nakagawa 2012). Furthermore, changes in GFAP expression and phosphorylation have been consistently reported in the central nervous system during neurodegenerative disorders such as Alzheimer's disease, frontotemporal dementia, and Parkinson's disease (PD) (Damier *et al.* 1993; Korolainen *et al.* 2005; Herskowitz *et al.* 2010; Kamphuis *et al.* 2014).

Astrocytes in the central nervous system are not the only cell type to express GFAP. In the early eighties, Jessen and Mirsky convincingly demonstrated that the glial cells in the enteric nervous system (ENS) were also immunoreactive for GFAP (Jessen and Mirsky 1980). This led to a reappraisal of the function and morphology of these glial cells, which were hitherto defined as Schwann cells. There is now a large body of evidence to support that the so-called enteric glial cells (EGCs) are in fact the digestive counterparts of central nervous system astrocytes (Gulbrandsen and Sharkey 2012). EGCs lie adjacent to the neurons in the enteric ganglia and envelop both the neuronal cell bodies and the axon bundles, an aspect that is highly reminiscent of the close relationship between astrocytes and neurons in the central nervous system (Jessen and Mirsky 1983). EGCs and astrocytes are also similar at the molecular and functional levels as they share electrophysiological and neuroprotective properties (Hanani 1993; Hanani *et al.* 2000; Abdo *et al.* 2010; Boesmans *et al.* 2013). In contrast to astrocytes, EGCs are readily accessible to biopsy and can therefore be analyzed in living patients (Lebouvier *et al.* 2010a; Neunlist *et al.* 2013). A routine colonic biopsy enables analysis of both mucosal and intraganglionic submucosal populations of EGCs and this approach has been used to demonstrate that GFAP is up-regulated in the gut of patients with inflammatory bowel disease (Boyen von *et al.* 2011).

It has become evident over the last 20 years that PD is not only a neurodegenerative brain condition but also a gut disorder (Cersosimo and Benarroch 2008; Derkinderen *et al.* 2011). Gastrointestinal symptoms are prominent non-motor manifestations of the disease (Edwards *et al.* 1993) and neuropathological studies showed the presence of Lewy pathology in the enteric neurons in the vast majority of patients (Beach *et al.* 2009). Our recent results showing an increase in total GFAP mRNA in the colon of PD patients suggest that enteric Lewy pathology in PD does not occur in isolation and may be accompanied by enteric glial reaction (Devos *et al.* 2013). In this study, we extended these preliminary results by determining whether this

increase in GFAP expression is specific for some isoforms and whether associated post-translational modifications are involved.

Patients and methods

Subjects

A total of 57 subjects participated in this study: 24 PD, six progressive supranuclear palsy (PSP), and six multiple system atrophy (MSA) patients as well as 21 healthy controls. PD patients aged 40–75 years were recruited from the movement disorder clinic at Nantes University Hospital, France. Diagnosis was made according to criteria provided by the United Kingdom Parkinson's Disease Survey Brain Bank (Hughes *et al.* 2002). PSP and MSA patients fulfilled the diagnostic criteria for possible or probable PSP (Litvan *et al.* 1996) and MSA (Gilman *et al.* 2008), respectively. Control subjects were healthy subjects who had a normal colonoscopy performed for colorectal cancer screening. All control subjects underwent a detailed 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*), conformed to the Code of Ethics of the World Medical Association (Declaration of Helsinki) and registered on ClinicalTrials.gov (identifier NCT00491062 and NCT01353183). Written informed consent was obtained from each patient and from each normal volunteer.

Endoscopic procedure and colonic biopsies

Four biopsies were taken in the sigmoid/descending colon during the course of a rectosigmoidoscopy for PD, PSP, and MSA patients and during a colonoscopy for control subjects. A follow-up call was scheduled 15 days after the endoscopic procedure. Biopsies were performed using standard biopsy forceps without needle (FB210K; Olympus co., Tokyo, Japan). Two biopsies were stored at -80°C in lysis buffer RA1 (Macherey-Nagel, Hoerd, France) for further analysis by real-time PCR and immunoblotting. The two remaining biopsies were snap frozen in liquid nitrogen at the time of collection and kept at -80°C .

Rat enteroglial cell line and treatment with serine/threonine phosphatases inhibitors

Enteric glial cell line was generated and cultured as previously described (Van Landeghem *et al.* 2011). At confluence, cells were treated with a cocktail of three phosphatase inhibitors for broad-spectrum inhibition of serine/threonine phosphatases including 1 μM okadaic acid, 5 μM cyclosporine A, and 6.75 μM sanguinarine (Sigma, Saint Quentin Fallavier, France) for 3 h or with vehicle (100 mM NaCl, 2 mM EDTA, 50 mM Tris-Cl, pH 7.4, and 50 mM NaF).

Human brain sample

A coronal frozen section of a human brain devoid of neurodegenerative pathological changes passing through the head of caudate nucleus (approximately 10 mm thick) was kindly provided by Pr Charles Duyckaerts, CRICM, Salpêtrière, Paris, France. Samples of frontal cortex and subventricular zone (SVZ) were taken and lysed in NETF (100 mM NaCl, 2 mM EDTA, 50 mM Tris-Cl, pH 7.4, and 50 mM NaF) buffer (protein concentration: 12 $\mu\text{g}/\mu\text{L}$ for undiluted cortex sample and 2 $\mu\text{g}/\mu\text{L}$ for undiluted SVZ sample) for western

blot analyses (see below). Because GFAP δ is mainly observed in the adult SVZ, a lysate of this brain region at a 1 : 10 dilution was used for the evaluation of the expression of this specific isoform (Roelofs *et al.* 2005). Lysate of frontal cortex either diluted at 1 : 10 or 1 : 1000 was used for the evaluation of the expression of all others GFAP isoforms.

Western blot

Following RNA isolation, total proteins from the two-pooled biopsies were precipitated and prepared for polyacrylamide gel electrophoresis (PAGE) using protein precipitator and resuspension buffer [Protein solving buffer and TCEP (tris(2-carboxyethyl) phosphine) reducing agent, PSB/TCEP] from NucleoSpin Triprep Kit (Macherey-Nagel, Hoerd, France) according to the manufacturer's instructions. For additional experiments on GFAP isoforms and phosphorylation, the two remaining biopsies that were dry frozen and stored at -80°C were lysed in NETF buffer containing 1% (v/v) IGEPAL CA-630 , 2 mM orthovanadate, phosphatase inhibitor cocktail II (Roche, Neuilly sur Seine, France) and a protease inhibitors cocktail (Roche) using the 'Precellys 24' tissue homogenizer (Bertin technologies, St Quentin-en-Yvelines, France) and followed by sonication with 'vibracell 75 186' device (Sonics, Newton, CT, USA). Human brain samples and EGCs were processed in the same way. Total protein was quantified using a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific, Cillebon sur Yvette, France) for samples prepared with PSB/TCEP and bicinchoninic acid protein assay kit (Pierce Thermo Scientific, Illkirch, France) for samples lysed in NETF buffer. Equal amounts of lysate were separated using the Invitrogen NuPage Novex Bis Tris MiniGels TM together with the 2-(N-morpholino)ethanesulfonic acid/sodium dodecyl sulfate (MES-SDS) running buffer before electrophoretic transfer to nitrocellulose membranes with the iBlot TM Dry Blotting System also from Invitrogen (Invitrogen, Cergy-Pontoise, France). In some experiments, the MES-SDS buffer was replaced by the 3-morpholinopropane-1-sulfonic acid/sodium dodecyl sulfate (MOPS-SDS) running buffer. Membranes were blocked for 1 h at 25°C in Tris-buffered saline (TBS) (150 mM NaCl, 15 mM Tris, 4.6 mM Tris Base, pH 7.4) with 5% non-fat dry milk and incubated overnight at 4°C with the primary antibodies. Details on the primary antibodies against GFAP are summarized in Table 1; mouse monoclonal anti- β -actin antibody was from Sigma and used at 1 : 10 000; rabbit anti-extracellular signal-regulated kinases was from Cell Signaling (Ozyme, Saint Quentin en Yvelines, France) and used at 1 : 1000. Bound antibodies were detected with horseradish peroxidase-conjugated anti-rabbit, anti-mouse antibodies (Amersham, Les Ulis, France; diluted 1 : 5000) or anti-goat

antibody (Santa Cruz Biotechnologies, Cliniscience, Nanterre, France; diluted 1 : 5000) and visualized by enhanced chemiluminescent detection (ECLPrime, Amersham). When necessary, membranes were stripped for 15 min in Reblot buffer Strong TM (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. The value of GFAP was normalized to the amount of beta-actin or extracellular signal-regulated kinases (ERKs), for comparison between biopsies and between biopsies and brain samples, respectively, in the same sample and expressed as a percentage of the average of controls. Phospho-GFAP immunoreactivity was normalized to GFAP immunoreactivity.

Membrane dephosphorylation

The specificity of the pSer13 antibodies (Table 1) was tested using western blotting and dephosphorylation treatment. Protein homogenates were electrophoresed, transferred to nitrocellulose membrane, and blocked for 1 h in TBS with 5% non-fat dry milk as performed above. The membrane to be dephosphorylated was placed in buffer (50 mM sodium acetate at pH 5.5 and 0.1% Tween 20) with 0.5 mg/mL acid phosphatase (Sigma) and incubated overnight at 37°C with gentle agitation. The control membrane was processed similarly but without acid phosphatase. Western blotting and stripping of membranes were performed as above.

Real-time PCR analysis

Total RNA (1.0 μg) was DNase I treated and used as a template to generate cDNA following the manufacturer's instructions (Quantitect Reverse Transcription Kit-Qiagen, Courtaboeuf, France) with a blend of oligo-dT and random hexamer primers. The reverse transcriptase reaction was incubated at 42°C for 30 min. The resulting cDNA was diluted 1 : 20 and served as a template in real-time qPCR assays (SYBR-Green PCR Master Mix; Applied Biosystems, Courtaboeuf, France). Primers were generated for the specific GFAP isoforms and tested for efficiency. The PCR signal was normalized against a set of reference genes to control for variability in the amount and quality of the RNA. Primer specificity and sensitivity are tested on dilution series of cloned GFAP isoforms to prevent unwanted cross-amplification of GFAP α , the most abundant transcript; details and primer sequences were described previously (Kamphuis *et al.* 2014).

Table 1 Human GFAP isoform-specific antibodies used in the present study

Name	Specificity	Epitope	Source and dilution
PanGFAP	α , δ , κ , $\Delta 135$, $\Delta \alpha 6$, $\Delta 164$	Full-length GFAP cow	Dako, Les Ulis, France, rabbit polyclonal Z0334 (1 : 2000)
GFAPmono	α , δ , κ , $\Delta 135$	ITIPVQTFNSNLQIR	Sigma, mouse monoclonal GA5 (1 : 1000)
GFAP N-term	α , δ , κ , $\Delta 135$	MERRRITSAARRSYVSSGEMMV	SCBT, mouse monoclonal F-2 (1 : 500)
GFAP C-term	α , $\Delta 135$	EMRDGEVIKESKQEHKDV	SCBT, goat polyclonal C-19 (1 : 500)
GFAP δ	δ	QAHQIVNGTPPARG	Millipore, rabbit polyclonal (1 : 500)
GFAP κ	κ	SLGAFVTLQRS	NIN, rabbit polyclonal (1 : 500)
pSer13	@Ser13	aa sequence around @Ser13	SCBT, rabbit polyclonal (1 : 1000)

Statistics

All data are given as the mean \pm SEM. For comparisons of means between groups, a Mann–Whitney test was performed. Differences were deemed statistically significant if $p < 0.05$.

