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Liste des abréviations

5-HT : sérotonine (5-hydroxytryptamine)

6-OHDA : 6-hydroxydopamine

ACh : acétylcholine

ADN : acide désoxyribonucléique

Calb : calbindine

CGE : cellule gliale entérique

cGMP : monophosphate cyclique de guanosine

CGRP : calcitonin gene-related peptide

ChAT : choline acétyltransférase

CIC : cellules interstitielle de Cajal

CL : corps de Lewy

DA : dopamine

DYN : dynorphine

ENK : enképhaline

GRP : gastrin releasing peptide

IPAN : intrinsic primary afferent neurons

MP : maladie de Parkinson

MPTP : 1-méthyl-4-phényl-1,2,3,6-tétrahydropyridine

NF : neurofilament

NL : prolongements de Lewy

NPY : neuropeptide Y

NO : monoxyde d'azote

NOS : nitric oxide synthase

PM : plexus myentérique

PSM : plexus sous-muqueux

SNpc : substance noire pars compacta

SNC : système nerveux central

SNE : système nerveux entérique

SP : substance P

TH : tyrosine hydroxylase

VIP : vasoactive intestinal peptide

INTRODUCTION

1 LA MALADIE DE PARKINSON: UNE MALADIE DU MOUVEMENT CARACTERISEE PAR UNE NEURODEGENERESCENCE DE LA SUBSTANCE NOIRE

La maladie de Parkinson (MP) est la seconde maladie neurodégénérative après la maladie d'Alzheimer. La prévalence de cette pathologie augmente avec l'âge, elle touche à présent environ 1% de la population au-delà de 65 ans et plus de 5% au-delà de 85 ans. L'âge moyen au diagnostic est de 57 ans environ (Martinez-Rumayor *et al.* 2009). Le nombre d'individus touchés croît d'années en années. Cette augmentation de la prévalence de la MP est à mettre directement en relation avec un meilleur diagnostic et l'allongement de l'espérance de vie. Dans les pays développés et en voie de développement, la prévalence devrait doubler d'ici 2030 (Dorsey *et al.* 2007). La MP affecte indifféremment l'ensemble des ethnies même si les populations caucasiennes semblent les plus affectées.

1.1 SIGNES CLINIQUES MOTEURS DE LA MALADIE

La MP est caractérisée par une tétrade symptomatique motrice résumée par l'acronyme *TRAP* : *Tremblement*, *Rigidité*, *Akinésie* et *instabilité Posturale*. Parmi ceux-ci les signes cardinaux de la maladie, l'akinésie et la rigidité, sont la conséquence de la dénervation dopaminergique du putamen suite à une destruction relativement sélective de la substance noire pars compacta (SNpc) (Jellinger 1999). Les prolongements des neurones dopaminergiques de la substance noire constituent en effet le tractus nigro-striatal qui projette dans les deux structures du striatum, putamen et noyau caudé. La

perte neuronale dans la SNpc est associée à la formation d'inclusions cytoplasmiques hyalines dans les neurones restants nommées corps de Lewy (CL). Quand la gêne motrice apparaît, on estime que 60% des neurones dopaminergiques ont dégénéré, et que le putamen a perdu 80% de la dopamine des terminaisons axonales nigro-striatales (Fearnley and Lees 1991; Hilker *et al.* 2005).

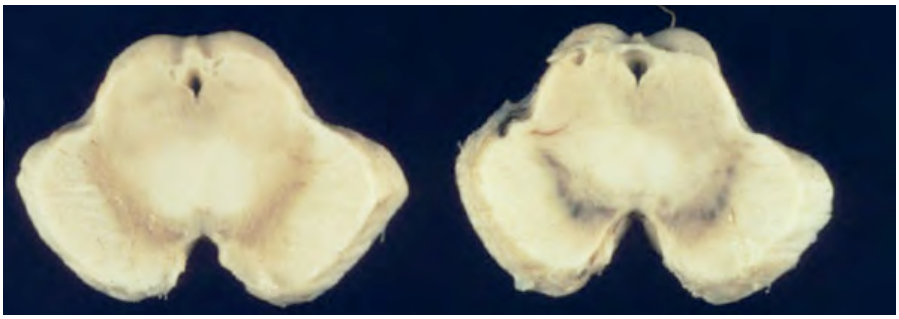
1.2 CARACTERISTIQUES NEUROPATHOLOGIQUES : ATTEINTE DE LA SUBSTANCE NOIRE ET CORPS DE LEWY

A ce jour, le diagnostic de certitude de maladie de Parkinson ne peut être posé qu'à l'autopsie, par la mise en évidence *a)* d'une perte neuronale et d'une dépigmentation de la SNpc, associée à *b)* la présence de CL dans les neurones restants. Cette mise en évidence reste justifiée pour corriger le diagnostic dans les formes atypiques de MP ; les critères diagnostiques actuels ont en effet une valeur prédictive positive excellente (99%) (Hughes *et al.* 2002) mais une sensibilité perfectible, estimée à environ 90%.

1.2.1 PERTE NEURONALE DE LA SUBSTANCE NOIRE

Le principal aspect caractéristique de la MP est la perte massive et relativement spécifique des neurones dopaminergiques de la SNpc (figure 1). Découverte en 1919 par Tretiakoff, cette caractéristique a depuis été confirmée comme l'un des aspects majeurs de la MP. Au sein même de la SNpc les neurones dopaminergiques présentent une susceptibilité différente en fonction de leur distribution : la perte neuronale prédomine dans la partie ventrale de la SNpc (Fearnley and Lees 1991). La susceptibilité à la dégénérescence des neurones dopaminergiques dépend de leur distribution au sein de compartiments de la SNpc définis par l'immunomarquage de la calbindine : la mort

neuronale est plus importante dans les nigrosomes (régions pauvrement marquées par la calbindine) que dans la matrice (fortement marquée) (Damier *et al.* 1999a, b).



(MEDEX-Northwest-Physician-Assistant-Objectives 2009)

Décoloration macroscopique de la substance noire chez un sujet parkinsonien (à gauche) en comparaison avec un sujet contrôle (à droite). Cette décoloration est la conséquence de la dégénérescence des neurones dopaminergiques contenant la neuromélanine qui donne cette couleur noire.

Figure 1 : Décoloration de la substance noire au cours de la maladie de Parkinson

1.2.2 AGREGATION PROTEIQUE : LES CORPS DE LEWY

La caractérisation de la MP est étroitement associée à la découverte d'agrégats protéiques, inclusions intraneuronales, qui ont été décrits pour la première fois en 1912 par Friedrich Lewy (Lewy 1912) au niveau du noyau de Meynert et du noyau dorsal du nerf vague. Ces agrégats protéiques sont longtemps restés de nature inconnue mais étaient identifiés grâce à leur affinité pour le marquage à l'éosine. Les progrès techniques en immunologie et spectroscopie de masse ont permis de mieux caractériser les protéines contenues au sein de ces CL.

Sur le plan ultrastructurel, les CL sont composés d'un noyau sphérique dense mesurant de 8 à 30 µm de diamètre entouré à sa périphérie d'éléments fibrillaires.

Les CL sont principalement retrouvés dans les neurones survivants de la SNpc au cours de la MP. Cependant ils ont été aussi détectés dans d'autres régions du système

nerveux central (SNC) tel que le locus cœruleus, les noyaux du raphé, les noyaux des nerfs crâniens, le cortex, le système limbique, l'hypothalamus ou encore le noyau dorsal moteur du nerf vague (Lewy 1912).

Le rôle exact de ces agrégats protéiques au cours de la MP reste encore mal défini et controversé. Les CL présents dans les neurones survivants de la SNpc pourraient être un signe avant-coureur de leur dégénérescence ou bien la raison de leur résistance (Lu *et al.* 2005; Manning-Bog *et al.* 2003; Tompkins and Hill 1997). Certaines formes familiales rares de la MP ne sont remarquablement pas associées à la formation d'inclusions protéiques du type des CL.

Plus d'une centaine de protéines composant les CL ont été répertoriées par immunohistochimie ou spectrométrie de masse. On retrouve parmi les principales protéines composant ces CL, outre l'α-synucléine, des protéines faisant partie du système ubiquitine-protéasome, des facteurs de transcription comme NFκB, des protéines du cytosquelette comme les neurofilaments ou bien encore des protéines chaperonnes comme DJ-1. La présence de ces protéines au sein des CL laisse supposer leur implication directe dans la physiopathologie de la maladie.

Le composant majeur de ces agrégats est l'α-synucléine (Spillantini *et al.* 1997 ; Wakabayashi *et al.* 1997) dont la présence au sein des CL a été montrée après la découverte d'une forme familiale, monogénique, de la MP due a une mutation du gène de cette protéine (Polymeropoulos *et al.* 1997). Depuis cette découverte deux autres mutations du gène codant pour l'α-synucléine ont été identifiées (Kruger *et al.* 1998 ; Zarranz *et al.* 2004), chacune de ces trois mutations pouvant être à l'origine d'une forme de MP similaire à la forme sporadique. Son expression est majoritairement neuronale bien qu'elle ait été décrite comme pouvant être présente mais pas synthétisée dans

d'autres types cellulaires comme les cellules gliales (Braak *et al.* 2007 ; Mori *et al.* 2002). Son rôle physiologique reste largement méconnu, mais l'α-synucléine pourrait être impliquée dans la plasticité synaptique, l'homéostasie des vésicules synaptiques, la régulation de la libération de la dopamine ou encore servir comme protéine chaperonne (Ahn *et al.* 2002 ; Cabin *et al.* 2002 ; Larsen *et al.* 2006). Il est supposé qu'elle serait principalement impliquée dans la physiologie vésiculaire et le transport axonal (Larsen *et al.* 2006; Lavedan 1998). Elle a ainsi été montrée liée aux membranes vésiculaires (Jensen *et al.* 1998). Son rôle majeur est identifiable grâce à la structure de la protéine (figure 2), constituée de 140 acides aminés chez l'homme et les rongeurs. Elle est caractérisée, entre autre, par un domaine N-terminal hautement conservé pouvant aisément se lier aux lipides.

Les trois mutations génétiques associées à des maladies de Parkinson sont indiquées : A30P, A53T et E46K. Le site de phosphorylation sur la sérine 129 considéré comme la forme pathologique de la protéine est aussi représenté (P).

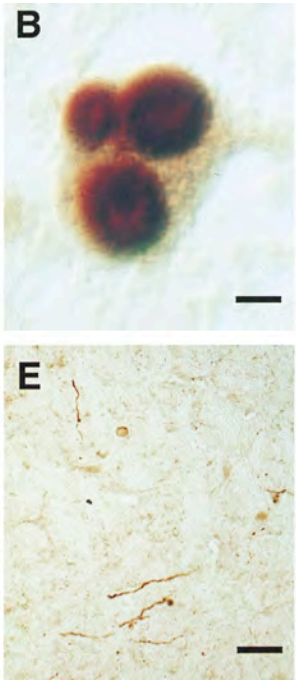
Figure 2 : structure de l'α-synucléine

Son implication dans la physiopathologie de la MP a été révélée lors de la découverte des formes familiales de la MP liée aux mutations de l'α-synucléine. De plus le fait qu'une surexpression liée à une duplication ou une triplication du gène de cette

dernière suffise à induire une MP chez l'Homme ne fait que renforcer le rôle de cette protéine dans la MP (Chartier-Harlin *et al.* 2004 ; Ibanez *et al.* 2004; Singleton *et al.* 2003), par analogie à d'autres maladies à duplication comme la maladie de Charcot-Marie-Tooth de type 1 (Hallam *et al.* 1992).

L'agrégation de cette protéine dans les CL est généralement précédée de nombreuses modifications post-traductionnelles comme une sumoylation, une nitration ou une glycosylation. La plus fréquente de ces modifications, en ce qui concerne la MP, est la phosphorylation de son résidu sérine 129 (Anderson *et al.* 2006; Fujiwara *et al.* 2002). Bien qu'il semble exister un niveau basal de phosphorylation sur la sérine 129 de l'α-synucléine en condition physiologique, cette modification est relativement spécifique de la forme agrégée (Anderson *et al.* 2006). En revanche la signification de l'agrégation de l'α-synucléine reste sujette à controverse avec un effet parfois considéré comme une perte de fonction protectrice (Chen and Feany 2005; da Costa *et al.* 2000 ; Manning-Bog *et al.* 2003) ou comme un gain de fonction toxique (Cookson *et al.* 2007 ; Galvin *et al.* 1999 ; Galvin *et al.* 2001). L'agrégation d'α-synucléine comme la présence des CL est retrouvée dans toutes les MP idiopathiques et la plupart des formes génétiques de la maladie.

Les inclusions d'α-synucléine au sein des neurones peuvent être présentes sous 2 formes distinctes : les CL lorsqu'elles sont au sein des somas, ou prolongements de Lewy (NL) lorsqu'elles sont dans les prolongements neuronaux (Figure 3).



(B) Immunomarquage pour l'α-synucléine et l'ubiquitine montrant un soma neuronal contenant 3 corps de Lewy dans la substance noire d'un patient parkinsonien. Echelle : 10µm
(E) Immunomarquage de l'α-synucléine montrant des prolongements de Lewy dans la substance noire d'un patient parkinsonien. Echelle : 100µm.
D'après Spillantini et al. *1998.*

Figure 3 : Corps de Lewy et prolongements de Lewy

1.3 ETIOPATHOGENIE DE LA MALADIE DE PARKINSON : DES FORMES FAMILIALES ET DES CAUSES ENVIRONNEMENTALES

1.3.1 LES FORMES GENETIQUES DE LA MALADIE DE PARKINSON

Depuis 1997 et la découverte de la première forme monogénique de la MP, liée à une mutation du gène SNCA codant pour la protéine α-synucléine, nombre d'autres formes héréditaires de la MP ont été décrites. Les formes monogéniques de MP sont à transmission autosomique dominante ou autosomique récessive. Des formes multigéniques ou à pénétrance incomplète sont responsables de modes de transmission plus complexes. La prévalence des formes monogéniques de la MP reste cependant restreinte, ne représentant que 5 à 8% des cas de MP (Biskup *et al.* 2008).

Les découvertes récentes et rapides de ces diverses formes génétiques de la maladie de Parkinson depuis 1997 ont changé l'idée que cette maladie était d'origine

purement environnementale. Ces découvertes ont aussi autorisé une avancée majeure dans la compréhension des divers mécanismes impliqués. Ainsi, par exemple le fait qu'une duplication du gène codant pour l'α-synucléine puisse induire une MP montre l'importance de cette protéine dans la MP.

Les mutations ainsi à l'origine de la majeure partie des MP monogéniques sont les mutations des gènes codant pour la leucine-rich repeat kinase (LRRK2) et les mutations du gène codant pour la protéine parkine (Abou-Sleiman *et al.* 2004; Hedrich *et al.* 2004). Les mutations de ces gènes impliquent respectivement les mécanismes d'apoptose pour la protéine LRRK2 (Farrer 2007) et le système ubiquitine-protéasome pour la protéine parkine (Shimura *et al.* 2000) dans l'étiopathogénie de la MP. Ces deux mutations sont responsables de formes précoces de la maladie, les symptômes moteurs se développant vers l'âge de 30 ans. Les autres mutations identifiées comme des formes monogéniques de la MP sont largement minoritaires mais suggèrent d'autres voies potentielles pour la physiopathologie de la MP. Les voies suspectées sont le stress oxydatif (DJ-1) capable d'induire une apoptose des neurones, le système ubiquitine-protéasome (UCHL 1, SCNA…) (Bonifati *et al.* 2003).

Cependant les diverses études réalisées sur les taux de concordance chez les jumeaux monozygotes et dizygotes (Burn *et al.* 1992; Tanner *et al.* 1999; Ward *et al.* 1983; Wirdefeldt *et al.* 2004) montrent qu'un facteur génétique ne peut sans doute pas être entièrement responsable de la MP, le facteur génétique ne peut pas non plus être entièrement exclu de l'origine de la maladie (Farrer *et al.* 2007).

Tableau I : Formes monogéniques majeures de la maladie de Parkinson

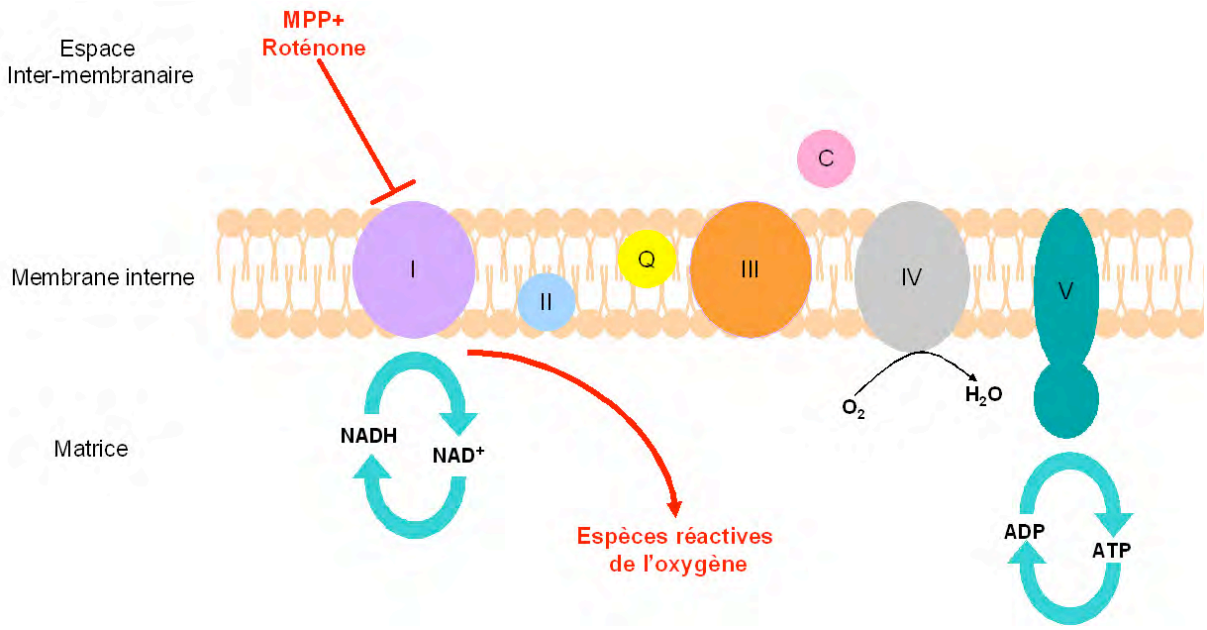
Gène	Locus	Transmission	Age moyen de début de la maladie	Mutations	Fonction de la protéine mise en jeu
PINK1	PARK6	Autosomique récessive	20-40 ans	40 mutations délétions	Kinase mitochondriale
DJ1	PARK7	Autosomique récessive	20-40 ans	10 mutations et délétion	Réponse au stress cellulaire
UCHL1	PARK5	Autosomique dominante	55-58 ans	1 mutation	Déubiquitinilase
SNCA (α-synucléine)	PARK1/PARK4	Autosomique dominante	30-45 ans	3 mutations faux sens / duplication ou triplication	Multiple rôles potentiels
Parkine	PARK2	Autosomique récessive	30 ans	Plus de 100 mutations différentes	Ubiquitine ligase
LRRK2	PARK8	Autosomique dominante	50 ans	Plus de 16 variants pathogéniques	Kinase de substrat inconnus

1.3.2 LES CAUSES ENVIRONNEMENTALES DE LA MALADIE DE PARKINSON

La MP dite idiopathique ou sporadique, représente la grande majorité des cas, plus de 90%. L'étude des facteurs de risque de la MP a débuté à la suite d'intoxications accidentelles de jeunes utilisateurs de drogues par un composé analogue à la mépéridine, le 1-méthyl-4-phényl-1,2,3,6-tétrahydropyridine (MPTP) (Langston *et al.* 1983; Langston and Ballard 1983). Le MPTP a provoqué chez ces personnes des altérations du contrôle des mouvements volontaires similaires à celles des parkinsoniens. Le tremblement de repos, l'akinésie et la rigidité étaient de plus corrigés, comme pour les patients, par la prise de L-DOPA. Ce composé a par la suite été caractérisé comme un précurseur d'inhibiteur du complexe I de la chaîne de transport

des électrons (Nicklas *et al.* 1985). Dès lors d'autres inhibiteurs mitochondriaux ont été suspectés comme les pesticides et les herbicides. Les études épidémiologiques (Butterfield *et al.* 1993; Elbaz and Tranchant 2007; Frigerio *et al.* 2006; Gorell *et al.* 1998) se sont tournées vers l'usage de ces composés, et ont montré des corrélations entre l'usage de ces substances et la survenue de la MP. Ainsi le rapport de risque entre l'exposition aux pesticides et la survenue de la MP est de 2,4 au Minnesota entre 1976 et 1995 (Frigerio *et al.* 2006). De nombreux autres facteurs de risques ont été évalués comme le travail dans l'industrie du métal (Elbaz 2007 ; Gorell *et al.* 1998), du papier (Rybicki *et al.* 1993).

Les résultats de ces études épidémiologiques ont grandement contribué au développement de modèles animaux expérimentaux de la MP dont nombres se sont révélés pertinents en reproduisant une partie des signes caractéristiques de la MP, perte de neurones dans la SNpc et agrégation protéique de type CL. Les inhibiteurs mitochondriaux couramment utilisés sont le MPTP et la roténone. Si leurs modes d'administration et d'action divergent, ces substances induisent toutes un important stress oxydatif qui va conduire à une perte de neurones dopaminergiques au sein de la SNpc. La roténone et le MPTP vont, par inhibition du complexe I de la chaîne de transport des électrons, induire une surproduction d'espèces réactives de l'oxygène (figure 4). Ces espèces réactives de l'oxygène vont induire un stress oxydatif. Bien que n'ayant pas la même spécificité cellulaire, le MPTP ainsi que la roténone induisent une neurodégénérescence préférentielle des neurones dopaminergiques de la SNpc (Betarbet *et al.* 2000 ; Bezard *et al.* 1997 ; Hoglinger *et al.* 2003). Cette destruction de la SNpc reproduisant en partie les symptômes moteurs de la MP par des agents toxiques suggère l'importance des facteurs environnementaux dans l'étiopathogénie de la MP.



L'inhibition du complexe I de la chaîne de transport des électrons de la mitochondrie par la roténone ou le MPP+, composé de dégradation du MPTP, va induire la surproduction d'espèces réactives de l'oxygène. I : complexe I ; II : complexe II ; III : complexe III ; IV : complexe IV ; V : complexe V ; Q : coenzyme Q ; C : cytochrome C.

Figure 4 : Inhibition du complexe I de la chaîne de transport mitochondriale des électrons par la roténone et le MPTP

1.4 SENSIBILITE DES SIGNES MOTEURS AU TRAITEMENT PAR LA LEVODOPA

La définition classique de la MP comme maladie du mouvement intègre de surcroît une sensibilité des troubles moteurs au traitement par la levodopa. L'administration de ce précurseur de la dopamine est utilisée pour compenser la diminution du niveau d'expression de la dopamine induite par la mort des neurones dopaminergiques de la SNpc et stimuler les récepteurs des cellules striatales. D'autres traitements à base d'agonistes des récepteurs à la dopamine comme l'apomorphine peuvent être utilisés mais apparaissent comme moins efficaces. La levodopa présente

une efficacité à long terme pour réduire le score de symptômes moteurs des patients de façon dose dépendante (Fahn *et al.* 2004).

2 SIGNES NON-MOTEURS DE LA MALADIE DE PARKINSON ET ATTEINTE EXTRA-DOPAMINERGIQUE

2.1 LES SIGNES NON-MOTEURS

Dès 1917, James Parkinson décrivait un certain nombre de symptômes non-moteurs de la maladie tels que l'incontinence urinaire, des troubles du sommeil, une constipation ou la démence. Depuis, de nombreux travaux ont été réalisés pour étudier ces signes non-moteurs de la MP et la liste a été complétée. L'ensemble de ces troubles non-moteurs regroupe désormais des symptômes neuropsychiatriques, des troubles du sommeil, des manifestations dites autonomes, des troubles digestifs, des symptômes sensitifs ainsi que diverses autres affections comme la fatigue et la perte de poids (Chaudhuri *et al.* 2006). Ces troubles non-moteurs représentent pour les patients parkinsoniens une réduction considérable de leur qualité de vie ainsi qu'un facteur supplémentaire d'hospitalisation (Aarsland *et al.* 2000). Bien que la prévalence de ces derniers soit relativement mal évaluée à cause de l'absence d'échelle de mesure exhaustive et reconnue de façon internationale, il est cependant admis que ceux-ci touchent une large majorité des patients et notamment les troubles digestifs. L'évolution de ces symptômes non-moteurs est proportionnelle à l'avancement de la maladie et domine le tableau clinique en fin d'évolution (Hely *et al.* 2005).

Contrairement à l'idée reçue, ces signes non-moteurs ne sont pas présents qu'en fin d'évolution de la maladie, ils peuvent même être pour certains d'entre eux préalables aux signes moteurs. Ainsi, les troubles de l'olfaction, de l'érection, la dépression et la

constipation peuvent être considérés comme des facteurs de risque de la MP (Chaudhuri *et al.* 2006 ; Hardoff *et al.* 2001 ; Tolosa *et al.* 2007).

De plus il apparaît que ces troubles non-moteurs (Chaudhuri and Schapira 2009 ; Goetz *et al.* 2005; Lees *et al.* 2009) et plus particulièrement les troubles digestifs (Soykan *et al.* 1997) ne sont pas améliorés, voire aggravés par le traitement dopaminergique. Ils présenteraient donc une origine probablement non-dopaminergique.

2.2 L'ATTEINTE EXTRA-DOPAMINERGIQUE POTENTIELLE RESPONSABLE DES SIGNES NON-MOTEURS

L'origine de ces troubles non-moteurs associés de la MP reste mal connue. Ils peuvent cependant être partiellement expliqués par des atteintes hors de la SNpc. Les altérations observées en dehors de la SNpc peuvent être de deux types, premièrement une perte neuronale et deuxièmement la formation de CL ou de NL couramment répertoriés dans diverses régions (Braak and Del Tredici 2008 ; 2009; Braak *et al.* 2003).

Ainsi des altérations de la viabilité neuronale ont été quantifiées dans le locus cœruleus, le noyau du raphé , le noyau pédonculo-pontin, le noyau basal de Meynert (Zarow *et al.* 2003) et le noyau dorsal du nerf vague (Del Tredici *et al.* 2002). La perte des neurones induit un déficit en acétylcholine, norépinephrine, et sérotonine. Une réduction de la production de ces neurotransmetteurs démontre une atteinte large des systèmes extra-dopaminergiques. De plus, il se développe en parallèle de ces altérations de la viabilité neuronale une pathologie de l'α-synucléine, marquée par la formation de CL et de NL, en relation avec ces troubles non-moteurs non-dopaminergiques. L'agrégation d'α-synucléine dans une région du système nerveux est un marqueur de son implication potentielle dans l'apparition des troubles non moteurs de la MP.

Il est ainsi possible de rapprocher les signes non-moteurs de la MP avec la formation d'agrégats protéiques. Les affections non-motrices induisant des troubles neuropsychiatriques peuvent ainsi être reliées avec des modifications du niveau de sérotonine (5-HT). Chez les patients parkinsoniens dépressifs une réduction du niveau de 5-HT a été mise en évidence (Mayeux *et al.* 1984). De plus la présence de CL au sein du noyau dorsal du raphé dont les neurones sont majoritairement sérotoninergiques pourrait expliquer cette diminution de 5-HT. Outre la dépression, la sévérité de la démence des patients parkinsoniens corrélée à un déficit cholinergique au niveau du cortex (Perry *et al.* 1990) est aussi corrélée à la densité de NL et de CL au niveau cortical (Churchyard and Lees 1997).

Les troubles du sommeil, et plus particulièrement ceux de la phase de mouvement oculaires rapides et la somnolence diurne excessive, peuvent être associés à des structures atteintes au cours de la MP : le locus cœruleus, le noyau du raphé et le noyau pédonculo-pontin entre autres.

L'anosmie, altération sensitive majeure de la MP, peut elle être étroitement associée à la présence de NL et de CL présents au sein du bulbe olfactif (Daniel and Hawkes 1992 ; Hawkes *et al.* 2007).

Les troubles digestifs peuvent être rapprochés de l'atteinte du noyau dorsal du nerf vague qui présente une perte de neurones cholinergiques ainsi que des inclusions de Lewy (Del Tredici *et al.* 2002). En effet, le nerf vague joue un rôle clé dans le contrôle des fonctions digestives.

Ces atteintes, perte neuronale ou inclusions de Lewy, pourraient expliquer l'apparition des symptômes non-moteurs de la MP. Cependant il reste difficile de justifier l'étendue de ces symptômes non-moteurs par ces seules altérations et en

particulier pour les fonctions digestives dont la régulation est principalement assurée par un système nerveux autonome et indépendant.

