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Recherche des facteurs génétiques à l'origine de la maladie de Parkinson dans la population canadienne-française du Québec

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Recherche des facteurs génétiques à l'origine de la maladie de Parkinson dans la population canadienne-française du Québec

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Résumé

La maladie de Parkinson (MP) est une affection neurodégénérative invalidante et incurable. Il est maintenant clairement établi que d'importants déterminants génétiques prédisposent à son apparition. La recherche génétique sur des formes familiales de la MP a mené à la découverte d'un minimum de six gènes causatifs (*SNCA*, *LRRK2*, *Parkin*, *PINK1*, *DJ-1* and *GBA*) et certains, par exemple *LRRK2*, contiennent des variations génétiques qui prédisposent également aux formes sporadiques. La caractérisation des protéines codées par ces gènes a mené à une meilleure compréhension des mécanismes moléculaires sous-jacents. Toutefois, en dépit de ces efforts, les causes menant à l'apparition de la MP restent inconnues pour la majorité des patients.

L'objectif général des présents travaux était d'identifier des mutations prédisposant à la MP dans la population canadienne-française du Québec à partir d'une cohorte composée principalement de patients sporadiques. Le premier volet de ce projet consistait à déterminer la présence de mutations de *LRRK2* dans notre cohorte en séquençant directement les exons contenant la majorité des mutations pathogéniques et en effectuant une étude d'association. Nous n'avons identifié aucune mutation et l'étude d'association s'est avérée négative, suggérant ainsi que *LRRK2* n'est pas une cause significative de la MP dans la population canadienne-française.

La deuxième partie du projet avait pour objectif d'identifier de nouveaux gènes causatifs en séquençant directement des gènes candidats choisis à cause de leurs implications dans différents mécanismes moléculaires sous-tendant la MP. Notre hypothèse de recherche était basée sur l'idée que la MP est principalement due à des mutations individuellement rares dans un grand nombre de gènes différents. Nous avons identifié des mutations rares dans les gènes *PICK1* et *MFN1*. Le premier code pour une protéine impliquée dans la régulation de la transmission du glutamate tandis que le second est un des acteurs-clés du processus de fusion mitochondriale.

Nos résultats, qui devront être répliqués, suggèrent que le séquençage à grande échelle pourrait être une méthode prometteuse d'élucidation des facteurs de prédisposition génétiques à la MP ; ils soulignent l'intérêt d'utiliser une population fondatrice comme les canadiens-français pour ce type d'étude et devraient permettre d'approfondir les connaissances sur la pathogénèse moléculaire de la MP.

Mots-clés : maladie de Parkinson, génétique, canadiens-français, séquençage de gènes candidats, formes sporadiques, mutations rares, *LRRK2*, *PICK1*, *MFN1*.

Abstract

Parkinson's disease (PD) is a complex neurological disorder with significant genetic predisposing factors which are extremely heterogeneous. Investigations of familial forms of the disorder revealed causative mutations in six different genes, namely *SNCA*, *LRRK2*, *Parkin*, *PINK1*, *DJ-1* and *GBA*, and functional analyses of these gene products pinpointed dysfunction of key molecular pathways involved in the neurodegenerative process of the disorder. Further sequencing and genome-wide association studies indicated that some of these genes, including *LRRK2*, also contain variants predisposing to sporadic forms of PD. Despite these significant breakthroughs, the vast majority of PD genetic predisposing factors remain unknown.

Our goal was to identify mutations predisposing to PD in the French Canadian (FC) population from a cohort mostly composed of late-onset sporadic cases. We therefore sequenced the two exons of *LRRK2* that contain most of the pathogenic mutations and we performed a case-control association study. Sequencing analysis did not reveal any reported or novel mutations and the case-control association study did not provide any positive signal, thus indicating that common variants in *LRRK2* are unlikely to be a significant cause of late-onset PD in the FC population.

Because of the allelic and non-allelic genetic heterogeneity observed for PD, we hypothesized that dozens of genes may carry rare pathogenic mutations. The second part of this research project was therefore aimed at identifying new PD causative genes by direct sequencing of genes functionally associated with the known causative gene pathways. Our screening uncovered several rare mutations likely pathogenic in the *PICK1* and the *MFN1* genes. PICK1 is involved in internalization of AMPA receptors whereas MFN1 is one of the core components of the mitochondrial fusion/fission machinery.

Although these observations will need to be replicated, our findings support the previously suspected pathogenic role for glutamate excitotoxicity and imbalanced mitochondrial dynamics in Parkinson's disease. They further emphasize the value of inbred populations in genetic studies of PD and provide new clues to the molecular pathogenesis of the disorder.

Keywords: Parkinson's disease, genetics, French-Canadian, candidate gene sequencing, sporadic forms, rare mutations, *LRRK2*, *PICK1*, *MFN1*.

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Acides aminés

A	Alanine
C	Cystéine
D	Aspartate
E	Glutamate
F	Phénylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Méthionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Sérine
T	Thréonine
V	Valine
W	Tryptophane
Y	Tyrosine

18F-dopa	Fluorine-18-L-dihydroxyphenylalanine
AD	Autosomique dominant
AMPA	α-amino-3-hydroxy-5-méthylisoazol-4-propionate
ap. J.-C.	Après Jésus-Christ

AR	Autosomique récessif
Arp2/3	Actin-related proteins 2 and 3
ATP13A2	ATPase type 13A2
av. J.-C.	Avant Jésus-Christ
Bcl-2	B-cell CLL/lymphoma 2
BAG5	BCL2-associated athanogene 5
BAR	Bin1/amphiphysin/Rvs167
CDK5	Cyclin-dependent kinase 5
CF	Canadien-français
DJ-1	Oncogene DJ1
DNM1L	Dynamin 1-like
Drp1	Dynamin-related Protein 1
EPS15	Epidermal growth factor receptor pathway substrate 15
FBXO7	F-box only protein 7
FC	French-Canadian
GBA	Glucocerebrosidase
GIGYF2	Grb10-Interacting GYF Protein-2
HGMD	Human Gene Mutation Database
HTRA2	HtrA serine peptidase 2
L-dopa	Levodopa
LRRK2	Leucine-rich repeat kinase 2
MAO-B	Monoamine oxydase B
MAPT	Microtubule-associated protein tau
MFN1	Mitofusin 1
MFN2	Mitofusin 2
MP	Maladie de Parkinson
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

NMDA	N-methyl-D-aspartate
PD	Parkinson’s disease
PDZ	PSD-95/Discs-large/ZO-1
PICK1	Protein interacting with PRKCA1
PINK1	PTEN induced putative kinase 1
PKCalpha	Protéine kinase C alpha
RNF11	Ring finger protein 11
SN	Substance noire
SNpc	Substance noire *pars compacta*
SNCA	Synuclein alpha
SNP	Polymorphisme d’un seul nucléotide
SPR	Sepiapterin reductase
TEP	Tomographie par émission de positons
UBE2L3	Ubiquitin-conjugating enzyme E2L 3 (UbcH7)
UBE2L6	Ubiquitin-conjugating enzyme E2L 6 (UbcH8)
UCHL1	Ubiquitin carboxyl-terminal esterase L1
UPDRS	Unified Parkinson's Disease Rating Scale
US	United States

À mes deux anges, Judith et Romy

« Le temps n'est pas un problème, c'est l'organisation du temps qui en est un. »

André Barbeau

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Chapitre 1 : Introduction

1.1 Manifestations cliniques, diagnostic et traitements

1.1.1 Manifestations cliniques

Au début du XIXème siècle, le médecin anglais James Parkinson fit la première description clinique de la maladie de Parkinson (MP) dans l'ouvrage *An Essay on the Shaking Palsy (1)*. Quelques décennies plus tard, le neurologue Jean-Martin Charcot proposa que l'on remplace le terme « paralysie agitante » par « maladie de Parkinson ».

Les trois symptômes initiaux les plus fréquents ou « triade parkinsonienne » sont un tremblement de repos souvent unilatéral des mains ou des jambes, une bradykinésie et une akinésie (ralentissement des mouvements et difficulté à initier des mouvements), et une rigidité du corps (hypertonie). D'autres symptômes inauguraux comme une micrographie, des troubles de la marche ou une posture fléchie peuvent également être observés *(2)*. Une perte de l'odorat *(3)* ou un trouble du comportement en sommeil paradoxal idiopathique *(4)* sont des signes précliniques souvent méconnus. Ils présentent néanmoins un grand intérêt à cause de leur tendance à apparaître plusieurs années avant les troubles moteurs. Au fil des ans, des complications motrices apparaissent, l'état général des patients se détériore et le handicap moteur s'accentue. À ce tableau clinique peuvent s'ajouter des troubles dysautonomiques, des troubles sensitifs, des troubles du sommeil, une grande fatigue, une démence, des troubles du comportement, de la dépression et des troubles de la parole *(5)*. L'espérance de vie moyenne des patients à partir du diagnostic se situe autour de 15 ans *(6)*. Ceux-ci présentent un risque de décès deux à trois fois plus élevé que les contrôles du même groupe d'âge *(7)*.

1.1.2 Diagnostic

Les critères minimaux nécessaires au diagnostic de la MP incluent généralement la présence de bradykinésie et au moins un des trois symptômes suivants : 1) une rigidité des muscles du tronc ou des membres ; 2) un tremblement postural ou de repos ; 3) une posture ou une démarche anormales *(2)*. De nombreuses échelles d'évaluation clinique existent, mais la *Unified Parkinson's Disease Rating Scale* (UPDRS) *(8)* est la plus largement

utilisée car elle permet une évaluation multidimensionnelle des signes cliniques de la MP. Les trois premières sections de l'UPDRS comportent une évaluation de l'état mental, une description des activités de la vie quotidienne et un examen moteur. La quatrième section évalue les complications associées au traitement. Les deux dernières sections sont deux autres échelles d'évaluation clinique : le stade de Hoehn et Yahr *(9)* et l'échelle de Schwab et England *(10)*. Le stade de Hoehn et Yahr estime la sévérité de la maladie suivant cinq stades; du premier stade caractérisé par une atteinte unilatérale sans déficit fonctionnel, au cinquième stade où le patient est alité ou en chaise roulante. L'échelle de Schwab et England évalue le degré d'autonomie des patients.