Results

Clinical features

Clinical features of the study population are shown in Table 2. Age and sex did not differ significantly between patients and control subjects. No complications occurred in the 57 patients included in this study, either during or after the endoscopic procedure.

Enteric GFAP expression is increased in PD but not in related disorders

In a first set of experiments, we evaluated the expression levels of GFAP in colonic biopsies by western blot with an antibody that recognizes the large majority of known isoforms and truncated forms of the protein (PanGFAP antibody, Table 1) (Middeldorp and Hol 2011; Kamphuis *et al.* 2012, 2014). The amounts of GFAP protein in colonic biopsies from PD patients were compared to samples from healthy subjects and patients with PSP and MSA, two atypical parkinsonian syndromes in which the ENS, in contrast with PD, is spared by the pathological process (Wakabayashi *et al.* 2010; Pouclet *et al.* 2012).

Detection of GFAP on western blots of protein samples from colonic biopsies with the PanGFAP antibody revealed one prominent and two weaker bands, migrating at 55, 50, and 45 kDa, respectively (Fig. 1a). When the density of all three GFAP-immunoreactive bands was assessed, a significant 1.2 fold increase in GFAP expression was observed in biopsies from PD patients as compared with controls (Fig. 1b). By contrast, the expression levels of GFAP in biopsies from PSP and MSA patients were significantly lower than in healthy controls (Fig. 1b). A separate analysis of each of the three GFAP-immunoreactive bands showed that the density of the 55-kDa band was significantly lower in patients with atypical parkinsonism compared with control subjects (Fig. 1c). A significant 1.6 fold increase in the density of the 50-kDa band was observed in PD patients when compared with controls (Fig. 1d), while the density of the 45-kDa band was not different between controls and patients (Fig. 1e).

GFAP κ is the main isoform expressed in enteric glia

To date, nine splice variants of GFAP have been described in the human central nervous system (Middeldorp and Hol 2011; Kamphuis *et al.* 2014). GFAP α , δ , and κ encode long isoforms of the protein of 432, 431, and 438 amino acids in length, respectively, that migrate together on PAGE at approximately 55 kDa (Fig. 2 and Table 3) (Middeldorp and Hol 2011; Kamphuis *et al.* 2014). GFAP Δ 135, Δ Ex6, and Δ 164 encode shorter isoforms of the protein of 387, 366, and 347 amino acids, respectively, with a faster migration profile on PAGE ranging between 40 and 45 kDa (Hol *et al.* 2003). All these isoforms are recognized by the PanGFAP antibody (Fig. 2) (Kamphuis *et al.* 2014). Regarding the three remaining known isoforms GFAP β , γ , and ζ , their detection at the protein level by PanGFAP or other anti-GFAP antibodies has never been tested (Zelenika *et al.* 1995; Condorelli *et al.* 1999). This suggests that the major 55-kDa migrating band we observed in Fig. 1 contains at least GFAP α , δ , and/or κ , whereas the two fastest migrating band at 50 and 45 kDa represent the alternatively spliced Δ 135, Δ Ex6, Δ 164 isoforms and/or truncated forms of GFAP that can be generated by N-terminal post-translational truncation (Lee *et al.* 2000; Zoltewicz *et al.* 2012).

To better identify the GFAP isoforms expressed by enteric glia, the immunoreactivity of five additional antibodies was analyzed in both colonic biopsies and human brain extracts using an adapted western blotting approach. Western blot experiments were performed using the MOPS-SDS running buffer as this buffer allowed a better separation of the GFAP isoforms when compared to the MES-SDS running buffer that was used in Fig. 1 (Fig. 3a). The first antibody we used (GFAPmono), which is specific for GFAP α , δ , κ , and Δ 135 (Table 1 and Fig. 2) (Middeldorp and Hol 2011; Kamphuis *et al.* 2014) detected one major band along with several faster migrating bands in human frontal cortex lysate (Fig. 3b and Figure S1). The pattern of immunoreactivity was different in colonic biopsies with one single major band migrating at 55 kDa in most samples (Fig. 3b). Faint faster migrating bands were visible in some biopsies lysates (see lane 1, Fig. 3b). The second antibody we used (GFAP C-term) is specific for the carboxy-terminal tail of GFAP α and the alternatively spliced variant GFAP Δ 135 (Table 1 and Fig. 2) (Middeldorp and Hol 2011; Kamphuis *et al.* 2014). This antibody detected one band at 55 kDa when the human frontal cortex lysate was diluted at 1 : 1000 (Fig. 3b) and

Table 2 Demographic data of control subjects and patients

	Controls ($n = 21$)	PD ($n = 24$)	PSP ($n = 6$)	MSA ($n = 6$)
Age, years	63.6 \pm 2 (39–75)	61.1 \pm 1.5 (44–71)	71 \pm 2 (63–75)	61 \pm 4.1 (51–71)
Gender, M/F	11/10	13/11	3/3	3/3

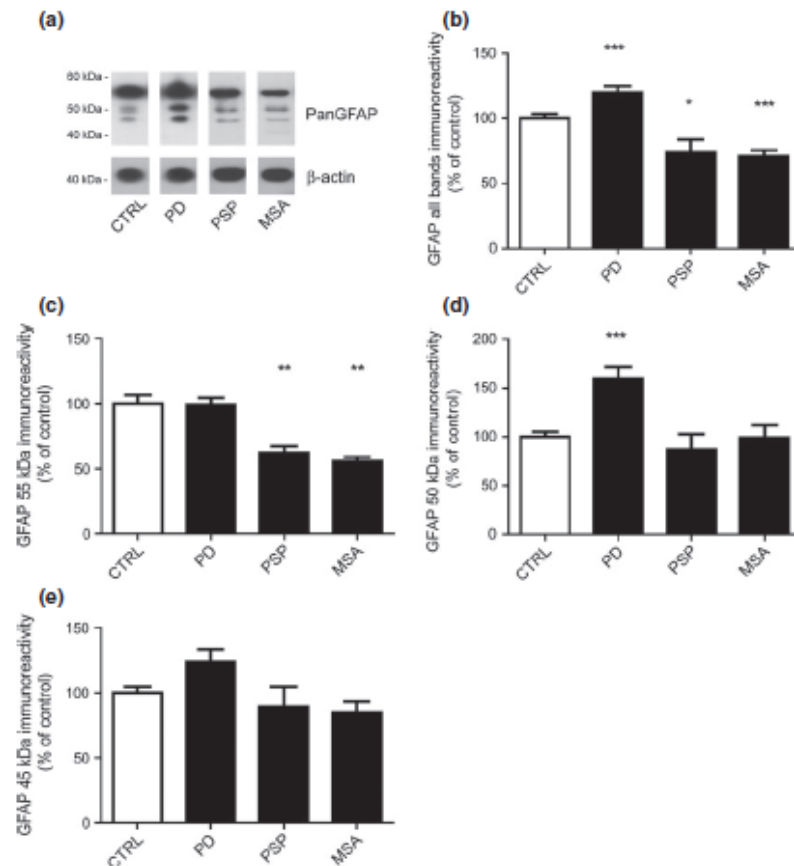


Fig. 1 Glial fibrillary acidic protein (GFAP) expression in colonic biopsies from patients with Parkinson's disease (PD), progressive supranuclear palsy (PSP), multiple system atrophy (MSA), and control subjects (CTRL). (a) Biopsies lysates (10 μ g of protein per sample) were subjected to immunoblot analysis using an antibody recognizing most known GFAP isoforms (GFAP, PanGFAP in Table 1) and beta-actin. The PanGFAP antibody detected three main bands in lysates of colonic biopsies, one major and two fainter bands, migrating at 55, 50, and 45 kDa, respectively. (b) Global quantification of all GFAP-immunoreactive bands. The optical densities of all three GFAP-immunoreactive bands were measured, normalized to the optical densities of beta-actin immunoreactive bands in the same

samples, expressed as percentages of controls and added. Data correspond to mean \pm SEM of 21 samples for control subjects (CTRL), 24 samples for PD patients and six samples for PSP and MSA. Patients versus control, * p < 0.05 and *** p < 0.001. (c, d, e) Individual quantification of the three GFAP-immunoreactive bands at 55, 50, and 45 kDa. The optical densities of each GFAP-immunoreactive band were measured, normalized to the optical densities of beta-actin immunoreactive bands in the same samples, and expressed as percentages of controls. Data correspond to mean \pm SEM of 21 samples for CTRL, 24 samples for PD patients and six samples for PSP and MSA. Patients versus control, * p < 0.05, ** p < 0.01 and *** p < 0.001.

two main bands migrating at 55 and 45 kDa when the 1 : 10 diluted lysate was used (Figure S1). In colonic biopsies, this antibody either detected a faint band at 55 kDa (Fig. 3b) or no specific band (Fig. 1). The third antibody (GFAP N-term), which recognizes intact GFAP α , δ , κ , and Δ 135 but not their amino-terminal cleavage products (Table 1 and Fig. 2) detected one band migrating at 55 kDa in human frontal cortex lysate diluted at 1 : 1000 (Fig. 3b) and two bands, one major and one minor migrating at 55 and 50 kDa, respectively, in the more concentrated brain lysate (Figure S1). A single major 55-kDa band was observed when this antibody was used in colonic biopsies (Fig. 3b and

Figure S1). An antibody specific for human GFAP δ (Table 1 and Fig. 2) that does not cross-react with other splice variants (Roelofs *et al.* 2005) failed to detect any specific band at the expected molecular weight in colonic biopsies (Fig. 3b), while it recognized a band migrating at the expected size in SVZ lysate (Fig. 3b). Finally, an antibody raised against a specific sequence of GFAP κ (Table 1) (Kamphuis *et al.* 2014) detected one major strong band migrating at 55 kDa in biopsies and a fainter band of approximately the same size in frontal cortex lysate (Fig. 3b). An additional faster migrating band was also observed in some samples (Fig. 3b). Taken together these

GFAP Alpha 432 aa, 49.9 kDa

```

1 merrrritsaa rrvsvvasgcm mvggglapgr lpggtrlsia rmpplptrv dflagalna
  61 gfketraser aemmelndrf asyiekvrfl eqqnkalaee lnqrakept kladvygae1
  121 relrlrdql tansarleve rdnlagdlat vrqklqdetn lrleaennla ayrqadeat
  181 larldlerki esleeeirfl rkiheeevre lqeqlarqqv hveldvakpd ltaalkeirt
  241 qyeamassnm heaeewyrsk fadltdaaar naellrqakh eandyrrqlq sltcdleslr
  301 gtnealerqm reqeerhvre aasyqealar leeeegskld emarhlqeyq dlnvklald
  361 ieiatyrkll egeenritip vgtfenlqir etsldtksvs eghikrnivv ktvmrrdqev
  421 ikeskqehkd vm
    
```