3 SYSTEME NERVEUX ENTERIQUE ET MALADIE DE PARKINSON

3.1 LE SYSTEME NERVEUX ENTERIQUE

La première description du système nerveux entérique (SNE) en tant que système nerveux indépendant a eu lieu en 1899 lorsque Bayliss et Starling (Bayliss and Starling 1899) ont découvert l'existence d'un réflexe coordonné conduit par un système nerveux local dans des anses intestinales isolées en réponse à un stimulus mécanique ou de façon spontanée. Ces travaux ont ensuite été confirmés par Langley et Magnus en 1905 qui ont affirmé que ces contractions coordonnées n'étaient pas un simple réflexe axonal du SNC (Langley and Magnus 1905). Ces observations fonctionnelles sont venues s'ajouter aux descriptions anatomiques faites par Auerbach et Meissner qui avaient déjà en 1864 et 1857 respectivement montré la présence de neurones dans la paroi intestinale (Timmermans *et al.* 1997).

3.1.1 STRUCTURE DU SYSTEME NERVEUX ENTERIQUE

Le SNE est un réseau dense de ganglions qui est présent dans l'intégralité du tube digestif, de l'œsophage jusqu'au rectum. Ces ganglions sont interconnectés entre eux par des fibres interganglionnaires qui associent fibres intrinsèques et fibres extrinsèques. Le SNE contient selon les estimations 2 à 6.10^8 neurones, c'est-à-dire autant que la moelle épinière (Goyal and Hirano 1996).

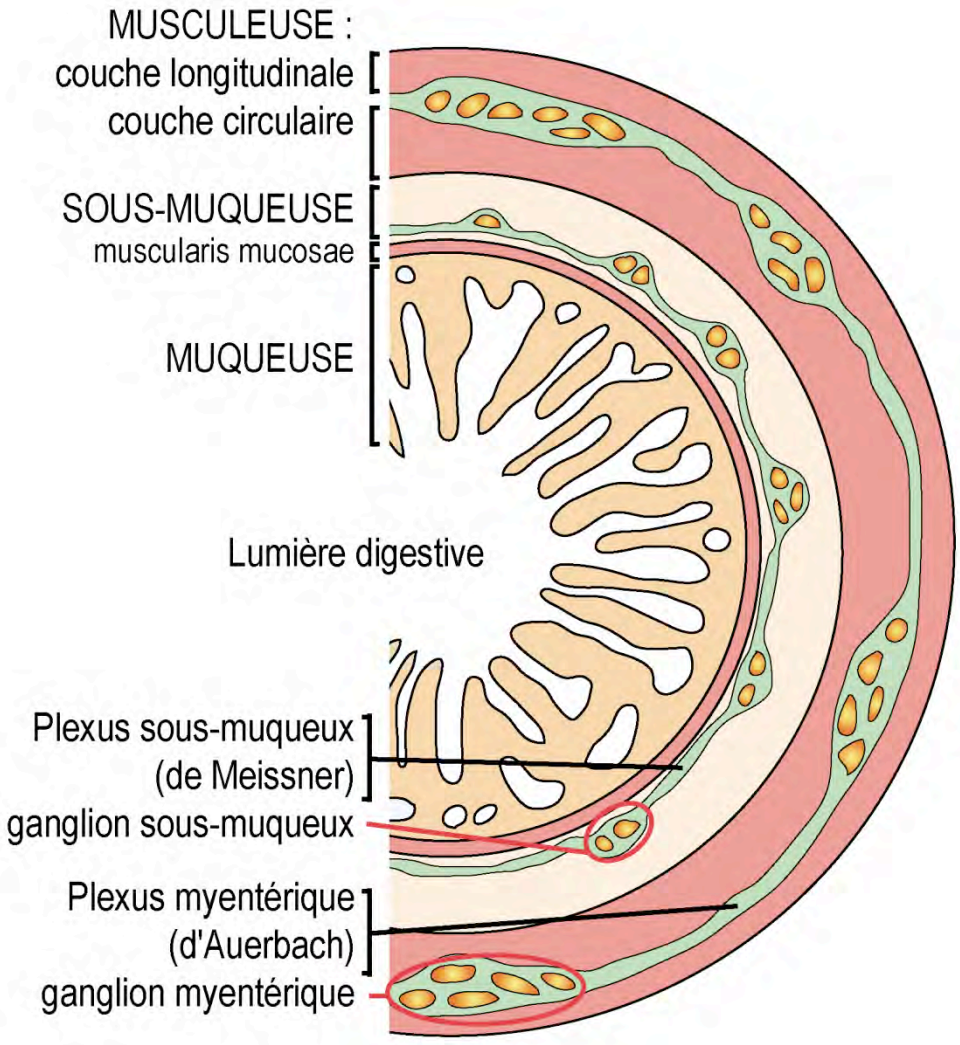
Le SNE est subdivisé en différents plexus présents en nombre variable en fonction des espèces, au nombre de 5 chez l'Homme. Cependant les deux plexus majeurs

du SNE sont présents chez l'ensemble des mammifères, le plexus sous-muqueux (PSM), ou de Meissner, et le plexus myentérique (PM), ou d'Auerbach (Figure 4). A partir de la lumière du tube digestif le premier plexus rencontré est le PSM, situé directement sous la muqueuse digestive ; le PM est compris entre les couches de muscles lisses circulaires et longitudinales. Ces différents plexus ne sont pas répartis de façon homogène le long du tractus digestif et présentent des différences morphologiques qui peuvent évoquer un rôle fonctionnel différent. Le PM est présent sur l'intégralité du tractus digestif tandis que le PSM est présent tout le long de l'intestin et du côlon mais absent de l'œsophage. De plus ces plexus ne présentent pas la même densité neuronale, la même morphologie ni la même composition neurochimique (Furness 2000 ; Timmermans *et al.* 1997 ; Wedel *et al.* 1999).

D'autre part, le nombre et l'organisation des plexus du SNE varient selon les espèces reflétant probablement une adaptation du SNE lors de l'évolution des espèces. Chez le cobaye, il n'existe qu'un seul PSM tandis que chez le porc, on en distingue 2 : le plexus de Meissner, localisé dans la sous-muqueuse proche de la couche *muscularis mucosae* et le plexus de Schabadasch, reposant sur la couche musculaire lisse circulaire. Chez l'homme, on distingue un 3^{e} PSM, le plexus intermédiaire, localisé entre le plexus de Meissner et le plexus de Schabadasch (Timmermans *et al.* 1997 ; Wedel *et al.* 1999)

Le PSM contrôle principalement les fonctions de la muqueuse intestinale (sécrétion, absorption, défense…) tandis que le PM régule principalement les fonctions de motricité du tube digestif. Cependant, des communications étroites existent entre les plexus permettant de contrôler les fonctions de sécrétion et de motricité (Cooke *et al.* 1993). Le SNE forme également des projections vers les ganglions cœliaques et mésentériques (Szurszewski *et al.* 2002) et innerve également d'autres organes tels que

le pancréas (Anglade *et al.* 1987 ; Kirchgessner *et al.* 1992) et la vésicule biliaire (Mawe and Gershon 1989).



Les principaux plexus du système nerveux entérique sont le plexus sous-muqueux situé entre la muqueuse et la sous-muqueuse, et le plexus myentérique situé entre les couches de muscle lisse circulaire et longitudinal. Ces plexus sont composés de ganglions (entourés en rouge) interconnectés par des fibres interganglionnaires.

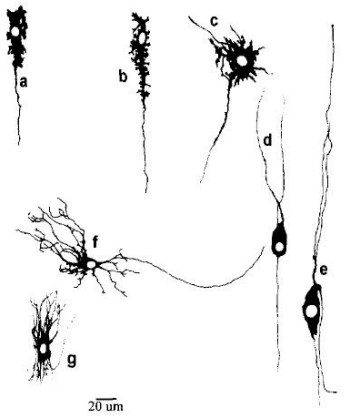
Figure 4 : Le système nerveux entérique

3.1.2 POPULATIONS CELLULAIRES DU SYSTEME NERVEUX ENTERIQUE

Le SNE dont les cellules sont originellement issues du segment vagal et sacré de la crête neurale, se forme par migration de ces cellules au cours de l'embryogénèse. Les cellules de la crête neurale migrent de l'extrémité orale vers l'extrémité anale du tractus digestif, puis se différencient soit en cellules gliales soit en neurones (Heanue and Pachnis 2007). La différenciation de ces cellules commence dès le début de leur migration (Heanue and Pachnis 2007), cependant ces cellules gardent des capacités prolifératives tout au long de leur migration même si elles expriment déjà des marqueurs de différenciation. Une deuxième vague de migration prend son origine au niveau sacré. Ces cellules de la crête neurale suivent une progression caudo-rostrale.

3.1.2.1 Les neurones

Les neurones du SNE ont été en premier lieu caractérisés et décrits par Dogiel en 1899 selon leur morphologie. La classification de Dogiel (figure 5), complétée par les travaux de Stach et de Furness, a permis de classer les neurones de type I à VII et neurones géants (Furness *et al.* 1988 ; Timmermans *et al.* 1997). La majorité des neurones entériques sont de type I à III (Hansen 2003). Les neurones de type I présentent un seul axone et de multiples dendrites courtes et lamellaires. Les neurones de type II possèdent plusieurs axones et n'ont pas de dendrites. Enfin, les neurones de type III sont uniaxonaux et présentent de longues dendrites.



***Principaux types morphologiques de neurones entériques de l'intestin grêle chez le cobaye selon la classification de Dogiel**. **a-c** : neurones de type I, uniaxonaux multidendritiques (courtes et lamellaires) ; **d-e** : neurones de type II, adendritiques multiaxonaux ; **f** : neurones de type III, uniaxonaux et multidendritiques (longues) ; **g** : neurones filamenteux. D'après Timmermans et al, 1997.*

Figure 5: Classification de Dogiel

Les propriétés électrophysiologiques des neurones définissent leur comportement lors de stimuli électriques. La réponse neuronale à ces stimuli régule la libération de neuromédiateurs (quantité et type de neuromédiateurs) à l'interface neuro-neuronale ou neuro-effectrice. On identifie deux grandes classes de neurones : les neurones « synaptiques » (S) et les neurones « after hyperpolarization »(AH) (Hirst *et al.* 1974). Les neurones de type S correspondent à des neurones recevant des signaux provenant d'autres neurones *via* des synapses. Ces neurones sont de type I selon la classification de Dogiel. Les neurones AH sont caractérisés par des potentiels d'action insensibles à la tétrodotoxine suivis d'une hyperpolarisation tardive. Ces neurones ont une morphologie de Dogiel de type II (Furness *et al.* 2004). Stimulés, ces neurones vont générer un potentiel d'action qui résulte de l'intégration des stimuli électriques et chimiques en une somme de potentiels post-synaptiques pouvant être excitateurs rapides ou lents ou encore inhibiteurs.

Les neurones entériques synthétisent et sécrètent des substances chimiques variées, les neuromédiateurs, qui leur permettent de communiquer entre eux et avec

leurs cellules cibles effectrices. Plus de 30 neuromédiateurs ont été répertoriés dans le SNE, et jusqu'à 11 neuromédiateurs différents ont été identifiés dans un même neurone (Tableau II) (Furness 2000). Ces neuromédiateurs sont de petites molécules telles que la 5-HT, l'acétylcholine ou l'ATP, des peptides comme le vasoactive intestinal peptide (VIP) ou encore des gaz tels que le monoxyde d'azote (NO). De nombreuses études associant des techniques d'immunohistochimie, de traçage neuronal couplées à des études fonctionnelles et pharmacologiques ont permis de relier le codage neurochimique du neurone à sa fonction.

Tableau II : Diversité du codage neurochimique des neurones entériques

Fonction	codage neurochimique
IPAN	ChAT, Calb, CGRP, SP
Interneurones ascendants	ChAT, Calret, ENK, SP
Interneurones descendants	5-HT, ChAT, DYN, GRP, NOS, somatostatin, VIP
Neurones moteurs excitateurs	ChAT, Calret, ENK, SP
Neurones moteurs inhibiteurs	DYN, ENK, GRP, NOS, VIP
Neurones sécrétomoteurs	ChAT, CCK, CGRP, DYN, NPY, somatostatin, VIP

Abréviations : 5-HT : sérotonine (5-hydroxytryptamine) ; ChAT : choline acétyltransférase ; Calb : calbindine ; Calret : calrétinine ; CGRP : calcitoningene-related peptide ; DYN : dynorphine ; ENK : enképhalines ; GRP : gastrin releasing peptide ; IPAN : intrinsicprimaryafferentneurons ; NOS : nitricoxidesynthase ; SP : substance P ; NPY : neuropeptide Y ; VIP : vasoactive intestinal peptide. D'après Hansen et al, 2003

3.1.2.2 Les cellules gliales

Les cellules gliales entériques (CGE) possèdent de nombreuses propriétés morphologiques et fonctionnelles communes avec l'astroglie du SNC. Quatre fois plus

nombreuses que les neurones, les CGE forment un dense réseau cellulaire au sein du tissu nerveux entérique, en contact étroit avec le pôle basal de l'épithélium digestif et avec les vaisseaux de la sous-muqueuse (Neunlist *et al.* 2008; Ruhl 2005). Une seule cellule gliale entoure plusieurs neurones (Gershon and Rothman 1991), de plus ces cellules sont à même de communiquer entre elles *via* des jonctions communicantes (Maudlej and Hanani 1992). Par ailleurs, comme pour les neurones entériques, il existe pour les CGE des différences notables entre les espèces mais aussi entre les différents plexus et leur localisation le long du tractus digestif. Ainsi la densité des CGE varie en fonction de l'espèce animale et de leur localisation au sein d'un ou de l'autre des plexus (Hoff *et al.* 2008). Chez le cobaye, le rapport nombre de CGE/nombre de neurones entériques est de 0,8-1,0 dans le PSM contre 1,7 dans le PM. Chez l'homme, le rapport nombre de CGE/nombre de neurones est de 1,3-1,9 dans le PSM tandis qu'il est de 5,9-7,0 le PM (Hoff *et al.* 2008). D'après cette étude le rapport nombre de CGE/nombre de neurones entériques semble augmenter selon un axe phylogénétique.

De nombreux travaux ont montré que les CGE jouent un rôle majeur dans la régulation de l'homéostasie du SNE. Leur fonction trophique et cytoprotectrice vis-à-vis des neurones dont elles régulent l'activité (par exemple en assurant la synthèse de précurseurs de neuromédiateurs) n'est que l'un des aspects de leur activité. En effet, les CGE jouent un rôle majeur dans le contrôle des fonctions neuronales. Elles sont ainsi capables de réguler le phénotype neurochimique des neurones entériques (Aube *et al.* 2006). Dans cette dernière étude l'ablation des cellules gliales induisait une augmentation du nombre de neurones cholinergiques et une diminution du nombre de neurones nitrergiques. Les CGE sont aussi mises en jeu dans la neuroprotection en particulier via la sécrétion de facteurs solubles (Abdo *et al.* 2010).

Les CGE sont aussi nécessaires au maintien des fonctions de la barrière épithéliale intestinale. En effet, dans un modèle murin transgénique, des lésions spécifiques des CGE entrainaient des altérations majeures de l'intégrité de la barrière épithéliale intestinale (Bush *et al.* 1998). Ces travaux ont été récemment confirmés par des études *in vitro* qui montrent que les CGE contrôlent la perméabilité paracellulaire de la barrière épithéliale intestinale via la production d'un facteur soluble le S-nitrosoglutathion (Savidge *et al.* 2007). Les CGE contrôlent aussi la prolifération et la différenciation des cellules épithéliales intestinales (Neunlist *et al.* 2007).

3.1.2.3 Autres populations

Les cellules interstitielles de Cajal (CIC), cellules *pacemaker*, sont localisées entre le muscle circulaire et le muscle longitudinal au niveau du PM. Elles génèrent spontanément des ondes lentes qui se propagent tout au long de l'intestin (Sanders *et al.* 2006). Les CIC forment des jonctions communicantes entre elles et avec les cellules du muscle lisse vers lesquelles elles transmettent les signaux électriques qu'elles génèrent (Ward and Sanders 2006; Ward *et al.* 2004). Par ailleurs, les CIC sont innervées par les neurones entériques et expriment différents récepteurs aux neuromédiateurs tels que des récepteurs muscariniques, des récepteurs aux neurokinines et au VIP, suggérant qu'elles participent directement à la transmission du signal neuronal vers les cellules musculaires (Ward *et al.* 2004) ou que leur activité est régulée par les neurones entériques.

3.1.2.4 Fonctions du SNE

Premier mécanisme mis en évidence démontrant l'autonomie du SNE (Bayliss and Starling 1899), le péristaltisme intestinal assure la grande majorité de la

progression du bol alimentaire le long du tractus digestif. C'est un mécanisme réflexe qui au travers du SNE conduit schématiquement à une relaxation des muscles lisses digestifs du versant anal et une contraction du versant oral. La répétition de ce réflexe induit des vagues de contraction relaxation le long du tractus digestif qui poussent le bol alimentaire vers le côté anal.

Le réflexe péristaltique est induit par des stimuli variés tel qu'une distension mécanique, le frottement du bol alimentaire sur la muqueuse digestive, la présence d'acides gras ou une variation de pH. Ces stimuli activent les cellules entérochromaffines, cellules épithéliales endocrines, qui libèrent alors la 5-HT qu'elles contiennent (Ahlman and Nilsson 2001). Cette sécrétion de 5-HT active les neurones afférents primaires intrinsèques (IPAN) de la muqueuse intestinale (Furness 2000).

Ces IPAN sont des neurones de type II selon la classification de Dogiel et ont un comportement de type AH. Ils expriment les neuromédiateurs ACh, calbindine, substance P et la CGRP chez l'animal (Furness 2000 ; Wolf *et al.* 2007). Ils sont connectés avec les interneurones du tube digestif qu'ils vont activer ou inhiber en fonction de leur propre orientation orale ou anale.

Les interneurones classés dans la catégorie de Dogiel II sont de type S ou AH (Brehmer *et al.* 2006 ; Hansen 2003). Ils expriment divers phénotypes neurochimiques selon leur propre orientation. Les interneurones ascendants sont principalement cholinergiques mais expriment aussi d'autres neuromédiateurs comme la calrétinine, ou les tachykinines (Brookes *et al.* 1997). Les interneurones descendants sont plus variés, ils expriment un panel différent de neurotransmetteurs selon qu'ils sont impliqués dans le réflexe péristaltique, Ach/NO/VIP, dans le réflexe sécrétomoteur, Ach/5-HT, ou

encore dans la conduction de complexes myoélectriques migrants, Ach/somatostatine (Furness 2000).

Les interneurones impliqués dans le réflexe péristaltique sont connectés aux neurones moteurs. Les neurones moteurs sont des neurones S et de type I selon la classification de Dogiel. Ces neurones sont soit excitateurs soit inhibiteurs et sont en contact avec la couche circulaire ou longitudinale de la *muscularis propria*, ou encore avec la *muscularis mucosæ*. Les motoneurones excitateurs sont principalement cholinergiques et tachykininergiques. Les motoneurones inhibiteurs sont principalement nitrergiques mais peuvent exprimer également le VIP et l'ATP entre autre.

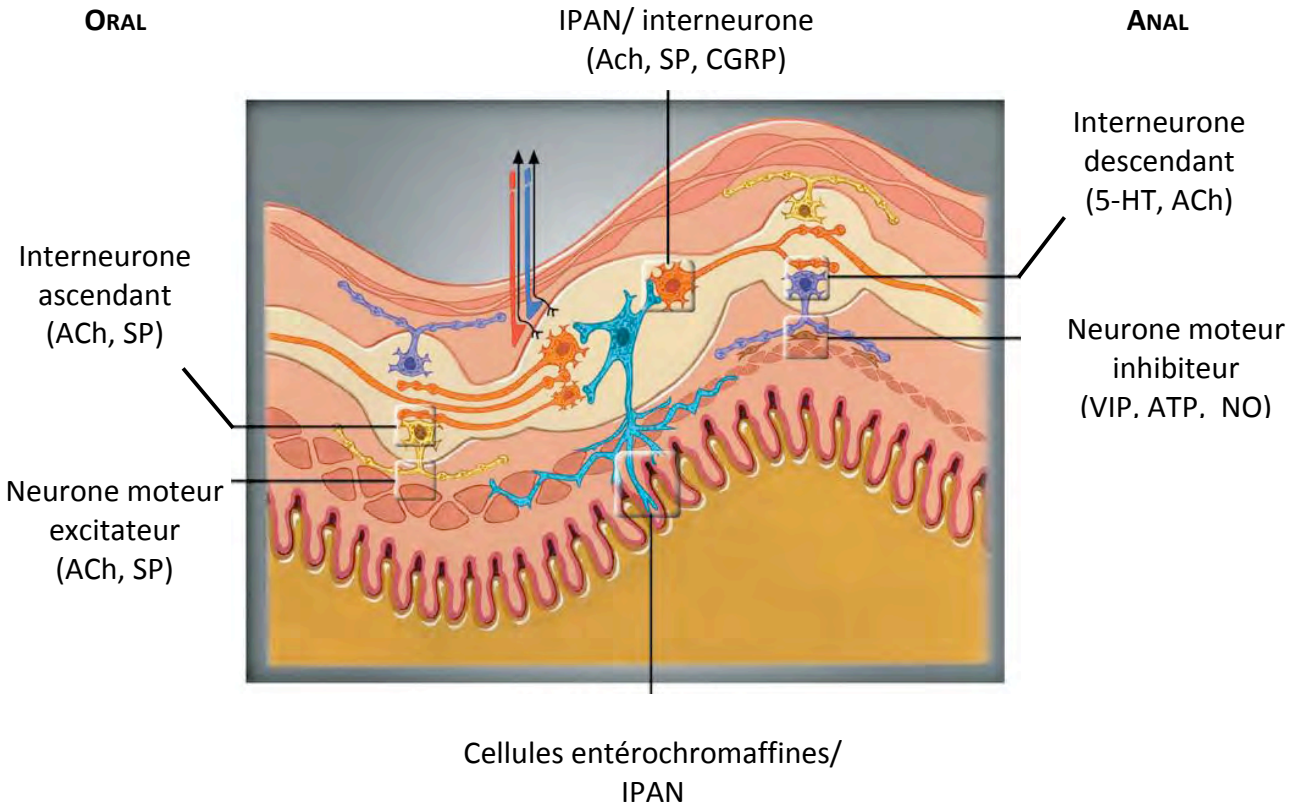


Figure 6 : Schéma des voies nerveuses intrinsèques impliquées dans le réflexe péristaltique

Suite à une stimulation au niveau de la muqueuse, la 5-hydroxytryptamine – sérotonine (5-HT) est libérée par les cellules entérochromaffines et active les intrinsic primary afferent neurons (IPAN). Les IPAN libèrent alors l'acétylcholine (ACh), la substance P (SP) et la calcitonin gene-related peptide (CGRP) qui activent les interneurones ascendants et descendants. Les interneurones ascendants activés vont libérer de l'ACh et de la SP activant ainsi les neurones excitateurs. Ces derniers libèrent alors de l'ACh et de la SP au niveau de la jonction neuromusculaire, ce qui induit la contraction musculaire. Par ailleurs, les interneurones descendants activés vont libérer de l'Ach, du monoxyde d'azote (NO), du vasoactive intestinal peptide(VIP) et de l'adénosine triphosphate (ATP) et ainsi activer les neurones moteurs inhibiteurs qui, en libérant du NO, du VIP et de l'ATP, provoquent une relaxation musculaire. D'après Hansen el al, 2003.

3.2 LES ATTEINTES DU SYSTEME NERVEUX ENTERIQUE AU COURS DE LA MALADIE DE PARKINSON

3.2.1 ATTEINTES FONCTIONNELLES

Parmi les nombreuses manifestations non-motrices répertoriées chez les parkinsoniens, les troubles digestifs sont sans doute les plus fréquents. Ils touchent potentiellement l'ensemble du tractus digestif (Pfeiffer 2003), de la bouche à l'anus.

La dysphagie est présente chez plus de 50% des patients (Edwards *et al.* 1994). Elle peut s'accompagner d'un phénomène d'hypersalivation qui serait observé chez 70% des patients parkinsoniens, reflet d'un défaut de la déglutition secondaire à une dysphagie. En effet, la production de salive est en réalité diminuée chez les patients (Pfeiffer 2003). Les patients parkinsoniens peuvent de plus présenter des perturbations de la motricité œsophagienne basse allant jusqu'à l'apéristaltisme (Castell *et al.* 2001). D'autres altérations motrices sont répertoriées dans l'œsophage tel que le ralentissement du transit, des spasmes ou des contractions spontanées de l'œsophage proximal.

Au niveau gastrique, une gastroparésie est rapportée chez 45% des patients (Edwards *et al.* 1994). Ainsi le temps de demi-vidange gastrique était de 44±11 min dans le groupe témoin et de 55±26 min dans le groupe des patients parkinsoniens modérés et de 64±29 min dans le groupe des patients plus atteints (Hardoff *et al.* 2001). Ce ralentissement de la vidange gastrique peut être à l'origine de nausées ou d'épigastralgies.

Pour l'intestin grêle relativement peu d'altérations sont répertoriées du fait de sa difficile accessibilité. Des modifications des aspects manométriques ainsi qu'une dilatation importante du grêle ont été rapportées (Bozeman *et al.* 1990; Lewitan and Nathanson 1954).

L'atteinte la plus fréquente du tube digestif rencontrée au cours de la maladie est la constipation (Edwards *et al.* 1994; Pfeiffer 2003), 60% des patients selon les critères de Rome III (Longstreth *et al.* 2006). Cette atteinte semble secondaire à une altération de la motricité colique. Il a, en effet, été montré une corrélation négative entre la fréquence des exonérations et le risque de survenue de MP (Abbott *et al.* 2001 ; Savica *et al.* 2009) (Tableau III).

Tableau III : Incidence de la MP en fonction de la fréquence des selles

Bowel movements/d	Sample size	Incident PD cases	Incidence, rate/10,000 person-years	
			Unadjusted	Age-adjusted
<1	289	10	19.6	18.9
1	4371	66	8.0	7.9
2	1704	17	5.2	5.4
>2	426	3	3.8	3.9
Test for trend	—	—	$p = 0.002$	$p = 0.005$
Overall	6790	96	7.5	—

L'incidence de la MP est inversement proportionnelle à la fréquence des défécationss, environ 0.2% des patients ayant moins d'une selle par jour ont développé une MP contre seulement 0.04% des patients ayant plus de deux selles par jour. D'après Abbott et al. *2001.*

D'autres facteurs peuvent intervenir et agir en synergie comme une plus faible absorption d'eau liée à une diminution de la sensation de soif ou encore un manque d'activité physique des patients. La constipation est parfois sévère pouvant aller jusqu'à une pseudo-obstruction ou un volvulus du sigmoïde (Marinella 1997; Rosenthal and Marshall 1987). Dans ces études un cas de pseudo-obstruction compliqué par une perforation caecale et quatre cas de volvulus du sigmoïde ont été rapportés chez des patients parkinsoniens.

Pour finir, les patients peuvent aussi souffrir d'une dysfonction anorectale liée à une mauvaise coordination entre contraction et relaxation des muscles anorectaux et abdominaux-pelviens.

Comme les autres signes non-moteurs de la maladie, les troubles digestifs sont parfois précurseurs des signes moteurs. Ceci en fait aussi un facteur de risque, en effet une fréquence de selles réduites représente une augmentation du risque relatif de développer la maladie de 2,48 (Abbott *et al.* 2001; Savica *et al.* 2009). De plus la régulation de la motricité du tube digestif, assurée par des muscles lisses, est sous la dépendance du SNE. Par déduction celui-ci peut présenter des altérations morphologiques potentiellement responsables de ces altérations fonctionnelles.

3.2.2 ATTEINTES MORPHOLOGIQUES

La première étude mettant en évidence la présence de CL au sein du SNE a été publiée en 1984 (Qualman *et al.* 1984). Cette étude de prélèvements autopsiques sur 3 patients parkinsoniens dysphagiques a mis en évidence la présence de CL au sein du PM œsophagien chez 2 d'entre eux. Cette étude comparait ces altérations à d'autres

observées chez des patients ayant une achalasie et concluait que les parkinsoniens dysphagiques et les patients achalasiques présentaient peut être le même mécanisme de neurodégénérescence responsable des troubles de motricité de l'œsophage car 2 patients achalasiques sur 8 patients présentaient aussi des CL. Cependant les inclusions retrouvées chez les patients achalasiques n'ont pas depuis été confirmées. De plus les 2 patients de cette étude pourraient être associés à un stade pré-symptomatique de la MP. Cette étude représente ainsi la première mise en évidence de CL au sein du SNE chez des patients parkinsoniens symptomatiques et peut être pré-symptomatiques.

Ensuite Kupsky *et al.* ont montré, en 1987, la présence d'inclusions éosinophiles similaires au CL au sein du PM et du PSM colique d'un patient parkinsonien ayant une colectomie pour un mégacôlon.