1.1.3 Traitements

La neurodégénérescence caractérisant la MP est irréversible et incurable. Aucun médicament neuroprotecteur n'est encore disponible, en dépit de certaines indications suggérant que des inhibiteurs de la monoamine oxydase B (MAO-B) ralentiraient la progression de la maladie lorsqu'administrés au cours de son premier stade *(11)*. Des traitements symptomatiques peuvent néanmoins durablement améliorer la qualité de vie des patients. Ces traitements ciblent les symptômes moteurs de la MP en s'appuyant sur le fait qu'elle est causée par un déficit dopaminergique sous-cortical. Trois stratégies thérapeutiques sont employées pour pallier à ce manque : 1) l'apport de dopamine exogène, 2) l'utilisation d'agonistes dopaminergiques, et 3) l'inhibition du catabolisme de la dopamine *(12)*. Le médicament le plus couramment utilisé, car le mieux toléré et le plus efficace contre les symptômes moteurs, est la levodopa (L-dopa), un précurseur de la dopamine. La L-dopa est combinée à un inhibiteur périphérique de la dopa-décarboxylase. La dopamine n'est pas directement administrée à cause de son incapacité à traverser la barrière hémato-encéphalique. Une fois dans le cerveau, la L-dopa est captée par les neurones dopaminergiques et décarboxylée en dopamine *(13)*. Malgré des effets bénéfiques évidents, cette médication présente plusieurs limites. À long terme, des fluctuations motrices et des dyskinésies (mouvements involontaires) apparaissent chez les patients

traités par la L-dopa. En outre, la dopathérapie n'est pas efficace contre les symptômes non moteurs *(14)*. Les agonistes dopaminergiques sont des molécules qui stimulent les récepteurs dopaminergiques grâce à leur structure analogue à la dopamine. Contrairement à la L-dopa, ils n'induisent pas de dyskinésies. Pour cette raison, ils sont souvent prescrits aux patients présentant une forme précoce de MP *(15)*. Par contre, ils sont généralement moins efficaces que la L-dopa pour contrer les symptômes moteurs de la MP. Le rôle des inhibiteurs de la MAO-B comme la sélégiline et la rasagiline est de réduire le catabolisme de la dopamine dans le cerveau. Généralement bien tolérés, ils possèdent un effet symptomatique au premier stade de la MP *(16-18)*. Grâce à des progrès importants en neurochirurgie, la chirurgie lésionnelle et la stimulation cérébrale profonde sont maintenant proposées pour traiter les formes invalidantes de MP pour lesquelles les traitements classiques ne suffisent pas *(19)*. La relative simplicité des lésions responsables de la MP et la disponibilité de bons modèles animaux combinées à des percées majeures en biologie cellulaire et moléculaire permettent aussi de tester de nouvelles stratégies thérapeutiques. La thérapie cellulaire et la thérapie génique sont encore largement à l'état expérimental mais sont généralement considérées comme les avenues les plus prometteuses pour arriver à enrayer le processus neurodégénératif de la MP *(20, 21)*.

1.2 Épidémiologie

La MP touche surtout les personnes âgées. L'âge de début médian, autour de 60 ans, est assez similaire entre les différentes études. L'apparition de la maladie avant l'âge de 20 ans (Parkinson juvénile) représente une forme héréditaire très rare. Dans la grande majorité des cas, l'âge de début oscille entre 50 et 75 ans. On estime à 5% ou moins la proportion de patients chez qui la MP apparaît avant 50 ans *(22, 23)*.

La prévalence et l'incidence de la MP varient d'une région à l'autre du monde *(24)*. Ces divergences, qui pourraient en partie être expliquées par des problèmes méthodologiques et/ou des critères de diagnostic non standardisés, compliquent la

comparaison entre les différentes études. Dans la population générale, la prévalence de la MP oscille entre 18 et 418 cas/100 000 habitants. On observe généralement de plus hautes prévalences dans les pays occidentaux que dans les pays africains et asiatiques *(24)*. Une méta-analyse a estimé la prévalence à 1,8% de la population européenne au-dessus de 65 ans et à 2,6% chez les 85-89 ans *(25)*. Une étude américaine a estimé le risque de développer la MP au cours de sa vie à 2% pour les hommes et 1,3% pour les femmes *(26)*. La MP est donc la deuxième maladie neurodégénérative la plus répandue après la maladie d'Alzheimer *(27)*. Les hommes pourraient être 1,5 fois plus à risque d'être atteints que les femmes mais ces observations varient d'une étude à l'autre *(28)*. Comme pour la prévalence, l'incidence est très variable. Selon les études, elle oscille entre 1,5-26 nouveaux cas/100 000 habitants par an *(28)*. Sans surprise, l'incidence augmente avec l'âge. Une étude épidémiologique américaine a ainsi rapporté une incidence de parkinsonisme de 114,7 cas/100 000 habitants par an dans le groupe des 50-99 ans. L'estimation de l'incidence allait de 0,8 chez les 0-29 ans à 304,8 chez les 80-99 ans *(29)*. En 2003, une revue de 25 études épidémiologiques a estimé l'incidence de la MP à 16 à 19 cas/100 000 habitants par an *(28)*.

1.3 Étiologie

1.3.1 Facteurs environnementaux

L'hypothèse voulant que des facteurs environnementaux soient à l'origine de la MP a longtemps été favorisée, notamment en raison de la description d'une « épidémie » de parkinsonisme chez des toxicomanes californiens suite à l'injection d'un opioïde de synthèse artisanale contaminé par du 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) *(30)*. Cette substance est métabolisée en 1-methyl-4-phenylpyridinium (MPP+) par l'enzyme MAO-B. Le MPP+ est capturé sélectivement par les neurones dopaminergiques de la substance noire (SN), il s'accumule dans les mitochondries, inhibe le complexe I de la

chaîne respiratoire, augmente la libération de radicaux oxygénés et induit un stress oxydatif *(31, 32)*.

De nombreux autres facteurs de risque environnementaux ont été suggérés, comme le fait de travailler dans une ferme, des traumatismes crâniens répétés *(33)*, l'exposition à des pesticides ou à des métaux lourds et la consommation d'eau de puits *(34-36)*. Cependant, aucun de ces éléments ne peut être considéré à lui seul comme un facteur causatif de la MP. Parmi eux, l'exposition aux pesticides est probablement le facteur de risque le plus reconnu. Une étude américaine basée sur plus de 140 000 personnes a notamment observé que l'incidence de la MP était plus élevée de 70% chez les personnes exposées aux pesticides. Par contre, aucune substance en particulier n'a pu être identifiée *(37)*. En ce qui concerne les facteurs protecteurs, une relation inverse dose-dépendante existe, sans qu'on en connaisse la cause, entre la consommation de tabac et le risque de développer la MP *(38, 39)*. Finalement, l'absence de différences temporelles ou géographiques marquées et convaincantes des taux de prévalence ou d'incidence de la MP ne plaide pas en faveur de l'hypothèse d'une exposition environnementale comme cause majeure de la MP *(13)*. Seul l'âge constitue un facteur de risque « environnemental » universellement reconnu.

1.3.2 Prédispositions génétiques

Sir William Richard Gowers fut le premier à soupçonner une origine génétique à la MP en observant que 15% de ses patients présentaient une histoire familiale de MP *(40)*. Depuis, de nombreuses études se sont penchées sur la proportion de cas familiaux/sporadiques au sein de la population parkinsonienne. Parmi les patients, la fréquence d'une histoire familiale de MP oscille entre 6,4 et 33%. Cette proportion varie de 1,4 à 14,8% chez les groupes contrôles *(41)*. À l'instar des études épidémiologiques présentées ci-haut, ces travaux souffrent de nombreux biais et sont difficilement comparables.

Les analyses de ségrégation familiale, dont le but est d'estimer la contribution des facteurs génétiques à la MP, ont généralement rapporté un mode de transmission autosomique dominant (AD) à pénétrance réduite *(42, 43)*. Des travaux plus récents penchent plutôt pour un mode de transmission multifactoriel avec une transmission polygénique combinée à la présence de facteurs de risque environnementaux *(33, 44)*. Quelques familles présentant un grand nombre d'individus atteints ont été décrites *(45-47)*. Il est néanmoins important de souligner que la vaste majorité des familles touchées par la MP ne contiennent qu'un petit nombre de sujets atteints.

La méthode la plus précise pour estimer la prédisposition génétique d'un trait donné consiste à comparer les taux de concordance entre jumeaux monozygotes et dizygotes. Par exemple, un trait génétique AD simple à pénétrance totale devrait correspondre à un taux de concordance de 100% chez les jumeaux monozygotes et de 50% chez les dizygotes. À la lumière des faibles taux de concordance (5-8%) obtenus autant chez les jumeaux monozygotes que dizygotes et en raison de l'absence de différences significatives entre les deux groupes, les premières études de ce type ont conclu à une non-participation des facteurs génétiques à la MP *(48-50)*. Il s'est plus tard avéré que ces travaux avaient tiré des conclusions prématurées en sous-estimant l'impact de l'âge de début très variable de la MP sur leurs observations *(51)*. Une étude postérieure a identifié des taux de concordance respectifs de 100% et 16% chez les jumeaux monozygotes et dizygotes dont l'âge de début de la MP était inférieur à 50 ans. Lorsque l'âge de début n'était pas pris en compte, les taux de concordance respectifs étaient de 16% et 11% chez les jumeaux monozygotes et dizygotes *(52)*. Ces observations confirment la contribution significative de facteurs génétiques aux formes précoces de MP et la complexité des formes plus tardives. La technique d'imagerie médicale de tomographie par émission de positons (TEP) avec la 18F-dopa a mené à l'identification d'un phénotype préclinique dans la MP, soit une diminution de la capture de ce traceur par les neurones dopaminergiques nigro-striataux. Cette méthode présente l'avantage de ne pas être biaisée par les écarts d'apparition des symptômes de la

MP entre les jumeaux. À l'aide de ce marqueur préclinique, des taux de concordance respectifs de 75% et 22% chez les jumeaux monozygotes et dizygotes ont été obtenus *(53, 54)*, ce qui démontre clairement le rôle central de facteurs de prédisposition génétiques dans la MP et met en évidence la présence de formes asymptomatiques (ou présymptomatiques).

1.4 Gènes mutés dans la maladie de Parkinson

L'analyse de liaison génétique du génome entier est la méthode la plus répandue pour identifier les gènes mutés à l'origine de maladies héréditaires. Cette approche permet d'identifier des régions chromosomiques liées à la maladie étudiée à partir de familles contenant plusieurs individus atteints. Le séquençage des gènes localisés dans la région chromosomique liée permet ensuite d'identifier la mutation causative. Au cours des dernières décennies, cette méthode a mené à la découverte d'un grand nombre de gènes responsables de maladies monogéniques. Elle a cependant montré ses limites dans le cas des maladies multifactorielles. La MP est un rare exemple de maladie neurologique complexe pour laquelle les études de liaison génétique se sont avérées fructueuses (**Tableau I**). Celles-ci ont mené à la découverte de deux gènes responsables de formes familiales à transmission AD (*SNCA*, *LRRK2*) *(55-57)* et de trois gènes responsables de formes familiales à transmission autosomique récessive (AR) caractérisées par un âge de début précoce (*Parkin*, *PINK1* et *DJ-1*) *(58-60)*.

1.4.1 *SNCA*

La mutation A53T dans le gène *SNCA* fut la première mutation pathogénique identifiée dans la MP *(56)*. À ce jour, seules trois différentes mutations faux-sens (A53T, A30P et E46K) présentes dans une poignée de familles AD à travers le monde ont été découvertes dans ce gène codant pour la protéine alpha-synucléine *(56, 61, 62)*. Aucune mutation ponctuelle de *SNCA* n'a été trouvée dans des cas sporadiques *(63)*. Les patients répondent bien à la L-dopa mais présentent une forme plus sévère de la MP avec un âge de

début dans la quarantaine, une progression de la maladie plus rapide et une fréquence accrue de démence et de troubles psychiatriques rappelant la démence à corps de Lewy *(62)*. Des duplications et des triplications de *SNCA* ont aussi été identifiées chez des patients parkinsoniens familiaux avec ou sans démence *(64-66)*, prouvant ainsi qu'une surexpression de l'alpha-synucléine de type sauvage est toxique pour les neurones *(67)*. Fait intéressant, les patients avec une duplication présentent une MP non différenciable de la forme idiopathique alors que les porteurs d'une triplication ont un âge de début autour de 35 ans et une fréquence élevée de démence, ce qui suggère un effet dose-dépendant *(64)*. Une étude a récemment trouvé des duplications de *SNCA* dans 1,5% des familles avec une MP classique de type AD et des triplications dans 4,5% des familles avec un parkinsonisme AD atypique *(68)*. Enfin, la découverte de duplications *de novo* dans 0,25-1% des cas sporadiques démontre que les mutations dans *SNCA* ne sont pas totalement exclusives aux formes familiales de la MP *(69)*.