GFAP Delta 431 aa, 49.5 kDa

```

1 merrrritsaa rrvsvvasgcm mvggglapgr lpggtrlsia rmpplptrv dflagalna
  61 gfketraser aemmelndrf asyiekvrfl eqqnkalaee lnqrakept kladvygae1
  121 relrlrdql tansarleve rdnlagdlat vrqklqdetn lrleaennla ayrqadeat
  181 larldlerki esleeeirfl rkiheeevre lqeqlarqqv hveldvakpd ltaalkeirt
  241 qyeamassnm heaeewyrsk fadltdaaar naellrqakh eandyrrqlq sltcdleslr
  301 gtnealerqm reqeerhvre aasyqealar leeeegskld emarhlqeyq dlnvklald
  361 ieiatyrkll egeenritip vgtfenlqir gqkstkdgen hkvtrykksl tirvipigah
  421 qivnqtppar g
    
```

GFAP Kappa 438 aa, 50.3 kDa

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1 merrrritsaa rrvsvvasgcm mvggglapgr lpggtrlsia rmpplptrv dflagalna
  61 gfketraser aemmelndrf asyiekvrfl eqqnkalaee lnqrakept kladvygae1
  121 relrlrdql tansarleve rdnlagdlat vrqklqdetn lrleaennla ayrqadeat
  181 larldlerki esleeeirfl rkiheeevre lqeqlarqqv hveldvakpd ltaalkeirt
  241 qyeamassnm heaeewyrsk fadltdaaar naellrqakh eandyrrqlq sltcdleslr
  301 gtnealerqm reqeerhvre aasyqealar leeeegskld emarhlqeyq dlnvklald
  361 ieiatyrkll egeenritip vgtfenlqir gqyrsasweg hwsppasra crltqtgtd
  421 qgkqqlslg avvtlqra
    
```

GFAP Δ135 387 aa, 44.5 kDa

```

1 merrrritsaa rrvsvvasgcm mvggglapgr lpggtrlsia rmpplptrv dflagalna
  61 gfketraser aemmelndrf asyiekvrfl eqqnkalaee lnqrakept kladvygae1
  121 relrlrdql tansarleve rdnlagdlat vrqklqdetn lrleaennla ayrqadeat
  181 larldlerki esleeeirfl rkiheeevre lqeqlarqqv hveldvakpd ltaalkeirt
  241 qyeamassnm heaeewyrsk fadltdaaar naellrqakh eandyrrqlq sltcdleslr
  301 gtnyqdlnv klaldieiat yrkllegeen ritipvgtfs nlqiretsld tksvseghik
  361 rnivkvtvm rdgevikesk qehkdvm
    