Puis Wakabayashi dans une série d'études débutée en 1988 a cartographié la présence de ces CL au sein du tractus digestif et du SNE. Les CL étaient retrouvés dans les plexus entériques de tous les étages du tube digestif des 7 patients autopsiés, de l'œsophage haut au rectum (Wakabayashi *et al.* 1988). Les CL sont majoritairement le plus présents dans le PM de l'œsophage distal. Cependant dans cette étude des CL étaient présents chez 8 des 24 témoins ; ce chiffre paraît surévalué en comparaison avec les travaux actuels. Les progrès en immunohistochimie, en particulier le marquage de l'α-synucléine lui ont permis ensuite à ces auteurs de proposer que ces inclusions apparaissaient préférentiellement dans les neurones entériques exprimant la tyrosine hydroxylase (TH) (Wakabayashi *et al.* 1989). Cependant dans un travail ultérieur il a démontré que les CL et NL se développaient majoritairement dans les neurones VIPergiques (Wakabayashi *et al.* 1990). En 1995 (Singaram *et al.* 1995) une autre publication a montré une altération importante de l'expression de la DA au niveau du

PM de patients parkinsoniens sans altérations de l'expression de la TH. Cette étude réalisée sur 11 patients parkinsoniens montrait une réduction majeure du niveau de DA chez 9 de ces patients. Par ailleurs les proportions de neurones TH et VIP immunoréactifs n'était pas modifiées. De plus des CL ont été retrouvés, par marquage à l'éosine, dans les neurones VIPergiques et dopaminergiques principalement. Depuis ces premiers travaux l'immunomarquage de l'α-synucléine (Spillantini *et al.* 1997) a permis d'effectuer un marquage plus précis et plus spécifique des NL et CL. L'immunohistochimie a permis alors de révéler la présence pour la première fois des NL au niveau du SNE de l'antre de patients parkinsoniens (Braak *et al.* 2006). Les fibres neuronales positives pour l'immunomarquage contre l'α-synucléine avaient un aspect dystrophique et étaient principalement localisées dans le PSM tandis que les CL étaient observés surtout dans le PM de l'estomac. Une étude a récemment montré qu'il existait un gradient rostro-caudal de l'expression d'α-synucléine chez le rat au sein du PM (Phillips *et al.* 2008). La densité d'α-synucléine était minimale dans le PM de l'estomac et maximale dans le PM du jéjunum.

Malgré ces études complètes sur la localisation des CL il n'a pas encore été mis en évidence de perte neuronale au sein du SNE spécifique chez l'Homme qui pourrait permettre d'expliquer les altérations des fonctions digestives observées chez les patients. La relative difficulté d'accès aux prélèvements autopsiques ou chirurgicaux représente un frein à la caractérisation des altérations du SNE au cours de la MP.

A l'inverse des autres altérations extra-dopaminergiques pouvant être impliquées au moins en partie dans l'altération de fonctions non-motrices comme la baisse du niveau de sérotonine et la dépression (Grinberg *et al.* 2010 ; Mayeux *et al.*

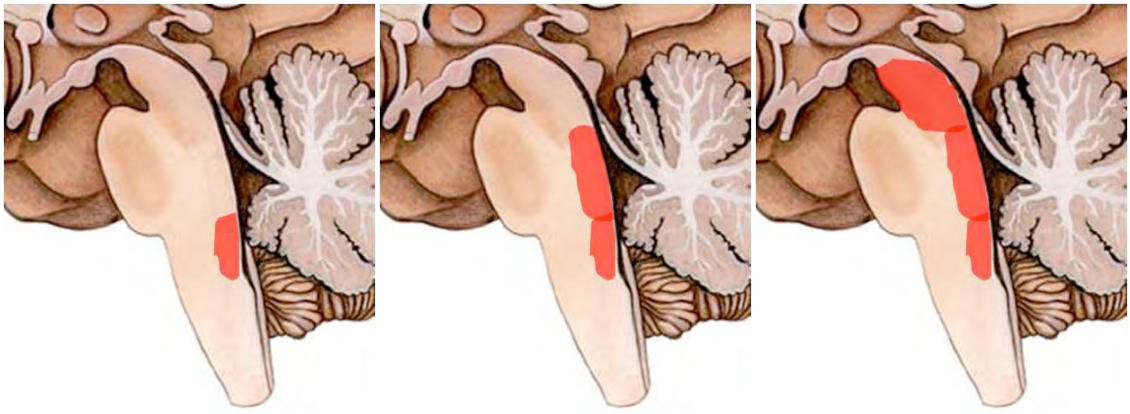
1984), il n'a pas été établi de corrélation entre des altérations extra-nigrales et les troubles digestifs des patients parkinsoniens.

Depuis 2006 et la série de travaux de Braak, un regain d'intérêt pour le SNE est constaté. En effet, en se basant sur le marquage de ces agrégats d'α-synucléine à la suite de larges études sur des prélèvements autopsiques, l'anatomopathologiste allemand Heiko Braak *et al.* ont proposé un modèle de progression temporo-spatiale des formes sporadiques de la MP (figure 7). Ce modèle est la conclusion d'une large étude transversale sur 168 patients.

Dans ce modèle, la MP débuterait au niveau du système nerveux périphérique soit au niveau du bulbe olfactif soit au niveau du SNE puis, suivant une progression rétrograde via le nerf vague, remonterait vers le SNC. Cette progression ascendante pourrait être divisée en 6 stades distincts. De façon schématique les premières inclusions apparaîtraient au stade 1 dans le noyau dorsal moteur du nerf vague et dans les bulbes olfactifs ; des agrégats d'α-synucléine sont alors déjà présents dans les centres sympathiques médullaire (Braak *et al.* 2007), dans les axones des efférences vagales et au niveau du SNE (Braak *et al.* 2006). Au second stade les inclusions progressent vers les noyaux du raphé, du tronc cérébral et la moelle allongée. Au troisième stade le mésencéphale et le prosencéphale sont touchés, mais ce stade représente aussi l'apparition de prolongements et de CL au sein de la SNpc, cependant sans perte neuronale. Au stade 4 le cortex présente lui aussi des inclusions d'α-synucléine. Au stade 5, les altérations progressent au sein du cortex mais aussi des autres structures préalablement atteintes. La perte neuronale dans la SNpc se révèle enfin lors du sixième et ultime stade.

Cette même équipe a ensuite proposé que la MP pourrait être déclenchée par un neurotoxique environnemental qui serait inhalé ou ingéré. Ce modèle reste discuté, il existe en effet diverses études (Attems and Jellinger 2008; Kalaitzakis *et al.* 2008) qui montrent que nombre de patients autopsiés ne peuvent être classés dans aucun des 6 stades proposés. Cependant une nouvelle étude réalisée par un consortium européen (Alafuzoff *et al.* 2009) a mis en évidence au moins 80% de cas correspondants aux critères de Braak. De plus de récentes études montrent que l'α-synucléine supposée responsable de cette progression centrifuge peut être sécrétée (Lee *et al.* 2005) et ainsi contaminer les neurones sains adjacents (Desplats *et al.* 2009). En outre l'existence d'une voie ininterrompue de neurones exprimant l'α-synucléine reliant le SNE au SNC a été identifiée (Phillips *et al.* 2008). Ces différentes études appuient l'hypothèse d'une propagation de la pathologie d'un système nerveux à l'autre. En outre la découverte d'inclusion au sein de neurones nouvellement greffés (Kordower *et al.* 2008 ; Li *et al.* 2008) pourrait s'expliquer en appliquant cette théorie.

L'ensemble de ces données réoriente vers une origine périphérique de la maladie, le SNE pourrait être atteint de façon primitive dans la MP. L'autre porte d'entrée supposée de la maladie serait le bulbe olfactif qui représente un autre système nerveux situé à proximité des muqueuses et du milieu extérieur. La pathologie progresserait ensuite de façon rétrograde vers le SNC de manière similaire à la progression du nouveau variant du prion.



Selon la théorie de Braak les altérations observées dans le tronc cérébral progresseraient de façon centripète affectant en premier lieu le noyau dorsal du nerf vague (panneau de gauche) puis progresserait pour toucher le locus cœruleus (panneau central). La substance noire ne serait atteinte que tardivement (panneau de droite).

Figure 7 : Progression temporo-spatiale des inclusions d'α-synucléine dans le tronc cérébral (d'après Braak)

3.3 L'ATTEINTE DU SYSTEME NERVEUX ENTERIQUE DANS LES MODELES ANIMAUX DE LA MALADIE DE PARKINSON

Malgré l'importance des troubles digestifs chez les patients parkinsoniens, peu de travaux ont été consacrés à la compréhension des mécanismes sous-jacents, et en particulier à l'analyse et à la caractérisation du SNE, principal régulateur de la motricité digestive. La fonction digestive, de même que le SNE, ont peu été étudiés dans les modèles déjà existants et reconnus de MP.

3.3.1 ATTEINTES FONCTIONNELLES

Certaines altérations des fonctions digestives ont déjà été étudiées dans plusieurs modèles animaux de MP. Ces études récentes tentent de caractériser les

atteintes fonctionnelles digestives de modèles toxiques ou génétiques connus pour reproduire au niveau du SNC une MP.

Une première étude réalisée dans un modèle murin d'intoxication au MPTP (Anderson *et al.* 2007) a montré des atteintes de la fonction colique *ex-vivo* avec une augmentation de la force de contraction des muscles longitudinaux et circulaires du côlon chez la souris. Cependant ces résultats *ex vivo* n'étaient pas confirmés par les expériences *in vivo* qui ne révélaient qu'une augmentation transitoire de la motricité colique.

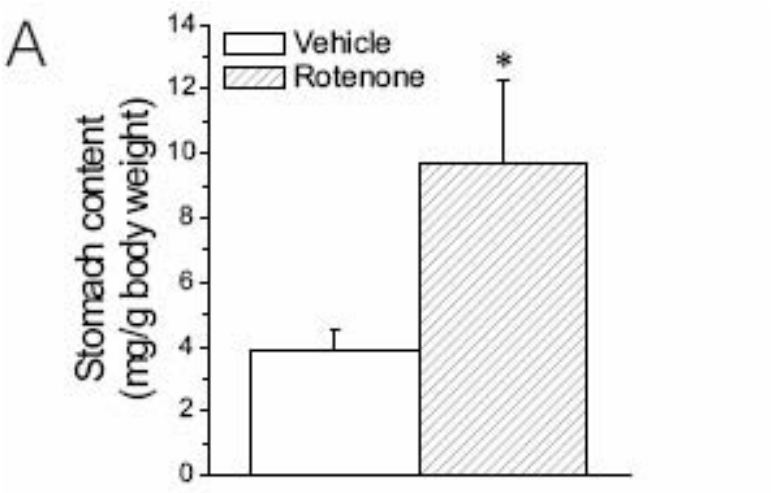
Plus récemment l'administration chronique de roténone chez des rats par voie sous-cutanée a permis d'observer un ralentissement de la vidange gastrique (figure 8) constaté chez les patients parkinsoniens associé à un ralentissement du rythme d'exonération des selles. Ces altérations *in vivo* ont été corrélées à des altérations *ex vivo* de la réponse contractile à la stimulation du SNE (Greene *et al.* 2009). Ces résultats sont appuyés par une autre équipe qui en utilisant le même toxique a démontré un ralentissement du transit global chez les rats traités (Drolet *et al.* 2009).

Une étude réalisée chez des rats ayant reçu une injection intracrânienne de 6-hydroxydopamine (6-OHDA) a montré une diminution significative du rythme d'exonération des selles chez ces animaux (Blandini *et al.* 2009).

Ces toxiques induisent un stress oxydant considéré comme un facteur majeur de la MP. Par ailleurs, une production d'espèces réactives de l'oxygène induite par des herbicides tels que le paraquat et le diquat ont été montrés comme pouvant altérer les fonctions digestives. En effet, le diquat induit une hypersécrétion intestinale médiée par les neurones ainsi qu'une inflammation (Anton *et al.* 1998).

Dans un modèle de souris transgéniques sur-exprimant l'α-synucléine sous le contrôle du promoteur THY-1 (spécifique des neurones dopaminergiques) une réduction de la motricité colique distale ainsi qu'un ralentissement de la fréquence de production des selles ont été mis en évidence (Wang *et al.* 2008).

Enfin, un modèle murin double transgénique a mis en évidence un lien entre α-synucléine et troubles du système digestif (Kuo *et al.* 2010). Les souris utilisée au cours de cette étude étaient d'une part invalidées pour le gène murin de l'α-synucléine et d'autre part porteuse du gène humain de l'α-synucléine, ce dernier étant muté ou non. Les résultats montraient que les souris porteuses du gène muté A53T et dans une moindre mesure A30P présentaient de nombreuses altérations du transit digestif. Ainsi le transit total, le transit colique et le transit colique distal étaient significativement réduits de façon stable jusqu'à 18 mois. En revanche aucun de ces paramètres n'était changé chez les souris exprimant la forme non mutée de l'α-synucléine humaine.



Réduction de la vidange gastrique chez des rats traités à la roténone. Le poids de nourriture restant dans l'estomac chez les rats traités à la roténone était significativement augmenté par rapport aux rats contrôles.

Figure 8 : Ralentissement de la vidange gastrique par un traitement chronique à la roténone (Greene *et al.* 2009)

L'ensemble de ces résultats et de ces études montrent que les modèles animaux toxiques ou génétiques ne permettent à l'heure actuelle de ne reproduire que partiellement les altérations des fonctions digestives humaines. En effet, parmi ces modèles un seul (roténone) permet d'induire des altérations du tractus digestif haut et du tractus digestif bas (Greene *et al.* 2009), or celui-ci ne reproduit pas au niveau du SNC les altérations caractéristiques de la MP ni même celles des autres études réalisées avec le même protocole (Betarbet *et al.* 2000; Sherer *et al.* 2003).

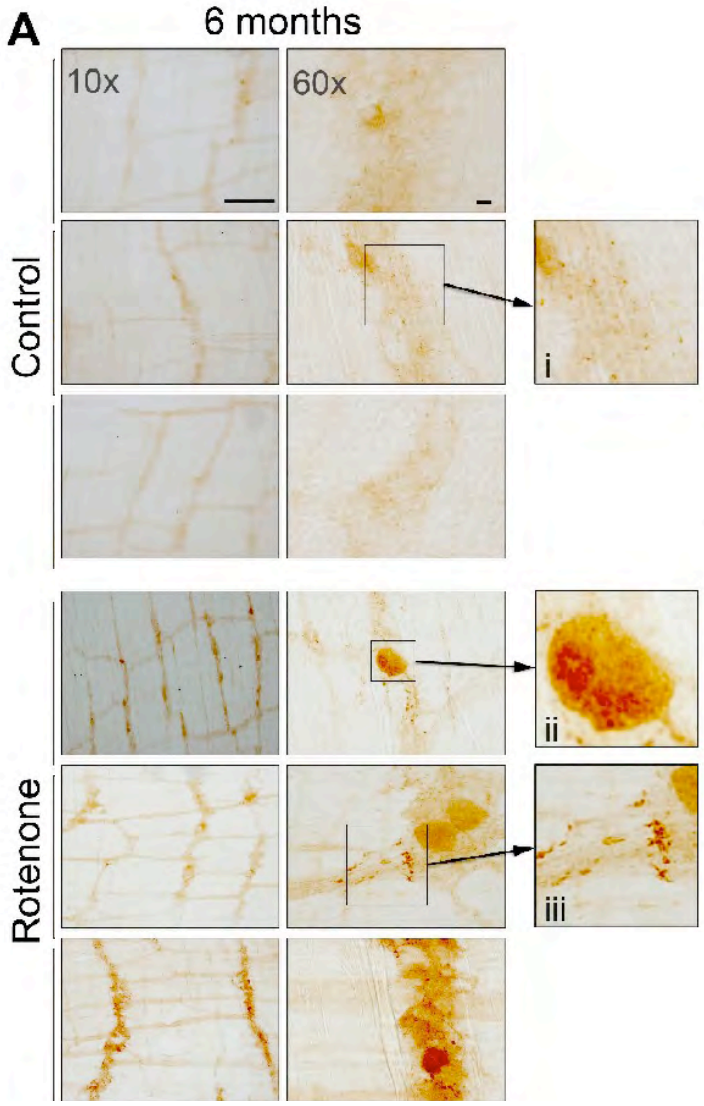
3.3.2 ATTEINTES MORPHOLOGIQUES

Au delà des atteintes fonctionnelles de ces modèles animaux, existe-t-il des atteintes spécifiques du codage neurochimique du SNE pouvant expliquer ces altérations ou bien encore les altérations de la fonction digestive humaine ? Jusqu'à maintenant un seul article fait état d'altérations du codage neurochimique du SNE chez l'homme (Singaram *et al.* 1995), mais les résultats qui y sont publiés n'ont pas encore été confirmés par d'autres études. Les modèles animaux tentent de répondre en partie à cette question mais de façon très diverse et avec des biais d'expérimentation inhérents aux méthodes d'induction d'une MP.

En 2008 une première étude a été réalisée en utilisant deux modèles d'intoxication différents, une injection intracrânienne de 6-OHDA chez des rats et une administration intra-péritonéale de MPTP chez des souris. Les résultats montraient une réduction de la proportion de neurones TH positifs dans les 2 modèles tandis que le niveau d'expression du transporteur à la dopamine n'était réduit que dans le modèle de rat (Tian *et al.* 2008).

Dans le modèle de souris traitées au MPTP (Anderson *et al.* 2007), le seul changement du SNE rapporté était une diminution de la proportion de neurones dopaminergiques au niveau du PM du côlon proximal.

Parmi les études publiées en 2009 une faisait état de modifications du SNE en lui-même avec une diminution de la densité ganglionnaire et de la densité neuronale 3 jours et 6 mois après intoxication chronique des animaux par la roténone (Drolet *et al.* 2009). En revanche l'autre étude publiée la même année ne montrait aucune modification de la densité neuronale aussi bien que des proportions des neurones VIPergiques, cholinergiques et nitrergiques (Greene *et al.* 2009).



Immunomarquage contre l'α-synucléine après traitement à l'acide formique du plexus myentérique de l'intestin grêle de rats contrôle (panneau supérieur) et de rats traités à la roténone (panneau inférieur). L'immunoréactivité était augmentée chez les rats traités à la roténone 6 mois après traitement.

Figure 9 : Agrégats d'α-synucléine dans le plexus myentérique d'intestin grêle de rats traités à la roténone (Drolet *et al.* 2009)

Finalement une diminution de la proportion de neurones nitrergiques a aussi été rapportée dans le modèle de rats traités à la 6-OHDA au niveau du PM de l'iléon

terminal et du côlon proximal de ces animaux (Blandini *et al.* 2009). Outre l'altération du codage neurochimique du SNE, l'étude de la présence d'inclusions d'alpha-synucléine a été rapportée dans plusieurs travaux sur les modèles animaux de MP. La présence d'agrégats protéiques pourrait conduire à un dysfonctionnement du système nerveux entérique indépendamment de son phénotype neurochimique.

La présence d'agrégats protéiques immunoréactifs pour l'alpha synucléine a été démontrée au sein du modèle de rats recevant des injections chroniques intra-péritonéales de roténone. Au sein de ce modèle, des agrégats d'α-synucléine résistant à l'acide formique ont été mis en évidence. L'intensité de leur marquage était réduite 3 jours après la fin du traitement et au contraire augmentée à 6 mois (figure 9) post-traitement (figure 9) (Drolet *et al.* 2009).

La capacité pro-agrégeante de la roténone vis-à-vis de l'α-synucléine a aussi été mise en évidence au niveau du SNE dans un modèle murin d'intoxication orale (Pan-Montojo *et al.* 2010). Une augmentation de l'expression de l'α-synucléine ainsi qu'une augmentation du nombre d'inclusions a été mise en évidence au niveau du duodénum et de l'iléon.

Un modèle de souris doubles transgéniques a aussi montré la présence d'agrégats immunoréactifs pour l'α-synucléine résistant chez les souris possédant le gène humain muté en A53T de la protéine. Ces agrégats ont été mis en évidence au sein des PSM et PM de l'iléon chez les souris portant le gène muté humain et pas chez les animaux portant le gène humain normal (Kuo *et al.* 2010). Les atteintes morphologiques et du codage neurochimique rapportées dans ces modèles sont, en l'absence d'études plus approfondies chez l'Homme, les meilleurs arguments permettant d'expliquer l'origine des troubles digestifs des patients parkinsoniens.

4 OBJECTIFS DE LA RECHERCHE

Les publications récentes de la littérature mettent en évidence et de façon croissante, l'importance des manifestations « non-motrices » de la MP. D'une part ces manifestations non-motrices contribuent à l'altération de la qualité de vie des patients, et d'autre part elles peuvent, pour certaines, faire évoquer des mécanismes étiopathogéniques de la maladie. Ces études montrent aussi la rareté des données concernant le SNE. Les études ayant caractérisé le SNE chez les patients parkinsoniens ont principalement été réalisées sur des prélèvements autopsiques. Elles ne font état que de la présence de CL et de NL, le plus souvent sans caractérisation du SNE. Les modèles animaux de MP sont en revanche mieux caractérisés en ce qui concerne les manifestations non-motrices digestives. Cependant les altérations du SNE en lui-même restent peu décrites ou imprécises. Ainsi les objectifs principaux de ces travaux ont été :

1/ de déterminer, en utilisant deux modèles animaux toxiques, les modifications induites au niveau du SNE, d'une part chez la souris traitée à la roténone et d'autre part chez le singe traité au MPTP.

2/ de caractériser les altérations fonctionnelles digestives pouvant se manifester dans le modèle murin d'intoxication à la roténone.

3/ de caractériser dans une étude pilote les modifications du SNE au cours de la MP chez l'Homme et d'y rechercher des caractéristiques spécifiques de la maladie.

Ces travaux seront présentés sous la forme de 4 articles dont 2 publiés et 2 en préparation.

Le premier article décrit les modifications du codage neurochimique du SNE dans un modèle de singes traités par le MPTP de façon chronique. Le protocole d'intoxication des singes à été réalisée en collaboration avec l'UMR 5527 du CNRS. J'ai en particulier contribué à la préparation des tissus digestifs ainsi qu'à l'analyse des marquages immunohistochimiques. Les résultats montrent des altérations importantes au niveau des deux plexus dans le côlon de ces animaux.

Le second article présente les altérations du SNE chez des souris traitées à la roténone ainsi que les conséquences sur la motricité digestive de ces animaux, caractérisées à l'aide d'une série de tests aussi bien *in vivo* qu'*ex vivo*. J'ai contribué à la mise en place de ce modèle ainsi qu'au développement des tests *in vivo* et à l'analyse des résultats.

Le troisième article présente les résultats préliminaires d'une étude visant à caractériser le phénotype neurochimique du PSM de patients parkinsoniens et à mettre en corrélation ces altérations avec le stade d'avancement de la maladie. La description de la méthode utilisée au cours de cette étude a aussi fait l'objet d'une publication située en annexe.

Le quatrième article complète ces résultats dans une série plus large de patients parkinsoniens et établit des corrélations avec les symptômes moteurs et la constipation. Pour ces deux derniers travaux, j'ai, en particulier, participé à l'étude de la faisabilité de cette approche ainsi qu'à la collection des prélèvements et à leur préparation.

Ces articles sont présentés successivement ; ils sont précédés d'un résumé en français pouvant contenir des résultats supplémentaires non publiés. Une discussion générale de synthèse des résultats de ce travail de thèse est présentée à l'issue des 4 articles.

RESULTATS

ARTICLE 1 : PLASTICITE NEURONALE DU SYSTEME NERVEUX ENTERIQUE DANS UN MODELE EXPERIMENTAL DE MALADIE DE PARKINSON CHEZ LE PRIMATE

Cet article a été publié dans le journal *neurogastroenterology and motility* en 2009.

Diverses études montrent l'importance majeure des troubles digestifs chez les patients atteints de MP. Ces troubles, constipation, gastroparésie ou dyspepsie, sont sans doute pour une bonne part liés à une dérégulation de la fonction motrice digestive. Une altération du SNE, principal structure de contrôle de la motricité, pourrait être à l'origine de ces changements chez les patients parkinsoniens. Cependant les changements survenant au sein du SNE au cours de la MP sont encore très largement inconnus, en particulier en ce qui concerne le phénotype neurochimique des neurones entériques.

Ainsi l'objectif de cette première étude était de caractériser les modifications du codage neurochimique des neurones entériques dans un modèle animal de MP, le singe traité au MPTP.

Nous avons d'abord évalué le nombre total de neurones par ganglion au sein des PM et sous muqueux du côlon proximal de singes traités avec du MPTP et de singes contrôles en réalisant un marquage immunohistochimique contre la protéine pan-neuronale Hu. Nous avons ainsi pu mettre en évidence une augmentation significative de la densité neuronale (nombre de neurones par ganglion) chez les animaux traités dans le PM mais pas dans le PSM. La densité ganglionnaire du PM n'était pas modifiée chez les animaux MPTP. Dans un second temps, au niveau du PM nous avons réalisé un

marquage contre le marqueur glial Sox-10 pour évaluer le nombre de cellules gliales présentes par ganglion. Ce nombre n'était pas modifié chez les singes traités par rapport aux singes contrôles. En revanche le ratio cellules gliales / neurones était réduit chez les singes traités au MPTP en raison de l'augmentation du nombre de neurones Hu immunoréactifs.

Dans un troisième temps nous avons déterminé les sous-populations neuronales dont la représentation était modifiée par le traitement au MPTP. Par marquages immunohistochimiques, nous avons mis en évidence une augmentation du nombre de neurones nitrergiques, une diminution du nombre de neurones dopaminergiques et une absence de modification du nombre de neurones cholinergiques et VIPergiques au niveau du PM colique des singes traités au MPTP. Au niveau du PSM, seule la proportion de neurones dopaminergiques était significativement réduite tandis que la proportion de neurones nitrergiques n'était pas modifiée.

En conclusion le MPTP en administration chronique, induit des modifications au niveau des PSM et PM du côlon proximal chez le primate non-humain qui pourraient être en partie responsables des troubles de la motricité digestive présents chez l'Homme au cours de la MP.

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Neurochemical plasticity in the enteric nervous system of a primate animal model of experimental Parkinsonism

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Abstract *Emerging evidences suggest that the enteric nervous system (ENS) is affected by the degenerative process in Parkinson's disease (PD). In addition lesions in the ENS could be associated with gastrointestinal (GI) dysfunctions, in particular constipation, observed in PD. However, the precise alterations of the ENS and especially the changes in the neurochemical phenotype remain largely unknown both in PD and experimental Parkinsonism. The aim of our study was thus to characterize the neurochemical coding of the ENS in the colon of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated monkeys, a well-characterized model of PD. In the myenteric plexus, there was a significant increase in the number of neurons per ganglia (identified with Hu), especially nitric oxide synthase immunoreactives (IR) neurons in MPTP-treated monkeys compared to controls. A concomitant 72% decrease in the number of tyrosine hydroxylase-IR neurons was observed in MPTP-treated monkeys compared to controls. In contrast no change in the cholinergic or vasoactive intestinal peptide-IR population was observed. In addition, the density of enteric glial cells was not modified in MPTP-treated monkeys. Our results demonstrate that MPTP induces major changes in the myenteric plexus and to a lesser extent in the submucosal plexus of monkeys. They further reinforce the observation that lesions of the ENS occur in the course of PD that might be related to the GI dysfunction observed in this pathology.*

Keywords *1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, colon, enteric nervous system, Parkinson's disease, tyrosine hydroxylase.*

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disease. The core of the neuronal lesions in PD is the progressive degeneration of dopaminergic neurons in the *substantia nigra*, which is responsible for the major motor symptoms of the disease.[1] Nevertheless, it has become increasingly evident that non-motor symptoms, which can occur early in the course of the disease, are frequent and disabling.[2] Gastrointestinal (GI) impairment, consisting mainly in gastroparesis, transit constipation and defecatory dysfunction, is one of the prominent non-motor feature of PD for which therapeutic options are of limited efficiency.[3,4] Constipation, mainly related to altered colonic motility, appears to be the most common GI symptom in PD patients.[5] However, the precise mechanisms of this colonic dysmotility in PD remain largely unknown.[6,7]

Neural regulation of GI functions is largely mediated by the enteric nervous system (ENS), which is a neuronal network organized in two major ganglionated plexuses, the myenteric plexus (MP) and submucosal plexus (SMP).[8] Enteric neurons and enteric glial cells (EGC) of the MP are mainly involved in the control of motor functions while that of the SMP are involved in the control of intestinal barrier functions.[8] Neuronal regulation of GI functions is due to the liberation of

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specific neuromediators synthesized by functionally defined neurons. In particular, vasoactive intestinal peptide (VIP) or nitric oxide is found in inhibitory muscle motoneurons while acetylcholine is found in excitatory motoneurons.[9] Dopamine has also been identified as an enteric inhibitory neuromediator of GI motility.[10]

Alterations of the neurochemical coding of enteric neurons and/or changes in the phenotype of EGC have been described in several GI pathologies such as inflammatory bowel disease, achalasia and constipation.[11–14] Lewy bodies, the pathological hallmark of PD in the central nervous system, have been also identified in the ENS of PD patients, presumably in VIPergic neurons.[15] However, the precise neurochemical alterations of the ENS during PD remain largely unknown, except a decrease in the number of dopamine-immunoreactive (IR) neurons in the colon of PD patients.[16] This paucity of data is due in large part to the limited access of whole mount preparations of the ENS in colonic tissues from PD patients, as the majority of the studies were performed using autopsy material.[17–19] Therefore to overcome the poor availability of human tissue, validated animal models of PD could prove themselves a valuable tool to characterize the lesions in the ENS during the disease.

The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been widely used to study PD in primates and rodents.[20] Repetitive administration of MPTP over time in monkeys initiates a process of neurodegeneration reminiscent of that seen in humans during PD.[21] A recent study in mice has shown that a single injection of MPTP leads to a rapid 40% decrease in the proportion of tyrosine hydroxylase (TH)-IR neurons in the ileum.[22] Nevertheless, whether chronic administration of MPTP also alters the colonic neuronal and glial phenotype is currently unknown.