1.4.2 *LRRK2*

Le locus PARK8, localisé sur le chromosome 8, fut identifié à partir d'une famille présentant une forme AD de la MP *(70)*. En 2004, deux études simultanées ont découvert des mutations faux-sens du gène leucine-rich repeat kinase 2 (*LRRK2*) dans plusieurs familles *(55, 57)*. Depuis, de nombreuses études se sont penchées sur ce gène dont les mutations ce sont avérées être la cause la plus commune de MP identifiée à ce jour. Diverses études basées sur des cas familiaux AD d'origine européenne on identifié des mutations pathogéniques dans 5-15% des patients *(71-73)*. La *Human Gene Mutation Database* (HGMD) fait état de 57 différentes mutations potentiellement pathogéniques (http://www.hgmd.cf.ac.uk). Cependant, d'après les analyses de ségrégation dans des familles et les résultats d'études fonctionnelles, seules six d'entre elles (R1441C, R1441G, R1441H, Y1699C, G2019S et I2020T), sont considérées comme des mutations définitivement pathogéniques *(74, 75)*. Deux mutations à effet fondateur retiennent particulièrement l'attention : dans le nord de l'Espagne, la mutation R1441G expliquerait

jusqu'à 20% des cas familiaux *(76, 77)*, alors que la mutation G2019S est présente dans 40% des patients d'Afrique du Nord et 18% des cas juifs ashkénazes, sporadiques ou familiaux *(78, 79)*. La plus vaste étude génétique de *LRRK2* (15 000 patients sporadiques et 5000 patients familiaux issus de 24 populations différentes) a démontré que la mutation G2019S est présente dans 1,3% des cas sporadiques, 4% des cas familiaux et 0,07% des contrôles appariés dans le monde *(80)*. En Europe, la fréquence des patients porteurs de mutations dans *LRRK2* est particulièrement élevée dans les pays méditerranéens et plus faible dans les pays d'Europe du Nord. Sur le plan clinique, les porteurs présentent un âge de début moyen de 58 ans et une MP asymétrique classique qui répond bien à la L-dopa. Leurs symptômes ne sont pas différenciables de ceux observés chez les patients sans mutations de *LRRK2*. Dans la population asiatique, une autre variation du gène, G2385R, est présente dans 6-10% des patients sporadiques et 3-5% des contrôles *(81-83)*. Les porteurs de ce polymorphisme auraient donc un risque deux à trois fois plus élevé de développer la MP. Cette observation est particulièrement intéressante car elle indique qu'un même gène peut contenir des mutations causatives à forte pénétrance causant des formes familiales et des allèles à risque moins pénétrants prédisposant à des formes sporadiques.

1.4.3 *Parkin*

Le locus de *Parkin* fut la seconde région chromosomique liée à la MP et la première liée à une forme récessive précoce *(84)*. Depuis la découverte de délétion exoniques récessives dans *Parkin (59)*, de nombreuses mutations de tous types ont été identifiées sur les cinq continents : des mutations faux-sens provoquant un changement d'un acide aminé, des mutations non-sens entraînant la production d'une protéine tronquée et des réarrangements exoniques (délétions, duplications ou triplications) *(85-89)*. La HGMD rapporte 204 mutations de tous types (http://www.hgmd.cf.ac.uk). Cette diversité nécessite l'utilisation de techniques différentes afin d'identifier les mutations pathogéniques, ce qui rend le diagnostique génétique plus complexe. Les mutations de *Parkin* expliqueraient jusqu'à 50% des formes récessives précoces de la MP, ce qui constitue de loin la cause la

plus commune de ces formes. Les patients ne présentent généralement pas de symptômes atypiques pouvant les distinguer des autres formes récessives précoces de la MP et ils répondent bien à la L-dopa. La progression de la maladie est lente et l'âge de début moyen est de 32 ans mais peut varier énormément d'un patient à l'autre, c'est-à-dire de l'enfance au troisième âge *(89)*. De rares réarrangements exoniques sont présents à l'état hétérozygote dans la population générale et pourraient être surreprésentés chez les patients sporadiques *(90)*. Le rôle de ces mutations hétérozygotes n'est cependant pas encore clairement établi *(91)*, mais il est possible qu'elles influencent l'âge de début *(92)*.

1.4.4 *PINK1*

Le locus PARK6 fut identifié dans une grande famille sicilienne présentant une forme récessive précoce de la MP ressemblant au phénotype des familles avec des mutations de *Parkin* : un âge de début entre 32 et 48 ans, une bonne réponse à la L-dopa et une progression lente de la maladie *(93)*. Deux mutations pathogéniques furent subséquemment identifiées dans le gène *PINK1 (60)*. Plusieurs études ont confirmé ces résultats par l'identification de nouvelles mutations récessives faux-sens ou non-sens *(94-97)* ainsi qu'une délétion complète du gène *(98)*. Ces mutations causeraient entre 1 et 8% des formes récessives précoces de la MP. Comme pour le gène *Parkin*, certains travaux ont suggéré que des mutations hétérozygotes de *PINK1* pourraient prédisposer à la MP *(94, 99, 100)*.

1.4.5 *DJ-1*

Des mutations du gène *DJ-1*, qui est situé sur le locus PARK7 *(101)*, constituent la troisième cause connue de MP récessive précoce. Une délétion des cinq premiers exons du gène et une mutation faux-sens furent d'abord identifiées dans les deux familles consanguines liées au locus PARK7 *(58)*. Depuis, le séquençage du gène chez un grand nombre de patients n'a révélé qu'un petit nombre de mutations. On estime à environ 1% la

proportion de formes AR de la MP dues à des mutations de *DJ-1 (102-106)*. Les manifestations cliniques des patients sont similaires à celles observées chez les autres formes de MP récessive précoce causées par des mutations de *Parkin* ou *PINK1*, à l'exception d'une famille italienne présentant un syndrome parkinsonien avec démence doublé d'une sclérose latérale amyotrophique *(107)*. Il n'existe à ce jour aucune autre famille présentant ce phénotype et des mutations de *DJ-1*.

1.4.6 *GBA*

La maladie de Gaucher est un rare désordre neurométabolique dû à un déficit enzymatique en glucocérébrosidase et caractérisé par une accumulation d'un glycolipide, le glucocérébroside, dans plusieurs organes *(108)*. Elle est causée par des mutations récessives dans le gène codant pour l'enzyme glucocérébrosidase (*GBA*) *(109)*. La prévalence particulièrement élevée de la maladie de Gaucher de type I dans la population juive ashkénaze est due à des mutations fondatrices dont le taux de porteurs hétérozygotes est de 6% *(110)*. En se basant sur l'observation clinique que les personnes atteintes de la maladie de Gaucher de type I sont plus fréquemment touchés par la MP que la population générale *(111)*, une étude a démontré que 31% des patients parkinsoniens juifs ashkénazes contre 6% des contrôles étaient porteurs de mutations dans *GBA (112)*. Ces résultats ont été largement répliqués dans plusieurs populations *(113)*. Chez les caucasiens, on estime maintenant que des mutations hétérozygotes de *GBA* sont présentes chez 4-6% des patients parkinsoniens et <2% des contrôles. Les porteurs de ces mutations auraient ainsi un risque deux à trois fois plus élevé de développer la MP. Ces patients présentent un âge de début moyen légèrement plus précoce (55 ans contre 59 ans), un taux d'histoire familiale de MP un peu plus élevé (24% contre 18%) mais des symptômes en général similaires à la forme idiopathique classique de la MP. *GBA* est le premier gène formellement associé à la MP qui n'a pas été identifié par des études de liaison génétique.

1.4.7 Autres gènes

Trois autres gènes responsables de formes de parkinsonisme caractérisées par un mode de transmission AR et un âge de début précoce sont maintenant connus. Cependant, ces formes ne sont pas considérées comme partie intégrante de la MP dite « pure » en raison de la présence de manifestations cliniques atypiques ne correspondant pas au spectre des symptômes généralement observés dans la MP. Des mutations dans *ATP13A2* causent le syndrome de Kufor-Rakeb, une forme de parkinsonisme associée à une dégénérescence pyramidale et une démence *(114)*. Des mutations de *FBXO7* sont à l'origine d'un syndrome parkinsonien pyramidal *(115)*. Enfin, *PLA2G6* est muté dans des familles présentant un parkinsonisme-dystonie *(116)*. En ce qui concerne les formes de parkinsonisme AD, des mutations du gène *MAPT* causent une démence frontotemporale avec ou sans parkinsonisme *(117)*.

Tel qu'indiqué dans le **Tableau I**, aucun gène causatif n'a encore été identifié dans certains des loci liés à la MP. Pour plusieurs d'entre eux cependant, des gènes responsables ont été proposés, mais leur véritable implication dans la MP reste controversée. C'est notamment le cas du gène *SPR*, situé sur le locus PARK3 *(118)*. Il code pour la sepiapterine reductase, une des enzymes responsables de la synthèse de dopamine. Malgré le fait qu'un haplotype incluant *SPR* a été associé à la variabilité de l'âge de début de la MP, aucune mutation pathogénique n'a été identifiée dans le gène *(119, 120)*. Une mutation faux-sens dans le gène *UCHL1* a été trouvée chez deux patients d'une même famille *(121)*. Depuis, aucune autre mutation de ce gène n'a été observée dans des patients parkinsoniens. Il a récemment été proposé que des mutations faux-sens du gène *GIGYF2* sur le locus PARK10 prédisposeraient à la MP *(122)*. Cette association n'a pu être reproduite par aucune des études de réplication subséquentes *(123)*, incluant les auteurs de l'étude originale *(124)*. Une mutation faux-sens et un polymorphisme du gène *HTRA2* ont été associés à la MP *(125)*. Ce gène code pour une protéine impliquée dans l'apoptose mitochondrie-dépendante

(126), un mécanisme particulièrement étudié dans la MP. Malheureusement, la plupart des études de réplication subséquentes ont exclu l'association entre des variations génétiques de *HTRA2* et la MP *(127, 128)*. Une seule étude de réplication a identifié chez un patient une mutation faux-sens dans le gène *(129)*.

Tableau I. Loci chromosomiques et gènes mutés dans la maladie de Parkinson.

Locus	Type	Gène	Notes	Références
PARK1	AD	*SNCA*	Premier gène identifié	*(56)*
PARK2	AR	*Parkin*	MP récessive précoce	*(59)*
PARK3	AD	(*SPR*)	Rôle incertain	*(45)*
PARK4	AD	*SNCA*	Duplications et triplications du gène	*(66)*
PARK5	AD	(*UCHL1*)	Rôle incertain	*(121)*
PARK6	AR	*PINK1*	MP récessive précoce	*(60)*
PARK7	AR	*DJ-1*	MP récessive précoce	*(58)*
PARK8	AD	*LRRK2*	Cause de la MP la plus fréquente	*(55, 57)*
PARK9	AR	*ATP13A2*	Syndrome de Kufor-Rakeb	*(114)*
PARK10	-	-	Locus modifiant l'âge de début de la MP	*(130)*
PARK11	AD	(*GIGYF2*)	Résultats non répliqués	*(122)*
PARK12	X	-	Chromosome X, modifiant l'âge de début	*(131)*
PARK13	AD	(*HTRA2*)	Résultats non répliqués	*(125)*
PARK14	AR	*PLA2G6*	Syndrome dystonie-parkinsonisme adulte	*(116)*
PARK15	AR	*FBXO7*	Syndrome parkinsonien-pyramidal	*(115)*
PARK16	-	-	Études d'association du génome entier	*(132, 133)*

Abréviations : AD. Autosomique dominant. AR. Autosomique récessif. Gènes entre parenthèses : gènes dont le rôle dans la MP reste controversé.