```

Fig. 2 Epitopes recognized by the different anti-gial fibrillary acidic protein (GFAP) antibodies in the four main human GFAP isoforms. GFAPmono (single underline); GFAP C-term (double underline); GFAP δ (dashed underline); GFAP N-term (thick underline); GFAP κ (bold wavy underline). NCBI reference sequence: GFAP α NP_002046.1; GFAP δ: NP_001124491.1; GFAP κ: NP_001229305.1. The expected molecular weights are indicated.

Table 3 Results of quantitative polymerase chain reaction assays

	C	C	C	PD	PD	PD	PD	PD	PD	PD	Mean ± SEM
α/α	0.28	2.40	0.78	1.28	1.32	0.18	0.13	4.94	3.83		1.68 ± 0.57
β/α	0.018	–	0.026	0.036	–	0.001	–	0.029	–		0.022 ± 0.006

Data are presented as ratio of GFAPκ and GFAPβ transcripts to GFAPα transcript.

results show that enteric glia does not express GFAPδ and Δ135, and that GFAPκ is only a minor component of the main 55 kDa band detected by PanGFAP. Furthermore, the results obtained with GFAPκ and GFAP N-term antibodies strongly suggest that GFAPκ accounts for most of the GFAP immunoreactivity observed at 55 kDa and that the fastest migrating bands observed in PanGFAP immunoblots represent truncated products of this isoform.

To further refine the GFAP isoforms that are expressed by EGCs, PCR analyses were performed in biopsies from three control subjects and six PD patients. Transcripts for GFAPκ and α were the only ones to be consistently detected in all nine samples with the κ transcripts being 1.7-fold more abundant than the α transcript (Table 3). GFAPβ transcript was detected in five of the nine samples with an average β/α ratio of 2% (Table 3). All other isoforms transcripts, including GFAPδ and Δ135, were not or barely detectable. As a whole, our results obtained at the transcript and protein

levels are consistent and demonstrate that GFAPκ is the main isoform expressed in enteric glia.

Enteric GFAP phosphorylation at serine 13 is decreased in PD

GFAP can be phosphorylated at multiple sites in its amino-terminal domain, including threonine 7, serine 8, 13, 17, and 34 (Inagaki *et al.* 1994). Among these sites, serine 13 has recently received much attention as it has been shown to be regulated during central nervous system neurodegeneration (Herskowitz *et al.* 2010). We have therefore studied the regulation of GFAP phosphorylation at this residue in the ENS using a rabbit polyclonal phospho-specific antibody (pSer13, Table 1). As a first step, we have validated the specificity of this antibody in enteric glia by treating rat enteroglia cells with a combination of serine/threonine phosphatase inhibitors (okadaic acid, ciclosporin A, and sanguinarine) and by incubating nitrocellulose membranes

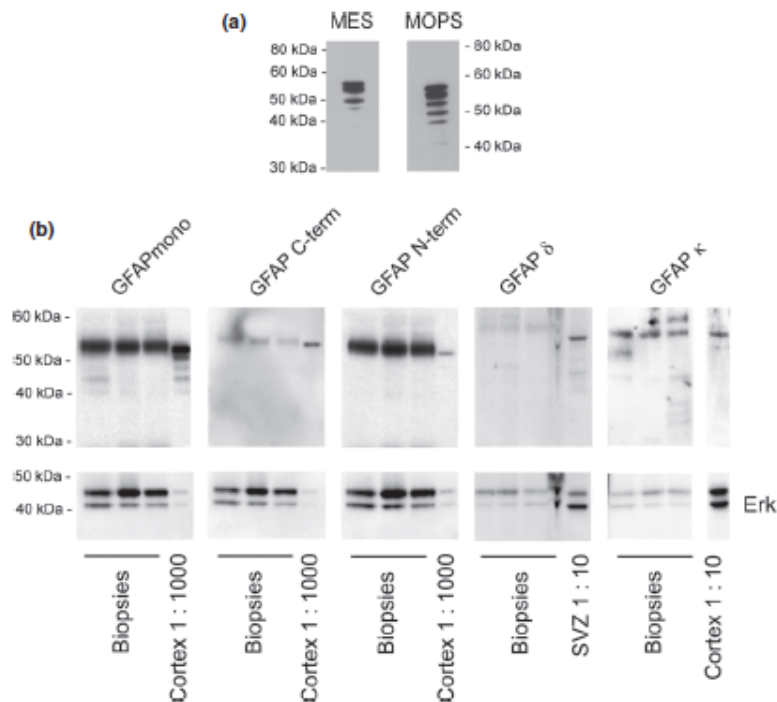


Fig. 3 Glial fibrillary acidic protein (GFAP) isoforms expressed by enteric glia. (a) Comparison of immunoblot profiles between MES-SDS and MOPS-SDS running buffers. A colonic biopsy from a control subject was subjected to PanGFAP immunoblot analysis using either MES-SDS running buffer (MES) and MOPS-SDS running buffer (MOPS). (b) Colonic biopsies and brain samples lysates from healthy subjects were subjected to immunoblot analysis using the MOPS-SDS running buffers and various antibodies against specific isoforms of GFAP: GFAPmono specific for GFAP α , δ , κ , and $\Delta 135$; GFAP C-term specific for the carboxy-terminal tail of GFAP α and of the alternatively spliced

variant GFAP $\Delta 135$; GFAP N-term, which recognizes intact GFAP α , δ , κ , and $\Delta 135$ but not their amino-terminal cleavage products; isoform-specific anti-GFAP δ and anti-GFAP κ . The human frontal cortex lysate (Cortex) was diluted at 1 : 1000 for experiments with GFAPmono, GFAP C-term, and GFAP N-term, as these antibodies detect GFAP α , the most abundant GFAP isoform in brain. Human frontal cortex and subventricular zone (SVZ) lysates were both diluted at 1 : 10 for the evaluation of the GFAP κ and GFAP δ isoforms, respectively. Extracellular signal-regulated kinases (ERK) immunoblot was used as a loading control between biopsies and brain samples.

with acid phosphatase. pSer13 detected one major band migrating at 55 kDa in rat enteroglial cells only in the presence of phosphatase inhibitors (Fig. 4a). No immunoreactivity for pSer13 was observed if the membranes were dephosphorylated, while immunoblotting for PanGFAP revealed that regular GFAP was detected in both the normal and the dephosphorylated membrane (Fig. 4a). In colonic biopsies, pSer13 detected two bands, one major and one minor at 55 and 50 kDa, respectively, that comigrated with the two matching bands labeled by PanGFAP (Fig. 4b). Although highly variable between individuals, quantification of the X-ray films revealed that the expression levels of GFAP phosphorylated at serine 13 were significantly lower in PD patients as compared with control subjects (Fig. 4b, c and Figure S2). By contrast, no significant changes in pSer13 immunoreactivity were observed between PSP and MSA patients when compared with controls (Fig. 4b, c). Because the RA1 buffer that was used for storage and lysis of biopsies does not contain any phosphatase inhibitors, additional

experiments were performed to show that our results were not the mere consequence of sample dephosphorylation. To this end, snap-frozen biopsies were lysed at 4°C in the presence of the tyrosine phosphatase inhibitor sodium orthovanadate and serine/threonine phosphatase inhibitors before being processed for western blotting. A decrease in pSer13 immunoreactivity was still observed in colonic biopsies from PD patients when this lysis method was used, confirming that our results were not because of artifactual dephosphorylation during sample preparation (Figure S3).

Discussion

Altogether, our previous results (Devos *et al.* 2013) and this study show that the expression of GFAP is increased at both mRNA and protein levels in mucosal and submucosal EGCs in PD. In analogy with the central nervous system, it has been proposed that GFAP up-regulation in the gut is induced by the activation of EGCs, a phenomenon known as reactive

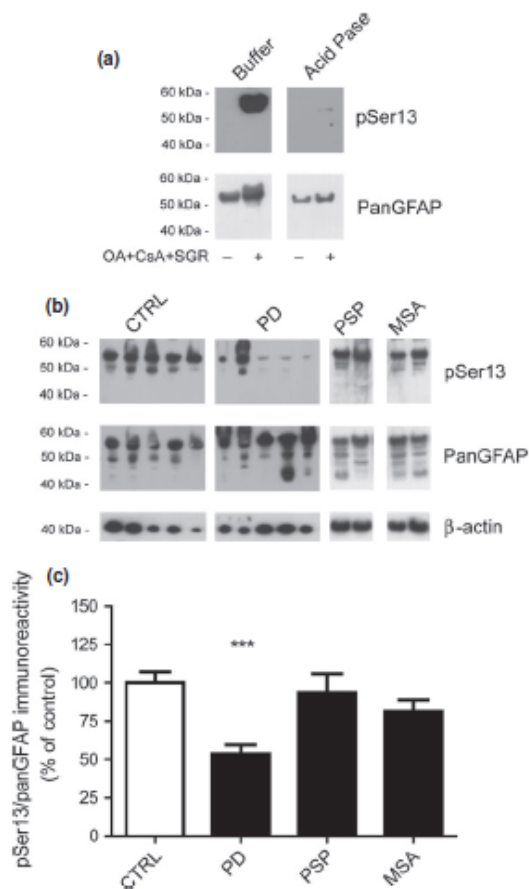


Fig. 4 Glial fibrillary acidic protein (GFAP) phosphorylation at serine 13 in colonic biopsies from Parkinson's disease (PD), multiple system atrophy (MSA) and progressive supranuclear palsy (PSP) patients and in control subjects. (a) The specificity of the antibody against the phosphorylated form of GFAP phosphorylated at serine 13 (pSer13) was assessed in enteroglial cells treated or not with a cocktail of three phosphatase inhibitors including 1 μ M okadaic acid, 5 μ M ciclosporine A, and 6.75 μ M sanguinarine (OA+CsA+SGR) for 3 h or with vehicle. Fifteen μ g of cell lysates were subjected to immunoblot analysis, with the nitrocellulose membrane being treated with acid phosphatase (acidPase) or not (buffer) before incubation with pSer13 antibodies. (b) Biopsies lysates (15 μ g of protein per sample) were subjected to immunoblot analysis using pSer13 antibody and reprobated with PanGFAP and beta-actin antibodies. The GFAP-Phospho-Ser13 antibody detects two bands at 55 and 50 kDa that comigrate with the two corresponding bands detected by PanGFAP antibody, either in biopsies from controls (CTRL), PD, PSP or MSA. (c) Quantification of the two immunoreactive bands at 55 and 50 kDa detected by the pSer13 antibody. The optical densities of the two pSer13-immunoreactive bands were measured, normalized to the optical densities of PanGFAP-immunoreactive bands in the same samples, expressed as percentages of controls and added. Data correspond to mean \pm SEM of 12 samples for CTRL, 19 samples for PD patients, 6 samples for MSA, and 5 samples for PSP patients (patients vs. control, *** p < 0.001).

gliosis. In the past few years, several studies have demonstrated that EGCs are critically involved in maintaining gut homeostasis (Neunlist *et al.* 2013) and especially in regulating gut inflammation (Cabarrocas *et al.* 2003; Ruhl 2005). Reactive gliosis as well as morphologically altered EGCs have been reported in the gut of patients with inflammatory bowel disease (Cornet *et al.* 2001; Boyen von *et al.* 2011) and studies in transgenic animals have showed that enteric glia ablation resulted in severe gut inflammation (Bush *et al.* 1998; Cornet *et al.* 2001; Aube *et al.* 2006). Further supporting the link between EGCs and inflammation, *in vitro* experiments obtained in cultured EGC showed that pro-inflammatory cytokines such as tumor necrosis alpha increase the expression levels of GFAP (Boyen von *et al.* 2004) and that once reactive, enteric glia is capable of secreting interleukin-6 (Ruhl *et al.* 2001). Regarding PD, we have recently shown that the expression levels of the main pro-inflammatory cytokines were increased in the colonic biopsies from PD patients and correlated with the expression of GFAP mRNA (Devos *et al.* 2013). By demonstrating the occurrence of glial reaction in the gut of PD patients, our results strongly support the assumption that PD is not restricted to the brain but is rather a systemic disorder that affects the peripheral autonomic networks and in particular the ENS (Braak and Del Tredici 2008; Beach *et al.* 2009). The data also reinforce the role of peripheral inflammation and associated glial reaction in the initiation and progression of the disease (Lema Tomé *et al.* 2013).

Various degrees of astrocytic reaction have been reported in the brain of parkinsonian syndromes, including PD, PSP, and MSA. In PD, reactive astrogliosis is usually mild, but abnormal synuclein deposition occurs in astrocytes, whereas MSA and PSP cases show marked astrocytic reaction, which is thought to contribute to neurodegeneration (Song *et al.* 2009). We show in the present report that, by contrast to PD, the levels of GFAP in colonic biopsies from PSP and MSA patients are either comparable or lower to the control subjects. This lack of increase in GFAP expression in the gut of MSA and PSP patients is a strong argument against the occurrence of enteric reactive gliosis and strongly suggests that the pathology in PSP and MSA is limited to the central nervous system. This is further supported by a small set of studies that showed the absence or paucity of pathologic lesions in the peripheral nervous systems in these two disorders (Wakabayashi *et al.* 2010; Pouclet *et al.* 2012).

Regarding the GFAP isoforms, our results were consistent between real-time PCR and western blot analyses and showed that GFAP κ , which is the most recently discovered GFAP isoform (Blechingberg *et al.* 2007), is by far the major isoform expressed by EGCs obtained from gastrointestinal biopsies. This stands in sharp contrast with the data that were recently obtained in human brain showing that the median expression level of GFAP κ transcript was 1.1% when the

level of GFAP α was set at 100% (Kamphuis *et al.* 2014). The GFAP κ protein has a C-terminal tail that is different from the C-terminal tails of the GFAP α and GFAP δ isoforms and it has been suggested that these differences may have a physiological consequence as GFAP κ , in contrast with GFAP α , has a low propensity to form homomeric intermediate filaments (Blechingberg *et al.* 2007). Whether the high expression level of GFAP κ in the gastrointestinal tract has an impact on the physiology of EGCs still needs to be studied. Furthermore, it remains to be determined if the EGCs from the myenteric plexus, which are not accessible to routine gastrointestinal biopsies, also preferentially express GFAP κ like their mucosal and submucosal counterparts.