Therefore, the aim of this study was to characterize the neurochemical phenotype of submucosal and myenteric neurons as well as EGC in monkeys chronically treated with MPTP.

MATERIALS AND METHODS

Animal study

All animal studies were carried out in accordance with European Communities Council Directive for the care of laboratory animals (86/609/EEC). Experiments were conducted according to previously published procedures and methods on 12 male rhesus monkeys (*Macaca mulatta*; SAH/Xierxin, Beijing, China). Six monkeys received once daily i.v. injections of MPTP hydrochloride (0.2 mg kg^{-1}) until they displayed parkinsonian symptoms including rigidity and bradykinaesia (mean cumulative dose of 3.7 mg kg^{-1}).[21] The remaining six animals received vehicle only (control group). Animals were then kept without dopaminergic supplementation for 5 months before killing. All MPTP-treated animals displayed a severe decrease in striatal DA transporter binding (4.3 ± 2.7 fmol mg^{-1} of equivalent tissue) compared to control animals (142.3 ± 9.1 fmol mg^{-1} of equivalent tissue) in the brain.[23] Stool consistency was monitored using Bristol stool scale[24] 1 week before killing. There was no difference in stool consistency between controls and MPTP-treated animals (data not shown).

Tissue collection and immunohistochemistry

Following euthanasia of animals, the ascending colon was removed, stretched and pinned flat on Sylgard-coated Petri dishes, and fixed overnight in 4% phosphate buffer saline (PBS) paraformaldehyde (Sigma-Aldrich, St Quentin Fallavier, France). The macroscopic aspect of bowel was not different between control and MPTP-treated animals. Layers of tissue containing the MP and the internal SMP (Meissner plexus) were then separated by microdissection.[13] Samples were permeabilized for 2 h in a 4% horse serum/PBS blocking buffer containing 1% Triton X-100 (Sigma-Aldrich), and incubated for 24 h with the following primary antibodies diluted in the blocking buffer: goat anti-choline acetyl transferase (ChAT) (1 : 200; Millipore, St Quentin en Yvelines, France), mouse anti-VIP (1 : 800; Euromedex, Mundolscheim, France), rabbit anti-nNOS (1 : 2000; COGER, Paris, France), rabbit anti-TH (1 : 500; Pel-Freez, Rogers, AR, USA), sheep anti-TH (1 : 500; Pel-Freez, Rogers, AR, USA), mouse anti-Hu C/D (1 : 200; Invitrogen, Cergy Pontoise, France), mouse anti-Sox-10 (1 : 500; M. Wegner, University of Erlangen, Germany), mouse antiactive caspase-3 (1 : 1000; Sigma-aldrich). Samples were washed with PBS and incubated for 3 h with a combination of donkey anti-rabbit IgG conjugated to carboxymethylindocyanine (CY3, 1 : 500; Immunotech, Marseille, France), donkey anti-mouse IgG conjugated to CY3 (1 : 500; Immunotech), donkey anti-sheep IgG conjugated to fluorescein isothiocyanate (FITC, 1 : 500; Immunotech), donkey anti-rabbit IgG conjugated to FITC (1 : 500; Interchim, Montluçon, France) and donkey anti-mouse IgG conjugated to CY5 (1 : 500; Immunotech).

Identification of neuronal cell populations and phenotypic analysis

Immunoreactive neurons for VIP, ChAT, nNOS, TH and Hu C/D were counted in at least 20 ganglia per condition and per animal (mean 757.0 ± 136.7 myenteric neurons per condition and per animal). The relative proportion of ChAT-, VIP-, nNOS- and TH-IR neurons was expressed as a percentage of the total number of neurons determined with the general neuronal marker Hu C/D. For each animal, the mean of the proportion of a given marker within a ganglion was calculated for all ganglia evaluated. Enteric glial cells counting (using anti-Sox-10 antibody) was performed in five ganglia per animal (mean 279.5 ± 59.7 myenteric glial cells per animals).

Statistical analysis

Data are represented as mean ± standard deviation. Analysis of the distribution was made using the Kolmogorov–Smirnov test, the homogeneity of variances was tested using the Levene's test for equality of variances. For comparison the two-tailed Student's *t*-test was used. *P*-values <0.05 were considered significant. Statistical analysis was performed using SIGMASTAT 3.10 for Windows (Systat Software, Erkrath, Germany).

RESULTS

Neuronal population increases in the myenteric but not in SMP in experimental parkinsonism

The general neuronal and glial populations were first studied in control and MPTP-intoxicated monkeys in both the MP and SMP.

In the MP of MPTP-treated monkeys, there was a significant 20% increase in the number of Hu-IR neurons (44.7 ± 7.2; $n = 6$) when compared to control (35.0 ± 4.9; $n = 6$; $P = 0.02$; Fig. 1A–C). In contrast, no change in the number of neurons was observed in the SMP between control and MPTP-treated animals (10.9 ± 0.7 Hu-IR neurons and 11.1 ± 0.9 respectively; $n = 5$; $P = 0.87$) (Fig. 1D–F). In addition, no change was observed in the density of myenteric ganglia in MPTP-treated monkeys (98.8 ± 24.7 ganglia cm^{-2}; $n = 5$) when compared to control (76.1 ± 29.7; $n = 5$; $P = 0.23$). No active caspase 3-IR neurons were observed in the MP or SMP of control or MPTP-treated animals (data not shown). Consistently, no degenerative signs such as reduced neuronal cytoplasmic size was observed in MPTP-treated monkeys when compared to control (412 ± 153 μm^2 *vs* 380 ± 110; $n = 5$; $P = 0.72$ respectively).

No significant difference was observed between the number of EGC (identified with Sox10) in the MP of control (262.9 ± 86.6; $n = 4$) and MPTP-treated animals (292.7 ± 31.8; $n = 5$; $P = 0.49$) (Fig. 1G–I). However, the ratio glia/neurons was significantly reduced in MPTP-treated monkeys when compared to control (5.4 ± 0.6 and 8.1 ± 1.7 respectively; $P = 0.019$).

Neurochemical phenotype of the ENS is affected in experimental Parkinsonism

Myenteric plexus The neurochemical coding of the MP of monkeys was assessed using triple immunohistochemical staining.

In control animals, the majority of colonic neurons were nNOS-IR (51.0 ± 4.1% of Hu-IR neurons; $n = 5$) and ChAT-IR (29.9 ± 5.8%; $n = 5$) (Fig. 2A–C). In addition, 11.2 ± 1.3% ($n = 5$) of myenteric neurons were TH-IR and 0.8 ± 0.3% ($n = 5$) VIP-IR (Fig. 2D–F). Analysis of colocalization was performed on the three major populations identified, i.e. nNOS, ChAT and TH. Choline acetyl transferase and nNOS formed neurochemically distinct populations with only 1.0 ± 0.3% ($n = 5$) of neurons expressing ChAT and nNOS-IR simultaneously. Regarding the TH-IR population, 98.3 ± 2.5% ($n = 5$) of TH-IR neurons were also nNOS-IR and almost none was ChAT-IR (0.03 ± 0.01%; $n = 5$) (Fig. 2G–H).

In MPTP-treated animals, significant changes in the phenotype of myenteric neurons were observed. First, there was a significant 25% increase in the number of nNOS-IR neurons per ganglion when compared to control (21.0 ± 3.1 and 17.3 ± 2.0 respectively; $P = 0.049$; Fig. 3A–C), although the proportion of nNOS-IR neurons (51.9 ± 5.6%; $n = 5$) (normalized to Hu-IR neurons) remained similar to control (51.0 ± 4.1%; $n = 5$; $P = 0.95$). Secondly in MPTP-treated animals, the number of TH-IR neurons per ganglion was significantly reduced by 64% when compared to control ($n = 5$; $P = 0.008$) (Fig. 3D–F) and the proportion of TH-IR neurons by 72% (3.1 ± 0.6% and 11.1 ± 3.0% respectively; $n = 5$; $P = 0.001$). The number of ChAT-IR neurons per ganglion remained similar in MPTP-treated monkeys when compared to control (10.0 ± 1.7 and 10.8 ± 1.9 respectively; $n = 5$; $P = 0.51$). Furthermore, the proportion of ChAT-IR neurons was not significantly reduced in MPTP treated monkeys when compared to control (25.9 ± 2.0% and 29.9 ± 5.8% respectively; $n = 5$; $P = 0.18$) (Fig. 3C). No significant change in the number per ganglia and proportion of VIP-IR neurons was observed in MPTP-treated monkeys when compared to control (Fig. 3F).

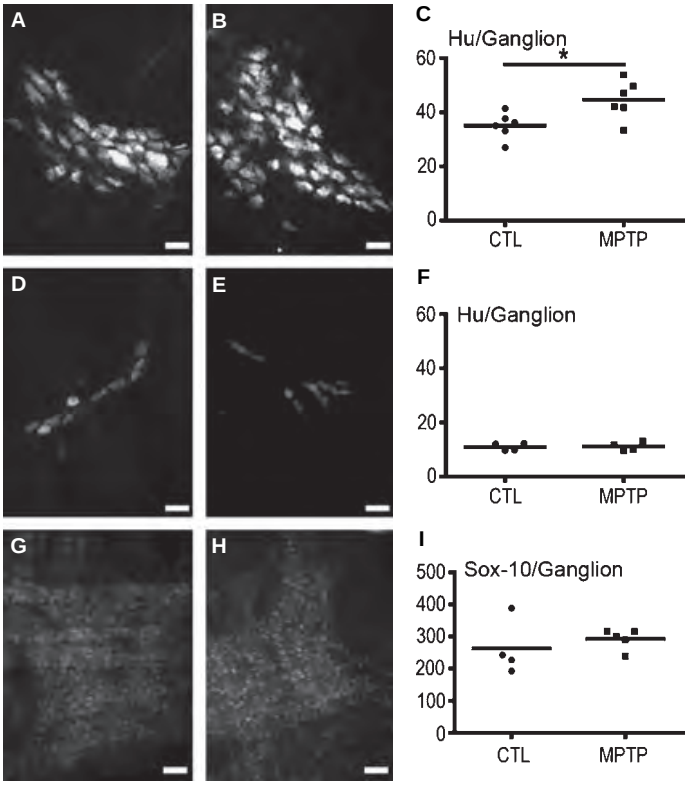

Figure 1 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) increases the number of myenteric but not submucosal neurons and does not change the number of myenteric enteric glial cells (EGC). Hu-immunoreactive (IR) myenteric neurons were identified in the colon of control (A) and MPTP-treated monkeys (B). MPTP treatment induced a significant increase in the number of Hu-IR myenteric neurons per ganglion (n = 6 controls and 6 MPTP-treated monkeys; P = 0.022) (C). Hu-IR submucosal neurons were identified in the colon of control (D) and MPTP-treated monkeys (E). MPTP treatment did not change the number of Hu-IR submucosal neurons per ganglion (n = 4 controls and 4 MPTP-monkeys) (F). Sox-10-IR EGC were identified in the myenteric plexus (MP) of control (G) and MPTP-treated monkeys (H). MPTP treatment did not change the number of Sox-10-IR EGC per ganglion in the MP (n = 4 controls and 5 MPTP-monkeys) (I). Each point represents a value from a monkey injected with vehicle (circle) or with MPTP (square). Horizontal bars represent the mean. Scale bar: 40 μm.

Submucosal plexus The neurochemical coding of the SMP of monkeys was analysed using triple immunohistochemical staining (Fig. 4A–C).

In control animals, 44.0 ± 2.4% of submucosal neurons was nNOS-IR and 38.1 ± 6.0% ChAT-IR. Analysis of colocalization was performed on the two major populations identified, i.e. nNOS and ChAT. Simultaneously ChAT- and nNOS-IR neurons formed 33.1 ± 6.7% of the Hu-IR neurons in control animals. In MPTP-treated animals, there was no significant change in these populations when compared to control. Indeed, nNOS-IR neurons formed 43.7 ± 1.9% of Hu-IR neurons (P = 0.85) and ChAT-IR neurons represented 35.3 ± 9.9% of Hu-IR neurons (P = 0.70) (Fig. 4E). Tyrosine hydroxylase-immunoreactive neurons were fainter in the SMP (Fig. 4D) when compared to the MP (Fig. 3D–E), and they formed 10.5 ± 1.8% of Hu-IR neurons in control animals (Fig. 4E). This proportion was significantly reduced by 49% in MPTP-treated animals (5.1 ± 1.7%; n = 5) when compared to control (P = 0.003).

DISCUSSION

This study was performed in monkeys chronically treated with MPTP according to a regimen that closely mimics the degeneration pattern of the *substantia nigra* of human PD, thereby producing a progressive parkinsonian state.[21] We showed profound and differential alterations of the neurochemical coding in the colonic MP when compared to the SMP. The changes in the MP were characterized by a significant decrease in the number of TH-IR neurons and a concomitant increase in the number of nNOS-IR neurons associated with an increase in the total number of neurons per ganglion. In contrast, only a decrease in the proportion of TH-IR neurons was observed in the SMP.

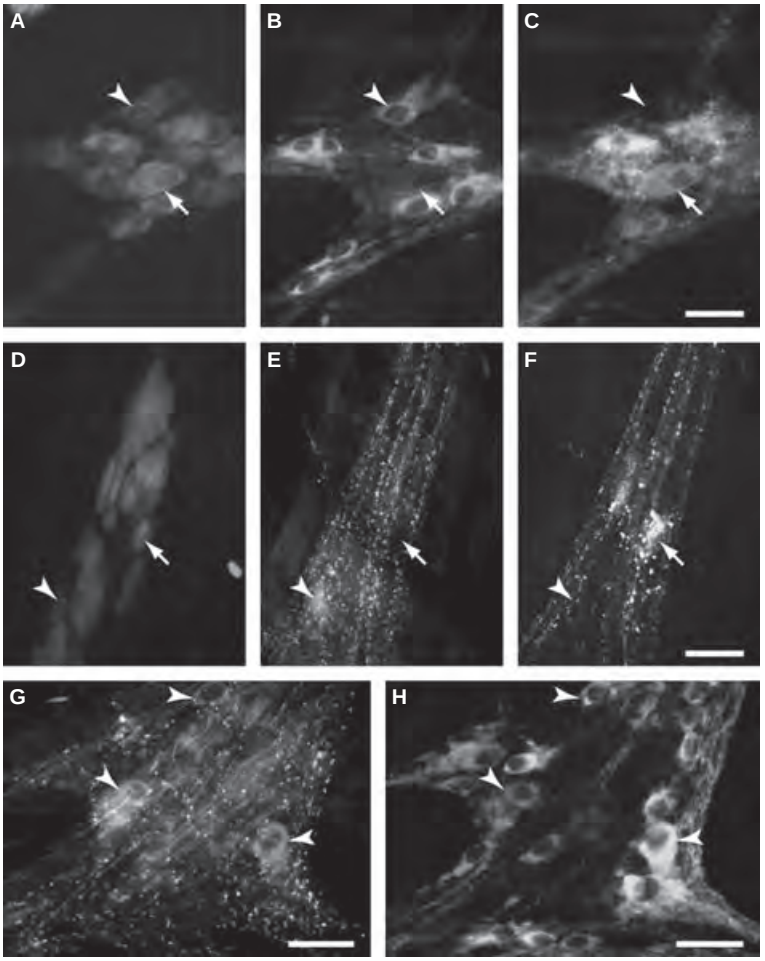

Figure 2 Immunohistochemical detection of transmitter coding of myenteric plexus from control monkeys. Triple labelling with antibodies against Hu (A), nNOS (B) and choline acetyl transferase (ChAT) (C) showed that the majority of neurons are either nNOS-immunoreactive (IR) (arrowhead) or ChAT-IR (arrow). Triple labelling with antibodies against Hu (D), tyrosine hydroxylase (TH) (E) and VIP (F) showed that few neurons are TH-IR (arrowhead) and exceptionally VIP-IR (arrow). Most of TH-IR neurons (arrowhead) (G) co-expressed nNOS (H). Scale bar: 40 μm.

An important result of this study was the first establishment of the neurochemical coding of the ENS in the colon of monkeys. Due to important interspecies differences in the ENS properties (neurochemical coding, electrophysiological properties), identification of animal species with a neurochemical coding similar to that of the human might be useful, especially for the study of human diseases. The proportions of major neuromediators of the ENS are similar to that observed in the human colon, in particular for NOS-IR (51%)[25] and for ChAT-IR neurons (34%).[13] Furthermore, the proportion of TH-IR neurons in the MP and SMP of monkey (11% and 10% respectively) was similar to that obtained in human colon (12% and 14.7% respectively).[16] Interestingly, all the TH-IR neurons were NOS-IR but not ChAT-IR. This is consistent with a study in human in which TH-IR cell body is found not to be cholinergic.[26] Finally, the ratio glia to neurons was also similar both in the colonic MP of the monkey (8) and in the human (5.9–7).[27] Taken together these data suggest a close similarity in the ENS phenotype between humans and this non-human primate model.

Our study characterized the alterations of the neurochemical phenotype induced by MPTP in the colonic ENS. It showed that the number of TH-IR neurons in the MP and SMP of monkeys treated with MPTP was reduced when compared to control. Although TH is a marker of both dopaminergic and noradrenergic neurons, the majority of TH-positive neurons with the cell bodies in the MP are considered to be dopaminergic.[28] The decrease of TH-IR neurons observed in our experiments could either represent a change in the neurochemical phenotype (i.e. downregulation of TH) or a loss of dopaminergic neurons. The increase in the total number of neurons per ganglia observed in the MP of MPTP-treated monkeys and the absence of active caspase-3-IR neurons argue against a loss of dopaminergic cells at the time of our experiments. However, one cannot exclude that cell loss of TH-IR neurons occurred earlier in the course of MPTP injection. Further reinforcing this hypothesis is the observation

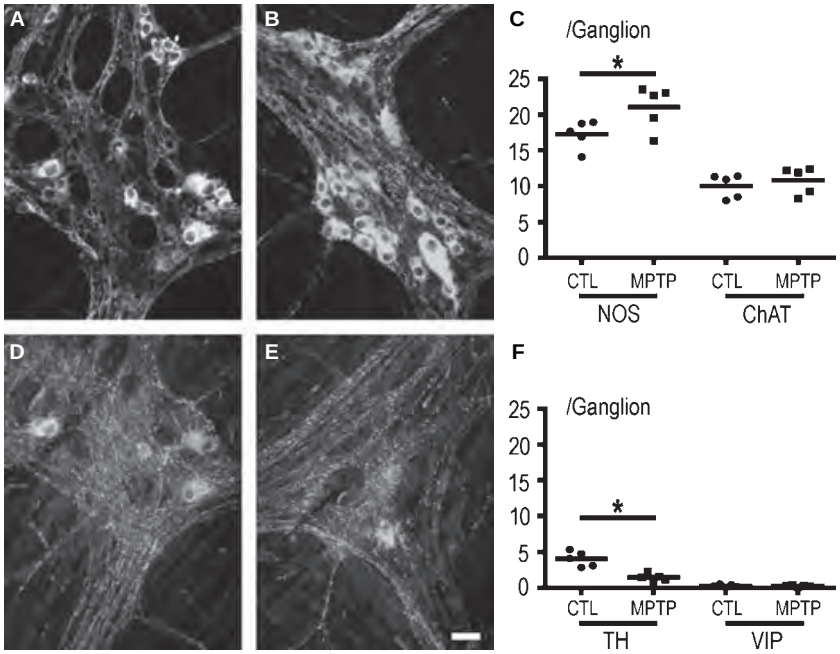

Figure 3 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) changes the neurochemical coding of the myenteric plexus. nNOS-immunoreactive (IR) myenteric neurons were identified in the colon of control (A) and MPTP-treated monkeys (B). MPTP treatment induced a significant increase in the number of nNOS- but not choline acetyl transferase-IR myenteric neurons per ganglion (n = 5 controls and 5 MPTP-monkeys; P = 0.049) (C). Tyrosine hydroxylase (TH)-IR myenteric neurons were identified in the colon of control (D) and MPTP-treated monkeys (E). MPTP treatment induced a significant decrease in the number of TH- but not VIP-IR myenteric neurons per ganglion (n = 5 controls and 5 MPTP-monkeys; P = 0.008) (F). Each point represents a value from a monkey injected with vehicle (circle) or with MPTP (square). Horizontal bars represent the mean. Scale bar: 40 μm.

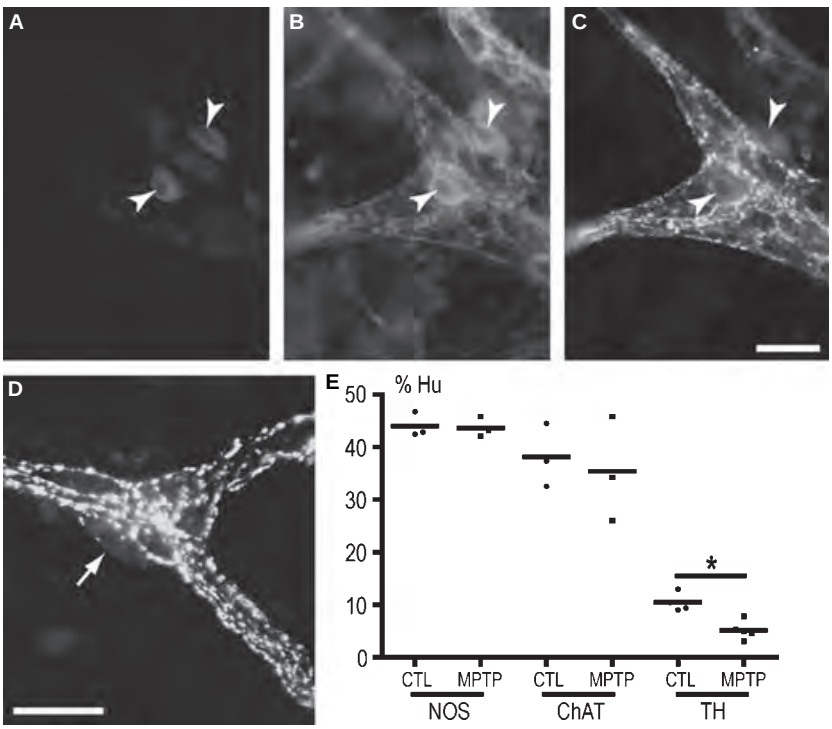

Figure 4 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) reduces the proportion of tyrosine hydroxylase-immunoreactive (TH-IR) submucosal neurons. Triple labelling with antibodies against Hu (A), choline acetyl transferase (ChAT) (B) and nNOS (C) showed that a large proportion of submucosal neurons were ChAT- and nNOS-IR (arrowheads). MPTP treatment induced a significant decrease in the proportion of TH- (arrow) but not of ChAT- and nNOS-IR submucosal neurons (n = 4 controls and 5 MPTP-monkeys; P = 0.003) (D, E). Each point represents a value from a monkey injected with vehicle (circle) or with MPTP (square). Horizontal bars represent the mean. Scale bar: 40 μm.

that MPTP can cause a loss in TH without necessarily destroying neurons in murine *substantia nigra*.[29] In MPTP-treated mice, Anderson *et al.*[22] suggested that the absence of TH-positive cell bodies in the ENS most likely represents a loss of cells rather than a mere downregulation of TH but no assessment of cell death nor cell counting were performed in these mice.

Parallel to the decrease in the number of TH-IR neurons, a significant increase in the number of nNOS-IR neurons was observed in the MP of monkeys treated with MPTP. This contrasts with the results obtained in MPTP-treated mice in which no change in the density of nitrergic neurons was evidenced in the MP.[22] These differences between the two studies could result from the fact that a single injection of MPTP was performed in mice while our protocol consisted of multiple injections and longer survival time allowing adaptation. In this context, it is tempting to speculate that the increase in the number of nNOS-IR neurons could represent an adaptative response to the drop in TH as both subsets of neurons exert an inhibitory effect on GI motility.[10]

A body of literature supports a critical role for astrocytes in protecting dopaminergic neurons in the CNS.[30] Recent experiments have shown *in vivo* that MPTP induced both activation and apoptotic cell death of astrocytes in the *substantia nigra*.[31] As EGC are likely to represent the ENS counterpart of CNS astrocytes,[32,33] it was critical to assess whether MPTP was toxic for EGC. Using Sox10 antibodies, a reliable marker for EGC,[27] no significant difference was observed in the number or in the phenotype of glial cells in the MP between MPTP-treated and control animals, suggesting that EGC are not a primary target of MPTP in the ENS. However, the ratio of astrocytes to neurons was significantly decreased by MPTP treatment suggesting that, under these conditions, myenteric neurons could be less protected by astrocytes during an insult and making them more sensitive to infectious or oxidative stress.

Our study also suggests that neuropathological processes are more limited to the MP when compared to the SMP. This observation is consistent with earlier descriptions mentioning that Lewy body were only present in the MP and absent or undetectable in the SMP.[18] However, further studies have shown that pathological changes also occur in the SMP, although to a lower extent than in MP.[17,19,34] In particular, using routine colonic biopsies obtained during the course of colonoscopy, we have identified aggregates of synuclein reminiscent of Lewy neurites in the SMP of PD patients but no change in the number of submucosal neurons, similarly to our observation in the SMP of MPTP-treated monkeys.[35] Although the changes were less pronounced in the SMP, both plexuses were targeted by MPTP in our model, further reinforcing the fact that the two structures are involved during the course of PD and that they should be systematically assessed in studies performed in PD patients and animal models of the disease.[6,36]

From a functional point of view, the changes of the neurochemical phenotype observed in the MP of PD patients and in animal models of the disease could account for the GI dysfunction, which is frequently encountered by parkinsonian patients.[3] Interestingly, increased proportion of NOS-IR neurons and decreased proportion of ChAT-IR neurons have recently been reported in the MP of patients suffering from slow transit constipation, a major GI dysfunction observed in PD patients.[25] However, the functional impact of the alterations of the ENS in the monkey could not be determined in this study due to technical constraints.

In summary, MPTP induced pronounced changes in the neurochemical coding in both the MP and the SMP of monkeys. Remarkably, in the MP, these changes were not restricted to dopaminergic neurons but also involved nitrergic enteric neurons. Our results open new insights into the understanding of GI dysmotility in PD and into the pathophysiology of this disease.

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COMPETING INTERESTS

The authors have no competing interests.

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Article 1 : Données et commentaires complémentaires

L'augmentation de la densité neuronale peut faire évoquer des modifications des mécanismes de prolifération ou de mort neuronale. Nous avons donc réalisé une étude immunohistochimique complémentaire en microscopie à fluorescence standard. Cette analyse a montré une superposition du marqueur neuronal Hu et du marqueur de prolifération Ki-67 (figure 10A) au sein du PM de tous les animaux traités au MPTP. Ce marquage était absent chez les animaux témoins. Cependant une analyse plus poussée en microscopie confocale n'a pas permis de mettre en évidence une réelle colocalisation du marqueur neuronal neurofilament (NF) et du marqueur de prolifération Ki-67, les cellules immunoréactives pour le marqueur Ki-67 pouvant être situées au sein des fibres interganglionnaires (figures 10B), ou bien être juxtaposées aux ganglions myentériques (figure 10C). De plus un marquage réalisé contre la protéine caspase-3 activée, marqueur de l'apoptose, n'a pas mis en évidence de phénomène de mort induite par le traitement au MPTP.

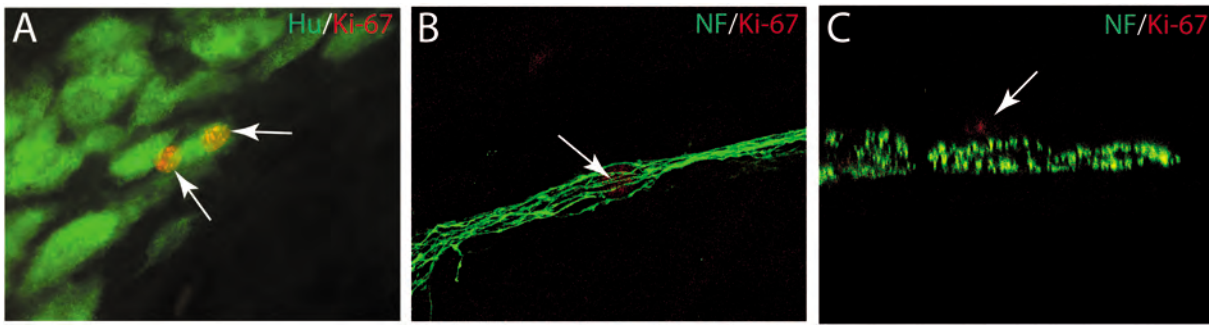

Figure 10 : Cellules Ki-67 immunoréactives chez les singes traités au MPTP

(A) Superposition des marqueurs Hu, marqueur neuronal, et Ki-67, marqueur de prolifération au sein du plexus myentérique colique de singes traités au MPTP en microscopie standard. (B) Projection d'un empilement de coupes photographiques réalisées en microscopie confocale au niveau du plexus myentérique colique. Une cellule immunoréactive (flèche) pour Ki-67 située à l'intérieur d'une fibre interganglionnaire révélée à l'aide du marqueur pour neurofilaments (NF). (C) Projection en Z d'un ganglion myentérique révélé par le marqueur NF en microscopie confocale. Une cellule Ki-67 immunoréactive est juxtaposée au ganglion. Flèches : cellules Ki-67 positives.