1.5 Études d'association du génome entier

Les mutations conférant un risque élevé de développer la MP sont généralement rares (à l'exception de certaines mutations fondatrices) et ont jusqu'ici été majoritairement trouvées chez des cas familiaux qui ne représentent que 15% de tous les patients touchés par la MP. Ce constat s'applique à de nombreux autres traits génétiques complexes. L'hypothèse « maladie commune/variant commun » (*common disease/common variant hypothesis*) a donc été proposée pour tenter d'expliquer la susceptibilité héréditaire aux maladies multifactorielles à prévalence élevée *(134, 135)*. Ce modèle se base sur l'idée que les facteurs de susceptibilité génétiques prédisposant aux traits complexes sont des variations génétiques fréquentes (polymorphismes) conférant individuellement un risque moins important que les mutations pathogéniques responsables des maladies mendéliennes. La fréquence élevée de ces polymorphismes dans la population serait due à leur présence dans la population fondatrice des humains actuels. L'explosion démographique et les migrations humaines auraient entraîné leur distribution à l'échelle planétaire. Si un de ces allèles est impliqué dans la susceptibilité à une maladie donnée, il devrait être surreprésenté (ou sous-représenté dans le cas d'un effet protecteur) chez les personnes atteintes. La récente amélioration des techniques de génotypage permet aujourd'hui de tester cette hypothèse à l'échelle du génome. Ce type d'analyse, communément appelé « étude d'association du génome entier » (*genome-wide association study* ou *GWAS*), s'appuie sur l'examen de centaines de milliers de polymorphismes d'un seul nucléotide (SNP) couvrant la totalité du génome dans des cohortes cas-témoins de plusieurs centaines, voire plusieurs milliers de personnes. La première génération d'études d'association du génome entier s'est avérée particulièrement utile dans la compréhension de nombreux traits héréditaires

complexes mais présente un bilan mitigé pour la majorité de ceux qui touchent le cerveau *(136)*.

La première étude du genre dans la MP a été publiée en 2005 *(137)*. Elle était basée sur l'analyse de 200 000 SNP génotypés dans 775 patients et 775 contrôles caucasiens. Les auteurs ont identifié une association marginale entre des SNP localisés dans *MAPT*, *SNCA* et d'autres loci chromosomiques précédemment liés à la MP. Cependant, aucun SNP n'a pu être clairement associé à la MP après correction statistique pour un test d'hypothèses multiples. La deuxième étude a génotypé un plus grand nombre de SNP mais n'a examiné qu'un petit nombre d'individus pour ce genre d'étude (276 patients et 276 contrôles) *(138)*. Dans ce cas-là aussi, aucun résultat n'était significatif après correction statistique. Le troisième groupe de recherche à avoir publié des résultats d'association génomique dans la MP a utilisé une approche différente en se concentrant exclusivement sur des cas familiaux. Encore une fois, aucun SNP n'a pu être associé de manière significative à la MP mais les auteurs ont rapporté une association marginale entre la MP et des SNP dans les loci chromosomiques de *MAPT* et *SNCA (139)*. Finalement, deux récentes méta-analyses ont échoué dans leur but de répliquer les résultats d'association obtenus par les deux premières études présentées ci-dessus *(140, 141)*. Le relatif échec de ces études d'association génomique n'exclue pas la possibilité que des polymorphismes soient des facteurs de risques pour la MP. Ces travaux ont probablement souffert d'un manque de puissance statistique et ont démontré la nécessité d'utiliser des cohortes de cas-témoins beaucoup plus importantes.

Tout récemment, soit en novembre 2009, deux nouvelles études du genre s'appuyant sur des cohortes de plusieurs milliers de cas-témoins ont été publiées simultanément *(132, 133)*. L'intérêt de ces travaux réside dans la réplicabilité des résultats. En effet, les deux études ont identifié de façon indépendante trois régions chromosomiques communes présentant des SNP associés à la MP. Deux de ces trois loci concernent les deux gènes responsables de formes AD de la MP (*SNCA* et *LRRK2*) alors que la troisième

région, qui est localisée sur le chromosome 1q32, n'avait auparavant jamais été associée à la MP. Cette dernière a été appelée PARK16 (**Tableau I**). Ces deux études concluent à la présence de polymorphismes associés à la MP dans quatre loci chromosomiques distincts dont trois contiennent des gènes porteurs de mutations prédisposant au parkinsonisme (*SNCA*, *LRRK2* et *MAPT*). Il est cependant important de noter que le risque relatif associé à chacun de ces polymorphismes ne dépasse jamais 1,5 et que l'héritabilité de la MP attribuable à ces régions chromosomiques serait de l'ordre de 25% *(133)*, suggérant ainsi que l'hypothèse maladie commune/variant commun est très loin d'expliquer à elle seule la prédisposition génétique aux formes sporadiques de la MP.

1.6 Neuropathologie

Sur le plan neuroanatomique, la principale caractéristique de la MP est une mort massive et relativement sélective des neurones dopaminergiques de la substance noire *pars compacta* (SNpc) identifiable par une dépigmentation de cette structure *(142)*. Ces neurones projettent leurs axones dans le ganglion de la base et forment des synapses dans le striatum, une structure responsable de l'initiation et du contrôle de mouvements *(143)*. La conséquence directe de cette perte neuronale est un déficit en dopamine dans le striatum *(144)*. Au moment de l'apparition des premiers symptômes de la MP, on estime que les patients ont déjà perdu environ 60% de leurs neurones dopaminergiques nigrostriataux et 80% du contenu dopaminergique striatal *(143)*. Dans une moindre mesure, d'autres systèmes neuronaux peuvent aussi être touchés. Ceux-ci sont responsables des manifestations cliniques non-motrices de la MP *(145)*.

L'autre signe neuropathologique caractéristique de la MP est la présence d'inclusions cytoplasmiques sphériques appelées corps de Lewy dans les neurones survivants des régions cérébrales touchées *(146, 147)*. Les corps de Lewy sont des agrégats éosinophiles de nature fibrillaire contenant de nombreuses protéines, dont l'alpha-synucléine, parkin, l'ubiquitine et des neurofilaments *(147, 148)*. Ils ne sont pas exclusifs à

la MP. On les retrouve également dans la maladie d'Alzheimer, la démence à corps de Lewy et chez des individus asymptomatiques d'âge avancé *(149)*. Ces inclusions ont longtemps été considérées comme une preuve définitive de MP lorsqu'associées à une dégénérescence des neurones dopaminergiques de la SNpc. Ce critère de diagnostic est aujourd'hui remis en question par l'absence de corps de Lewy chez les patients avec des mutations de *Parkin (59, 150)*.

1.7 Pathogénèse moléculaire

En dépit de la rareté de la majorité des mutations responsables de la MP, la découverte de formes monogéniques a énormément contribué à une meilleure compréhension des mécanismes moléculaires impliqués dans la pathogénèse de la maladie. La formation de corps de Lewy par l'agrégation d'alpha-synucléine, le système ubiquitine-protéasome, certaines fonctions mitochondriales et la réponse au stress oxydatif sont les principaux mécanismes moléculaires considérés comme centraux dans le processus neurodégénératif de la MP *(151, 152)*.

1.7.1 Alpha-synucléine et agrégation protéique

Des agrégats protéiques anormaux sont observés dans plusieurs maladies neurodégénératives dont la maladie d'Alzheimer, qui est caractérisée par la présence de plaques amyloïdes extracellulaires et d'enchevêtrements neurofibrillaires *(153, 154)*. La protéine alpha-synucléine (codée par le gène *SNCA*) est potentiellement impliquée dans la régulation de la transmission synaptique *(155)* mais c'est surtout le constituant principal des corps de Lewy *(148)*. Il est important de noter que la forme sauvage a naturellement tendance à former des protofibrilles et des fibrilles insolubles *(156)*. Cependant, la mutation ponctuelle A53T ou une surexpression de la protéine sauvage dans des modèles cellulaires accélèrent le processus d'agrégation *(156, 157)*. La découverte de duplications ou de triplications du gène *SNCA* chez des patients parkinsoniens a permis de confirmer

l'hypothèse que la surexpression d'alpha-synucléine est impliquée dans la pathogénèse de la MP *(65, 66)*. En dépit du constat que l'apparition d'inclusions fibrillaires est associée à une neurodégénérescence dans la plupart des modèles transgéniques étudiés, le lien de cause à effet entre la formation de ces inclusions et la dégénérescence des neurones dopaminergiques demeure controversé *(147)*. Certains arguent que la formation de ces agrégats pourrait être un mécanisme de défense des neurones contre les petits oligomères solubles d'alpha-synucléine qui seraient en fait les formes les plus toxiques *(158)*.

1.7.2 Système ubiquitine-protéasome

L'ubiquitinylation est un système post-traductionnel dont le rôle principal est de marquer les protéines pour qu'elles soient dégradées par le protéasome. Le système ubiquitine-protéasome permet à la cellule de se débarrasser de protéines qui ont tendance à s'agréger, induire une cytotoxicité et/ou interférer avec des processus cellulaires clés. Des enzymes spécifiques sont responsables de l'activation des monomères d'ubiquitine et de l'étiquetage des protéines à dégrader. Les protéines marquées sont ensuite transportées vers le protéasome où elles sont désubiquitinylées et dégradées par le complexe 26S *(159)*. La sélectivité de ce mécanisme est assurée par les nombreuses E3 ubiquitin ligases dont le rôle est de poly-ubiquitinyler des substrats spécifiques. Le gène *Parkin* code pour une de ces enzymes, ce qui suggère qu'une capacité réduite à marquer et à dégrader des protéines mal repliées pourrait causer la MP. Cette hypothèse est appuyée par l'observation d'une accumulation de substrats de parkin non-ubiquitinylés dans la SNpc de patients ayant des mutations du gène *Parkin (160)*.

1.7.3 Mitochondries et stress oxydatif

Le lien entre un dysfonctionnement mitochondrial et la MP a été intensément étudié depuis la découverte de l'induction d'un syndrome parkinsonien par le MPTP, un inhibiteur du complexe I de la chaîne respiratoire mitochondriale (section 1.3.1) *(161)*. Le

MPTP tue sélectivement les neurones dopaminergiques de la SNpc chez l'humain, le singe ainsi que dans d'autres espèces, ce qui en fait un bon modèle expérimental pour étudier la MP *(143)*. L'inhibition du complexe I par le MPTP provoque une augmentation de la libération de radicaux libres et un stress oxydatif *(32)*. Depuis cette découverte, une réduction de l'activité de ce complexe a été observée dans le cerveau, les plaquettes et les muscles squelettiques de patients parkinsoniens idiopathiques *(162, 163)*. Le remplacement des mitochondries de cellules en culture par des mitochondries dérivées de plaquettes de patients (cybrides) induit aussi une réduction de l'activité du complexe I *(164)*. À la lumière de ces observations, les auteurs de ces travaux ont suggéré que des anomalies génétiques dans le génome mitochondrial pourraient être responsables de la MP. Cela a été confirmé chez des patients par la découverte d'un taux plus élevé de délétions dans l'ADN mitochondrial des neurones de la SN *(165)*. Au niveau fonctionnel, l'inhibition du complexe I dans les neurones dopaminergiques de la SNpc induit une production élevée de radicaux libres qui endommagent l'ADN, les lipides, et les protéines; un épuisement des réserves en ATP, et une activation de l'apoptose mitochondrie-dépendante *(143, 166)*.