The phosphorylated residues located at the amino terminus of GFAP, including threonine 7, serine 8, 13, 17, and 34, are involved in the regulation of the protein self-assembly (Inagaki *et al.* 1994). Phosphorylation of GFAP at its amino-terminus residues causes disassembly of the intermediate filaments and conversely its dephosphorylation restores its potential to assemble (Takemura *et al.* 2002). As the integrity of the cytoskeleton is essential for normal astrocyte function, it has been suggested that GFAP phosphorylation may be an important regulatory mechanism in central nervous system disorders. In spite of this, there are very few studies that have examined the phosphorylation state of GFAP in pathological conditions. Using two-dimensional immunoblotting, Korolainen and collaborators convincingly showed that the total amount of phosphorylated GFAP was increased in Alzheimer's disease brains (Korolainen *et al.* 2005). Only two reports used a phospho-specific antibody to examine GFAP phosphorylation in brain neurodegeneration or insults. Using a phosphoproteomic analysis, Herskowitz *et al.* showed that the phosphorylation of GFAP at serine 13 was increased in the brain of individuals with frontotemporal lobar degeneration, a result that was validated by immunoblot with a phospho-specific antibody for GFAP phosphorylated at serine 13 (Herskowitz *et al.* 2010). Recently, Sullivan *et al.* (2012) demonstrated that the occurrence of hypoxia in pig brains was associated with increased amount of phosphorylation of GFAP at serine 13. Our findings showing GFAP hypophosphorylation at serine 13 in enteric glia during PD are thus apparently at odds with previous observations in brain damage. Nevertheless, given the role of GFAP phosphorylation in the plasticity of glia cytoskeleton, it could be argued that glial cells respond differently depending on the neurodegenerative process and that, by contrast to Alzheimer's disease and frontotemporal dementia, reactive gliosis in PD is associated with a drop in GFAP phosphorylation. In this respect, it is worth noting that the phosphorylation state of GFAP has hitherto not been investigated in the brain of PD patients. As a logical follow-up of our study, it would be logical to study whether GFAP is also hypophosphorylated in brain astrocytes during PD.

There is increasing interest in bidirectional signaling between the gut microbiota and brain and the potential impact of this communication on the development of psychiatric and neurological disorders, leading to the concept of a microbiota-gut-brain axis (Forsythe *et al.* 2012). Although the precise mechanisms through which signals from gut bacteria are communicated to the brain are still largely unknown, evidence obtained from vagotomy experiments point toward a key role for the vagus nerve in the interplay between the microbiota and the brain (Forsythe *et al.* 2012). In this context, the enteric neurons, which are embedded in the wall of the gastrointestinal tract, show some unique features that make them prime candidates to act as a first relay between gut microbiota and the brain as: (i) some of them, located in the submucosal plexus send axons to the gut mucosa that are only micrometers away from the gut lumen and thus from the gut flora, (ii) their neurochemical phenotype and electrophysiological properties can be modulated by changes in the composition of gut microbiota (Kunze *et al.* 2009), and (iii) they synapse with both afferent and efferent vagal neurons (Walter *et al.* 2009). With regard to PD, Braak suggested that the involvement of the enteric neurons by Lewy pathology was an early event in the development of the disease. This led to the assumption, the so-called Braak's hypothesis, that PD pathology may in fact begin in the gastrointestinal tract further spreading to the central nervous system via the vagal pre-ganglionic innervation of the gut and thus following the brain-gut axis (Braak *et al.* 2006). Our results, showing significant changes in EGC during PD, further reinforce a possible role of the enteric nervous system in the initiation or the progression of the disease. Further work will be needed to determine whether changes in gut microbiota occur in PD and whether these changes are capable of modifying enteric neurons and EGC.

Despite technological advances in the field of molecular genetics and in *in vivo* imaging, 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. The observations that demonstrated that Lewy pathology is not limited to the central nervous system but also involves peripheral tissues accessible to biopsies including skin, salivary glands, and gut, provide new opportunities to develop original histopathological markers of the disease that will directly assess the pathological process *in vivo* (Lebouvier *et al.* 2010c). Remarkably, by contrast to skin and salivary glands, a gut biopsy does not only contain post-ganglionic neuronal processes but a dense network of neurons and EGCs (Lebouvier *et al.* 2010a,b). By showing differences in the expression and phosphorylation of GFAP, our results support the use of a single colonic biopsy as an original source of biomarkers in PD beyond the sole assessment of Lewy pathology.

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All experiments were conducted in compliance with the ARRIVE guidelines.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1. Immunoblots of colonic biopsies and brain samples lysates diluted at 1 : 10 with GFAPmono, C-Term, and N-term antibodies.

Figure S2. Representative immunoblots of colonic biopsies lysates showing the heterogeneous phosphorylation of GFAP at serine 13 between PD patients.

Figure S3. GFAP phosphorylation at serine 13 in colonic biopsies lysed in NETF buffer.

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Article 6 : Les cellules gliales entériques : une participation nouvelle dans la maladie de Parkinson ?

VIEWPOINT

Enteric Glial Cells: New Players in Parkinson's Disease?

Thomas Clairembault, BSc,^{1,2,3} Laurène Loclair-Visonneau, MD,^{1,2,4} Michel Neunlist, PhD,^{1,2,3} and Pascal Derkinderen, MD, PhD^{1,2,4}

¹Inserm, U913, Nantes, F-44093, France

²University Nantes, Nantes, F-44093, France

³CHU Nantes, Institut des Maladies de l'Appareil Digestif, Nantes, F-44093, France

⁴Inserm, CIC-04, Nantes, F-44093, France

ABSTRACT: Lewy pathology has been described in neurons of the enteric nervous system in nearly all Parkinson's disease (PD) patients at autopsy. The enteric nervous system not only contains a variety of functionally distinct enteric neurons but also harbors a prominent component of glial cells, the so-called enteric glial cells, which, like astrocytes of the central nervous system, contribute to support, protect, and maintain the neural network. A growing body of evidence supports a role for enteric glial cells in the pathophysiology of gastrointestinal disorders such as

inflammatory bowel disease and chronic constipation. We have recently shown that enteric glial cell dysfunction occurs in PD. In the present review, we discuss the possible implications of enteric glia in PD-related gut dysfunction as well as in disease initiation and development. © 2014 International Parkinson and Movement Disorder Society

Key Words: Parkinson's disease; enteric nervous system; enteric glial cells; GFAP

Over the last 15 years, that Parkinson's disease (PD) is a gut disorder has become evident.¹ Gastrointestinal symptoms occur in almost every PD patient at some point and are among the most debilitating non-motor features of the disease.² These clinical data have been supported by several pathological studies demonstrating the presence of Lewy bodies and neurites (together referred to as Lewy pathology) in the enteric nervous system. Since the first demonstration of Lewy bodies in the enteric nervous system in 1984,³ Lewy pathology has been confirmed in the enteric neurons in nearly every case examined pathologically.^{4,5}

The enteric nervous system also contains a population of glial cells, the so-called enteric glial cells, which are likely to represent the digestive counterpart of brain astrocytes.⁶ Several recent studies have

indicated that the enteric glial cells participate in the regulation of gastrointestinal functions and that they may be critically involved in the pathophysiology of gastrointestinal disorders such as inflammatory bowel disease and chronic constipation.^{6,7} We have recently shown that biochemical changes suggestive of glial dysregulation occurs in enteric glial cells in PD.^{8,9} This led us to propose that the enteric glial cells might be involved in PD-related gastrointestinal dysfunction as well as in disease initiation and development.

Enteric Glial Cells, the Gut Astrocytes

Dogiel was the first to describe the presence of glial cells within the enteric nervous system at the end of the nineteenth century.⁶ Nevertheless, for more than 70 years, enteric glia were largely ignored and frequently defined as Schwann cells. In his detailed anatomical description of the guinea-pig myenteric plexus, Gabella noted that enteric glial cells, such as astrocytes, carry extensive branchings and irregular processes that mingle with neuronal cell bodies and the axon bundles.¹⁰ A turning point came in the early 1980s when Jessen and Mirsky demonstrated that enteric glial cells were immunoreactive for the two

*Correspondence to: Dr. Derkinderen, Inserm U913, 1 place Gaston Veil, 44000 Nantes, France. E-mail: derkinderen@yahoo.fr; pascal.derkinderen@chu-nantes.fr

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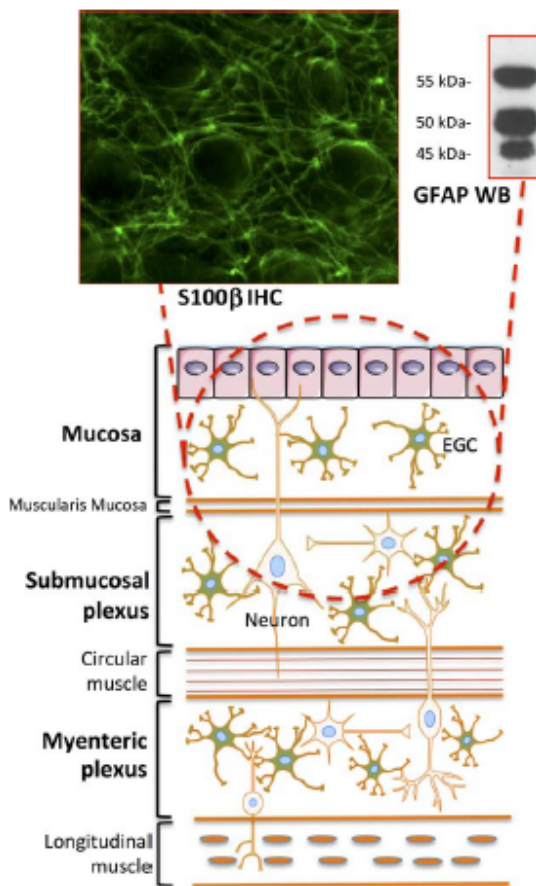


FIG. 1. Populations of enteric glial cells within the gut wall. A subpopulation of enteric glial cells is located directly underneath the epithelial cells and is involved in the regulation of intestinal epithelial barrier function. Enteric glial cells of the submucosal and myenteric plexus are mingled with neurons and are involved in the regulation of enteric neurotransmission. Enteric glial cells lying under the mucosa and in the submucosal plexus are readily analyzable using routine gastrointestinal biopsies (dashed line). Following microdissection of the biopsy, the glial network can be analyzed by immunohistochemistry (S100 β immunostaining). The whole biopsy also can be processed by Western blot for the analysis of the expression levels of glial markers, for instance, GFAP. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

canonical astrocyte markers, namely glial fibrillary acidic protein (GFAP) and S100-beta (Fig. 1).¹¹ More recent studies showed that the enteric glial cells and astrocytes are also similar at the functional levels, as they share electrophysiological and neuroprotective properties. Likewise, their central nervous system counterparts, enteric glial cells, possess a number of voltage-gated ion channels¹² and express neurotransmitters receptors such as purinergic, adrenergic, glutamate metabotropic receptors and are therefore capable of modulating synaptic transmission.^{13,14} Reduced glutathione and prostaglandins have been identified as factors secreted by the enteric glial cells protecting neurons from dopamine-induced oxidative stress.¹⁵

The glial population of the gut is unlikely to be homogenous, and unique populations of enteric glial cells may reside at multiple levels through the gut wall, the unique microenvironments of the gastrointestinal tract defining the phenotype of these cells (Fig. 1).⁶ Thus, enteric glial cells that lie in the mucosa directly underneath the epithelial cells influence epithelial cells and intestinal epithelial barrier function, whereas enteric glial cells of the submucosal or myenteric plexus embed neurons and are involved in the regulation of neurotransmission (Fig. 1).⁶ By contrast to the central nervous system astrocytes, the enteric glial cells are readily accessible and analyzable through routine gastrointestinal biopsies. We have shown that the microdissection of a routine colonic biopsy enables an immunohistochemical assessment of the glial network located either in the mucosa or in the submucosal plexus (Fig. 1).¹⁶ Biopsies also can be processed by polymerase chain reaction and Western blot, which can be used to measure quantitative differences in glial markers (Fig. 1).^{8,9,16}

Enteric Glial Cells in Gastrointestinal Physiology and Pathology

The intestinal epithelium forms a regulated barrier between the blood circulation and the contents of the intestinal lumen, preventing the passage of noxious contents while allowing the absorption and secretion of nutrients.¹⁷ Increasing evidence suggests that factors secreted by the enteric glial cells that are located directly underneath the intestinal epithelial cells (Fig. 1) are involved in the differentiation of epithelial cells and, as such, regulate gut barrier function.¹⁸