ARTICLE 2 : ALTERATIONS DIGESTIVES DE LA MALADIE DE PARKINSON DANS UN MODELE MURIN D'ADMINISTRATION ORALE DE ROTENONE

Cet article est en préparation, des résultats complémentaires sur le SNC et le comportement moteur des animaux devraient y être ajoutés ultérieurement.

Plusieurs études épidémiologiques ont mis en évidence un lien entre les pesticides tel que la roténone et la MP. En effet, l'usage de tels produits augmente le facteur de risque de développer la maladie. De plus, l'hypothèse de l'origine périphérique de la MP implique directement le rôle du SNE dans l'initiation du processus pathologique par ingestion de produits toxiques. La roténone a été régulièrement utilisée afin de développer des modèles animaux de MP. Cependant, un seul mode d'administration, l'intoxication par gavage oral, correspond au potentiel contact habituel de l'Homme avec ce contaminant.

Les objectifs de cette étude étaient de caractériser les altérations digestives induites par l'intoxication orale chronique de souris par la roténone, de découvrir si ces altérations pouvaient être liées à des modifications du phénotype neurochimique du SNE et de décrire les modifications de l'expression d'α-synucléine dans le SNE dans ce modèle.

Nous avons en premier lieu mis en évidence la présence d'altérations des fonctions digestives, et en particulier d'un ralentissement du transit total chez les souris traitées à la roténone. Cette altération était associée à une diminution de la fréquence de

production des selles, mais pas a un ralentissement de la vidange gastrique, ni à un ralentissement du transit colique distal. La perméabilité paracellulaire totale du tube digestif était significativement réduite chez les souris traitées à la roténone.

Cependant aucune modification du phénotype neurochimique n'était présente au niveau du PM du côlon proximal des souris traitées à la roténone. Le nombre de neurones ainsi que leur densité n'était pas modifiée. Les proportions des principales sous-populations neurochimiques, cholinergique et nitrergique, n'étaient pas modifiées par le traitement à la roténone.

Le marquage de l'α-synucléine a révélé une tendance à l'augmentation de son expression dans le côlon proximal des souris traitées. Cette augmentation s'est révélée statistiquement significative après quantification par Western Blot.

Ces résultats mettent en évidence des altérations digestives reproduisant en partie les atteintes humaines lors de la MP. De plus, la surexpression d'α-synucléine pourrait être à l'origine des CL présents dans le SNE des patients parkinsoniens.

Digestive features of Parkinson's disease in a mouse model of oral rotenone intoxication

Short title: Enteric nervous system and Parkinson's disease

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Abbreviations: PD, Parkinson's disease; ENS, enteric nervous system; EFS, electrical field stimulation; AUC, area under the curve

Key words: Enteric nervous system, Parkinson's disease, Lewy bodies, α-synuclein, rotenone

Abstract

Parkinson's disease is a late onset neurodegenerative pathology which main characteristic are motor symptoms induced by a dopaminergic loss in the substantia nigra. Parkinson's disease is also characterized by non-motor symptoms among which digestives troubles appear to be the most frequent. Digestive transit is ruled mainly by the enteric nervous system. Therefore we investigated the digestive dysfunctions in a mouse model of parkinsonism. C57/Bl6 mice received an oral 28 days administration of rotenone (30mg/kg/days) and were evaluated for gastric emptying, fecal pellet output and colonic transit. Immunohistochemistry and western blot were performed to characterize enteric nervous system neurochemical coding and α-synuclein expression. Rotenone-treated mice displayed a slowed total transit time, a reduced fecal pellet output and an increased colonic α-synuclein expression. These results, taken together, describe large similarities with digestive symptoms of Parkinson's disease patients.

Introduction

Parkinson's disease is a late-onset progressive neurodegenerative pathology mainly caracterised by a loss of dopaminergic neurons in the substantia nigra and the aggregation of fibrillar proteins namely Lewy bodies, in the surviving neurons among which the major component is the α-synuclein (Spillantini *et al.* 1997) (Wakabayashi *et al.* 1997).

Clinically PD is mainly characterized by motor symptoms such as bradykinesia, akinesia and rest tremor. But PD also induces non-motor features (Dubow 2007) like anosmia, sleep disorders or mood disorders. Among these non-motor features of PD the very most common are digestive impairments (Cersosimo and Benarroch 2008). They occur in more than 60% of the PD (Longstreth *et al.* 2006) and therapeutics options are still of limited efficiency (Natale *et al.* 2008). Digestive impairments could have several aspects affecting the whole digestive tract. In PD the most frequent digestive symptoms are gastroparesis and constipation (Natale *et al.* 2008), mainly related to altered colonic motility. However, the precise mechanisms of this colonic dysmotility in PD remain largely unknown.

Gut motility is mainly controlled by the enteric nervous system (ENS), divided in two major ganglionated plexuses, the submucosal plexus which control mainly the mucosal functions and the myenteric which rules the motility of the GI tract. This control of the digestive motility is mediated by the release of specific inhibitory or excitatory neurotransmitters, nitric oxide and acetylcholine, respectively, are the major ones (Schemann and Neunlist 2004). Moreover neurochemical coding changes have already been linked to gut pathologies like achalasia, constipation or inflammatory bowel disease (Bruley des Varannes *et al.* 2006) (De Giorgio *et al.* 2004).

Those non-motor symptoms are often described to occur early in the course of the disease. Moreover it has been shown in several studies that the first structure of the central nervous system affected by the pathology is the dorsal motor nucleus of the vagus nerve which innervate the ENS (Braak et al. 2003). From those observations Braak and colleagues hypothesized that the peripheral nervous system and particularly the enteric nervous system could be affected early or even primarily in the course of the disease (Hawkes *et al.* 2007) (Hawkes *et al.* 2009). Moreover Lewy bodies could also be found within the (ENS) (Braak *et al.* 2006) reinforcing the idea that ENS is affected.

Few is known about the ENS in PD patients but also in experimental animal models of PD. Epidemiologic studies have shown that pesticides such as rotenone could be responsible for a large number of PD. Rotenone, a membrane-permeable component, is a widely use pesticides known to induce an experimental parkinsonism in animal models (Betarbet *et al.* 2000; Sherer *et al.* 2003). Rotenone targets the complex I of the mitochondrial respiratory chain inducing an increase in reactive oxygen species and then lead to oxidative stress. Furthermore it has been shown an increased oxidative stress in the brain of PD patients (Jenner 2003). Therefore pesticides-induced oxidative stress pesticides has been supposed to be highly involved in the PD process and is one of the major factors used to induce PD in animal models.

As PD could affect the peripheral nervous system and in particular the ENS, recent studies have tried to identify ENS alterations in PD animal models. Rotenone effects have already been studied in two different rats models injected with rotenone s.c. or i.p. (Greene *et al.* 2009) (Drolet *et al.* 2009). It was observed a decreased gastric emptying, a transient decreased stool frequency and a modified longitudinal muscle response to ENS stimulation by electrical field stimulation (EFS) (Greene *et al.* 2009). The second study showed α-synuclein pathology, impaired GI transit time and a loss of enteric neurons density. These studies are based on injection models of rotenone, either s.c. or i.p. and if PD is at least partially induced by pesticides or other toxics, the way of contamination is more likely to be during ingestion of "dirty" fruits or vegetables than by systemic injection. This oral administration of rotenone have already been studied in the CNS showing all the pathological hallmarks of PD, a loss of substantia nigra neurons associated with an α-synuclein pathology in the surviving neurons (Inden *et al.* 2007). However the ENS alterations are still unknown in this model.

This model was here used to investigate whether the digestive functions could be impaired and whether those alterations could be relevant to the human pathology. We also studied if these impairments could be related to alterations of the neurochemical coding or neurochemical content of the ENS. Finally we tried to detect the putative aggregation or dysregulation of α-synuclein in the ENS.

Materials and methods

Animal care and rotenone administration

All animal studies were carried out in accordance with European Communities Council Directive for the care of laboratory animals (86/609/EEC). C57Bl/6 eight weeks old mice were used as control and rotenone treated animals. Rotenone (Sigma-Aldrich, St Quentin Fallavier, France) was administered daily and orally by gavage during 28 days at a dose of 30 mg/kg. Rotenone was dissolved in 4% chloroform (MP biomedical, illkirch, France), 0.5% carboxymethyl cellulose sodium salt (CMC, Nacalai Tesque, Kyoto, Japan). Vehicle mice received an orally administration of 4% chloroform, 0.5% of CMC daily.

In vivo distal colonic transit

Briefly, mice were slightly anaesthetized with isoflurane (1–2min; ABBOT, Rungis, France) and a 2 mm-diameter glass bead (Sigma-Aldrich) into the distal colon (2 cm from the anus margin) using a fire-polished glass rod. After insertion of the bead, mice were isolated in a cage without food and water. Distal colonic transit was assessed by measuring the time required for the expulsion of the glass bead (bead latency).

Fecal pellet output

Animals were placed in individual clean cages without food and water. Pellets were collected, counted and weighted (wet weight) each 15 min for 2H. Pellets were then dry and weighted again (dry weight). The percentage of water content was then calculated using the dry and wet weight.

Gastric emptying

Following a 12 h fast, mice received food *ad libitum* for 1 h. The consumed quantity of food was calculated with the weight of food before and after. 1H30 after food removal, animals were sacrificed and the stomach contents were weighed. The percentage of food remaining in the stomach was calculated.

Whole gut paracellular permeability

Animals received an oral gavage with cell-impermeant sulfonic acid conjugated with fluorescein. Blood was collected 10 and 30 minutes after gavage and fluorescence detected in plasma after 3000 rpm 20 min centrifugation. Fluorescence intensity was measured using a plate reader (multilabel counter, Wallac 1420 Victor, PerkinElmer, Courtaboeuf, France).

Western blot

After euthanasia striatum, segments of colon and ileum were fastly dissected, removed and frozen. Protein extraction was realized in a 1% SDS buffer and mechanically disrupted. Striatum protein extraction was realized in a RIPA lysis buffer (0.5M Tris-HCl, pH 7.4, 1.5M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10mM EDTA) (Millipore; St Quentin en Yvelines, France) at 4°C. Tissues were mechanically disrupted. Lysate was centrifuged at 11500 g for 10 min. Total protein content in the supernatant was quantified using Pierce BCA Protein Assay (Thermo, Brebière, France) and an equal amount of protein was loaded on a NuPAGE Novex 4-12% Bis-Tris gel (Invitrogen, Cergy-Pontoise, France). Electrophoretic transfer onto nitrocellulose membrane (Hybond Pure; GE Healthcare, Orsay, France) was realized using iBlot® Dry Blotting System (Invitrogen). Membranes were incubated for 10 min in 10% acetic acid then for 1 h at room temperature in Tris-buffered saline (100 mM, 1 NaCl, 10 mM, 1 Tris, pH 7.5) with 5% non-fat dry milk. Membranes were then incubated overnight at 4 °C with either rabbit anti protein gene product 9.5 (PGP 9.5) antibodies (1:1000; Ultraclone, Cambridge, UK), rabbit anti α-synuclein (Tebu-bio, Le perray-en-Yvelines, France). After three washes, membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated antirabbit or antimouse antibodies (Jackson ImmunoResearch, purchased from Immunotech, Marseille, France; diluted 1:10 000). Bound antibodies were visualized by enhanced chemiluminescence detection (GE Healthcare, Fairfield, CT, USA). Lane's intensity was evaluated using ImageJ software (National institute of health, Bethesda, MD) and normalized

to PGP 9.5 expression to give relative α-synuclein expression level. Control relative α-synuclein level was normalized to 100%.

Tissue collection and immunohistochemistry

Following euthanasia ascending colon was removed, stretched and pinned flat on Sylgard-coated Petri dishes, and fixed overnight in 4% phosphate buffer saline (PBS) paraformaldehyde (Sigma-Aldrich). Layers of tissue containing the myenteric and the submucosal plexus were then separated by microdissection. Samples were permeabilized for 2 h in a 4% horse serum/PBS blocking buffer containing 1% Triton X-100 (Sigma-Aldrich). Before Hu immunostaining endogenous peroxidase activity was blocked by incubating preparations with 3% H_2O_2 for 20 min. Endogenous biotin was blocked with a commercial streptavidin/biotin blocking kit (vector laboratories, Burlingame, CA, USA) according to the manufacturer's instruction. Tissues were then washed 3 times in PBS before overnight incubation with mouse anti-Hu conjugated with biotin (1:50; Invitrogen). Tissues were incubated with streptavidin conjugated with carboxymethyl indocyanine-3 (CY3) (1:500; Invitrogen). For other immunostainings tissues were incubated for 24 h with the following primary antibodies diluted in the blocking buffer: goat anti-choline acetyl transferase (ChAT) (1:200; Millipore), rabbit anti-neuronal nitric oxid synthase (nNOS) (1:2000; COGER, Paris, France), rabbit anti-α-synucleine (1:1000; Santa cruz biotechnology, CA, USA). Samples were washed with PBS and incubated for 3 h with suitable secondary antibody: donkey anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) (1:500; Immunotech, Marseille, France), donkey anti-rabbit IgG conjugated to carboxymethyl indocyanine-5 (CY5) (1:500; Interchim, Montlucon, France) for nNOS or anit-rabbit conjugated carboxymethyl indocyanine-3 (CY3) for α-synuclein. Images were acquired with a digital camera (model DP71; Olympus, Rungis, France) coupled to a fluorescence microscope (Olympus IX 50) and analyzed with the Cell B software (Soft Imaging System; Olympus).

Identification of neuronal cell populations and phenotypic analysis

Immunoreactive neurons for ChAT, nNOS, and Hu were counted in at least 20 ganglia per condition and per animal. The total number of neurons determined with the general neuronal marker Hu and the number of neurons was calculated for each marker.

Statistical analysis

Data are represented as mean ± standard error to mean (SEM) or individually plotted, therefore horizontal bars represented mean. Statistical analyses were realized using a t-test. P-values <0.05 were considered significant. Statistical analysis was performed using Graphpad prism 5.00 for windows (Graphpad software, CA, USA).

Results

1. Rotenone induced gastrointestinal impairments

Total transit time was significantly increased in rotenone animals as compared to controls (vehicles-treated animals) (119.9 ± 9.276 min; n=18 for controls and 164.4 ± 10.82 min; n=19 for rotenone; p=0.0038) (fig 2A). Chronic rotenone administration did not modify gastric emptying to solids between controls and rotenone animals (fig 2B). Total colonic transit time was measured by the fecal pellet output. The number of pellet produced by the mice was reduced by 36.8% in rotenone animals as compared to control during a 2 hours collection (fig 2C). Controls produced 18.06 ± 1.279 pellets (n=18) whereas rotenone animals produced only 11.42 ± 1.437 pellets (n=19; p= 0.0015). Moreover the water content, expressed in % of the total wet weight of the pellets, was significantly reduced in rotenone-treated animals (72.07 ± 1.835 %; n=18 in control vs 52.57 ± 3.476 %; n=19 in rotenone; $p<0.0001$) (fig 2C). The distal colonic transit was not altered by rotenone as shown in figure 2D. The whole gut permeability to fluorescein sulfonic acid was significantly reduced at 30 min (fig 2E). Permeability flow-through was 22.69 ± 3.141 10^{-5}ng/mL; n=8 for controls and 15.26 ± 0.8310 10^{-5}ng/mL; n=10 for rotenone animals (p=0,0223).

2. α-synuclein expression level was increased by rotenone treatment in the ENS

Quantitative immunohistochemistry of α-synuclein expression revealed a trend to an increased expression in rotenone animals as compared to controls (fig 4 A and B). α-synuclein expression level quantified by western blot, show an increased relative level (compared to PGP 9.5) of α-synuclein was observed in the colon by western blot (100.0 ± 30.28; n=6 for control vs. 210.5 ± 25.24; n=6 for rotenone; p=0.0173) (fig 4 C and D).

3. Neurochemical phenotype was not modified by rotenone

The number of immunoreactive neurons per ganglia for Hu was counted in each condition in the myenteric plexus of both antrum and stomach (fig 5A and E). This number was not changed in rotenone treated animals (12.83±1.50; n=4) as compared to control (12.08±1.64; n=5) in the antrum nor in the colon (18.79±1.14; n=6 and 16.37±1.50; n=7 respectively). The number of immunoreactive neurons per ganglia for nNOS (fig 5B), ChAT (fig 5C) and TH (fig 5D) were also evaluated in the same parts of the digestive tract. No change occurred in the rotenone-treated animals (fig 5E).

Discussion

The development of new animal models of PD appears to be necessary 1) to reproduce and understand the mechanism of digestives troubles and in particular for ENS 2) to be able to study new therapeutic targets as well in the CNS as in the ENS.

Rotenone is widely used to induce an experimental parkinsonism but ENS studies remain rare. Oral intoxication is one of the most logic ways of contact between nervous system and pesticides which have been suspected for long to induce Parkinson's disease. In this study we described the ENS functions and coding in a mice model of chronic oral intoxication with rotenone. Taken together our results indicate that an environmental factor could be responsible for digestives troubles recorded in humans during PD.

We first showed that rotenone-treated animal exhibit a delayed total transit time by nearly 37%. To discover which parts of the digestive tract were affected we assessed gastric emptying. The gastric emptying was unchanged between rotenone and control animals. The decreased fecal pellet output in rotenone animals as compared to controls reveals a decrease in the colonic motility. Moreover this colonic slow-down transit would probably be due to an alteration in the proximal colon as the bead latency test did not show any difference between rotenone and control animals. The altered digestive function observed in our study is in accordance with previous published study by Drolet *et al.* where rotenone-treated rats exhibit an increased total transit time. This slow digestive transit and decreased stool frequency reproduces one of the main features of PD. However we didn't reproduces the slow-down gastric emptying in this model. These results need to be confirmed, it could not be exclude that this effects could be mediated by a direct action of the rotenone on the muscles cells.

The immunohistochemical staining of the main neurochemical subpopulations, nitrergic and cholinergic neurons, revealed no differences. Neither the number of neurons per ganglion nor the density of neurons were modified (data not shown). There are few studies on the effect of rotenone-induced parkinsonism on the ENS. However our results are in accordance with a previous study on the rotenone effect on the ENS (Greene *et al.* 2009) where no change was observed in the proportion of VIP, nitrergic and cholinergic neurons. It could also be compared to a study of our laboratory (Chaumette *et al.* 2009) were 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated monkeys presented an increased number of neurons per ganglia and in particular an increased number of nitrergic neurons. Few are known about the alterations of the human ENS during PD. In a recent study we described that the modification in the submucosal plexus were rare (Lebouvier *et al.* submitted). Moreover in human myenteric plexus it has only been shown a loss of dopamine without any modification number of TH-immunoreactive neurons (Singaram *et al.* 1995).

One of the main characteristic of PD is the formation of protein aggregates in PD. Those protein aggregates are mainly composed of α-synuclein. We investigated for a dysregulation of α-synuclein expression by western blot and immunohistochemistry and showed a significant up-regulation of α-synuclein by western blot in the proximal colon of the rotenone animals. This observation is in accordance with the results of rat rotenone model (Drolet *et al.* 2009). It still remains to be proven that α-synuclein

overexpression could directly be responsible for digestive functions impairments. This study has been designed for short term experimentation. Animals were sacrified 1 week after the end of the intoxication. As shown in other studies {drolet} the effect of chronic rotenone administration could reproduce the progression of the human disease. It would be thus relevant to characterize the long-term consequences of the modifications observed in our model and in particular the α-synuclein overexpression..

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Figures legends

Figure 1. Slowed digestive total transit and reduced water content in pellets in rotenone treated animals

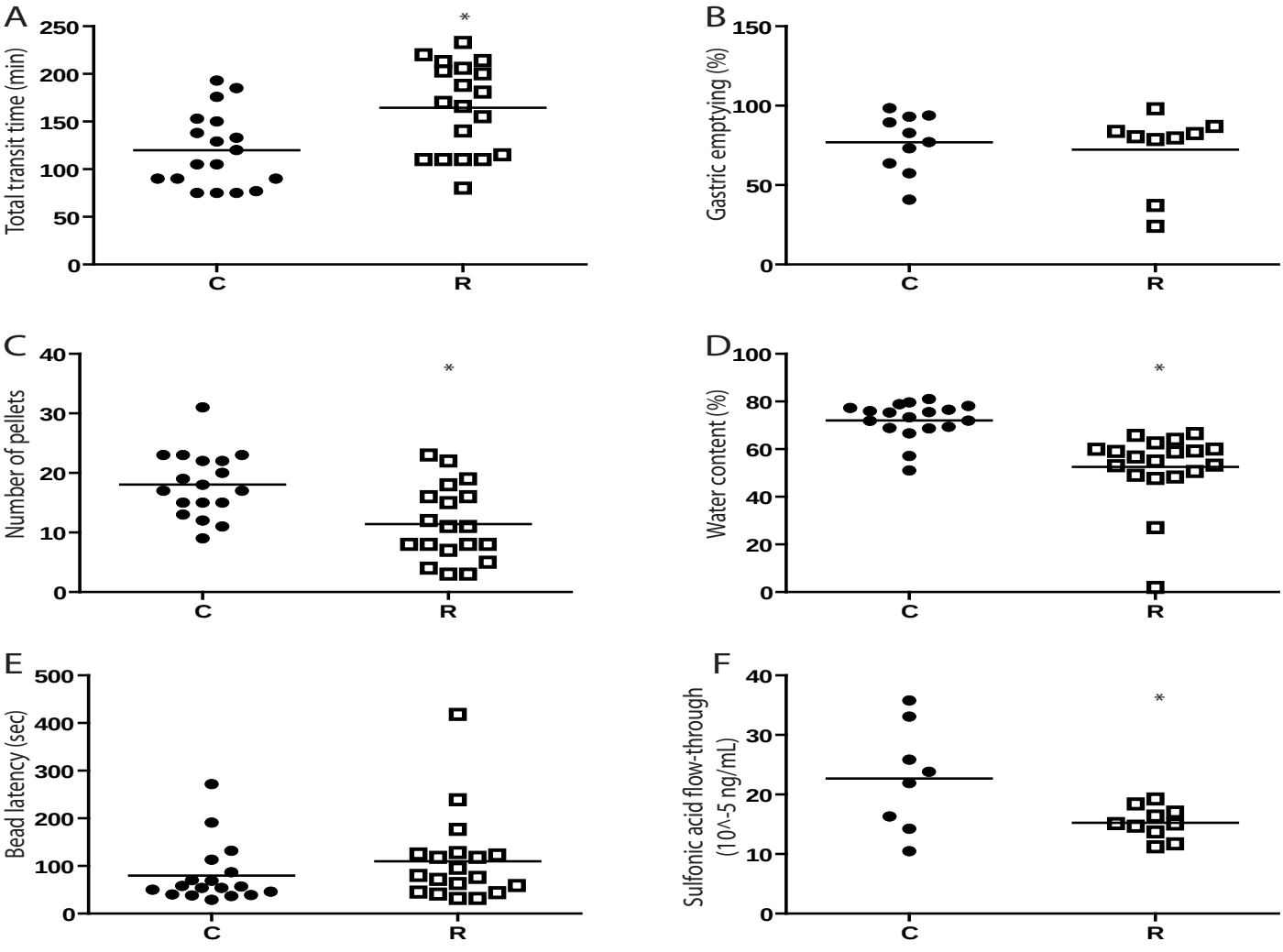
(A) Total transit time assessed by carmin red was significantly increased in rotenone (R) animals as compared to control animals (V) (p= 0.0038). (B) Gastric emptying of solids meal expressed in % of ingested food after 1H30. (C) Number of pellets produced by mice during 2H (n=18 for V mice and n=19 for R mice). The number of pellets in R-treated animals was significantly reduced (p= 0.0015). Moreover in (D) the water content in R-treated animal was significantly reduced by 27 % compared to V-treated mice. (E) Bead latency time recorded after the insertion in the colon of a glass bead at 2 cm of the anal margin and before its expulsion. No difference was observed between V- and R-treated animals. (F) Whole gut transit permeability at 30 min after gavage with fluorescein sulfonic acid in R-treated animals was decreased as scompared to V-treated (p=0.0223). Each point represents a mouse injected with control (circle) or with rotenone (square). Horizontal bars represent the mean.

Figure 2. Rotenone treatment induced significant increase in α-synuclein expression in proximal colon

Representative photographs of α-synuclein immunoreactivity in the myenteric plexus of control (A) and rotenone (B) animals. Scale bars represent 40µm. Quantitative analysis of fluorescence intensity level of α-synuclein immunoreactivity (C). Relative quantification of α-synuclein expression normalized by PGP 9.5, a neuronal marker, in control and in rotenone treated animals in the colon (D). Representative photography of western blot lanes of α-synuclein and PGP 9.5 in the colon (E). Error bars represent the SEM.

Figure 3. Major enteric neurotransmitters expression is not modified in the proximal colon

(A, B, C, and D) Photography of a control animal whole mount of myenteric plexus. Staining was realized with antibodies against PGP 9.5 (A), nNOS (B), ChAT (C) and TH (D). Scale bar represent 40µm. (E) Table presenting the number of neurons per ganglia for different markers in the myenteric plexus of the proximal colon. The number of neurons was not different between control and rotenone animals. PGP 9.5: protein gene product 9.5. nNOS: neuronal nitric oxid synthase. ChAT: choline acetyl transferase. TH: tyrosine hydroxylase. Arrows show an immunoreactive neuron. Each point represents a mouse injected with control (circle) or with rotenone (square).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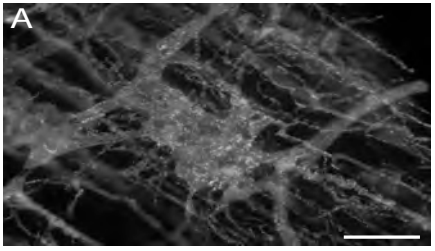
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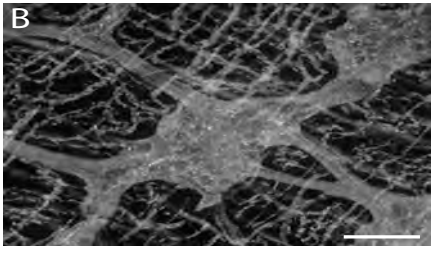
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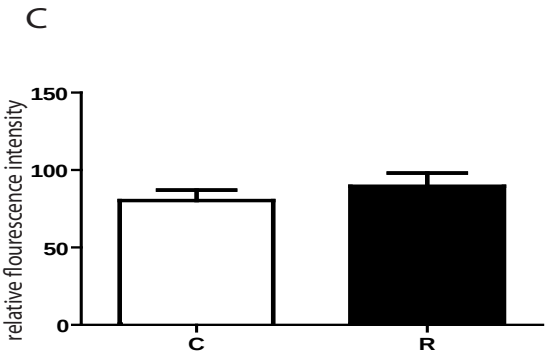
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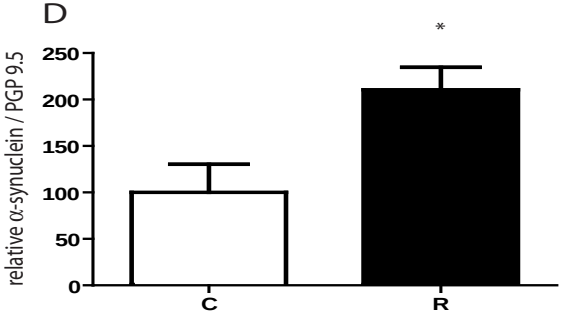
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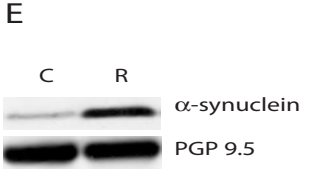
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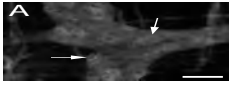

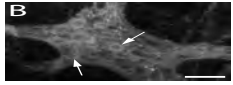

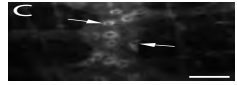

D

	Control	Rotenone
Hu	48.45±2.02 (n=6)	50.18±3.01 (n=6)
ChAT	18.33±1.45 (n=6)	18.53±1.39 (n=6)
NOS	20.01±0.57 (n=6)	21.38±1.46 (n=6)

ARTICLE 3 : LESIONS PATHOLOGIQUES DANS DES BIOPSIES COLIQUES AU COURS DE LA MALADIE DE PARKINSON

Cet article a été publié dans le journal *gut* en 2008.

La maladie de Parkinson, maladie neurodégénérative est caractérisée par la perte de neurones au sein de la SNpc et la présence d'agrégats protéiques dont le principal composant est l'α-synucléine. Ces altérations sont responsables des troubles moteurs, cependant la MP est désormais considérée comme une maladie multicentrique affectant d'autres structures. Si des altérations du SNE on pu être observées, la chronologie de leur apparition n'a pas été étudiée. Par conséquent démontrer la faisabilité d'une étude du SNE sur des biopsies coliques standard et la présence de lésions caractéristiques de la MP pourrait permettre de mieux comprendre la physiopathologie de cette maladie et d'en avancer le diagnostic.