Chacun des cinq gènes mutés dans la MP (*SNCA*, *Parkin*, *PINK1*, *DJ-1*, *LRRK2*) interagit avec les mitochondries *(167)*. Une inhibition du complexe I suite à l'administration de MPTP induit une accumulation d'alpha-synucléine dans les neurones nigraux des babouins *(168)* et les souris dont le gène *SNCA* a été invalidé sont résistantes aux effets du MPTP *(169)*. Une étude récente a démontré que l'alpha-synucléine est importée dans les mitochondries, qu'elle s'associe à la membrane interne mitochondriale et perturbe spécifiquement l'activité du complexe I. Cet effet est augmenté par la mutation pathogénique A53T. De plus, l'analyse *post mortem* de patients indique que les mitochondries issues de la SNpc présentent une accumulation d'alpha-synucléine associée à la membrane interne mitochondriale *(170)*.

Au cours de la division cellulaire, la protéine parkin est importée dans les mitochondries et participe à la transcription et à la réplication de l'ADN mitochondrial

(171). Parkin joue aussi un rôle anti-apoptotique en retardant la libération de cytochrome c *(172)*. Récemment, l'étude de la drosophile comme modèle animal de la MP a mené à des avancées importantes dans la caractérisation des fonctions mitochondriales de parkin, PINK1 et DJ-1 *(173)*. Les cellules musculaires des mouches sans *Parkin* ou surexprimant une forme mutée du gène dégénèrent par un processus apoptotique et présentent des mitochondries ballonnées caractérisées par une désintégration des crêtes de leur membrane interne *(174, 175)*. Une dégénérescence partielle des neurones dopaminergiques a aussi été observée dans le cerveau de ces mouches *(176)*. Toujours chez la drosophile, une perte de fonction de *PINK1* induit un phénotype moteur et une morphologie mitochondriale similaires. Les mutants présentent des niveaux d'ATP et un contenu d'ADN mitochondrial réduits. Les mitochondries des neurones dopaminergiques ont aussi une morphologie anormale. Une surexpression de *Parkin* renverse le phénotype pathologique observé chez les mouches mutantes pour *PINK1* mais une surexpression de *PINK1* ne compense pas la perte de *Parkin*. Une double invalidation de *Parkin* et *PINK1* ne cause pas un phénotype plus sévère *(177-179)*. Ces résultats indiquent que ces deux gènes agissent de concert dans une voie linéaire responsable du maintien de l'intégrité et des fonctions mitochondriales et que *Parkin* agit en aval de *PINK1*. Le fait que les formes de MP causées par des mutations de *Parkin* et *PINK1* ne sont pas différentiables suggère que cette voie moléculaire est aussi présente chez l'humain *(180)*. En outre, l'expression du gène *PINK1* humain supprime le phénotype pathologique causé par une perte de fonction de *PINK1* chez la drosophile *(179)*.

Une perte de fonction de *DJ-1*, le troisième gène impliqué dans la MP récessive précoce, rend les cellules plus vulnérables au stress oxydatif alors que la surexpression du gène a un effet protecteur *(181, 182)*. Même si elle est principalement localisée dans le cytosol, la protéine DJ-1 est également trouvée dans la matrice mitochondriale ou dans l'espace inter-membranaire *(183)*. La protéine migre vers la mitochondrie en réponse à un stress oxydatif *(184)*. Chez la drosophile, la surexpression de *DJ-1* ne compense pas la perte de fonction de *PINK1*, suggérant ainsi que DJ-1 n'agit pas dans la même voie que

PINK1 et parkin *(179)*. Par contre, des études *in vitro* ont proposé une interaction physique entre DJ-1 et parkin sous des conditions de stress oxydatif *(185)*.

La protéine cytosolique LRRK2 est aussi partiellement associée à la membrane externe mitochondriale *(186)* et des mutations pathogéniques du gène induisent une mort cellulaire par apoptose mitochondrie-dépendante *(187)*. Chez la drosophile, la surexpression de la protéine humaine sauvage ou du mutant G2019S provoquent une perte sélective des neurones dopaminergiques et un dysfonctionnement locomoteur répondant à la L-dopa. Ce phénotype est plus sévère pour le mutant G2019S *(188)*. Le mécanisme pathogénique est encore flou, même si de récents travaux suggèrent une interaction génétique entre *LRRK2* et *Parkin*, *PINK1* et *DJ-1 (189)*.

Récemment, l'hypothèse d'une perturbation de la dynamique mitochondriale dans la MP a été soulevée *(190)*. Cette théorie sera plus longuement décrite au chapitre 3. Conjointement, les études épidémiologiques, toxicologiques, génétiques et fonctionnelles convergent de plus en plus vers un dysfonctionnement mitochondrial comme mécanisme primaire à l'origine de la MP *(167, 191)*.

1.8 La population canadienne-française du Québec

1.8.1 Une population fondatrice

La structure génétique des populations est intimement liée aux flux migratoires et à divers facteurs culturels, socioéconomiques et environnementaux *(192, 193)*. Depuis 1492, la découverte et la colonisation du Nouveau-Monde par les européens ont profondément bouleversé la génétique des populations nord-américaines *(194)*. Parmi celles-ci, il est maintenant clairement établi que la population canadienne-française (CF) du Québec présente des caractéristiques démographiques uniques qui favorisent la recherche en génétique humaine *(195)*. Le Québec compte 7,6 millions d'habitants dont environ 6 millions sont de descendance CF. La première colonie française en Nouvelle-France fut

établie en 1608 à Québec. Entre 1608 et 1759 (date de la conquête anglaise), la Nouvelle-France reçut plus de 25 000 colons, majoritairement d'origine française. Seulement 8500 d'entre eux dont 1600 femmes s'y sont installés de manière permanente *(196)*. Les 2600 colons établis au Québec avant 1680 auraient contribué aux deux tiers du patrimoine génétique actuel des CF *(197)*. Après 1759, le flux migratoire d'origine française cessa et fut remplacé par une immigration majoritairement composée d'irlandais, d'anglais et d'écossais *(198)*. Pour des raisons socioéconomiques, religieuses et linguistiques, les populations CF et britannique évoluèrent pratiquement sans se mélanger. Un fort taux de natalité provoqua une croissance soutenue de la population CF qui doubla tous les 30 ans entre 1750 et 1875 *(199)*. La population fondatrice CF du Québec présente donc trois traits démographiques centraux qui expliquent sa relative homogénéité génétique actuelle : 1) un petit nombre d'immigrants fondateurs, 2) une période de croissance démographique soutenue, et 3) une expansion de la population en vase relativement clos. Plusieurs facteurs sont néanmoins responsables du fait que l'ensemble de la population CF n'est pas aussi homogène qu'on pourrait le penser à prime abord : 1) l'hétérogénéité du noyau fondateur, 2) un effet fondateur distinct entre les fronts pionniers à l'origine d'une certaine stratification régionale, et 3) une consanguinité relativement faible *(200, 201)*. Il est néanmoins clairement établi que la relative homogénéité génétique des CF du Québec influence de manière importante la prévalence de plusieurs maladies héréditaires. Certaines sont beaucoup plus fréquentes dans la population CF que dans d'autres populations. Fait important à noter, plusieurs de ces maladies génétiques sont spécifiques à une région du Québec alors que d'autres sont présentes dans l'ensemble de la province. Au moins 27 maladies héréditaires sont plus fréquentes au Québec que dans le reste du monde ou présentent des caractéristiques distinctes chez les CF *(202)*. Ce phénomène est attribuable à un effet fondateur, c'est-à-dire que des mutations dites fondatrices sont surreprésentées lorsqu'une population est issue d'un nombre relativement restreint de pionniers. Ces 27 maladies génétiques sont presque exclusivement des traits héréditaires monogéniques. Peu de données sont disponibles sur l'existence de mutations fondatrices à l'origine de traits

complexes chez les CF, à l'exception notable des gènes *BRCA1* et *BRCA2* pour le cancer du sein et des ovaires *(203)*. À notre connaissance, aucun exemple de mutations fondatrices CF n'existe pour des maladies multifactorielles qui affectent le cerveau.

1.8.2 Études antérieures sur la maladie de Parkinson au Québec

Il est impossible de parler de la recherche sur la MP sans mentionner les travaux d'André Barbeau (1931-1986), clinicien-chercheur et fondateur du laboratoire de neurologie de l'Université de Montréal. Le Dr Barbeau a dédié une partie de sa carrière à la recherche sur l'étiologie, la pathogénèse et le traitement de la MP. Sa contribution a commencé par la découverte inopinée d'une baisse significative de l'excrétion urinaire de dopamine chez des patients parkinsoniens *(204, 205)*. Ces observations l'ont amené à formuler l'hypothèse que la MP serait causée par une déficience en dopamine dans le cerveau. Il fut parmi les premiers au monde à démontrer les effets bénéfiques de la dopathérapie *(206)*. André Barbeau a aussi exploré l'hypothèse de l'existence de facteurs de risque environnementaux en démontrant que l'exposition aux pesticides dans les zones rurales du Québec était associée à une plus forte prévalence de la MP *(207, 208)*. Ses travaux suggéraient également la présence de facteurs de susceptibilité génétiques d'après l'observation que les patients parkinsoniens ont plus fréquemment une histoire familiale de MP ou de tremblement essentiel que les contrôles *(209)*. Vers la fin de sa carrière, il proposa un nouveau concept, intégrant hérédité et environnement qu'il nomma l'«écogénétique », une théorie qui reste d'actualité dans la MP *(210)*.

1.9 Problématique

1.9.1 Analyse du gène *LRRK2*

L'objectif général des travaux présentés dans cette thèse était d'identifier des mutations prédisposant à la MP dans la population CF du Québec à partir d'une cohorte de

patients parkinsoniens représentative des patients vus en pratique clinique, c'est-à-dire un groupe de cas majoritairement sporadiques et caractérisés par un âge de début tardif. Deux approches ont été mises en pratique. La première, présentée dans le chapitre 2, visait à trouver des mutations fondatrices CF dans le gène *LRRK2*, considéré comme la cause la plus fréquente de MP d'apparition tardive identifiée à ce jour.

1.9.2 Séquençage de gènes candidats

Le deuxième volet de ce projet de recherche, détaillé aux chapitres 3 et 4, visait à identifier de nouveaux gènes responsables de la MP dans la population CF en séquençant directement des gènes candidats et de tenter d'élargir ces résultats à d'autres populations.

Notre projet de recherche s'appuyait sur trois hypothèses centrales:

1) Des mutations rares prédisposent à la MP (*multiple rare allele hypothesis*).

2) Le séquençage direct de gènes candidats est l'approche la plus appropriée pour identifier ces mutations.

3) Ces mutations affectent des gènes impliqués dans des mécanismes moléculaires associés à la pathogénèse de la MP.