Increased expression of glial markers and especially of GFAP is a hallmark of reactive astrocytes in the central nervous system.¹⁹ Changes in the expression levels of the glial markers GFAP and S100-beta have been observed in various gastrointestinal disorders associated with barrier dysfunctions, including inflammatory bowel disorders. Both GFAP and S100-beta are up-regulated in the colons of patients with ulcerative colitis, whereas their expression levels are either unchanged or inconsistently increased in Crohn's disease.²⁰ This strongly suggests that the pattern of enteric glial dysregulation is different depending on the underlying pathological process and that ulcerative colitis might therefore represent a prototypical disorder for reactive enteric gliosis. Whether the reactive gliosis observed in inflammatory bowel disease is only a bystander effect or whether it participates actively in the inflammatory process remains to be elucidated. Slow-transit constipation, which defines patients with markedly delayed gut transit refractory to usual therapeutics, is another gastrointestinal pathology in which a role for the enteric glial cells has been suggested.

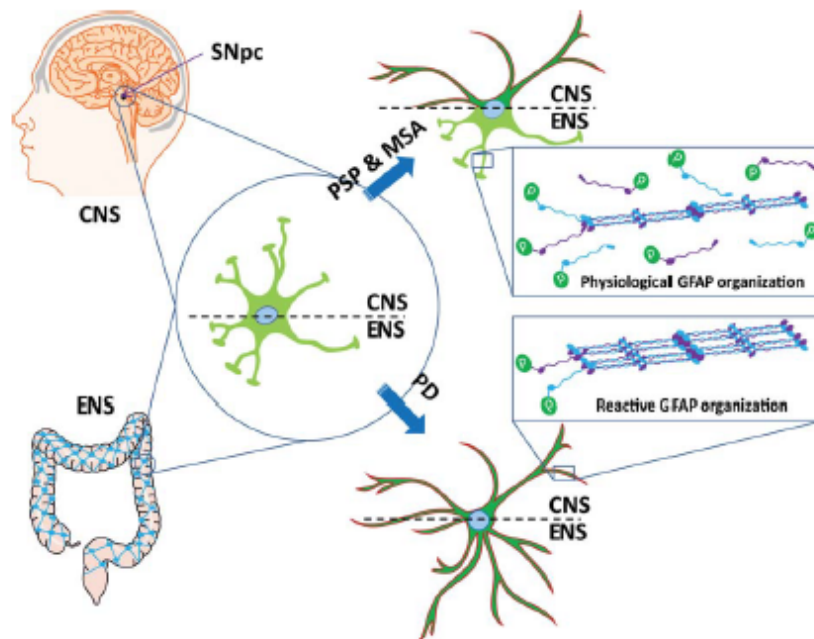


FIG. 2. Changes in enteric GFAP phosphorylation in PD. Astrocytic reactions characterized by morphological or biochemical changes of astrocytes have been described in the central nervous system (CNS) in PD, progressive supranuclear palsy (PSP), and multiple system atrophy (MSA) (rimmed cells). In the enteric nervous system (ENS), enteric glial cells exhibit major biochemical changes in PD (rimmed cells) but not in PSP and MSA (unrimmed cells). The biochemical changes observed in enteric glial cells in PD are exemplified by the phosphorylation status of GFAP. In physiological condition as well as in PSP and MSA, phosphorylation disassembles enteric GFAP filaments shifting the equilibrium to the soluble form of the protein and protecting it from proteolytic degradation (physiological GFAP organization). In PD, enteric GFAP is hypophosphorylated and therefore has a greater potential to assemble and a greater sensitivity to proteolytic cleavage. We suggest that these changes in phosphorylation regulate the structural plasticity of glial filaments and eventually functions of enteric glial cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Bassotti et al.²¹ investigated 26 patients with severe slow transit constipation who required colectomy and found that the density of enteric glial cells, as labeled by S100-beta was decreased when compared with control subjects.²¹ This led to the proposal that chronic constipation is an enteric neuro-gliopathy that might be explained by a defective neurotransmission induced by the loss of enteric glial cells.²¹

Enteric Glial Cells Dysregulation in PD

The emerging critical role of enteric glial cells in gastrointestinal function prompted us to investigate whether enteric Lewy pathology in PD was associated with enteric glial cells dysfunction. A first critical step was to study whether changes in the expression levels of the three commonly used enteric glial markers, GFAP, S100-beta, and Sox-10, were present in PD. Using quantitative polymerase chain reaction in colonic biopsies, we showed that the expression levels of GFAP and Sox-10, but not S100-beta, were increased in the gastrointestinal tract of PD patients.⁸ The GFAP and Sox-10 levels strongly correlated with the amount of several pro-inflammatory cytokines,

including interleukin-6,⁸ which is released after enteric glial cells activation.²² These first observations strongly supported the idea that glial reaction occurs in the gastrointestinal tract during PD. We were nevertheless struck by the heterogeneity of GFAP levels between PD patients, some displaying a level comparable to controls, whereas others had a more than a threefold increase in GFAP messenger RNA. Levels of glial markers were negatively related to disease duration, suggesting that enteric glial reaction is high at disease onset and decreases over time.⁸ Enteric glial reaction may be high at disease onset when the activated cells release their cytokines, then decreasing while maintaining ongoing disease activity as already shown in the brain for microglial activation.²³

Glial fibrillary acidic protein is a phosphoprotein and phosphorylation of serine, and threonine residues from its amino-terminus are involved in the regulation of the protein self-assembly (Fig. 2).²⁴ Because the stability of the cytoskeleton is essential for normal astrocyte function, GFAP phosphorylation may be critically involved in central nervous system disorders. Changes in GFAP phosphorylation and especially at serine 13 residue has been reported in the brain of patients with Alzheimer's disease and frontotemporal dementia^{25,26} as well as in a pig model of brain hypoxia.²⁷ We

therefore investigated whether the GFAP phosphorylation status was affected in the gastrointestinal tract during PD. By using two different antibodies specific for GFAP phosphorylated at serine 13, we showed that GFAP was hypophosphorylated at this residue in colon biopsy specimens from PD patients when compared with healthy controls (Fig. 2).⁹

Taken together, the biochemical changes in GFAP observed in the colon of PD patients are highly suggestive of reactive gliosis.¹⁹ Interestingly, these glial changes were specific to PD because no changes in GFAP expression or phosphorylation were observed when colon biopsy samples from progressive supranuclear palsy and multiple system atrophy patients were analyzed.⁹ By demonstrating the occurrence of glial reaction in the gastrointestinal tract of PD patients but not in related disorders, our results are consistent with the idea that PD is not limited to the central nervous system but is in fact a widespread neuronal disorder affecting peripheral autonomic networks and in particular the enteric nervous system. Moreover, they also suggest that the pathology in progressive supranuclear palsy and multiple system atrophy is limited to the central nervous system and does not affect the peripheral nervous systems.^{28,29}

Possible Roles of Enteric Glial Cells Dysregulation in PD

In light of these findings, what are the likely pathological outcomes of enteric glial dysregulation observed in PD? First, these enteric glial changes might be involved in the gastrointestinal symptoms often seen in PD.² Results from autopsy study have indicated no overt neuron loss or changes in the neurochemical phenotype in the gut in PD, suggesting that neuropathology in the enteric nervous system is unlikely to be a causative factor in PD-related gastrointestinal dysfunction.^{30,31} One might therefore postulate that the biochemical changes of enteric glial cells in PD might induce glial dysfunction, which in turn would lead to synaptic dysfunction and altered gastrointestinal motility as already suggested for chronic constipation.²¹ Second, the occurrence of enteric glial cells dysfunction in PD provides an argument to support Braak's hypothesis. Braak has proposed that PD may be triggered by a hitherto unknown neurotropic agent that breaches the intestinal epithelial barrier to initiate α -synuclein (α -Syn) aggregation in the terminal axons of the submucosal plexus.³² Thereafter, according to the hypothesis, α -Syn pathology would propagate in a prion-like manner to the central nervous system via the vagal preganglionic innervation of the gut.³² In such a scenario, the enteric glial reaction observed in PD may play a key role by modulating intestinal permeability as greater gut permeability has

been observed in PD patients^{33,34} and in experimental parkinsonism.³⁵ Equally intriguing is the possible role of enteric extracellular α -Syn,³⁶ which might promote local glial reaction, as already demonstrated in the central nervous system.³⁷ The enteric glial reaction would in turn induce local pro-inflammatory cytokines secretion and inflammation, thereby facilitating the spreading of PD pathological process.³⁸

Conclusion and Perspective

A pressing need exists for biomarkers not only to differentiate PD from related disorders but also to assess disease severity and progression. Despite technological advances in the field of neuroimaging, no fully validated biomarker for PD is available yet. The observations demonstrating that PD pathology was not limited to the central nervous system but also involved peripheral neuronal networks such as the enteric nervous system have provided new opportunities for the development of novel biomarkers of the disease. To date, search for enteric nervous system biomarkers in PD has focused on the identification of enteric nerve α -synuclein deposits.³⁹ The identification of enteric glial cells dysfunction in PD opens the way for the discovery of enteric biomarkers, which can go beyond the mere assessment of Lewy pathology.³⁹ Providing that, similarly to astrocytes,⁴⁰ the enteric glial cells are involved in the earliest disease changes, they might be considered as a source of biomarkers for prodromal PD. ●

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Article 7 : Que peut nous apprendre une biopsie gastro-intestinale sur la maladie de Parkinson ?

REVIEW ARTICLE

What a gastrointestinal biopsy can tell us about Parkinson's disease?

A.-G. CORBILLÉ,^{*,†,‡,§} T. CLAIREMBault,^{*,†,§} E. CORON,^{*,†,§} L. LECLAIR-VISONNEAU,^{*,†} C. PRETERRE,^{*,‡} M. NEUNLIST,^{*,†,§} & P. DERKINDEREN,^{*,†,‡}

^{*}Inserm, U913, Nantes, France

[†]Nantes University, Nantes, France

[‡]Department of Neurology, CHU Nantes, Nantes, France

[§]Institut des Maladies de l'Appareil Digestif, CHU Nantes, Nantes, France

Key Points

- Lewy bodies and neurites, the pathological hallmarks of Parkinson's disease are found in the enteric neurons of nearly all parkinsonian patients.
- A routine gastrointestinal biopsy contains epithelial cells, enteric glial cells, and enteric neurons.
- A substantial amount of research has been done to detect Lewy bodies and neurites in gastrointestinal biopsies in order to develop a *premortem* histopathological marker of the disease.
- Aside from neurons, the enteric glial cells and epithelial cells, which are present in a gastrointestinal biopsy, are also dysregulated in Parkinson's disease.
- A gastrointestinal biopsy could represent a unique window to assess the neuropathology in living patients with Parkinson's disease.

Abstract

Background The intraneuronal inclusions called Lewy bodies and neurites, which represent the characteristic pathological changes in Parkinson's disease, are found in the enteric neurons in the great majority of parkinsonian patients. This observation led to a substantial amount of research over the last few years in order to develop a minimally invasive diagnostic procedure in living patients based on gastrointestinal (GI) biopsies. **Purpose** In this review, we will begin by discussing the studies that

focused on the detection of Lewy bodies and neurites in GI biopsies, then broaden the discussion to the pathological changes that also occur in the enteric glial cells and intestinal epithelial cells. We conclude by proposing that a GI biopsy could represent a unique window to assess the whole pathological process of the brain in Parkinson's disease.

Keywords enteric glial cells, enteric nervous system, enteric neurons, gastrointestinal biopsy, Parkinson's disease.

Address for Correspondence

Pascal Derkinderen, Inserm U913, 1 place Alexis Ricordeau, Nantes 44035, France.

Tel: +33(0)240087924; fax: +33(0)240087506; e-mails:

derkinderenp@yahoo.fr and pascal.derkinderen@chu-nantes.fr

[†]Equal contributors.

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The first descriptions of Lewy bodies and neurites in the enteric nervous system (ENS) in Parkinson's disease (PD) reported in the 1980s found relatively little resonance in comparison to the amount of literature devoted to the lesions in the central nervous system.^{1–3} Nevertheless, it became obvious over the last 15 years that PD is a gut disorder. Symptoms such