Cette étude pilote a montré qu'il était possible d'isoler, à l'aide de biopsies prélevées au cours d'une coloscopie, le PSM, et, à l'aide de marquages immunohistochimiques, de caractériser les neurones de façon individuelle. Ainsi cette étude a montré l'absence de modification du nombre de neurones par ganglion immunoréactifs pour Hu, marqueur général des neurones, chez les patients parkinsoniens en comparaison avec des témoins sains et des témoins constipés. La proportion de neurones dopaminergiques au sein de ces trois groupes n'était pas modifiée ; en revanche, ce travail préliminaire a mis en évidence la présence chez 4 des 5 patients parkinsoniens de fibres neuronales, parfois dystrophiques, positives pour la forme phosphorylée de l'α-synucléine. Cette immunoréactivité était absente dans les autres groupes de l'étude.

Ces premiers résultats ont montré la possibilité de réaliser à l'aide de biopsies coliques standard une caractérisation du PSM colique.

(Sanofi-Winthrop, Riells y Viabrea, Spain) for 30 days. Criteria for eligibility, randomisation of patients and blinding were those used in our previous randomised study.[1] Following laparoscopic cholecystectomy, GB specimens were collected for routine histology and immunohistochemisty, the latter performed by the streptavidin–biotin method. Mouse monoclonal antibodies against COX-2 protein (clone CX-294; Dako, Glostrup, Denmark) and mouse monoclonal antibodies against CD68 (clone M7103; Dako) were used. Part of the GB tissue was collected for muscle cell contraction to cholecystokinin 8 (CCK-8) studies as well as for measurement of prostaglandin (PG) E2 levels as described elsewhere.[1]

Following randomisation, eight patients received UDCA and 11 received placebo, for 30 days. Staining with haematoxylin & eosin revealed chronic cholecystitis, with mild inflammatory infiltrates, in all GBs with gallstones, treated with UDCA or placebo. No histopathological lesions were observed in GBs from a control group, represented by 10 alithiasic GBs removed from patients with neoplastic diseases not involving the GB. The number of CD68 positive macrophages in the muscle layer of GBs from gallstone patients was significantly higher compared to that in control patients. In UDCA-treated patients, the number of CD68 positive macrophages, in GB muscle, was significantly lower when compared to that in placebo-treated patients. Positive COX-2 expression was almost exclusively present in macrophages within the muscle layer (fig 1). The number of COX-2 positive cells was higher in muscle from symptomatic gallstone patients compared to controls and, likewise macrophages, significantly lower following UDCA treatment (table 1, fig 1). A direct and significant correlation was observed between positivity for CD68 and COX-2 (Spearman's $\rho = 0.7$, $p<0.01$). As in our previous study, the production of PGE2 was significantly lower, following UDCA than after placebo. Furthermore, muscle contraction, induced by increasing concentrations of CCK-8 (assessed in four patients in each group) was significantly higher in the UDCA, compared to placebo, group (maximal contraction to CCK-8 10^{-8} mol/l was 25.1 (SD 3) vs 12 (SD 4)%, respectively; $p<0.001$). Our more recent data show that an inflammatory macrophage infiltrate is present in the GB muscle layer of cholesterol gallstone patients. UDCA decreases the presence of macrophages in the muscle layer and confirms improvement in GB muscle cell contraction. These results suggest that activated macrophages play a role in muscle cell dysfunction and add insight into the anti-inflammatory action of UDCA, which may explain some of the therapeutic effects of this bile acid in liver diseases as well as other gastrointestinal inflammatory conditions.

Table 1 Mean number (with SD in parentheses) of positively stained cells in 10 consecutive microscopic fields

	Controls	Placebo	UDCA
CD68	11.9 (8.1)	36.2 (11)*	19.6 (6)**
COX-2	10.5 (6.8)	30.6 (12)*	15.2 (5.5)**

*$p<0.001$ vs controls; **$p<0.01$ vs placebo.
COX-2, cyclooxygenase-2; UDCA, ursodeoxycholic acid.

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Competing interests: None.

Ethics approval: This study was approved by the Ethics Committee of Campus Bio-Medico University on 21 June 2001.

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Pathological lesions in colonic biopsies during Parkinson's disease

Parkinson's disease (PD) is a neurodegenerative condition that affects 1% of the population over 65 years of age. The two pathological hallmarks of PD are a loss of dopaminergic neurons in the substantia nigra (SN) and the presence of cytoplasmic eosinophilic inclusions termed Lewy bodies (LBs), whose main component is phosphorylated α-synuclein.[1] This degeneration of SN neurons leads to a dopamine deficiency

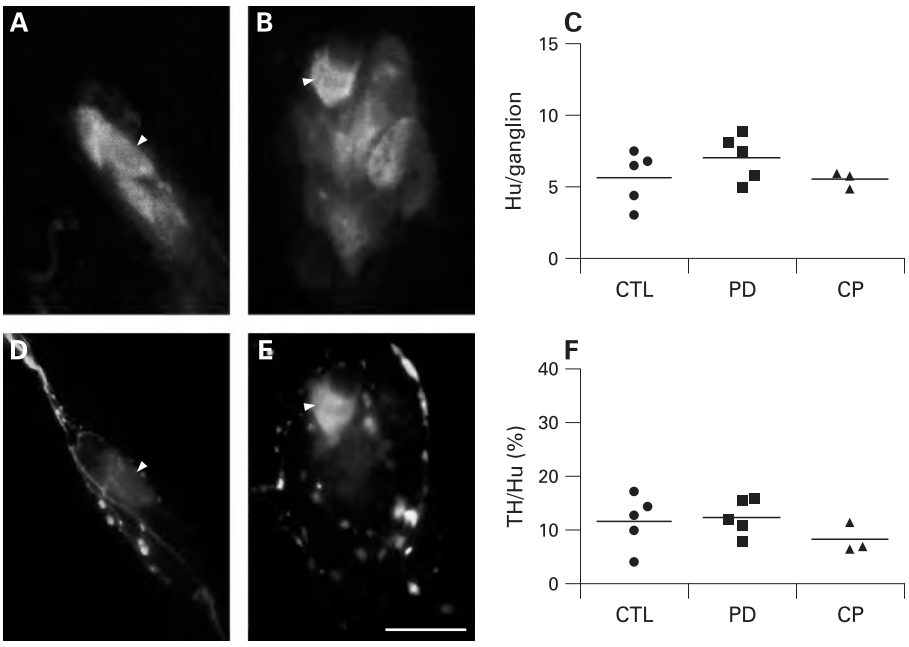

Figure 1 Submucosal neuron counts and dopaminergic phenotype are unchanged in patients with Parkinson's disease (PD). Hu-immunoreactive (IR) submucosal neurons were identified in the colon of controls (n = 5) (A), PD patients (n = 5) (B) and constipated patients (n = 3). There was no change in the number of Hu-IR submucosal neurons per ganglion in the three conditions (C). Double labelling with antibodies against Hu (A,B) and tyrosine hydroxylase (TH) (D,E) showed that occasional submucosal neurons were TH-IR (arrow heads). No significant decrease in the proportion of TH-IR submucosal neurons occurred in PD and in constipated patients (F). Each circle, square and triangle represents one control, PD or constipated patient, respectively. Horizontal bars represent the mean. Scale bar: 20 μm. CTL, control; CP, constipated patient.

responsible for the major motor symptoms. Nevertheless, it has become increasingly evident that PD is a multicentric neurodegenerative process that also affects neuronal structures outside the SN.[2] In this context, various reports performed on surgical or autopsy specimens have shown that the enteric nervous system (ENS) is affected during PD.[3 4] However, it is still a matter of debate whether these alterations occur early in the course of the disease. This is mainly due to a lack of accessibility to the ENS in the living patients. Therefore, demonstrating (1) the ability to study the ENS using routine colonic biopsies and (2) the presence of lesions characteristics of PD could be relevant for an early diagnosis of the disease and to better understand its pathophysiology.

We therefore performed routine colonic biopsies in five PD patients complaining of functional constipation (63 (SD 7) years, three men; all had disease duration >5 years). Five healthy age-matched patients (61 (SD 6.5) years, one man) requiring a total colonoscopy for colorectal cancer screening were included as controls. They had no known neurological disease. None suffered from functional digestive symptoms. In order to avoid any specific role for chronic constipation, we included three additional non-age-matched patients (52 (SD 5) years, no men) who underwent total colonoscopy for the assessment of a chronic intractable constipation as additional controls. Written consent was obtained according to the principles of the Declaration of Helsinki.

Four biopsies were taken from the ascending colon during colonoscopy. Biopsies were performed using standard biopsy forceps without needles (FB210K; Olympus, Tokyo, Japan). Samples were immediately immersed in 4°C saline solution and microdissected in order to separate the submucosa (containing the internal submucosal plexus) from the mucosa. The submucosa were then fixed in 4% paraformaldehyde. Imunohistochemical studies were then performed on these tissues using a combination of antibodies against rabbit anti-tyrosine hydroxylase (TH) (1:500, Pel-Freez, Rogers, Arkansas, USA), rabbit anti-dopamine-β-hydroxylase (DBH) (1:250, Millipore, Saint-Quentin-en-Yvelines, France), mouse anti-Hu C/D (1:200, Invitrogen, Cergy-Pontoise, Fance), rabbit anti-phosphorylated α-synuclein (1:5000, WAKO, Osaka, Japan) or rabbit anti-neurofilament 200 kDa (1:250; Millipore) as previously described.[5]

In control patients, individual biopsies contained 11.2 (SD 7.9) ganglia and each ganglia contained 5.6 (SD 1.9) Hu-immunoreactive (IR) neurons. In PD patients, the number of ganglia per biopsies was similar to controls (13.6 (SD 5.3); p = 0.22). In addition, the number of Hu-IR neurons per ganglion in PD was unchanged as compared to controls (7.0 (SD 1.6); p = 0.25) (fig 1A,C). Constipated controls did not differ from PD patients in the number of ganglia per biopsy (11.3 (SD 1.5); p = 0.57) or in the number of neurons per ganglion (5.5 (SD 0.6); p = 0.19) (fig 1C).

In healthy controls, 11.6 (SD 5.0)% of Hu-IR neurons were TH-IR. In PD patients, the proportion of TH-IR neurons was unchanged as compared to controls (12.3 (SD 3.3)%; p = 0.80) (fig 1D–F). In constipated patients, the proportion of TH-IR neurons was similar to the one of PD patients (8. (SD 2.7)%; p = 0.12) (fig 1F). In all groups no neuronal body was DBH positive, suggesting that all TH-IR neurons in the submucosal plexus were dopaminergic. These results are consistent with a previous report by Singaram *et al*[6] showing the absence of loss of TH-IR neurons in the submucosal and myenteric plexuses of PD patients, suggesting that it is not a marker of choice for detecting PD lesions in the ENS.

However, immunohistochemical staining with an antibody against phosphorylated α-synuclein, revealed that 4 out of 5 PD patients had phospho-α-synuclein-IR neurites (identified with neurofilament (NF) in the submucosa (fig 2A,F). These phospho-α-synuclein-IR neurites were absent in both control and constipated patients. In some cases, large aggregates were observed in dystrophic NF-IR neurites (fig 2E), a pattern reminiscent of Lewy neurites.

Taken together, our pilot study showed that routine colonic biopsies can be used to study the submucosal plexus of the ENS. In addition, we identified for the first time in the gut of living PD patients lesions similar to the ones observed in the brain. This technique could be a reliable tool to detect early lesions in the gut during the course of PD in order to better understand the pathogenesis of the disease and/or to identify novel biomarkers.

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Competing interests: None.

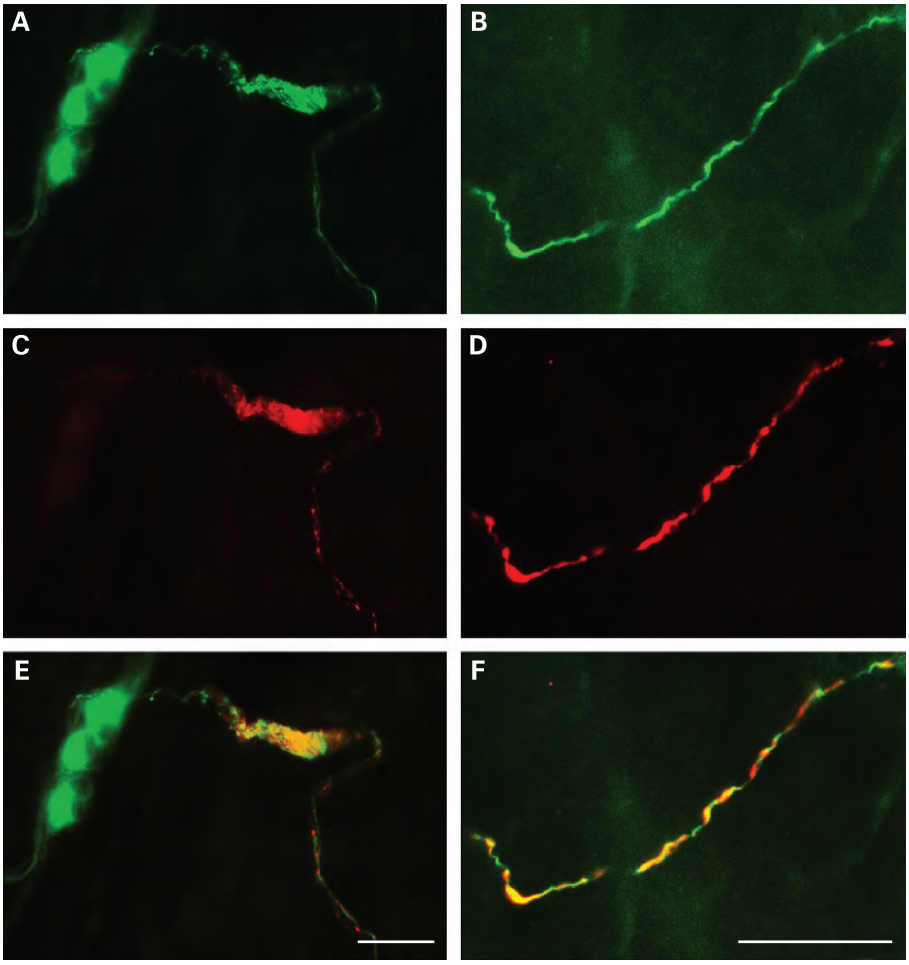

Figure 2 Phospho-α-synuclein-positive submucosal neurites differentiate Parkinson's disease patients from controls. Double labelling with antibodies against neurofilament (NF) (A,B) and phosphorylated α-synuclein (C,D) revealed that some NF-immunoreactive (IR) neuritic structures were also phospho-α-synuclein-IR (merged image in E,F) in the majority of Parkinson's disease patients, but in none of the controls. Occasionally the inclusion-bearing neurites displayed dystrophic alterations (A,C,E). Scale bar: 30 μm.

Ethics approval: The study protocol was approved by the local Committee on Ethics and Human Research on 27 February 2007.

TC and TL as well as PD and MN contributed equally to this work.

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CORRECTION

doi:10.1136/gut.2007.119446corr1

R Spiller, Q Aziz, F Creed, *et al*. Guidelines on the irritable bowel syndrome: mechanisms and practical management (*Gut* 2007;**56**:1770–98). In paragraph 4.4.1 the sentence "This in turn acts on the adrenal medulla, resulting in cortisol secretion into the circulation" should read "This in turn acts on the adrenal cortex, resulting in cortisol secretion into the circulation".

Editor's quiz: GI snapshot

ANSWER

From the question on page 1673

The patient had a large inflammatory abdominal aortic aneurysm. The abdominal CT scan shows a large infrarenal aortic aneurysm with a maximum diameter of 7.5 cm extending into the iliac vessels. There is an enhancing soft-tissue cuff surrounding the anterolateral margin of the aneurysm. The aneurysm appears to compress the third part of the duodenum (fig 1 below), which, however, was not detected at endoscopy. These CT findings were suggestive of an inflammatory aneurysm. Inflammatory abdominal aortic aneurysms represent 3–10% of all abdominal aortic aneurysms and occur predominantly in men.[1] They differ from atherosclerotic aneurysms in that patients often present with abdominal symptoms or anorexia, weight loss, and raised inflammatory markers. CT has a specificity of 99.7% for diagnosis of inflammatory aneurysms,[2] usually showing periaortic fibrosis as a cuff of enhancing soft tissue surrounding the anterolateral margin of the aneurysm. If periaortic fibrosis is extensive, adjacent abdominal structures may be compressed and adherent, most commonly the third part of the duodenum.[1] Although rare, inflammatory abdominal aortic aneurysms should be kept in mind as a cause of abdominal pain and/or anorexia, weight loss, and raised inflammatory markers. The natural history of inflammatory abdominal aortic aneurysms remains unknown, with 3.3–14% patients presenting with acute or chronic rupture.[1] As regards to management, the literature supports an operative approach with a 30 day operative mortality rate of up to 9%.[1] Complete regression of fibrosis and inflammatory process occurs in up to one-half of patients at long-term follow-up post-operatively. Clinical symptoms (such as weight loss and gastrointestinal symptoms) reverse in 93% of the patients after an operation.[3] Endovascular therapy is also a potential treatment

ARTICLE 4 : RELATION ENTRE SYMPTOMES DE LA MALADIE DE PARKINSON ET LESIONS DU SYSTEME NERVEUX ENTERIQUE DANS DES BIOPSIES COLIQUES

Ce manuscrit est actuellement en révision dans le journal PLoS ONE.

Cette étude fait directement suite à la précédente, la complète par un plus grand nombre de patients et apporte de nouveaux éléments cliniques. L'analyse du PSM des biopsies coliques a été réalisée chez 30 patients parkinsoniens et 10 témoins. Ces résultats ont ensuite été corrélés aux symptômes moteurs de la maladie et à la sévérité de la constipation des patients.

La réalisation d'un marquage contre la forme phosphorylée de l'α-synucléine a mis en évidence la présence de NL chez 72% des patients parkinsoniens. Aucun NL n'était observé chez les témoins.

L'évaluation du nombre de neurones par ganglion, immunoréactifs pour le marqueur NF, montrait une diminution significative chez les patients parkinsoniens. Par la suite les patients ont été répartis en 3 groupes, aucun NL, une densité modérée de NL et une forte densité. Cette répartition a mis en évidence une absence de perte neuronale chez les patients ne présentant aucun ou une densité modérée de NL. En revanche le groupe présentant une forte densité de NL avait une forte diminution du nombre de neurones par ganglion.

L'étude de la densité de NL en fonction de l'âge a montré que ce paramètre pouvait être confondant, en effet, la sévérité de l'atteinte augmente avec l'âge des patients. C'est pourquoi l'ensemble des corrélations ultérieures a été ajusté à l'âge des patients.

La densité de NL était positivement corrélée avec le score axial, marqueur des symptômes moteurs, et négativement corrélée avec la réponse au traitement par la levodopa.

La densité de NL n'était en revanche pas significativement corrélée avec le score de constipation après correction par l'âge des patients.

Cette étude montre que l'analyse du PSM des patients parkinsoniens par biopsies coliques pourrait permettre un diagnostic neuropathologique de la maladie.

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PLoS one

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

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Abstract

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

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

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

Citation: Lebouvier T, Neunlist M, Bruley des Varannes S, Coron E, Drouard A, et al. (2010) Colonic Biopsies to Assess the Neuropathology of Parkinson's Disease and Its Relationship with Symptoms. PLoS ONE 5(9): e12728. doi:10.1371/journal.pone.0012728

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Competing Interests: The authors have declared that no competing interests exist.

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9 These authors contributed equally to this work.

¶ These authors also contributed equally to this work.

Introduction

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

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

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

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

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

Methods

Subjects

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

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

Patient evaluation

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

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

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

Colonoscopy biopsies

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

Immunohistochemistry

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

Neuronal cell counting and scoring

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

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

Statistical analysis

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

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

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

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

Results

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

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

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

**md*: missing data.

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

Lewy neurites in colonic biopsies from PD patients

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

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

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

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

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

Neurofilament and tyrosine hydroxylase-expressing neurons in the submucosal plexus

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

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

Clinicopathological correlations

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

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

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

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

Discussion

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

Lewy pathology in the SMP of PD patients

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

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

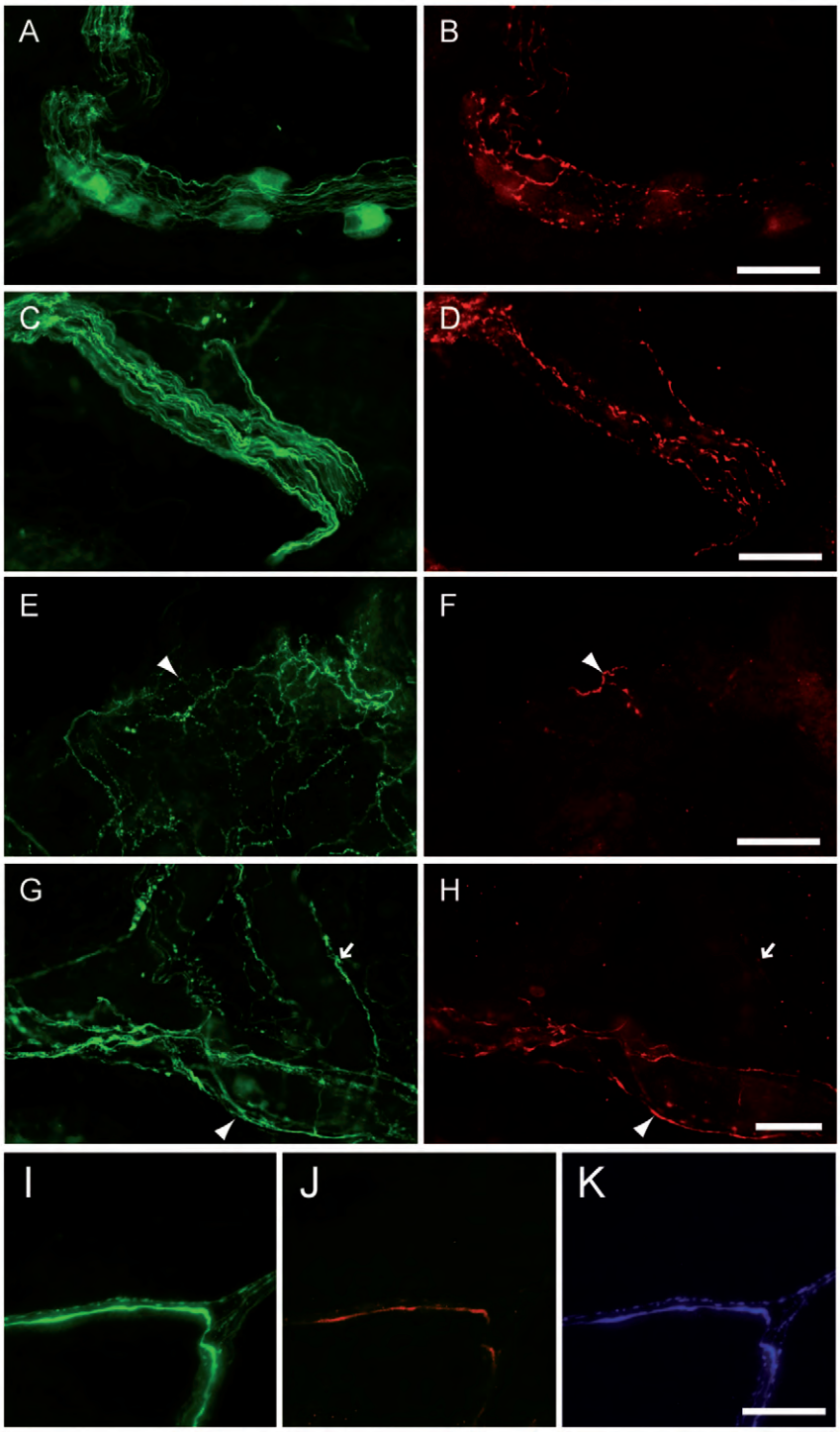

Figure 1. Phospho-α-synuclein-positive submucosal neurites in PD patients. Labeling with antibodies against neurofilament (NF) (**ACEGI**) and phosphorylated α-synuclein (**BDFHJ**) revealed that some NF-immunoreactive (IR) neurites were also phospho-α-synuclein-IR. Occasionally, these phospho-α-synuclein-IR neurites were present amidst a submucosal ganglion (**AB**). Some of these structures formed bundles (**D**) while others were isolated (**arrowhead in F**). Thirty seven percents of the phospho-α-synuclein-IR neurites were perivascular (**GH**). Triple immunostaining with antibodies against tyrosine hydroxylase (**K**) revealed that 60% of LN were also TH-immunoreactive (**IJK**). Scale bar: 30 μm.
doi:10.1371/journal.pone.0012728.g001

respectively, and that 51% are DBH-IR, suggesting that many TH-IR LN belong to postganglionic sympathetic neurons. Thus, a significant proportion of the enteric pathology reflects the widespread sympathetic degeneration seen in other systems [30,31].

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

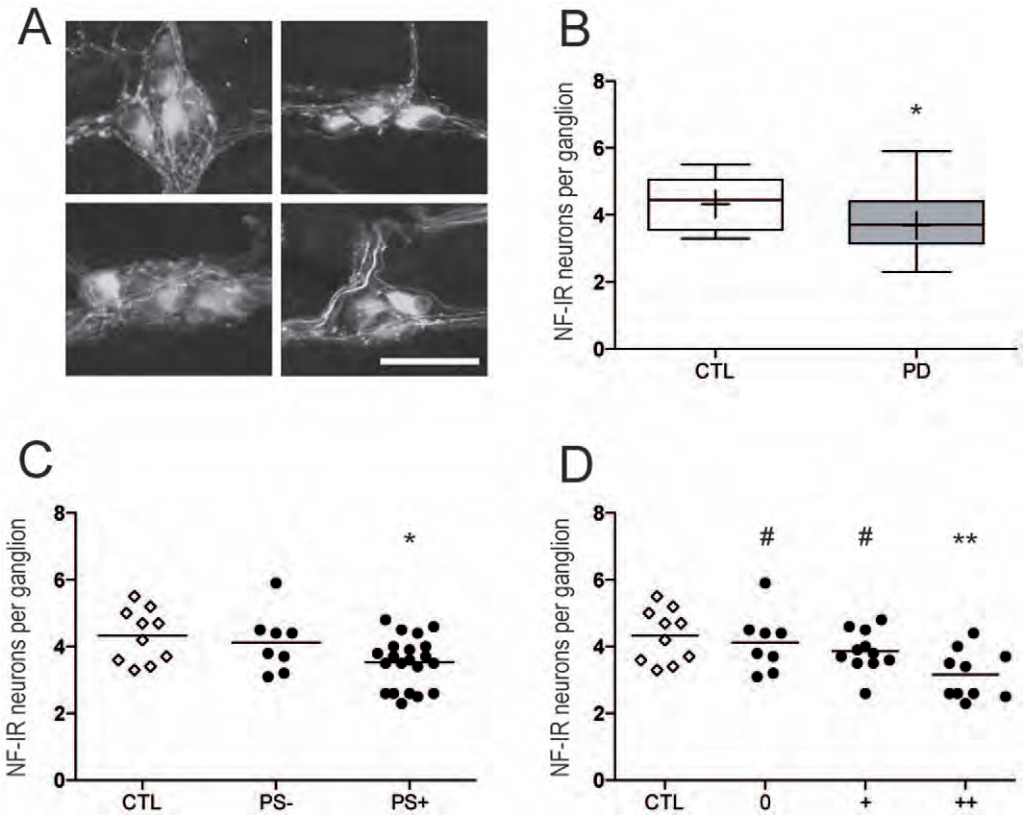

Figure 2. Count of neurofilament-positive neurons in the submucosal plexus of PD patients. A. Neurofilament-immunoreactive (NF-IR) submucosal neurons were counted in every available ganglion from colonic biopsies. Representative photographs of ganglia from PD patients (left panels) and controls (right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

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

Table 3. Clinico-pathological correlations.