Tel que discuté en introduction, on considère généralement que la MP est un désordre neurodégénératif fréquent causé par des interactions complexes entre de nombreux facteurs de susceptibilité génétiques et environnementaux ayant à long terme un effet dommageable sur le cerveau vieillissant. Cette perception entre cependant en contradiction avec le fait que de plus en plus de formes sporadiques sont expliquées par des facteurs génétiques majeurs (*SNCA*, *LRRK2*, *GBA*) alors que peu de données convaincantes existent pour démontrer un rôle prépondérant des facteurs de risque environnementaux en dépit de décennies d'études épidémiologiques. Malgré des progrès évidents en recherche génétique, les mutations identifiées à ce jour concernent seulement 5 à 10% des patients parkinsoniens

(211). L'identification de nombreux loci chromosomiques liés à la MP et de plusieurs gènes causatifs souligne l'importante hétérogénéité allélique (de multiples mutations dans le même gène) et non allélique (des mutations dans des gènes différents) de la MP. Les résultats mitigés obtenus par les études d'association du génome entier suggèrent que l'hypothèse « maladie commune/variant commun » n'est pas en mesure d'expliquer à elle seule l'héritabilité de la MP. Prises ensembles, ces observations indiquent qu'un grand nombre de gènes pourraient être impliqués dans la pathogénèse de la MP et que les mutations identifiées à ce jour n'en sont que la pointe de l'iceberg. En effet, si on part du principe que la plupart des formes de MP sont dues à des anomalies génétiques et que des mutations dans six gènes n'expliquent que 5 à 10% des patients, on peut formuler l'hypothèse que des mutations individuellement rares et à relativement forte pénétrance dans des dizaines, voire des centaines de gènes pourraient en théorie prédisposer à la MP. À l'origine, cette hypothèse a été proposée pour expliquer la prédisposition génétique à la schizophrénie *(212)*.

Le nombre limité de familles parkinsoniennes assez grandes pour faire des études de liaison génétique souligne la nécessité de développer d'autres approches afin d'identifier de nouvelles mutations prédisposant aux formes familiales et sporadiques de la MP. Sachant que ces mutations seraient généralement trop rares pour être détectées par des études d'association du génome entier *(136)*, Le séquençage direct de gènes candidats constituerait donc l'approche la plus appropriée pour identifier ces mutations. Il est important de noter que les nouvelles technologies de séquençage génomique n'étaient pas disponibles au moment de l'initiation de ce projet de recherche.

Les protéines codées par les gènes responsables de la MP semblaient à priori avoir des fonctions distinctes et être impliquées dans des voies moléculaires diverses. Cependant, il est aujourd'hui de plus en plus évident que la plupart de ces protéines sont impliquées dans des voies moléculaires communes et ont des rôles qui s'entrecroisent (voir section 1.7.3). Pour sélectionner les gènes candidats à séquencer, nous sommes donc simplement

partis de l'hypothèse que des mutations dans des gènes interagissant avec les gènes responsables de la MP ou ayant un rôle dans les voies moléculaires dysfonctionnelles dans la MP pourraient aussi contenir des mutations pathogéniques.

Chapitre 2 : Analyse du gène *LRRK2*

Présentation de l'article

Tel que discuté en introduction (section 1.4.2), les mutations du gène *LRRK2* représentent la plus fréquente cause de la MP identifiée à ce jour. La distribution et la fréquence des mutations de *LRRK2* sont très variables à travers le monde, ce qui confirme l'idée que d'importants déterminants génétiques de la MP sont spécifiques à certaines populations. Ceci est particulièrement vrai pour les populations isolées. Dans le nord de l'Espagne, jusqu'à 20% des patients d'origine basque présentant une forme familiale de la MP sont porteurs de la mutation fondatrice R1441G *(76, 77)*. Les porteurs de cette mutation auraient un ancêtre commun ayant vécu au VII^{ème} siècle *(77)*. Probablement originaire du Paléolithique supérieur *(213)*, la population basque est restée relativement isolée à travers les siècles *(214)*, ce qui expliquerait pourquoi la mutation R1441G est quasi-exclusive au nord de l'Espagne. La fréquence élevée de la mutation G2019S chez les patients parkinsoniens nord-africains (41% des patients sporadiques, 37% des patients familiaux) et juifs ashkénazes (13% des patients sporadiques, 30% des patients familiaux) est aussi due à un effet fondateur *(78, 79)*. Les porteurs seraient des descendants d'un ancêtre commun proche-oriental ayant vécu il y a 2250 ans *(215)*. Cette estimation concorde avec la période des diasporas juives (de 586 av. J.-C. à 70 ap. J.-C.) et se situe à une époque où le peuple juif ancestral et certaines populations arabes coexistaient dans la même région. Les diasporas juives et l'expansion de l'islam sont les deux évènements historiques probablement responsables de la dissémination de la mutation en Europe et dans le bassin méditerranéen.

Les données obtenues à partir de ces populations fondatrices nous ont poussé à vérifier la présence de mutations de *LRRK2* chez les patients parkinsoniens d'origine CF. Notre hypothèse était basée sur le scénario d'un effet fondateur dû à la présence de mutation(s) de *LRRK2* chez un ou plusieurs pionniers établis au Québec entre 1608 et 1759. À partir d'une petite cohorte de 125 patients parkinsoniens d'origine CF, nous avons directement séquencé les deux exons du gène *LRRK2* dans lesquels la majorité des mutations pathogéniques ont été observées (voir section 1.4.2). Nous avons également testé l'association entre la MP et des polymorphismes dans le gène. Nos données suggèrent

qu'aucun effet fondateur significatif n'a mené à l'apparition de mutation(s) fréquente(s) de *LRRK2* chez les CF.

Contribution des auteurs

ND : conception du projet de recherche sur la MP dans la population CF, suggestion et supervision de l'étude de *LRRK2*, recrutement des patients, analyse des données cliniques et rédaction de l'article.

JBR : conception et réalisation des expériences, analyse des données génétiques et rédaction de l'article.

RHM : collaboration à la mise sur pied de la partie clinique du projet.

PP, **EP** et **FE** : recrutement des patients.

GAR : supervision du projet et rédaction de l'article.

LRRK2 is not a significant cause of Parkinson's disease in French-Canadians

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2.1 Abstract

Background: An old founder mutation (G2019S) was found with high frequency in the North African Arabs (30%) and Ashkenazi Jews (18%). **Objective**: Demonstrate if mutations in the *LRRK2* gene are a significant cause of Parkinson's disease (PD) in the French-Canadian founder population. **Methods**: Cases were recruited through a designated movement disorder clinic in Quebec City. Every index case had to meet the Ward and Gibb criteria for PD. Controls consisted of a non-disease group of similar age and ethnicity as the cases. Exons 31 and 41 of *LRRK2* were amplified by PCR with intronic primers in all 125 PD cases and directly sequenced on an ABI 3700 sequencer. Six single nucleotide polymorphisms were typed in 125 PD cases and 95 normal controls. Associations between unrelated cases and matched controls were analyzed. Single marker analysis and haplotype association tests were performed. **Results**: Sequencing analysis did not reveal any reported or novel mutations in exons 31 and 41 of *LRRK2*. The G2019S mutation as well as mutations affecting amino acid 1441 were absent in the 125 patients. The case-control association study performed to detect the presence of a common variant in *LRRK2* did not provide any positive signal. Single-marker and haplotype analyses systematically gave non-significant P values. **Conclusions**: We performed a case-control association study in 125 French-Canadian (FC) patients with PD and 95 FC controls and found that common variants in *LRRK2* are unlikely to be a significant cause of late-onset PD in this founder population.

Résumé: *LRRK2* n'est pas une cause significative de la maladie de Parkinson chez les Canadiens français. **Contexte** : La fréquence d'une mutation fondatrice ancienne (G2019S) est élevée chez les Arabes de l'Afrique du Nord (30%) et chez les Juifs Ashkenazi (18%). **Objectif** : Déterminer si des mutations dans le gène *LRRK2* sont une cause importante de la maladie de Parkinson (MP) dans la population canadienne-française. **Méthodes** : Les cas ont été recrutés dans une clinique de troubles du mouvement à Québec.

Chaque cas index devait satisfaire aux critères de la MP de Ward et Gibb. Le groupe témoin était composé de sujets sains du même âge et de la même origine ethnique que les cas. Les exons 31 et 41 du gène *LRRK2* ont été amplifiés par PCR au moyen d'amorces introniques chez les 125 patients atteints de MP et séquencés directement au moyen d'un séquenceur ABI 3700. Six polymorphismes d'un seul nucléotide (SNPs) ont été analysés chez les 125 patients et chez 95 témoins normaux. Un test d'association a été effectué pour chaque marqueur et également pour les haplotypes, entre les cas non apparentés et les témoins appariés. **Résultats** : L'analyse du séquençage n'a pas révélé la présence de mutations déjà connues ou de nouvelles mutations dans les exons 31 et 41 du gène *LRRK2*. La mutation G2019S ainsi que les mutations de l'acide aminé 1441 n'étaient pas présentes chez les 125 patients. L'étude cas-témoins pour détecter la présence d'une variation fréquente du gène *LRRK2* était négative. Les valeurs de P des analyses de marqueurs uniques et d'haplotypes ont toutes été non significatives. **Conclusions** : Notre étude cas-témoins chez 125 patients d'origine canadienne-française atteints de MP et chez 95 témoins de la même origine ethnique démontre qu'il est peu probable qu'une variation fréquente du gène *LRRK2* soit une cause significative de la MP à début tardif dans cette population fondatrice.

2.2 Introduction

The minimum criteria for the diagnosis of Parkinson's disease (PD) include the presence of bradykinesia and at least one of the other three primary features: truncal and limb muscle rigidity, resting or postural tremor, and postural instability or gait disorder. At death the brain of patients with PD is characterized by degeneration of the dopamine-containing cells in the substantia nigra and depletion of the dopamine content in terminal areas in the basal ganglia. The cause of PD remains unknown, though evidence points toward a multifactorial etiology, most likely involving a genetic susceptibility to the effects of environmental agents or trauma. A number of environmental risk factors have been implicated in the etiology of PD, yet none have been unequivocally identified as causal

agent. Several inherited forms of PD are recognized, and these may have substantial genotypic and phenotypic heterogeneity:[1] PARK1 (*α-synuclein*, 4q21-23); PARK2 (*Parkin*, 6q25.2-27); PARK3 (2p13); PARK4 *(α-synuclein*, 4q21); PARK5 (*UCHL1*, 4p14); PARK6 (*PINK1*, 1p36); PARK7 (*DJ-1*, 1p36); PARK8 (*LRRK2*, 12q12); PARK9 (*ATP13A2*, 1p36); PARK10 (1p); PARK11 (2q36); PARK12 (Xq21-q25). One of the most recently identified of these, *LRRK2*, has been shown to have differing mutations of varying frequency among different ethnic groups. The high frequency of the G2019S mutation in North African Arabs (30%) and Ashkenazi Jewish PD subjects (18%) supports the presence of an ancient founder mutation in these populations.[2,3] A significant proportion of mutations in *LRRK2* reported to date are located in exons 31 and 41 of *LRRK2*, particularly mutations affecting amino acids 1441 and 2019 (R1441G, R1441C, R1441H, G2019S, and I2020T).[4-7] We screened these two exons in a sample of French Canadian (FC) PD patients. A case-control association study was also performed to evaluate whether any other common genetic variations within *LRRK2* were associated with PD in the FC founder population.

2.3 Methods

Cases were recruited through a designated movement disorder clinic in Quebec City (Quebec, Canada), located at the CHAUQ (Enfant-Jésus). Every index case was seen by a neurologist specialized in movement disorders and had to meet the Ward and Gibb criteria for PD.[8] Additionally, they had to be dopa-responsive. A detailed standardized clinical assessment form was completed for each subject. All cases signed a consent form approved by the ethics committee of the CHAUQ prior to being enrolled in the study. A structured questionnaire was used during the interview to assess environmental, medical, lifestyle, and familial risk factors. Controls consist of a non-disease group of similar age and same ethnicity as the cases. Patients and controls were collected from the same base population.