as dysphagia, nausea, and distension as a result of impaired gastric emptying, and bowel dysfunction, including both reduced bowel movement frequency and difficulty defecating, are among the most common non-motor symptoms of PD.^{4,5} Pathologically, Lewy bodies and neurites have been found in the ENS in nearly every case examined.⁶ These autopsy findings prompted a substantial amount of research on the detection of PD pathology using gastrointestinal (GI) biopsies in order to achieve *in vivo* pathological diagnosis of the disease (reviewed in Ref. 7). Most studies to date have therefore focused on the optimization of immunodetection of α -synuclein, the main component of Lewy bodies and neurites, in the enteric neurons.^{8–13} In this review, we will discuss recent evidence indicating that pathological changes in the gut in PD are not limited to enteric neurons but also involve the enteric glial cells (EGCs) and the intestinal epithelial cells and put into perspective the use of routine GI biopsy as a window on brain neurodegeneration in PD.

GI BIOPSY GIVES ACCESS TO THE DIGESTIVE NEURONAL–GLIAL–EPITHELIAL UNIT

On a routine basis, GI biopsies are primarily intended for analyzing the intestinal epithelial cells for the detection of digestive disorders such as colorectal cancer, inflammatory bowel disease, and peptic ulcer. Nevertheless, there is also mounting evidence to suggest that a routine GI biopsy enables easy access to the two neuronal populations of the gut, namely the EGCs and the enteric neurons. The EGCs that are lying just below the epithelial cells lining called the mucosal EGCs, are in close proximity (in the range of 1 μ m) to the intestinal epithelial cells and are therefore easily captured by the biopsy-forceps (Fig. 1).^{14,15} The same is true for the neuronal processes sent out by neurons of both the submucosal and myenteric plexus, which terminate in direct proximity with the intestinal crypts (Fig. 1).¹⁶ We have recently proposed to name the anatomical unit between these 3 cell types the 'digestive neuronal–glial–epithelial unit' (NGEU), which might be considered as the digestive counterpart of the neuronal–glial–endothelial unit of the blood–brain barrier.¹⁷ In addition, routine biopsies can also give access to the EGCs and the neuronal cell bodies and processes of the submucosal plexus (Fig. 1). It should be, however, noted that the submucosal neurons and EGCs might be missed by the forceps because of the fishnet-like architecture of the plexus and/or because of the biopsy being too superficial (Lebouvier, Pouclet, Clairembault, and Derkinderen, unpublished).

Once the biopsy has been performed, the NGEU can be analyzed both morphologically and functionally. In order to prevent RNA and protein degradation and dephosphorylation, the biopsy may be immediately quick-frozen in liquid nitrogen and kept at -80°C until further analysis by Western Blot or PCR.^{18,19} Such an approach has proven to be useful for studying the expression levels of major proteins of the intercellular contacts between epithelial cells, such as ZO-1 and occludins,²⁰ of enteric glial markers such as S100- β and glial fibrillary acidic protein (GFAP)^{19,21} and neuronal markers such as PGP9.5 (Fig. 1).^{18,22} The biopsy can be also microdissected for a separate immunohistochemical analysis of the submucosa and the mucosa.^{9,18,23} Submucosal enteric neurons can be visualized and counted by immunohistochemistry using general neuronal markers such as Hu, PGP9.5 and high-molecular weight neurofilament.^{9,18} A whole-mount preparation of submucosa obtained from one single biopsy contains an average of 35 ganglia with three to five neurons per ganglion, thus allowing the evaluation of approximately 150 neurons.¹⁸ In parallel, the distribution of tight junctions proteins and pericryptal neuronal processes can be readily analyzed on whole-mount preparation of submucosa (Fig. 1).^{20,23} From a functional point of view, the intestinal epithelial barrier may be evaluated *ex vivo* by measuring the diffusion of fluorescent probes through biopsy samples in Ussing chambers.²⁴

MODEST NEURONAL LOSS AND LEWY PATHOLOGY IN GI BIOPSIES IN PD

PD is pathologically characterized by a loss of dopamine-containing neurons in the substantia nigra pars compacta along with the presence of intracytoplasmic inclusions, termed Lewy bodies and neurites, in the remaining surviving neurons.²⁵ Since the discovery in the late 1990s that Lewy bodies and neurites had a strong α -synuclein immunoreactivity, α -synuclein immunostaining has become the method of choice for the detection of PD pathology.^{26,27} Several autopsy studies, including one recent survey in which α -synuclein immunohistochemistry was optimized, showed that the distribution of Lewy pathology is much greater than formerly appreciated, extending to peripheral autonomic neuronal networks and especially to the ENS.^{6,28,29} This logically led several groups to develop specific methods for the detection of Lewy pathology in routine GI biopsies for the development of biomarkers that will directly assess the pathological process in living patients (see

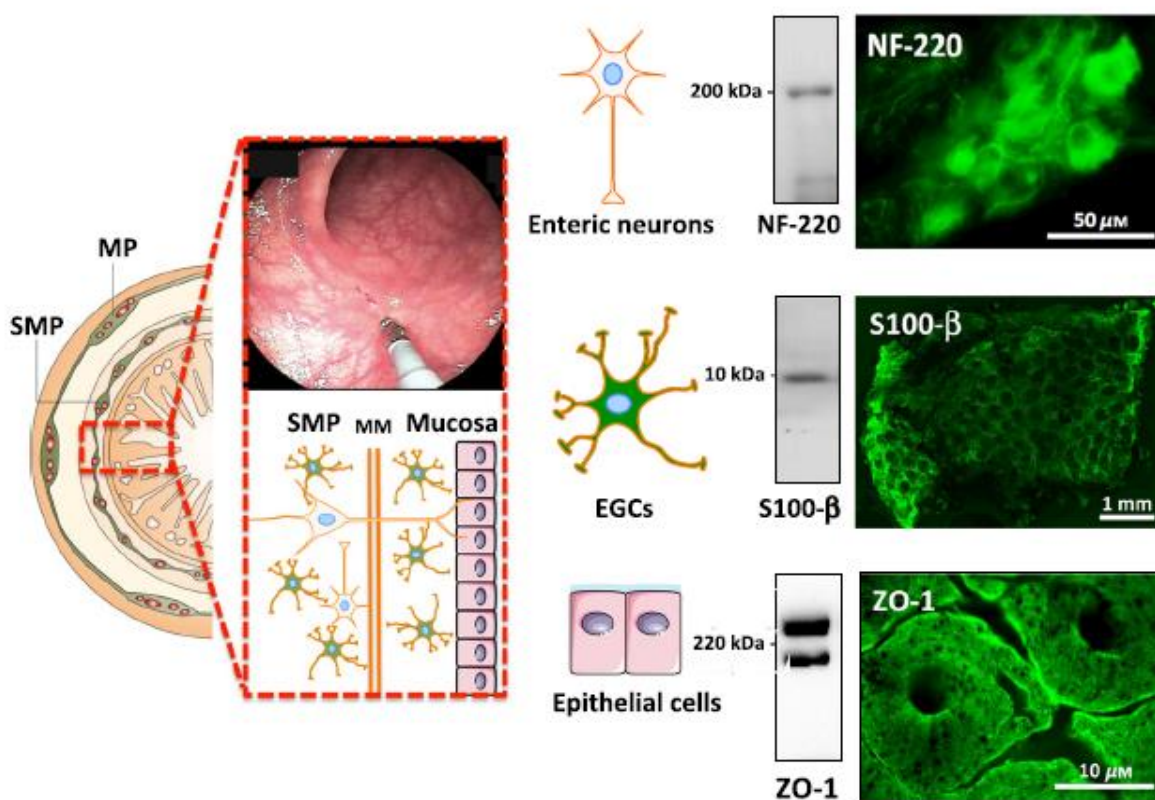


Figure 1 Cell types that can be analyzed using a routine GI biopsy. The biopsy forces used for routine GI biopsies are capable of capturing the intestinal epithelial cells as well the enteric glial cells (EGCs) and the neuronal processes that are present in the mucosa. In addition, biopsies can also give access to the EGCs and the neuronal cell bodies and processes from the submucosal plexus (SMP). The expression levels and distribution of neuronal markers such as high-molecular weight neurofilament protein of 220 kDa (NF-220), enteric glial markers such as S100- β and tight junction proteins such as ZO-1 can be analyzed by Western blot and immunohistochemistry. MM, muscularis mucosae; MP, myenteric plexus.

Ref. 7 for a recent review). In our seminal survey on GI biopsies in PD, we have used the above-mentioned whole-mount preparation of submucosa for the detection of Lewy pathology. Twenty-nine PD patients with disease duration ranging from one to 24 years were enrolled together with 10 control subjects who had undergone colonoscopy for colorectal cancer screening.⁹ Biopsies from 21 out of the 29 PD patients showed Lewy neurites in their submucosal plexus, whereas no Lewy pathology was observed in any of the controls (Fig. 2).⁹ The clinical relevance of these findings was supported by a correlation between pathological burden and the amount of axial and dopa-unresponsive symptoms, which reflect disease progression.⁹ We further showed that the presence of α -synuclein deposits within enteric neurons is specific to PD as it was not observed in patients with atypical parkinsonism such as multiple system atrophy and progressive supranuclear palsy.³⁰

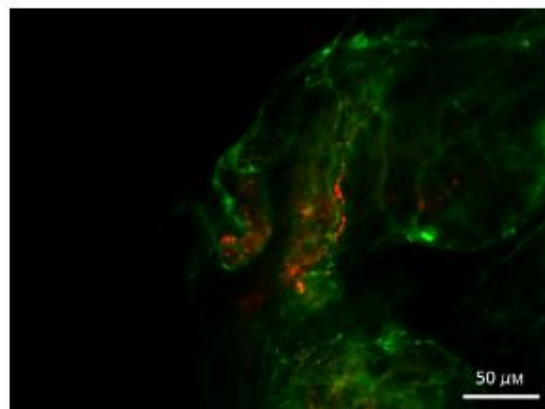


Figure 2 Analysis of the submucosal plexus by immunohistochemistry in PD. Submucosa whole mount obtained from a colonic biopsy of PD patient was stained with antibodies against PGP9.5 (green) and phosphorylated α -synuclein (red). The photomicrograph shows some co-localization of these proteins in neuritic structures reminiscent of Lewy neurites; scale bar: 50 μ m.

Although the microdissection technique we have developed provides outstanding information on the morphology of enteric neurons, it nonetheless has several limitations as it needs to be performed immediately after the endoscopic procedure and requires a skilled technician.^{9,18} Several subsequent studies have used GI biopsies that were formalin-fixed and paraffin-embedded in order to overcome the limitations of the whole mount dissection procedure. Despite the lower prevalence of Lewy pathology in the lower compared to the upper gut,^{3,6,31} the sigmoid colon and rectum have been by far the most commonly studied sites. This is likely explained because these portions of the GI tract are easily accessible via a flexible sigmoidoscope, a safe procedure that can be performed in 5–10 min without the need for sedation and colon cleansing.³² Regarding the lower GI tract, a first study demonstrated the presence of α -synuclein aggregates in the sigmoid colon of PD patients with a high sensitivity and specificity,¹⁰ but other groups did not confirm these results.^{12,13} Recently, a Spanish group performed two upper GI tract biopsies in 28 PD patients during device implantation for continuous levodopa (Duodopa).¹¹ They observed α -synuclein deposits in 17 of 28 PD patients and in only 1/23 control subjects, but another study found a lower sensitivity.¹² Although encouraging, these different studies that used paraffin-embedded samples have produced discrepancies regarding the prevalence and abundance of Lewy pathology per biopsy, either in the upper or lower GI tract. This might be partly explained by the diversity of methodology as some laboratories used total α -synuclein antibody¹⁰ while others used either phosphorylated or total α -synuclein antibodies.^{11,12} Moreover, all of these studies suffered from several limitations including the absence of replication by independent laboratories and the lack of double staining to confirm the neuronal nature of the α -synuclein immunoreactive structures.

Experience from the central nervous system strongly suggests that the motor symptoms in PD are driven primarily by neuronal loss rather than Lewy pathology.³³ As such, an evaluation of the density of enteric neurons that control GI motility and secretion is a critical step toward understanding the pathophysiology of GI symptoms in PD. Using cross sections from GI tract autopsy samples, a recent study has convincingly demonstrated that there was no neuronal loss in the myenteric plexus in PD, either in the lower or upper gut.³⁴ Concerning the submucosal plexus, immunohistochemical analysis of whole mount-preparations of endoscopically obtained biopsies revealed that there is a modest but significant 15% decrease in the submucosal neuronal density in PD patients.⁹ This moderate decrease in total neuronal

density was not associated with differences in the relative proportions of individual submucosal neuron phenotype and especially of dopaminergic neurons.²² Altogether, the results obtained with autopsy samples and biopsies show the absence of overt neuronal loss and changes in neurochemical phenotype in both the myenteric and the submucosal plexus. This suggests that the loss of intrinsic innervation of the gut is unlikely to be solely responsible for the GI manifestations so frequently encountered by PD patients and that extrinsic inputs and especially the dorsal motor nucleus of the vagus are critically involved.^{35,36}

GFAP IS OVEREXPRESSED IN GI BIOPSIES IN PD

Enteric glial cells are now regarded as the digestive counterpart of the central nervous system astrocytes (see Ref. 37 for a recent review). Indeed, both cell types are not only morphologically similar but they also share electrophysiological properties^{38,39} and express common key markers, including S100- β and the intermediate filament GFAP.^{40,41} Astrocytes respond to all forms of central nervous system insults, including neurodegeneration, through a process referred to as reactive astrogliosis.⁴² Although the denomination reactive astrogliosis encompasses a large spectrum of molecular, cellular, and functional changes, one of its key features is an increase in GFAP expression.⁴³ Whether changes in GFAP expression occurs in PD brain is still a matter of debate as existing reports are conflicting (see the discussion in Ref. 44). This might be explained by certain difficulties inherent with using *postmortem* brain tissue, such as difficulty in obtaining brain samples and the delay between death and tissue processing, which is known for inducing GFAP proteolysis.⁴⁵ These two limitations can be overcome when using GI biopsies, which, as stated above, can be easily performed and immediately frozen during the endoscopic procedure.¹⁸ By analyzing the colonic biopsies of 43 patients with an established diagnosis of PD (mean disease duration 9.8 years) and 25 age-matched healthy participants, we showed that the expression levels of GFAP was increased in the GI tract of PD patients both at the transcripts and protein levels.^{19,21} The upregulation of GFAP was accompanied by an increase in the expression of the main pro-inflammatory cytokines, including interleukin-6 which is secreted by reactive enteric glia.⁴⁶ Levels of glial markers and interleukin-6 mRNA were variable between patients and were negatively correlated with disease duration¹⁹ suggesting that GI inflammation may be higher in the early stages of the disease as