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

doi:10.1371/journal.pone.0012728.t003

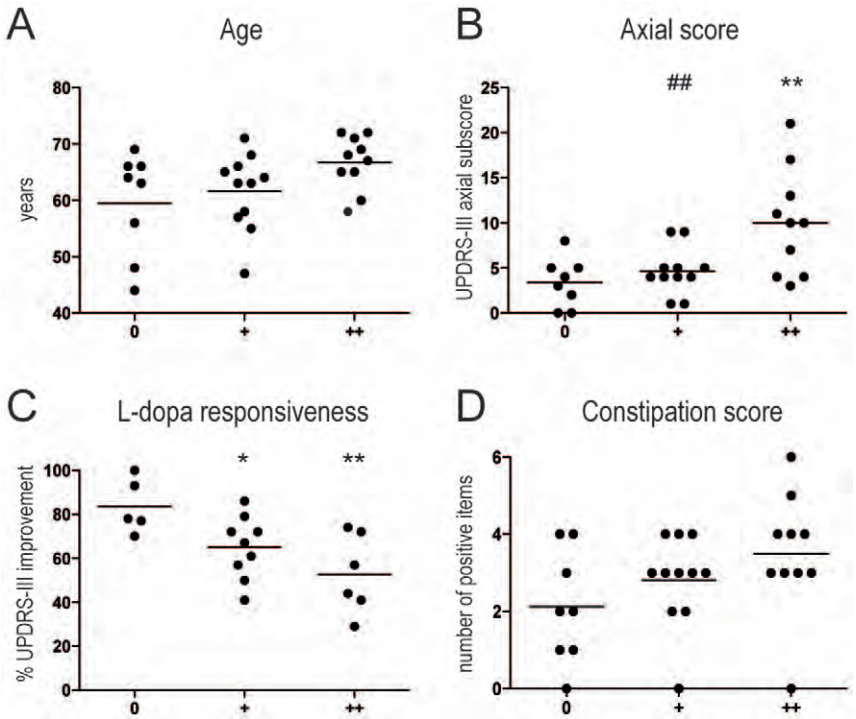

Figure 3. Correlation of clinical symptoms with pathology burden. A. Measure of pathology burden using a quantitative score correlated with age, which appeared as a potential confounding factor. Subsequent correlation analysis was performed after adjusting the data for age. **B.** Pathology burden positively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

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

Lewy pathology burden and constipation

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

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

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

Lewy pathology burden is correlated with PD progression

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

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

The ENS as biomarker in PD

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

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

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

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

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

Supporting Information

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

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

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Author Contributions

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

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DISCUSSION

L'ensemble de ces résultats permet de préciser les altérations du SNE dans les modèles expérimentaux de la MP. Les études chez l'Homme permettent aussi d'effectuer des parallèles entre les modèles animaux et la réalité de la pathologie, et ainsi d'évaluer la pertinence de ces modèles.

MODELES ANIMAUX ET MALADIE DE PARKINSON, NECESSITE D'ALLER AU-DELA DES LESIONS DOPAMINERGIQUES

D'un point de vue chronologique, les modèles animaux de MP ont été en premier lieu développés suivant l'idée que la perte de neurones dans la substance noire était le marqueur principal de cette pathologie. Ainsi ont émergé des modèles d'intoxication par des neurotoxines telles que le MPTP ou la 6-OHDA, affectant principalement les neurones dopaminergiques de la SNpc. Cependant il apparaît de plus en plus nettement que la maladie se caractérise aussi par une atteinte extérieure à la substance noire. Même si ces lésions extra-nigrales restent encore imparfaitement connues, de nombreux troubles dits non-moteurs en sont considérés comme la conséquence directe. Nombre de ces lésions observées dans le SNC ou dans les systèmes nerveux périphériques sont corrélées avec les manifestations non-motrices de la MP (Lim *et al.* 2009).

Les premiers modèles animaux développés étaient centrés sur la perte de neurones dopaminergiques de la SNpc. Cependant afin d'appréhender l'ensemble de la physiopathologie de la maladie l'étude de ces lésions extra-nigrales est nécessaire. Parce qu'elles ont été moins étudiées, jusqu'à maintenant peu de lésions extra-nigrales ont été rapportées dans les modèles animaux.

Le MPTP bien qu'ayant une spécificité dopaminergique théorique induit des altérations pouvant être comparées aux manifestations non-motrices de la MP. Ainsi une administration de MPTP dans un modèle de primate non-humain induit un trouble de la phase de mouvements oculaires rapides du sommeil apparaissant avant les manifestations motrices (Barraud *et al.* 2009), des désordres cognitifs (Decamp and Schneider 2004; Schneider and Kovelowski 1990) et des altérations de l'odorat (Miwa *et al.* 2004). De plus l'administration de MPTP induit des lésions du système nerveux sympathique cardiaque chez la souris (Takatsu *et al.* 2000). Ces études montrent que l'atteinte liée à l'administration de MPTP serait plus diffuse que ne le laisse supposer sa spécificité dopaminergique théorique, et indiquent de possibles altérations digestives.

L'effet du MPTP sur le SNE en particulier était inexploré. Cependant l'intérêt de cette caractérisation est grandissant comme l'attestent les publications de 2 articles récents étudiant l'impact du MPM sur le SNE (Anderson *et al.* 2007 ; Tian *et al.* 2008). Les résultats de ces deux études montrent la susceptibilité des neurones entériques dopaminergiques vis-à-vis de cette neurotoxine.

Le modèle roténone ne présente quant à lui pas de spécificité pour les neurones dopaminergiques. Leur perte préférentielle s'explique par leur sensibilité au stress oxydatif. Ce modèle est donc plus à même de reproduire les lésions extra-nigrales de la MP. Ainsi, des troubles de la phase de mouvements oculaires rapide du sommeil ont été rapportés chez des rats suite à l'injection de roténone (Garcia-Garcia *et al.* 2005). De plus, une agrégation d'α-synucléine a été rapportée dans les noyaux intermédiolatéral et dorsal moteur du nerf vague (Pan-Montojo *et al.* 2010).

L'ensemble de ces données tend à montrer que les modèles toxiques de MP pourraient induire des altérations significatives du système digestif et en particulier du SNE.

MODELES ANIMAUX DE LA MALADIE DE PARKINSON ET SYSTEME NERVEUX ENTERIQUE

L'administration chronique de MPTP réalisée chez les singes induit des altérations du SNC similaires à celles observées dans la MP (Bezard *et al.* 2001; Bezard *et al.* 2003). Au niveau du SNE nos résultats montrent que cette intoxication induisait de nombreuses modifications du phénotype neuronal. Le nombre de neurones TH-immunoréactifs était diminué, résultat qui démontre la sensibilité des neurones dopaminergiques du SNE, et donc leur potentielle implication dans la MP.

De façon surprenante, le nombre total de neurones par ganglion était augmenté suite à l'intoxication au MPTP. Nous avons alors pu montrer, par marquage des cellules en prolifération, qu'il existait une activité mitotique augmentée au niveau du PM colique chez les singes traités. Cependant aucune colocalisation n'a pu être établie entre les marqueurs neuronaux Hu et NF et le marqueur de prolifération Ki-67. Cette augmentation de densité neuronale pourrait avoir deux explications distinctes : a) le sacrifice des animaux ayant été réalisé 7 mois après la fin de l'intoxication, il est possible que la perte des neurones dopaminergiques ait induit, par un mécanisme compensatoire, une différenciation de précurseurs neuronaux ; b) l'administration de MPTP peut avoir été directement responsable de cette prolifération. Ces hypothèses sont étayées par plusieurs études montrant que le MPTP peut modifier le phénotype mitotique des neurones dopaminergiques (Hoglinger *et al.* 2007 ; Shan *et al.* 2006), mais ces données restent discutées (Tande *et al.* 2006).

Nous avons par la suite identifié la sous-population nitrergique comme étant significativement augmentée en condition MPTP. Un lien peut être directement établi

entre ce résultat et les résultats rapportés en 2006 au niveau du SNC (Chalimoniuk *et al.* 2006). Cette étude met en évidence une augmentation de la voie NO/cGMP lors d'un traitement par le MPTP chez la souris au niveau de la SNpc. Cette voie pourrait être modifiée de façon similaire dans le SNE. En outre les neurones TH sont considérés comme des neurones inhibiteurs du transit digestif (Li *et al.* 2006) au même titre que les neurones nitrergiques ; cette augmentation du nombre de neurones nitrergiques pourrait simplement permettre de rééquilibrer la balance neurones excitateurs/neurones inhibiteurs.

Cette étude met aussi en avant des différences importantes entre PM et PSM, tant en condition physiologique qu'en condition pathologique, entre le nombre de neurones par ganglion et les diverses proportions des sous-populations neuronales. La différence de modifications induites par le MPTP suggère que les deux plexus présentent des capacités de plasticité différentes. Le PSM et le PM pourraient être affectés de façon différentielle au cours de la MP.

La seconde partie de ce travail s'est attachée à la description des effets d'une autre toxine couramment utilisée pour induire la MP, la roténone. Celle-ci ne présente pas, au contraire du MPTP, de spécificité dopaminergique, ni même neuronale. Dans les études précédemment publiées utilisant ce modèle, la sélectivité de la mort des neurones dopaminergiques de la substance noire est expliquée par la sensibilité de ces neurones au stress oxydatif induit par la roténone. Au début de notre étude, aucune étude n'avait évalué les effets de la roténone sur le SNE. Depuis, deux études ont montré l'effet pro-agrégeant de la roténone sur l'α-synucléine du SNE (Drolet *et al.* 2009; Greene *et al.* 2009). Une des originalités de notre modèle repose sur la voie d'administration utilisée. En effet, en condition habituelle, les contacts humains avec les divers pesticides potentiellement impliqués dans l'induction de la MP sont limités. L'ingestion de

pesticides avec la nourriture, fruits et légumes principalement, semble être une des explications logiques de cette contamination. Ce modèle a été étudié après la mise en évidence de la possibilité d'induire au niveau du SNC une MP par administration orale de roténone en 2007 chez la souris (Inden *et al.* 2007).

Nos résultats montrent des altérations des fonctions digestives ainsi qu'une augmentation de l'expression d'α-synucléine au niveau du côlon proximal chez les souris traitées à la roténone. Ces observations laissent supposer un état au moins pré-symptomatique de la maladie. Les troubles digestifs sont considérés comme précurseurs des troubles moteurs chez les patients parkinsoniens. La MP est une pathologie progressive, l'apparition des symptômes moteurs n'a lieu que plusieurs années après son initiation (Hilker *et al.* 2005). L'augmentation de l'expression de l'α-synucléine au sein du SNE dans notre second modèle pourrait représenter un événement précoce de la maladie. Les altérations présentes dans notre modèle peuvent ainsi être interprétées comme soutenant la théorie de Braak et collaborateurs qui suggèrent que la MP pourrait être initiée dans les systèmes nerveux périphériques en contact avec l'environnement comme le SNE et le bulbe olfactif. Un événement lié à un pathogène affectant directement le système nerveux périphérique pourrait alors induire une pathologie centrale en progressant de façon rétrograde vers le SNC. De plus, les étroites relations entre les maladies neurodégénératives et l'inflammation (Amor *et al.* 2010), l'inflammation et les dysfonctions du tube digestif (Anton *et al.* 2000) renforcent l'idée qu'une inflammation digestive pourrait être à l'origine d'une MP.

Ainsi ces 2 études caractérisent les altérations du SNE dans des modèles validés de MP, et mettent en évidence des similitudes avec la MP humaine. Cependant comme dans la plupart des modèles, aucun des modèles de MP développés et caractérisés n'a, à ce jour, pu reproduire l'ensemble des symptômes rapportés chez l'Homme.

L'ALPHA-SYNUCLEINE COMME BIOMARQUEUR DE LA MALADIE DANS LE SYSTEME NERVEUX ENTERIQUE

Evaluer la pertinence des modèles animaux vis-à-vis de la MP au niveau du SNE reste délicat compte-tenu du faible nombre d'études réalisées. Si la présence d'inclusion de type CL et NL a été rapportée tant au sein du PM que du PSM (Braak *et al.* 2006; Kupsky *et al.* 1987 ; Qualman *et al.* 1984 ; Wakabayashi *et al.* 1988), les caractéristiques des atteintes restent mal connues (perte neuronale, sous-population neuronale affectée…).

En 1990, une étude a montré que les neurones présentant ces inclusions étaient VIPergiques (Wakabayashi *et al.* 1990). Ce résultat n'a cependant pas été confirmé depuis. Une seconde étude a montré dans le PM colique de 9 des 11 patients parkinsoniens une perte massive de DA mais pas de perte associée de neurones exprimant la TH, la perte de DA n'atteignant pas la significativité dans le PSM (Singaram *et al.* 1995). A notre connaissance, seules ces 2 études apportent un début de réponse concernant les modifications du phénotype neurochimique du SNE des patients parkinsoniens. La rareté de ces études est liée à la difficulté d'accès au tissu humain ; ces travaux ont en effet été réalisés sur des prélèvements autopsiques ou des pièces de résection chirurgicales.

La troisième partie de ce travail a visé, à l'aide de biopsies coliques prélevées de façon habituelle au cours d'une coloscopie à rechercher d'éventuelles altérations au niveau du PSM des patients parkinsoniens. De façon similaire à l'étude de Singaram *et al.* nous n'avons pas observé de modification du nombre de neurones TH. Cependant ces études ont été réalisées sur des plexus différents (myentériques dans l'étude de

Singaram *et al.* et sous muqueux dans notre étude). De plus, l'étude précédemment décrite sur le singe traité au MPTP a montré que les plexus pouvaient être altérés de façon différente. Ainsi il n'est pas possible d'extrapoler l'absence de modification du codage neurochimique du PSM vers le PM. Ces observations situent l'étendue de la méconnaissance des mécanismes et des conséquences de la MP au niveau du système digestif. Notre étude permet cependant d'envisager, au moyen de biopsies standard, l'évaluation d'une grande partie du PSM du tube digestif à l'aide d'un moyen peu invasif.

Réalisée sur un plus grand nombre de patients, l'analyse du PSM colique a permis de mettre en évidence une corrélation positive entre la densité des NL et la perte neuronale, et la densité de NL et les symptômes moteurs (score axial).

L'observation au niveau du SNE d'altérations au cours d'une maladie neurodégénérative, autrefois décrite comme principalement centrale, implique le fait que le SNE puisse à terme être utilisé comme une « fenêtre » sur le SNC. Il est envisageable d'utiliser cette technique d'exploration du SNE au cours d'autres maladies neurodégénératives si des biomarqueurs peuvent être identifiés.

Dans leur ensemble, les résultats des études réalisées sur l'Homme pointent un marqueur principal de la maladie au niveau du SNE : l'α-synucléine. Au regard de cette constatation les modèles animaux de la MP voient leur pertinence modifiée. Le MPTP, sauf dans de rares études (Kowall *et al.* 2000 ; McCormack *et al.* 2008), n'induit pas d'agrégation de l'α-synucléine. Il semble donc que ce modèle d'induction de la MP ne s'adapte que difficilement à cette théorie de synucléopathie. En revanche le modèle roténone s'accorde mieux à cette théorie. En effet, comme cela a été publié dans d'autres travaux (Drolet *et al.* 2009 ; Greene *et al.* 2009; Pan-Montojo *et al.* 2010), notre étude met en évidence une augmentation de l'expression de l'α-synucléine au niveau du SNE.

Cependant il n'a pas encore été établi de lien direct et formel entre la présence de CL ou la surexpression d'α-synucléine avec une dérégulation des fonctions du SNE.

CONCLUSION GENERALE ET PERSPECTIVES

Les résultats de nos travaux et les données de la littérature montrent que l'approche de la MP par des modèles animaux n'en est encore qu'à ses débuts en particulier en ce qui concerne le SNE. Les travaux de cette thèse ont permis de montrer que les modèles animaux de la MP développés pour étudier le SNC pouvaient aussi être utilisés pour caractériser le SNE. Chez le singe traité au MPTP on observe des altérations du codage neurochimique du SNE. Cependant le lien entre ces altérations et les troubles digestifs rencontrés chez les patients parkinsoniens est loin d'être établi. Nous avons également montré que la roténone induisait des altérations du transit digestif qui n'étaient pas associées à des modifications du codage neurochimique du SNE pouvant les expliquer. Même si l'on observe une surexpression d'α-synucléine, son rôle dans la physiologie digestive reste trop mal connu pour expliquer les altérations de ce modèle. Les études conduites chez l'Homme ont permis de préciser quelles devraient être les attentes envers les modèles animaux de la MP au niveau du SNE. Il apparaît dès lors important d'effectuer une caractérisation plus complète des atteintes du SNE chez l'Homme au cours de la MP, celle-ci ne pouvant cependant être effectuée que sur des prélèvements autopsiques ou des pièces de résections chirurgicales.

Le développement de traitements des symptômes digestifs de la MP nécessite le développement de modèles animaux même s'ils ne reproduisent pas l'ensemble des symptômes. Une récente étude réalisée dans un modèle génétique a montré qu'il était possible, en induisant une surexpression de l'α-synucléine humaine mutée, de reproduire une grande partie des troubles digestifs des patients parkinsoniens (Kuo *et al.* 2010). Cependant les modèles génétiques semblent ne pas représenter les meilleurs candidats pour induire une MP si l'on se réfère à leurs faibles similitudes avec la maladie

au niveau du SNC. Une nouvelle approche de l'étiologie de la maladie pourrait cependant permettre d'obtenir des modèles plus pertinents. En effet, la maladie de Parkinson semble d'origine multiple, les divers mécanismes potentiellement impliqués en sont le reflet. Il est envisageable qu'une combinaison entre facteur génétique et facteur toxique et/ou inflammatoire puisse en être la cause (Sulzer 2007). Quelques modèles combinatoires ont d'ores et déjà été développés (Gao *et al.* 2003 ; Gao *et al.* 2008), mais ils doivent encore être caractérisés au niveau du système digestif.

ANNEXES : AUTRES PUBLICATIONS REALISEES DURANT LE TRAVAIL DE THESE

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

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

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

INTRODUCTION

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

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MATERIAL AND METHODS

Patients

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

Colonoscopy and tissue collection

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

Obtention of whole-mount from colonic biopsies

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

Immunohistochemistry

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

Western Blot analysis

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

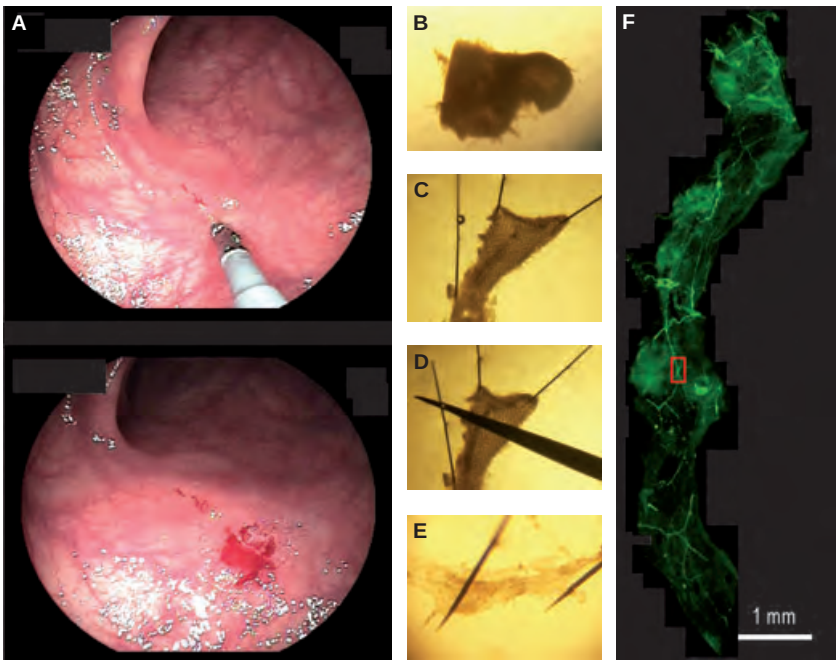

Figure 1 Obtention of whole-mount of the submucosal plexus (SMP) from colonic biopsies. (A) Biopsies are performed using standard biopsy forceps without needle. (B) Unstretched biopsies adopt a 'corn-flake' appearance in the Petri. (C) Biopsies are stretched and pinned flat under a stereomicroscope with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

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

RESULTS

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

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

DISCUSSION AND PERSPECTIVES

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

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

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

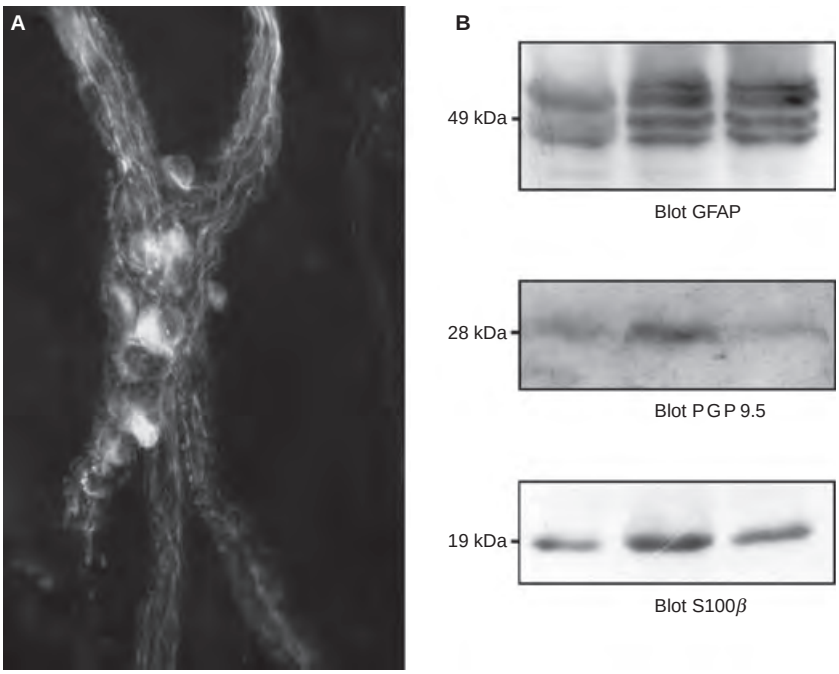

Figure 2 Analysis of the submucosal plexus (SMP) by immunohistochemistry and immunoblot. (A) Staining of a submucosal ganglia using NF 220 antibody allows a qualitative and quantative analysis of enteric neurons. (B) Four colonic biopsies for each patient were homogenized in NETF buffer. 50 μg of protein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

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

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

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

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REVIEW ARTICLE
The second brain and Parkinson's disease

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

Abstract

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

Introduction

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

The ENS is a second brain

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

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

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

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

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

The ENS is connected to the CNS

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

Afferent pathways

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

Efferent pathways

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

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

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

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

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

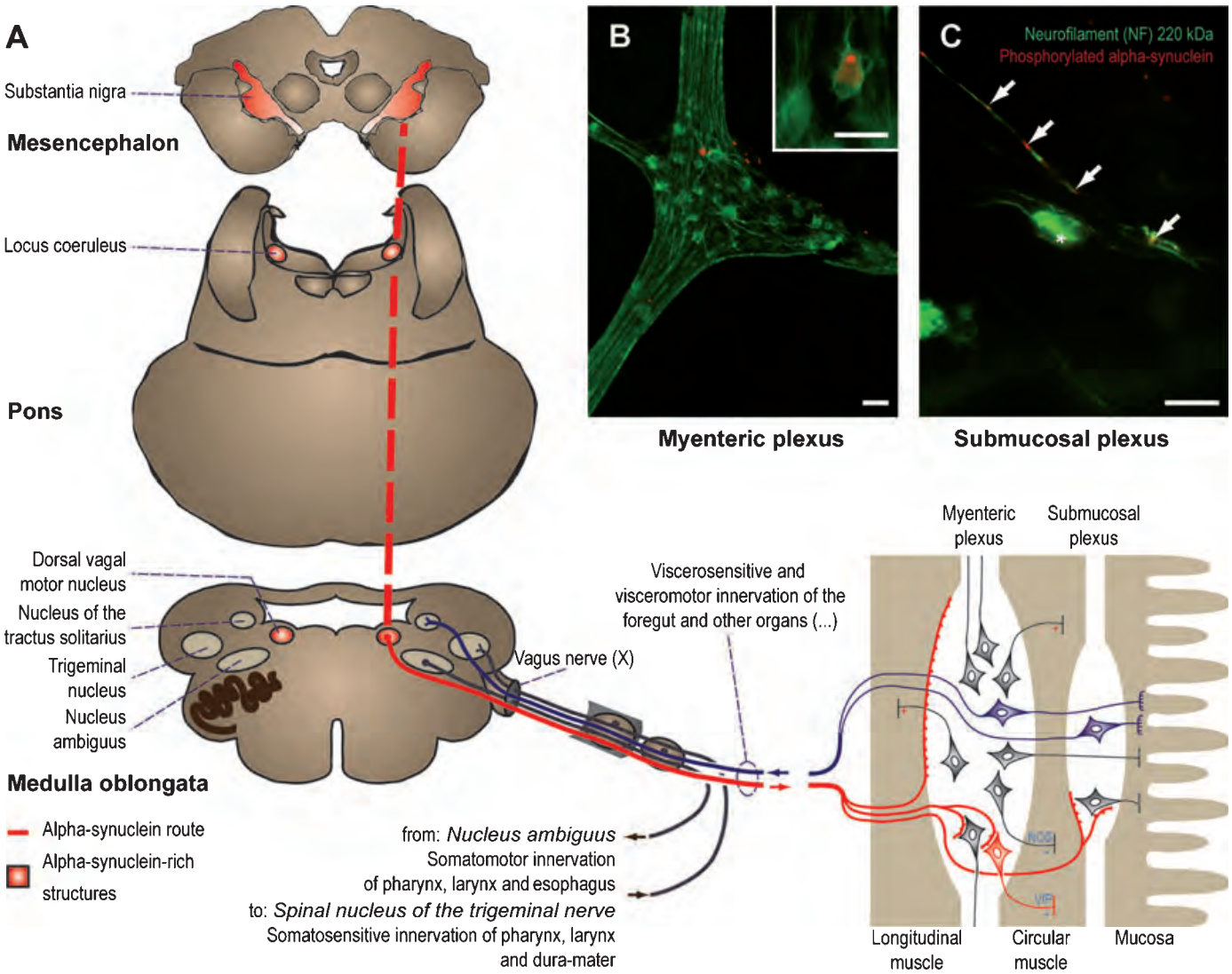

FIG. 1. (A) α-Synuclein pathway. A high endogenous content of α-synuclein seems to predispose the neural structures to the degenerative changes observed in Parkinson's disease (PD). Within the brainstem, the dorsal motor nucleus of the vagus nerve, locus coeruleus and substantia nigra (shaded red) are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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Conclusion

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

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Abbreviations

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

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Neuroplasticity and neuroprotection in enteric neurons: Role of epithelial cells

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ABSTRACT

Neurons of enteric nervous system (ENS) regulate intestinal epithelial cells (IEC) functions but whether IEC can impact upon the neurochemical coding and survival of enteric neurons remain unknown. Neuro-epithelial interactions were studied using a coculture model composed of IEC lines and primary culture of rat ENS or human neuroblastoma cells (SH-SY5Y). Neurochemical coding of enteric neurons was analysed by immunohistochemistry and quantitative PCR. Neuroprotective effects of IEC were tested by measuring neuron specific enolase (NSE) release or cell permeability to 7-amino-actinomycin D (7-AAD). Following coculture with IEC, the percentage of VIP-immunoreactive (IR) neurons but not NOS-IR and VIP mRNA expression were significantly increased. IEC significantly reduced dopamine-induced NSE release and 7-AAD permeability in culture of ENS and SH-SY5Y, respectively. Finally, we showed that NGF had neuroprotective effects but reduced VIP expression in enteric neurons. In conclusion, our study identified a novel role for IEC in the regulation of enteric neuronal properties.

Introduction

The enteric nervous system (ENS) is an integrative neuronal network organized along the gut which regulates gastrointestinal functions. Part of this regulation occurs via the liberation of mediators to the different target cells innervated by the enteric neurons such as intestinal epithelial cells (IEC). In particular, recent studies have shown that enteric neurons control intestinal epithelial barrier (IEB) function such as electrolyte secretion, barrier resistance and intestinal cells proliferation [1]. Conversely, enteric neurons are also under the control of its cellular microenvironment and in this context the IEB plays a crucial role. In particular, it is now well admitted that the IEB, and in particular enterochromaffin cells are able to transduce luminal signal to the enteric neurons via their secretion of serotonin and to activate enteric reflexes controlling intestinal peristaltism or mucosal secretion [2,3]. Also, mucosal application of short chain fatty acids or acidity induced the activation of myenteric neurons which depends on the presence of the mucosa [4]. Mechanical deformation of the mucosa is also able to activate myenteric neurons [5]. Further, supporting this regulation of neuronal excitability by the mucosa is the observation that *ex vivo* mucosal removal leads to a reduced neuronal excitability [6]. Besides these short term effects, emerging evidence suggest that the IEB can also impact over long term upon neuronal functions. It has been shown that erbB2 signalling in the colonic epithelia is required for the postnatal survival of enteric neurons [7]. In addition, Caco-2 IEC lines have been shown to favour neurite outgrowth in PC12 cells [8]. However, whether IEC can exert neuroprotective effects is currently unknown. In addition, a recent study has shown that IEC can impact on the ENS by regulating neuronal synthesis of chemokines. In particular, under basal condition, IEC reduced neuronal mRNA expression of IL-8 and MIP-1β in neuronal cell lines [9]. Following stimulation of the IEC by TNF-α/IFN-γ, IEC directly increased neuronal production or mRNA expression of cytokines [9]. However, besides regulation of neuronal production of chemokines, it remains currently unknown whether IEC can also regulate the neuronal expression of neuromediators. Therefore, using coculture of IEC lines and primary culture of rat ENS or neuroblastoma cell lines, we aimed at characterizing whether IEC can modulate neuromediators expression and exert neuroprotective effects.

Materials and methods

Cell culture. Primary cultures of rat ENS were obtained as previously described [10]. Human neuronal-like SH-SY5Y cells were

Abbreviations: ENS, enteric nervous system; IEB, intestinal epithelial barrier; IEC, intestinal epithelial cell; NGF, nerve growth factor; nNOS, neuronal nitric oxyde synthase; NSE, neuron specific enolase; VIP, vasoactive intestinal peptide.

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cultured as previously described [11]. Caco-2, T84, IEC-6 (ATCC) were seeded in 12-well Transwell® filters (Corning, NY, USA) at a density of 2×10^5 cells/insert and cultured for 13 days to obtain confluence as previously described [12]. All cells were maintained in 95% air, 5% CO_2 at 37 °C.