Upon receipt of informed consent, blood samples were obtained from 125 affected individuals and 95 controls. DNA was extracted from peripheral blood by standard methods. Exons 31 and 41 of *LRRK2* were amplified by PCR with intronic primers in all affected individuals and directly sequenced on an ABI 3700 sequencer, according to the manufacturer's recommended protocol (Applied Biosystems, Foster City, CA). Six single nucleotide polymorphisms (SNPs) were typed in all affected individuals and non-disease controls. The SNPs were selected using Applied Biosystems SNPbrowser™ Software and the HapMap project Linkage Disequilibrium (LD) map included within the software. At least three SNPs per Linkage Disequilibrium Unit (LDU) were chosen for a complete coverage of *LRRK2*. Due to the high LD of this locus, only six SNPs were sufficient for the purpose of this association study (**Table 2.1**). Associations between unrelated cases and matched controls were analyzed by the program COCAPHASE included in the software package UNPHASED.[9] This software is based on likelihood ratio tests in a log-linear model. Single marker analysis and haplotype association tests were performed. Haplotype sliding windows containing from 2 to 6 SNPs were analyzed (**Table 2.2**).

2.4 Results

A summary of the clinical data can be found in **Table 2.3**. Sequencing analysis did not reveal any reported or novel mutations in exons 31 and 41 of *LRRK2*. Neither non-reported synonymous coding changes nor non-synonymous coding changes were found. The G2019S mutation as well as mutations affecting amino acid 1441 were absent in all 125 affected individuals. The case-control association study on 125 affected individuals and 95 controls did not provide any positive signal that would suggest the presence of a common variant of *LRRK2* in the FC founder population. As shown in **Table 2.2**, single-marker and haplotype analyses systematically gave non-significant P values.

2.5 Discussion

The Quebec population contains about six million FC who are descendants of around 8500 permanent French settlers who colonized "Nouvelle-France" between 1608 and 1759. For socioeconomic, religious, and linguistic reasons the descendants of the initial settlers did not mix with other immigrants for over three centuries. There was sustained demographic growth, with doubling of the population every 25-30 years, thereby giving rise to a founder population undergoing rapid expansion. The relative contribution of each founder to the FC population varies depending on when they arrived in North America, in that the 2600 founders who settled before 1680 contributed approximately 70% of the present gene pool. From the above, we can conclude that a large segment of the FC population shares a relatively homogeneous genetic background, thereby reducing the possible number of disease producing susceptibility alleles present. The genetic risk factors involved in PD have been examined in the FC population mainly through the work of Andre Barbeau. In a survey of 300 consecutive FC cases with PD and 300 age-matched controls, 13% of PD patients and 5% of controls had one or more first degree relatives with either probable PD or essential tremor. In an additional survey of 135 cases of PD (onset before 40 years) and 30 controls, 45% of PD patients and 3% of controls had one or more first degree relatives with either probable PD or essential tremor.[10] These findings support the presence of important genetic factors for PD in this population, and our study is the first molecular study to be performed in FCs with this disease. In light of the characteristics of the FC population, we had anticipated that, as for the North African Arab and Ashkenazi Jewish populations, a significant proportion of PD could be related to mutations in *LRRK2*. To the contrary, we have observed no contribution of this gene to PD in the FC population. Sequencing of exons 31 and 41 of *LRRK2* demonstrated that none of our patients carried the common mutations. Our data show that G2019S is unlikely to be a common cause of PD in this founder population. However, a larger sample would be needed to assess the exact frequency of G2019S in this population. We are also aware that the association

results presented here should be taken with caution due to the small sample size of the study. A larger sample of FC patients would be needed to completely exclude the hypothesis of a common variant within *LRRK2* predisposing to PD in the FC population. Similarly to our study, genetic analysis of the Nurr1 gene and the neurofilament M gene in Canadian and FC patients with PD failed to identify common genetic variants predisposing to PD. These data, together with ours tend to confirm that PD is genetically extremely heterogeneous even in a sample originating from an isolate population.[11,12]

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2.8 Tables

Table 2.1. SNPs selected in the *LRRK2* gene. CEU MAF: allele frequency determined in the HapMap project using 60 CEPH Utah from Northern and Western Europe. CEU LDU: Linkage disequilibrium units based on 60 CEPH Utah from Northern and Western Europe.

SNP ID	Position	CEU MAF	CEU LDU	SNP type
rs10878245	38918058	40	964.3	Synonymous coding
rs7132171	38936016	43	964.58	Intron
rs11175784	38945801	49	964.85	Intron
rs10878368	39001052	38	965.11	Intron
rs4768232	39023507	12	965.48	Intron
rs3886747	39048218	33	965.55	Untranslated

Table 2.2. *P* values of single-marker and two to six-marker haplotype sliding window association tests. Window n: marker haplotype sliding window.

	SNP ID	Single marker	window 2	window 3	window 4	window 5	window 6
1	rs10878245	0.053	0.063	0.1	0.15	0.19	0.26
2	rs7132171	0.51	0.2	0.31	0.49	0.54	
3	rs11175784	0.2	0.35	0.32	0.86		
4	rs10878368	0.19	0.34	0.84			
5	rs4768232	0.44	0.62				
6	rs3886747	0.87					

Table 2.3. Characteristics of the PD population recruited.

Clinical data	No of affected / 125 (%)
Male sex	89 (71%)
Originate from Eastern Quebec	125 (100%)
French-Canadian ancestry	125 (100%)
Age of onset < 40	11 (9%)
Positive family history (first degree relative)	13 (10%)
Positive family history (overall)	28 (22%)
Age of onset, mean (range)	55.3 (30-79)
Duration of symptoms, mean	8.0
Hoen & Yahr stage at assessment, mean	1.90

Chapitre 3 : Séquençage de gènes candidats codant pour des partenaires d'interaction de parkin

Présentation de l'article

Tel que discuté en introduction, nous avons formulé l'hypothèse que les principaux facteurs génétiques prédisposant à la MP sont des mutations rares dans un grand nombre de gènes (section 1.9.2). En partant du principe que la méthode la plus directe pour identifier ces mutations serait de séquencer un certain nombre de gènes candidats chez des patients parkinsoniens, la question centrale était de tenter de prédire quels types de gènes sur les 20 000 ou 25 000 que comporte le génome humain *(216)* pourraient être plus susceptibles de contenir des mutations prédisposant à la MP. À ce jour, des mutations dans cinq gènes ont été invariablement liées à la MP (*SNCA*, *LRRK2*, *Parkin*, *PINK1*, *DJ-1*) *(55-60)*. Plus la caractérisation des produits de ces gènes progresse, plus il devient clair qu'ils agissent en partie dans des voies moléculaires communes (voir section 1.7.3). L'exemple le plus frappant est le lien fonctionnel entre *Parkin*, *PINK1* et *DJ-1 (177, 178, 217)*. Il a aussi été démontré à partir de neurones en culture que LRRK2 interagit avec parkin *(218)*. Chez la drosophile, la surexpression de parkin protège les neurones dopaminergiques contre la dégénérescence induite par la surexpression de versions mutantes de la protéine LRRK2 humaine *(219)*. De plus, il semble que LRRK2 phosphoryle la sérine 129 de l'alpha-synucléine *(220)*, un mécanisme qui serait en partie responsable de la formation des corps de Lewy *(221, 222)*.

À la lumière de ces observations, nous en sommes arrivés à la conclusion que les gènes les plus susceptibles de contenir des mutations prédisposant à la MP codent pour des protéines 1) exprimées dans le cerveau, 2) impliquées dans les voies moléculaires dysfonctionnelles dans la MP et/ou 3) qui interagissent avec parkin, LRRK2, alpha-synucléine, PINK1 ou DJ-1. Nous avons donc séquencé la région codante de 25 gènes respectant ces critères chez 95 patients CF et nous avons analysé plus en détail les gènes présentant des mutations potentiellement pathogéniques chez des patients et des contrôles additionnels. L'article présenté ci-dessous décrit les résultats obtenus pour huit de ces gènes (*BAG5*, *CDK5*, *DNM1L*, *EPS15*, *PICK1*, *UBE2L3*, *UBE2L6*, et *RNF11*). Chacun d'entre eux code pour une protéine fonctionnellement associée à parkin. En plus de son rôle dans le système ubiquitine-protéasome *(160)*, on sait maintenant que parkin interagit avec des

protéines impliquées dans la transmission synaptique *(223)* et régule certaines fonctions mitochondriales *(173, 224, 225)*. Les gènes sélectionnés illustrent bien ces diverses fonctions. *UBE2l3* et *UBE2L6* codent pour UbcH7 et UbcH8, des enzymes E2 de conjugaison de l'ubiquitine qui interagissent avec parkin *(160)*. RNF11 est une E3 ubiquitin ligase retrouvée dans les corps de Lewy *(226)*. BAG5 inhibe l'activité E3 ubiquitin ligase de parkin, promeut sa séquestration dans des agrégats protéiques et augmente la dégénérescence des neurones dopaminergiques chez les souris traitées au MPTP *(227)*. CDK5 phosphoryle parkin *(228)* et régule la fission mitochondriale *(229)*. L'interaction de parkin avec EPS15 *(230)* et PICK1 *(231)* indique que parkin régule l'internalisation de divers récepteurs membranaires. Enfin, il a récemment été démontré que la perte de parkin augmente le processus de fission mitochondriale dépendant de Drp1 (encodé par *DNM1L*) *(225)*. Il faut noter que notre approche n'est pas systématique. En effet, ces huit gènes ne constituent pas tous les partenaires d'interaction de parkin. Nous avons cependant sélectionné les gènes qui, à nos yeux, étaient les plus susceptibles d'être impliqués dans les mécanismes moléculaires sous-tendant la MP d'après les données fonctionnelles disponibles sur les protéines qu'ils encodent.

La recherche génétique sur la MP se concentre surtout sur les études de liaison génétique dans des grandes familles pour expliquer les formes mendéliennes de la maladie et les études d'association pour comprendre les formes sporadiques. De ce fait, le séquençage direct de gènes candidats dans des patients majoritairement sporadiques allait à contre-courant de la majorité des recherches actuelles. L'obtention des ressources nécessaires à un séquençage à grande échelle passait donc nécessairement par un projet pilote basé sur un nombre limité de gènes.

Contribution des auteurs

JBR : conception de l'étude, acquisition et analyse des résultats génétiques et rédaction de l'article.

AG : acquisition et analyse des résultats fonctionnels et rédaction de l'article.

JS : acquisition et analyse des résultats génétiques.

AN : soutien technique.

SC, **EP**, **MP**, **NJ**, **VS**, **ML**, **PP**, **SD**, **CK** et **ND** : recrutement des patients.

EAF : sélection des gènes candidats, supervision du projet et rédaction de l'article.

PD et **GAR** : supervision du projet et rédaction de l'article.

Sequencing of candidate genes of the parkin pathway in Parkinson's disease reveals rare mutations in the *PICK1* gene

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Manuscrit en préparation.

3.1 Abstract

Background: Parkinson's disease is a complex neurodegenerative condition characterized by extreme genetic heterogeneity. Although a number of disease-causing mutations in several genes have been uncovered, the vast majority of genetic predisposing factors remain unknown. The identification of causative genes has nonetheless pointed towards several key molecular mechanisms involved in the neurodegenerative process of the disorder and we hypothesized that mutations in additional genes, functionally associated with the known causative gene pathways, may account for the genetic predisposition of some remaining cases with Parkinson's disease.