reported in the brain.⁴⁷ Interestingly, no changes in GFAP expression were observed in colonic samples from progressive supranuclear palsy and multiple system atrophy patients, two neurodegenerative parkinsonian syndrome in which the ENS is pathologically spared.^{30,48} Taken together, these biochemical changes suggest that the changes observed in the gut in PD, contrasting with other parkinsonian syndromes, are not limited to Lewy pathology but also involve the EGCs. It still remains to be determined how the EGCs become activated in PD. One obvious candidate is α -synuclein, which is secreted by enteric neurons⁴⁹ and known for inducing astrocyte reaction in the central nervous system.⁵⁰

TIGHT JUNCTIONS STRUCTURE IS ALTERED IN GI BIOPSIES IN PD

Both neurons and EGCs have been increasingly recognized as major regulators of the intestinal epithelial barrier. Overall, activation of enteric neurons has been shown to result in the reinforcement of intestinal epithelial barrier functions⁵¹ while EGCs are critical for the maintenance of its integrity.^{52,53} These observations along with Braak's hypothesis proposing that PD pathology may originate in the gut⁵⁴ prompted several groups to study intestinal epithelial barrier permeability in parkinsonian patients. Three studies, which have all used absorption of sugar probes to investigate the paracellular permeability in PD subjects led to inconclusive and conflicting results.^{55–57} We, therefore, used another approach to evaluate the intestinal epithelial barrier. Colonic biopsies from PD patients were analyzed functionally using Ussing chambers and morphologically for the expression and localization of tight junction proteins.²⁰ Although the paracellular permeability in PD was not different from controls, we observed that occludin, one of the main tight junction protein, was down-regulated and redistributed from the membrane to the cytosol in biopsies

from PD subjects.²⁰ Remarkably, these changes in occludin expression and distribution were also noted in a subset of 5 patients who had never received levodopa, suggesting that they were not caused by chronic PD treatment. Although the clinical consequences of these morphological changes are still to be clarified, they support the assumption that a more permeable gut may be a starting point for neurodegeneration in PD.⁵⁴

GI BIOPSY AS A SOURCE OF BIOMARKERS IN PD

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 10–15 years.⁵⁸ The diagnosis of PD and the follow-up of disease progression are mainly based on clinical criteria and there is therefore a critical need for the development of biomarkers that will help to better diagnose the condition, define the subtypes of disease, and follow its course independently of any symptomatic drug effects.⁵⁹ In recent years, new initiatives have been undertaken to accelerate the search for new biomarkers in PD with the hope and expectation that they will be able to help diagnose PD, track its clinical course, and predict disease complications. For instance, both the National Institute of Health and the Michael J Fox Foundation for Parkinson's research have launched collaborative projects to identify new biomarkers.⁶⁰ These projects are mainly based on advanced brain-imaging techniques and analysis of body fluids. It is unlikely that one single biomarker will be capable of providing information on early diagnosis, differential diagnosis, and monitoring disease progression.^{61,62} In this context, the demonstration that the peripheral autonomic neuronal circuits are affected by the pathology, early, and specifically in a large proportion of patients has opened new insights into the development of novel and original biomarkers

Table 1 Advantages and limitations of GI biopsies for the diagnosis of PD

Pros	Cons
Sigmoid colon is easily accessible through rectosigmoidoscopy, a safe procedure that can be performed in few minutes without sedation and preparation ⁷⁴	Because of the rostro-caudal distribution of Lewy pathology in the GI tract ⁶ , use of upper digestive tract biopsies might be preferable. This is limited by the potential risk of inhalation during the procedure ⁷⁵
Lewy pathology can be detected in GI biopsies using whole mount preparations with a good specificity and sensitivity ^{9,20}	Whole-mount dissections need to be performed immediately after the endoscopic procedure. ¹⁸ This is a limitation to multicenter and/or retrospective studies
The SMP contains neurons and glial cells that are readily accessible to routine GI biopsies ^{18,20}	The MP, which is also a target of the disease, is not accessible to routine GI biopsies. It would be of critical interest to determine whether this plexus shows comparable abnormalities to those reported in the SMP

MP, myenteric plexus; SMP, submucosal plexus.

Table 2 Similarities between brain and gut in PD

	Brain	Gut
Lewy pathology	In all subjects except in some Parkin-associated familial PD ²⁵	In nearly every case examined pathologically (85–100%) ^{6,28}
Reactive glia	Changes in GFAP expression consistently reported in the SN (see ⁴⁴)	Changes in GFAP expression and phosphorylation in the colon ²¹
Inflammation Barrier	IL-6, IL-1 β and TNF- α are elevated in the striatum ^{71,72} Functional changes of the BBB in the striatum ⁷³	IL-6, IL-1 β , IFN- γ and TNF- α are elevated in the colon ¹⁹ Morphological and functional changes of the IEB in the colon ^{21,57}

BBB, blood brain barrier; IEB, intestinal epithelial barrier; SN, substantia nigra.

for PD that could directly evaluate the pathological process in living patients.^{6,63–65}

The ENS is not the only peripheral neuronal network affected by Lewy pathology that is accessible to biopsies. Because α -synuclein is also deposited in the autonomic circuits that innervate the skin and the submandibular glands, there has been an increasing interest in biopsying one of these sites for *in vivo* diagnosis of PD (reviewed in Ref. 7). Recent studies have indeed confirmed that skin and submandibular gland biopsies enable the detection of α -synuclein deposits in PD with a good sensitivity and specificity.^{66,67} Nevertheless, despite the potential interest of these two sites, we believe that the specific organization of the ENS makes it the best candidate tissue for developing biopsy-derived markers of PD (advantages and limitations of GI biopsies are discussed in Table 1). In contrast to skin and salivary glands, the ENS does not only contain postganglionic neuronal processes but is an integrated neuronal network that contains neuronal cell bodies and glial cells.⁶⁸ The data we have acquired so far using colonic biopsies in patients with PD enabled us to demonstrate that this whole neuronal network along with its microenvironment are dysregulated in PD.^{20,21} These observations support the hypothesis that the NGEU, by mirroring brain pathol-

ogy, might constitute an unparalleled source of biomarkers in PD (Table 2). Further work is needed to identify new digestive PD biomarkers that will demonstrate a superior sensitivity to the ones that have been proposed so far. Possible strategies might include a joint transcriptomic and proteomic analysis of the biopsies along with the analysis of biopsy supernatant, an approach that has already proven to be useful in irritable bowel syndrome.^{69,70}

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CONFLICTS OF INTEREST

The authors have no competing interests.

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Thèse de Doctorat

Laurène LECLAIR-VISONNEAU

Etude physiopathologique de la diffusion de la maladie de Parkinson au système nerveux autonome

Parkinson's disease spreading to the autonomic nervous system: a pathophysiological study

Résumé

Au-delà de la triade motrice liée à la dégénérescence de la substance noire, les symptômes non moteurs dans la maladie de Parkinson (MP) reflètent une large diffusion du processus pathologique (agrégation d'alpha-synucléine). Ainsi, la dysautonomie, les troubles du sommeil ou les troubles cognitifs sont fréquents au cours de la MP, la constipation et le trouble du comportement en sommeil paradoxal (TCSP) pouvant même précéder les signes moteurs. Le tractus digestif pourrait jouer un rôle dans la pathogénie de la MP. L'objectif de ce travail de thèse était d'explorer l'atteinte du système nerveux autonome (SNA) dans l'histoire naturelle de la MP. Nous avons réalisé une exploration fonctionnelle et morphologique de la barrière épithéliale intestinale sur des biopsies coliques. Nous avons montré une désorganisation structurale des jonctions serrées, non corrélée à la synucléinopathie dans le système nerveux entérique. Chez des patients parkinsoniens avec et sans TCSP, marqueur de sévérité de la MP et de diffusion au tronc cérébral, nous avons étudié la perméabilité et la charge lésionnelle entériques. La perméabilité ne différait pas, mais la synucléinopathie était plus fréquente chez les patients avec TCSP, supportant l'existence de formes plus diffuses de MP. Enfin, nous avons réalisé une étude systématique du SNA par des explorations cliniques, fonctionnelles et histologiques (biopsie cutanée). L'atteinte des modalités du SNA montrait une distribution hétérogène, suggérant une progression éparse et erratique. Seules la constipation et l'hypotension orthostatique étaient associées à une altération cognitive, confirmant leurs liens avec la sévérité de la maladie.

Mots clés

Maladie de Parkinson, système nerveux autonome, système nerveux entérique, alpha-synucléine, barrière épithéliale intestinale, trouble du comportement en sommeil paradoxal, dénervation cutanée, altération cognitive

Abstract

Beyond cardinal motor symptoms due to the degeneration of *substantia nigra* neurons, non motor symptoms are major features of Parkinson's disease (PD), displaying the large spread of alpha-synuclein histopathology. Dysautonomia, sleep problems or cognitive alteration are frequent in PD; constipation and rapid eye movement sleep behavior disorder (RBD) may even precede motor symptoms for years. Gastrointestinal tract might be involved in PD pathogenesis. The aim of the current research was to explore the autonomic nervous system (ANS) in the natural history of PD. We first performed a functional and morphological study of the intestinal epithelial barrier (IEB) in colonic biopsies. We observed a structural disorganization of tight junction, unrelated to ENS alpha-synuclein pathology. In PD patients with and without RBD, a disease severity and brainstem diffusion marker, we evaluated IEB permeability and enteric alpha-synuclein pathology. No difference in IEB permeability was observed between the two groups, but ENS alpha-synuclein pathology was more frequent in PD patients with RBD, suggesting that RBD may mark a more widespread alpha-synuclein-driven pathophysiology. Finally, we undertook a systematic assessment of ANS components, with clinical, functional and histological (skin biopsy) measures. ANS components were impaired in a heterogeneous pattern, thereby suggesting an erratic rather than a stepwise progression. Only constipation and orthostatic hypotension were associated with cognitive alteration, which reinforce their relationship with disease severity.

Key Words

Parkinson's disease, autonomic nervous system, enteric nervous system, alpha-synuclein, intestinal epithelial barrier, rapid eye movement sleep behavior disorder, cutaneous denervation, cognitive impairment-