Table 1
List of primary antibodies.

Raised against	Host species	Source or reference	Dilution
Hu proteins (HuC/D)	Mouse	Molecular Probes, OR, USA	1:200
VIP	Mouse	US biological, MA, USA	1:800
nNOS	Rabbit	Alexis Biochemicals, CA, USA	1:2000
P75	Rabbit	MC192 (gift from Dr. E.G. Johnson) [13]	1:3500
trkA	Rabbit	Kindly provided by Dr. L. Reichard [14]	1:5000
Active caspase-3	Rabbit	Sigma, Saint-Quentin Fallavier, FR	1:500

Table 2
List of secondary antibodies.

Antibody	Source or reference	Dilution
FluoroProbes488 donkey anti-mouse	Interchim, Montluçon, FR	1:200
Cy3-conjugated donkey anti-mouse	Jackson Immunoresearch, BA, USA	1:500
Cy5-conjugated goat anti-rabbit	Jackson Immunoresearch, BA, USA	1:500
Cy3-conjugated donkey anti-rabbit	Jackson Immunoresearch, BA, USA	1:500

Neuro-epithelial coculture model. Forty-eight hours prior to coculture with primary ENS or SH-SY5Y, IEC media were replaced by primary ENS or SH-SY5Y media, respectively. Coculture were performed by maintaining primary ENS or SH-SY5Y cells in presence or absence of IEC for 3 days (neurochemical coding experiments) or 1 day (neuroprotection experiments).

Oxidative stress. Oxidative stress was induced during 24 h with dopamine. Preliminary experiments show that neurotoxic effect in SH-SY5Y cells reaching a maximum at a concentration of dopamine of 1.2 mM (data not shown). This dose was chosen for the rest of the study.

Immunohistochemical analysis. At the end of the experiments, primary culture ENS was processed for immunohistochemical studies as previously described [10]. Primary culture of ENS was incubated in primary antibodies (Table 1) for 90 min. Following 3×10 min washes with PBS, they were incubated for 30 min with secondary antibodies (Table 2). In a second step, all neurons were stained with antibodies against the neuronal marker HuC/D. The number of VIP-, nNOS-, active caspase-3- and Hu-IR cells was counted in at least 20 ganglia per condition. The data were expressed as a percentage normalized to the total Hu-IR neurons.

Quantitative PCR analysis. Total RNA isolation and PCR were performed based on previously described methods [10]. The following primers were used: S6 forward: 5′-CCA AGC TTA TTC AGC GTC TTG TTA ACT CC-3′, S6 reverse: 5′-CCC TCG AGT CCT TCA TTC TC TTG

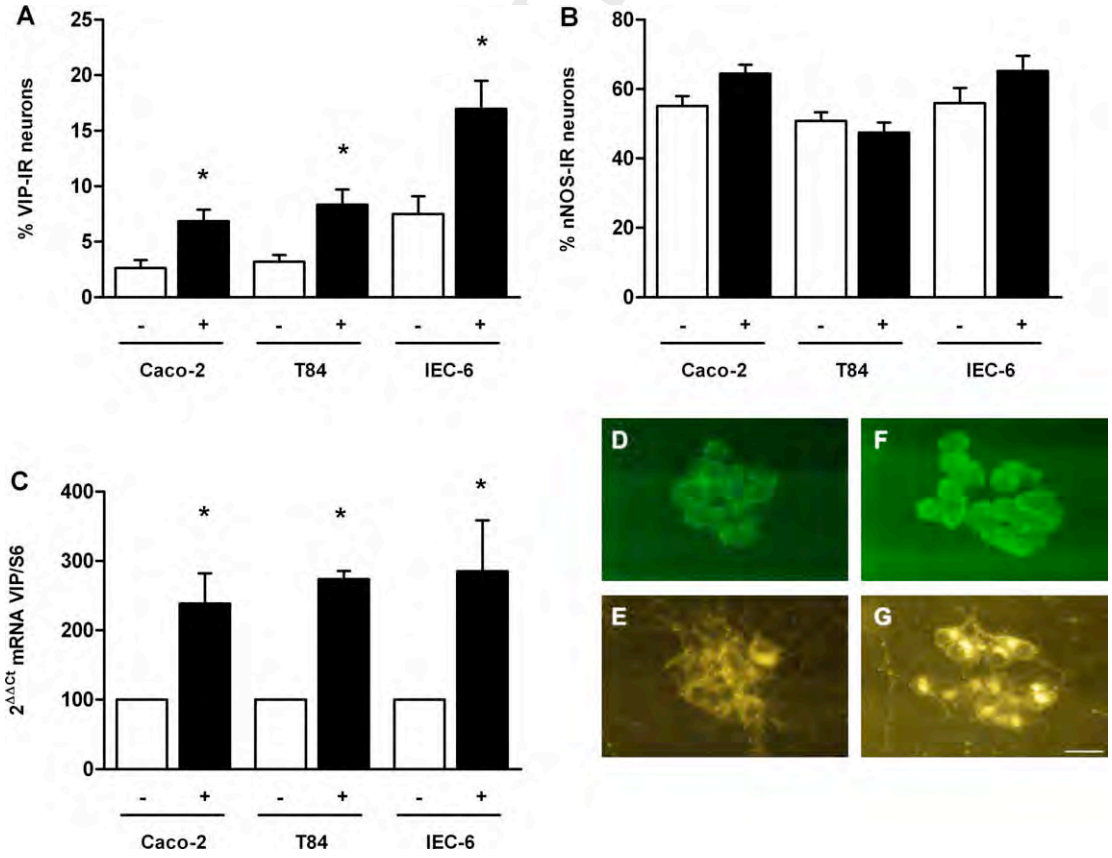

Fig. 1. Intestinal epithelial cells (IEC) modulate the neurochemical coding of the ENS. Primary cultures of ENS were cultured with or without IEC (Caco-2, T84 or IEC-6 cells) for 72 h. Culturing IEC with ENS induced a significant increase in the proportion of VIP-immunoreactive (IR) (A) but not nNOS-IR neurons (B) ($n = 6$, paired t test, $^*p < 0.05$ compared to control). VIP mRNA levels in ENS cultured with IEC were significantly larger than in primary ENS alone (C) ($n = 6$, paired t test, $^*p < 0.05$ compared to control). Values represent means ± SEM. Double immunohistochemical labelling of primary ENS cocultured with or without IEC. Images showed that a larger number of neurons (identified with Hu) were VIP-IR in presence of IEC (F,G, respectively) as compared to control (D,E, respectively). Scale bar = 20 μm.

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GC-3′, VIP forward: 5′-TTG GCA AAC GAA TCA GCA GTA G-3′, VIP reverse: 5′-ATT TGC TTT CTA AGG CGG GTG TA-3′. VIP mRNA expression was quantified using the 2-[Delta]-[Delta] CT method [13] with S6 as the internal control. Data are representative of six experiments performed in duplicate.

Assessment of cell viability. At the end of experiment, SH-SY5Y cells were collected both in the supernatant and on the bottom of the wells following treatment with trypsin–EDTA. Membrane permeability was evaluated by flow cytometry (BD FACSarray (Le pont de Claix, France) following incubation of cell suspension with 7-amino-actinomycin D (7-AAD). Results were expressed as percentage of total cells.

Detection of NSE. Neuron specific enolase (NSE) released in the culture medium was quantified by immunoradiometric assay (Prolifigen® NSE IRMA, Diasorin; Stillwater, USA). Results are expressed in ng/mL.

Chemicals. NGF (100 ng/ml; Sigma) was added in the culture medium for 72 or 24 h.

Statistical analysis. Analyses were done with GraphPad Prism 4.0c (GraphPad, San Diego, CA, USA). All data were expressed as means ± SEM. Conditions were compared using a one-way analysis of variance for repeated measures for multiple comparisons, or using a paired Student's *t* test. Difference were considered significant when $p < 0.05$.

Results

Intestinal epithelial cells modulate the neurochemical phenotype of the ENS

Coculturing Caco-2 with primary ENS induced a significant +161% increase in the proportion of VIP-IR neurons as compared to control (*i.e.*, ENS cultured alone; Fig. 1A, $n = 6$, $p < 0.05$). In contrast, the proportion of nNOS-IR neurons was not affected by the coculture (Fig. 1B). Furthermore, coculture of ENS with Caco-2 did not modify neuronal viability as (1) the number of neurons per ganglia was identical in both conditions (19.6 ± 0.7 *vs.* 19.5 ± 0.5 Hu-IR neurons/ganglia, $n = 6$) and (2) NSE levels in the culture supernatant was not modified by the culture with IEC compared to control (1.0 ± 0.2 *vs.* 0.6 ± 0.1 ng/mL, respectively; Fig. 2A, $n = 5$).

To determine whether other IEC lines can modify the phenotype of the ENS, we characterized the effect of ENS coculture with other transformed (T84) and non-transformed (IEC-6) IEC. Under these conditions, both IEC lines reproduced the effects of Caco-2 onto the proportion of VIP- and NOS-IR neurons (Fig. 1A and B). We next sought to determine whether the increase in the proportion of VIP-IR neurons was associated with an increase in VIP mRNA expression. Indeed, coculture of ENS with all IEC lines used induced a significant increase in VIP mRNA expression (Fig. 1C, $p < 0.05$).

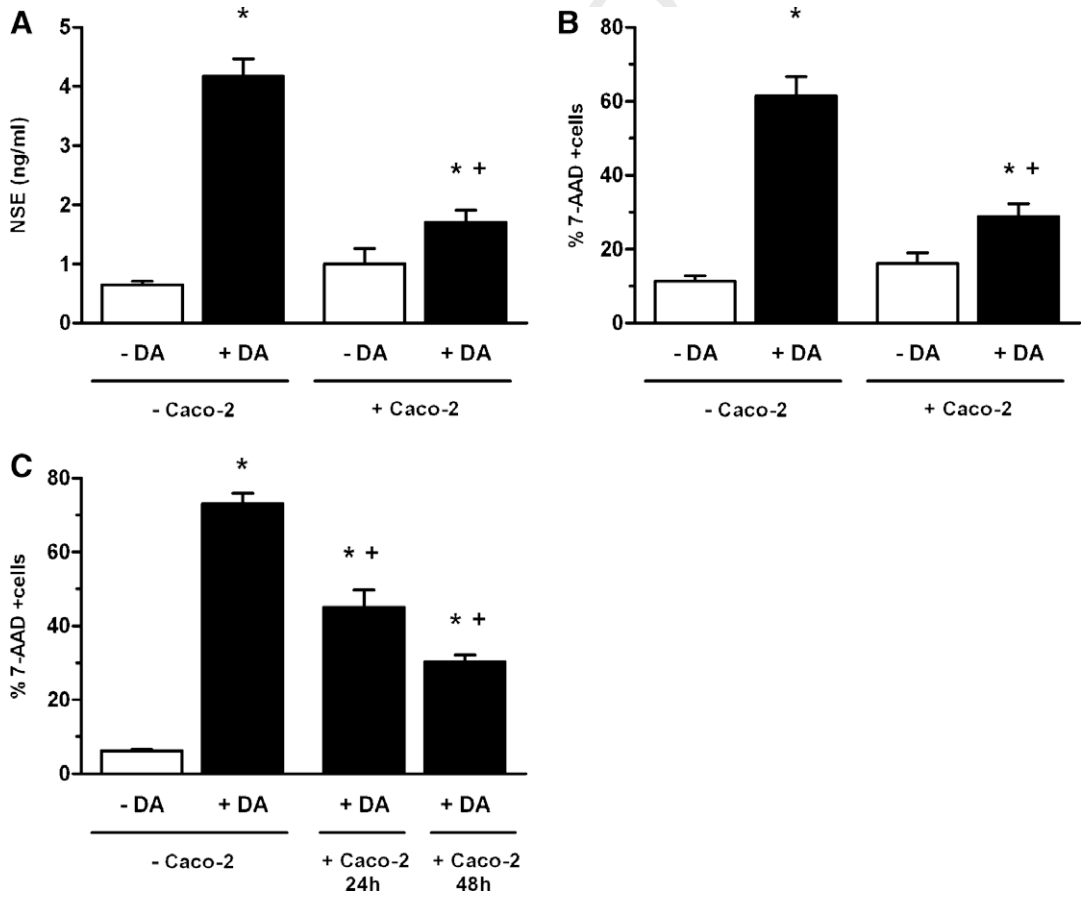

Fig. 2. Intestinal epithelial cells (IEC) prevent neuronal cell death induced by dopamine in primary cultures of ENS and in neuroblastoma cell lines. Treatment of primary culture of ENS with dopamine (1.2 mM) for 24 h induced a significant increase in extracellular level of NSE as compared to control (without dopamine). In presence of Caco-2, dopamine-induced NSE release was significantly reduced as compared to ENS alone treated with dopamine (A) ($n = 5$; $^{*}p < 0.001$ compared to control; $^{+}p < 0.05$ compared to ENS treated with dopamine alone; one-way ANOVA). Treatment of SH-SY5Y cells with dopamine (1.2 mM) for 24 h induced a significant increase in 7-amino-actinomycin D (7-AAD) permeability as compared to control (without dopamine). In presence of Caco-2, dopamine-induced 7-AAD permeability was significantly reduced as compared to SH-SY5Y cultured alone and treated with dopamine (B) ($n = 7$; $^{*}p < 0.001$ compared to control; $^{+}p < 0.05$ compared to SH-SY5Y cells treated with dopamine alone; one-way ANOVA). Culturing SH-SY5Y with Caco-2 prior to dopamine treatment (but not during oxidative stress) was sufficient to significantly reduce dopamine-induced increase in cell permeability as compared to SH-SY5Y cultured alone and treated with dopamine. Increasing the duration of coculture prior to oxidative stress increased the protective effect of IEB. ($n = 4$, $^{*}p < 0.001$ compared to control, $^{+}p<0.05$ compared to SH-SY5Y cells treated with dopamine alone; one-way ANOVA). Values represent means ± SEM.

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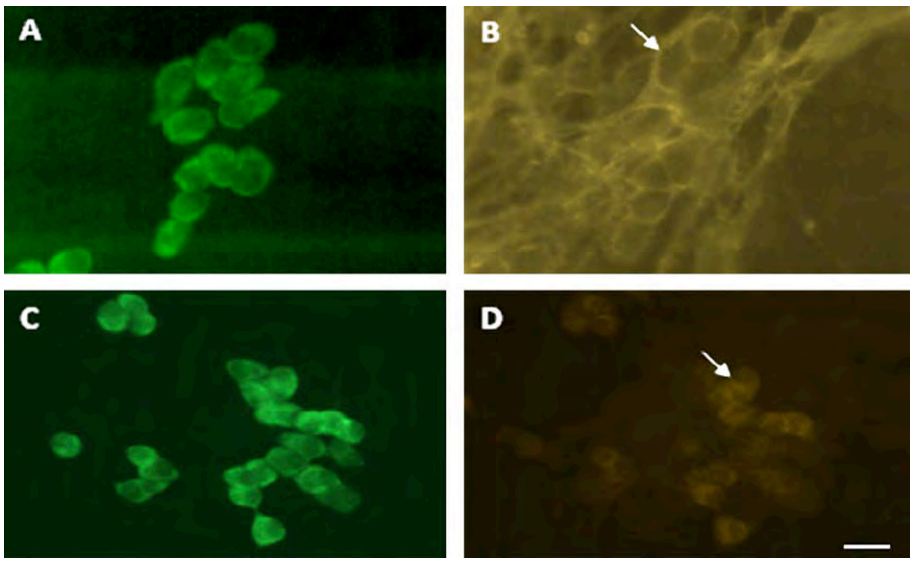

Fig. 3. Primary cultures of ENS express the receptors of NGF P75 end trkA. Double immunofluorescence staining of primary cultures of ENS with anti-Hu, anti-p75 or anti-trkA antibodies. Double immunohistochemical staining revealed enteric neurons (identified with Hu; A) with p75-immunoreactivity which was found solely on the plasma membrane of neurons (arrow, B). In addition, double immunohistochemical staining revealed enteric neurons (identified with Hu; C) with cytoplasmic trkA-immunoreactivity (arrow, D). Scale bar = 20 μm.

Intestinal epithelial cells protect enteric neurons during oxidative stress

Addition of dopamine to primary ENS induced a significant release of NSE in the culture medium as compared to control (4.2 ± 0.3 *vs.* 0.6 ± 0.1 ng/mL, respectively; Fig. 2A, $n = 5$, $p < 0.001$). In contrast, coculture with Caco-2 for 24 h prior the oxidative stress prevented the effects of dopamine upon the release of NSE (1.7 ± 0.2 *vs.* 4.2 ± 0.3 ng/mL, respectively; Fig. 2A, $n = 5$, $p < 0.001$). The direct neuroprotective effect of IEC was then evaluated using the neuroblastoma cell line SH-SY5Y. Dopamine induced a significant increase in cell permeability in SH-SY5Y compared to SH-SY5Y alone (control) (61.4 ± 5.2% *vs.* 11.4 ± 1.4% 7-AAD positive cells/total cells, respectively; Fig. 2B, $n = 7$, $p < 0.001$). This dopamine-induced increase in cell permeability was prevented when SH-SY5Y were cocultured with Caco-2 monolayer for 24 h prior oxidative stress as compared to control (28.9 ± 3.5% *vs.* 61.4 ± 5.2% 7-AAD positive cells/total cells, respectively; Fig. 2B, $n = 7$, $p < 0.001$). Such neuroprotective effects of Caco-2 did not require their presence during oxidative stress. Indeed, we still observed a neuroprotective effect of IEC upon SH-SY5Y if Caco-2 were cocultured with SH-SY5Y only 24 h prior the addition of dopamine but were not present during oxidative stress as compared to control (45.0 ± 4.8% *vs.* 73.0 ± 2.8% of 7-AAD positive cells/total cells, respectively; Fig. 2C, $n = 4$, $p < 0.001$). These preventive neuroprotective effects of Caco-2 were more effective if IEC were cocultured with SH-SY5Y for 48 h prior oxidative stress compared to 24 h time (30.3 ± 1.8% *vs.* 45.0 ± 4.8% of 7-AAD positive cells/total cells, respectively; Fig. 2C, $n = 4$, $p < 0.05$).

The effects of IEC upon neuronal functions are mediated in part by NGF

We finally sought to identify putative mediators secreted by IEC involved in their effects upon the ENS. We tested the effects of NGF which has been previously shown to be secreted by IEC [14,15]. We first confirmed that IEC also synthesize NGF in our conditions (data not shown). We next showed that both low-affinity receptor p75 and high-affinity trkA receptor for NGF were expressed in enteric neurons of primary ENS (Fig. 3). Concerning the role of this neurotrophin in the regulation of neurochemical coding, addition of NGF to primary ENS reduced significantly the proportion of VIP-IR neurons compared to control (−66%, Fig. 4A, $n = 6$, $p < 0.01$) without modifying the one of nNOS (Fig. 4A; $n = 6$). The number of neurons per ganglion was not modified by the addition of NGF (data

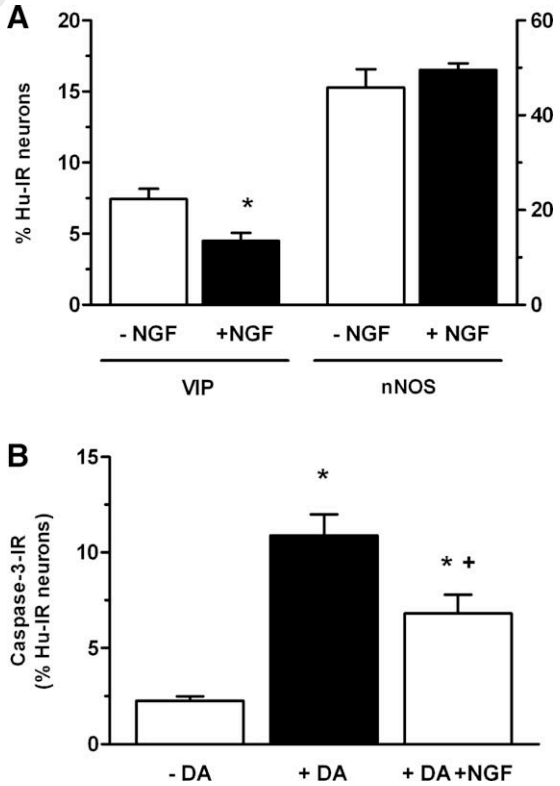

Fig. 4. Intestinal epithelial cells (IEC) effects upon the ENS are mediated in part by NGF. Primary cultures of ENS were treated or not with NGF 100 ng/ml for 72 h. The percentage of VIP-immunoreactive (IR) but not NOS-IR in the ENS was significantly decreased by NGF. (A) ($n = 6$; $^{*}p < 0.05$ compared to control; one-way ANOVA). ENS was treated with or without dopamine in absence or in presence of NGF. The proportion of caspase-3-IR neurons induced by dopamine was significantly reduced in presence of NGF as compared to ENS alone treated with dopamine. (B) ($n = 5$, $^{*}p < 0.001$ compared to control; $^{+}p < 0.05$ compared to primary ENS treated with dopamine alone; one-way ANOVA). Values represent means ± SEM.

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not shown). Concerning its neuroprotective role, we showed that preincubation of NGF with primary ENS significantly reduced the dopamine-induced neurotoxicity (−60% of caspase-3 positive neurons; Fig. 4C, $n = 5$, $p < 0.05$).

Discussion

This study identified a novel role of IEC in the control of neuronal functions. In particular, we showed that IEC can modulate neurochemical phenotype and exhibit neuroprotective effects. We also identified NGF as being as one of the mediators involved in the control of ENS properties by the IEB.

Emerging evidences suggest that the IEB can regulate the properties of enteric neurons and in particular its excitability, in response to luminal and mechanical stimulation of the mucosa. This regulation occurs over short period of time, *i.e.*, milliseconds to seconds. However, much less is known about the long-term regulation of ENS properties by the IEB. Our coculture model based on the use of primary culture of ENS allows extensive physiological studies of the role of the IEB upon various ENS properties. Although being based on the use of primary culture of rat ENS, we showed that rat IEC lines could reproduce the effects of human IEC lines in this model.

Using this coculture model, we first demonstrated that the IEB specifically modified the neurochemical coding of enteric neurons, by increasing the proportion of VIP-IR neurons and VIP mRNA expression without modifying nNOS-IR. This effect was not associated with an increased cell proliferation as the total number of neurons per ganglia remained unchanged in presence of IEC as compared to control. The exact mechanisms involved in this crosstalk between IEC and enteric neurons have not been identified in this work. Previous studies have shown that neuronal activity increased the proportion of VIP-IR neurons in our primary ENS culture model [10]. Whether such induction is regulated by neuronal activity is unknown, although removal of mucosa has been shown to reduces neuronal activity in the ENS [6]. Such impact of IEC upon the neurochemical coding of the ENS has never been reported and its signification remains to be determined. However, it is tempting to speculate that such interactions could be in part responsible for the increased proportion of VIP-IR neurons observed in the submucosal plexus (which are in close proximity to IEB) as compared to the one in the myenteric plexus (which are more distant from the IEB). [16,17] Although our study did not identify the function of these VIPergic neurons, previous study showed that submucosal VIPergic neurons regulate several functions of the intestinal epithelial barrier, such as electrolytes and mucus secretion, permeability and proliferation [1]. Besides regulating the neurochemical coding, our study also revealed that IEB could have neuroprotective effects upon the enteric neurons. To our knowledge this is the first report of neuroprotective functions directly attributed to IEC. A previous study has already hinted to such a role for IEC by demonstrating that erbB2 (receptor of neuregulins) signalling in colonic epithelial cells was necessary for ENS postnatal survival [7]. In this study, we extend this observation by demonstrating that IEC via the liberation of soluble factor have direct neuroprotective effects. Neuroprotective effects of IEC are probably due in part to the induction in neurons of an increased resistance to oxidative stress, as preincubation alone of ENS with IEC-conditioned medium prior to oxidative stress was sufficient to induce neuroprotection. Although currently unknown, the neuroprotective mechanisms of IEC could be due to the regulation of survival/death factors (Bcl-2; Bcl-xl; Bax...) or neuroprotective substances (glutathion,...) in enteric neurons.

Finally, a major result obtained in this study is that we identified NGF as being a likely mediator involved in the effects of IEC upon ENS properties. We first showed that NGF is expressed by IEC as previously reported by other studies [14,15]. We then showed that NGF receptors (p75 and trkA) were present in the neurons of primary ENS culture. These results are consistent with data showing a wide distribution and often coexpression of trkA and p75 receptors in myenteric neurons [18]. However, in their studies the functional consequence of the activation of these receptors remained unknown. In view of our study we can hypothesize that NGF secretion by IEC under physiological condition (as in our model) could have neuroprotective effects, via the activation of high-affinity trkA receptor. However, under pathological conditions, such as inflammatory bowel disease, large amounts of NGF released by IEC [19] could activate p75 receptors and induce enteric neuronal cell death [20]. Besides regulating cell survival, our study also suggests that NGF regulates the neurochemical phenotype of enteric neurons. Indeed, we showed that NGF reduced the proportion of VIP-IR enteric neurons. This observation is in agreement with data showing an increase in VIP-IR in the myenteric plexus of ileum from rats treated with NGF antiserum compared with controls [21]. Similarly, in rats injected with an antiserum against NGF, increased levels of VIP protein and mRNA expression have been observed in sensory neurons in lumbar dorsal root ganglia [22]. It remains currently unknown which mediator is responsible for the increase in the proportion of VIP-IR neurons. The regulation by NGF of increased neuronal survival and reduced VIP expression can also be viewed from an integrated view to limit functional redundancy. Indeed, as VIP has neuroprotective effect in the ENS [23,24] downregulation of its expression would by NGF which has the same properties have no impact on the survival of the neurons under physiological conditions.

In conclusion, this study extends the role of IEB in the control of neuronal functions (neurochemical content, survival) and also suggests that alterations in IEB phenotype, such as those observed during infectious or inflammatory insults, could impact upon ENS phenotype. This study also suggests that targeting the IEB could be of interest in the prevention/treatment of enteric neuropathies.

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D. G. Munoz and J. G. de Yebenes (2004). "The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia." Ann Neurol **55**(2): 164-73.

Etude du système nerveux entérique au cours de la maladie de Parkinson : contribution des modèles animaux.

Résumé :

La maladie de Parkinson (MP) est une maladie neurodégénérative multicentrique caractérisée par des symptômes moteurs mais aussi non-moteurs parmi lesquels les troubles digestifs sont les plus fréquents. Les fonctions digestives sont principalement régulées par le système nerveux entérique (SNE), qui semble anatomiquement et cliniquement affecté de façon précoce par la maladie. Néanmoins, les mécanismes et conséquences de la MP sur le SNE sont encore mal connus. Les objectifs de ce travail de thèse visaient à 1) caractériser les lésions du SNE et leur impact fonctionnel dans 2 modèles animaux toxiques et 2) à caractériser les lésions du plexus sous-muqueux chez les patients parkinsonien. Dans un modèle d'intoxication chronique de primate au 1-méthyl-4-phényl-1,2,3,6-tétrahydropyridine, nous avons montré une plasticité du SNE caractérisée par un augmentation du nombre de neurones et en particulier des neurones nitrergiques dans le plexus myentérique mais pas dans le plexus sous-muqueux. De plus une perte de neurones dopaminergiques a été montrée au sein de ces 2 plexus. Dans le modèle murin d'intoxication par la roténone nous n'avons pas mis en évidence les particularités précédemment décrites. En revanche, nous avons montré un transit total et une fréquence d'exonération diminués ; ainsi qu'une augmentation de l'expression d'α-synucléine. Enfin dans une étude pilote, suivie d'une étude plus complète, nous avons mis en évidence la présence d'α-synucléine phosphorylée chez 70% des patients parkinsoniens. Ce travail montre l'implication du SNE dans la physiopathologie de la MP et suggère un rôle majeur de l'α-synucléine dans le développement des symptômes digestifs.

Mots clés : système nerveux entérique, maladie de Parkinson, α-synucléine, corps de Lewy

The enteric nervous system during Parkinson's disease: insights from animal models.

Abstract:

Parkinson's disease (PD) is a multicentric neurodegenerative pathology characterized by motor and non-motor symptoms among which digestive troubles are the most frequent. Digestive functions are mainly ruled by the enteric nervous system (ENS) which seems to be primarily affected during the course of the disease. However consequences and precise action mechanism of PD on the ENS aren't fully known. The aims of the study were 1) to characterize ENS lesions and their functional consequences in two toxic animal models and 2) to characterize sub-mucosal alterations in living patients. In a chronic monkey model of intoxication with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine we have shown a plasticity of the ENS characterized by an increased number of neurons and in particular nitrergic neurons in the myenteric but not in the sub-mucosal plexus. Moreover we showed a decreased number of dopaminergic neurons in both plexus. In the rotenone-treated mouse model we didn't showed neurochemical changes. However we showed a slowed total transit time, a decreased stool frequency and an increased α-synuclein expression. Finally in a pilot, followed by a more complete study, we showed phophorylated α-synuclein in 70% of the parkinsonian patients. Our data show the implication of the ENS in the physiopathology of PD and suggest a major role for α-synuclein in digestive troubles development.

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