Methods: To test this assumption, we directly sequenced in 95 French Canadian individuals with Parkinson's disease the coding regions of eight candidate genes encoding proteins known to be functionally associated with parkin, an E3 ubiquitin ligase involved in mitochondrial function. Two of the genes that displayed potentially pathogenic variants, namely *UBE2L3* and *PICK1*, were further screened in an extended cohort of French Canadian and German patients and controls.

Results: Our genetic screening uncovered four rare mutations in the *PICK1* gene that were carried by five unrelated patients and absent in controls subjects. According to functional studies, at least one of these mutations is likely pathogenic through a dominant negative effect. Given that PICK1 is involved in internalization of AMPA receptors these findings provide the first direct genetic evidence that glutamate neurotransmission may be disrupted in Parkinson's disease.

Conclusions: Our findings further support the previously suspected pathogenic role for glutamate excitotoxicity in Parkinson's disease as well as the emerging evidence that drugs modulating the activity of glutamate receptors may improve symptoms of Parkinson's disease.

3.2 Introduction

Parkinson's disease is a complex neurodegenerative disorder with significant genetic predisposing factors that are extremely heterogeneous. The vast majority of affected individuals are late-onset sporadic cases but familial forms also exist.[1] To date, five "core" causative genes (*SNCA*, *LRRK2*, *Parkin*, *PINK1* and *DJ-1*) widely-believed to be central to the pathogenesis of Parkinson's disease have been uncovered.[2-7] Heterozygous mutations in *GBA* have also consistently been associated with the disorder.[8] Although most of the known pathogenic mutations account for rare familial forms of Parkinson's disease, these genetic breakthroughs have shed light on the pathogenesis of the disorder at the molecular level. Functional analyses of these gene products pinpointed dysfunction of key molecular pathways involved in the neurodegenerative process of Parkinson's disease, including dysfunction in the ubiquitin system and alpha-synuclein aggregation/Lewy body formation, alteration of oxidative stress response and disruption of mitochondrial function.[9] The E3 ubiquitin ligase parkin has a central role in these pathways. Indeed, in addition to its role in proteasomal-mediated degradation,[10] parkin has been shown to regulate mitochondrial function through the same molecular pathway as PINK1.[11, 12] Furthermore, a functional relationship has been observed between parkin, PINK1 and DJ-1 as the three proteins are thought to form an E3 ubiquitin ligase complex that promotes the degradation of parkin targets.[13]

A key issue in the genetics of Parkinson's disease remains the identification of causative genes underlying the more common sporadic form of the disease. Two schools of thought propose opposite, but not exclusive, models to explain the heritability of multifactorial disorders such as Parkinson's disease. The "common disease-common variant" hypothesis posits that common and low penetrant polymorphisms detectable by genome-wide association studies significantly account for the genetic predisposition of complex traits. The "common disease-rare variant" hypothesis assumes that such disorders

are characterized by extreme genetic heterogeneity and may be caused by relatively high penetrant but individually rare mutations.[14] Although two recent and promising genome-wide association studies identified several common polymorphisms associated with Parkinson's disease, these variants only confer small increments in risk and cannot by themselves alone explain the disease heritability.[15, 16] Because of the allelic and non-allelic genetic heterogeneity observed for this disease, dozens of genes may carry rare pathogenic mutations. We hypothesized that rare mutations in additional genes functionally associated with the known causative genes may account for the genetic predisposition of Parkinson's disease. As a proof of principle, we directly sequenced the coding regions of eight candidate genes (*BAG5*, *CDK5*, *DNM1L*, *EPS15*, *PICK1*, *UBE2L3*, *UBE2L6*, and *RNF11*) encoding proteins functionally associated with parkin (**Table 3.1**) in a cohort 95 French Canadian individuals with Parkinson's disease from the province of Quebec and mostly composed of late-onset sporadic cases. Genes displaying potentially pathogenic variants were further investigated in an extended cohort of patients and control subjects from Quebec and Germany.

3.3 Methods

3.3.1 Sample collection

Five-hundred patients were recruited through designated movement disorder clinics in Quebec and Germany. Index cases were diagnosed according to the Ward and Gibb criteria for Parkinson's disease[17] and the United Kingdom Parkinson's disease Brain Bank Criteria. Standard protocol approvals, registrations, and patient consents protocols were approved by the local ethics committees on human experimentation. Written inform consent was obtained from all cases prior to being enrolled in the study. General characteristics of the French Canadian (Quebec) and German cohorts of patients are shown in **Table 3.2**. We also recruited 476 healthy spouses of patients or ethnically and age-matched controls subjects including 190 French Canadian and 286 German individuals.

They underwent the same neurological examination as the patients. Blood samples were obtained and DNA was extracted from peripheral blood cells using standard methods.

Our research team had previously screened the *PICK1* gene in 380 individuals (mainly French Canadian and European Caucasians) as part of a large-scale resequencing project known as S2D and not related to Parkinson's disease but rather aimed at identifying mutations in autism, schizophrenia and mental retardation.

3.3.2 Amplicon design and gene screening

We identified the full mRNA sequence of all potential transcriptional isoforms of the eight selected genes and determined the exon/intron structures based on alignment from the publicly available human genome assembly (UCSC Genome Browser, http://genome.ucsc.edu/). Primers were designed using the "Exon Primer" software from the UCSC genome browser to amplify 200–700 bp fragments covering coding exons plus at least 50-bp into introns before and after each exon. We used 77 pairs of primers in order to amplify fragments covering the entire open reading frames of the eight candidate genes. Polymerase chain reaction products were sequenced at the Genome Quebec Centre for Innovation according to standard procedures. Variants identified were confirmed by a second polymerase chain reaction amplification followed by forward and reverse resequencing. We concentrated on coding variants, as these are more likely to be functional and are easier to validate. We verified the presence of each one of the variants in the NCBI single nucleotide polymorphism database (dbSNP, http://www.ncbi.nlm.nih.gov/snp/).

3.3.3 Statistical analysis

Fisher's exact test was performed with the use of the 2BY2 program from the "UTIL package" (from Dr Jurg Ott, Rockefeller University, NY) to compare the rates of non-synonymous *PICK1* mutations in 500 patients with Parkinson's disease and in 856 control subjects (including the 380 samples from the S2D project).

3.4 Results

3.4.1 Stage 1

Firstly, we screened the entire coding sequence of *BAG5*, *CDK5*, *DNM1L*, *EPS15*, *PICK1*, *UBE2L3*, *UBE2L6*, and *RNF11* in 95 unrelated French Canadian individuals randomly selected from our cohort of 212 cases with Parkinson's disease (Table 3.2). Sequencing analysis revealed nineteen exonic variants, including one frameshift insertion, one in-frame deletion, five missense and twelve silent changes. Among those, eleven were novel variants not reported in dbSNP (**Table 3.3**). None of the genes tested presented a high number of non-synonymous coding variants. No coding variants were found in *UBE2L6* and *RNF11*; no unreported non-synonymous coding variants were identified in *BAG5* and *CDK5* whereas *DNM1l* and *EPS15* displayed a single unreported missense variant each. Thus, sequencing analysis did not provide sufficient evidence to suspect that mutations in any of these six genes may predispose patients to Parkinson's disease. Conversely, sequencing data from *UBE2L3* and *PICK1* warranted a more detailed investigation. One patient was found to carry a heterozygous 79-bp frameshift insertion (c.358_359ins79; V120EfsX9. Fig. 1A-B) located within an alternatively spliced C-terminal exon of one of the two isoforms of *UBE2L3* (NM_198157). Direct sequencing of this exon indicated that the mutation was absent from our 190 French Canadian control subjects.

Sequencing of *PICK1* identified two unreported variants carried by one patient each, namely a 6-bp in-frame deletion (c.1158_1163del6; E387_E388del) predicted to withdraw two amino acid residues from the C-terminal acidic region of PICK1 and a missense change (c.473G>A; R158Q) located within the BAR domain (Table 3.3). Interestingly, the previous sequencing of *PICK1* in 380 individuals from the S2D project (see the *Methods* section for details) had not revealed any non-synonymous coding change

in the gene. The number of non-synonymous coding changes in the 95 individuals with Parkinson's disease was found to be higher than that in the series of individuals with autism spectrum disorders, schizophrenia or mental retardation tested for *PICK1* mutations (Fisher's exact test, P = 0.04).

3.4.2 Stage 2

In attempt to further assess the role of *UBE2L3* in Parkinson's disease, we sequenced the entire coding region of the gene in 117 additional French Canadian affected individuals (for a total of 212 French Canadian patients) and in 288 unrelated cases from Germany (Table 3.2). No additional coding variants were identified in the gene, either suggesting that causative mutations in *UBE2L3* are extremely rare, or that this heterozygous frameshift mutation is not pathogenic for Parkinson's disease.

Screening of the entire coding sequence of the *PICK1* gene in our additional 405 French Canadian and German patients with Parkinson's disease revealed another missense change (c.554G>A; R185Q) and a nonsense mutation (c.1236G>A; W412X) carried by two and one patients, respectively. Overall, four distinct rare non-synonymous coding variants of *PICK1* were identified in five out of 500 patients with Parkinson's disease from two distinct populations (**Figure 3.1**, **Table 3.4**, and **Supplementary figure 3.1**). Sequencing of the age-matched control subjects revealed three single missense changes carried by three individuals (V64G, S181L and E388dup; Table 3.4), further confirming, as previously observed from the S2D cohort, that *PICK1* only tolerates a low degree of sequence variation. None of the four coding changes identified in the five patients were found in the 476 age-matched control subjects and the series of 380 patients with autism spectrum disorders, schizophrenia or mental retardation (1712 total chromosomes). The rate of *PICK1* mutations in our sampled cases of Parkinson's disease (1%) was 2.8 times higher than that observed in the other groups (0.35%). This difference was nonetheless non-

significant (Fisher's exact test, $P = 0.15$) due to the limited size of our cohort to achieve sufficient statistical power to evaluate the contribution of such rare mutation rates.

The two missense mutations found in patients (R158Q and R185Q) are predicted to substitute an uncharged glutamine residue for a positively charged arginine residue at codons 158 and 185. Both are well conserved amino acid residues (Supplementary figure 3.1) located within the functional BAR domain of PICK1 which is involved in the subcellular localization of the protein through the binding to phospholipid membranes.[18] BAR domains are enriched in positively-charged arginine and lysine residues that bind to negatively charged phosphoinositides at the plasma membrane. In dimeric proteins, BAR domains form banana-shaped structures that interact with vesicles and invaginating membranes.[19] Substitutions of negatively-charged glutamate for lysine residues located within the BAR domain of PICK1 have been shown to disrupt the lipid binding capability of the protein[18, 20] but such a role for arginine residues in positions 158 and 185 has never been assessed.

3.5 Discussion

Parkin and its associated E2 ubiquitin conjugating proteins UbcH7 and UbcH8 have been shown to ubiquitinate a variety of different substrates.[21] The identification of a mutated form of UbcH7 (encoded by *UBE2L3*) in one patient originally suggested a possible link between mutations in *UBE2L3* and Parkinson's disease. Unfortunately, the lack of additional mutations in the gene in our extended cohort of patients failed to support this hypothesis. The patient bearing the frameshift mutation is a French Canadian individual that presented a typical idiopathic sporadic Parkinson's disease with an age of onset at 53 years and a positive response to levodopa (data not shown). These inconclusive data share similarities with the unreplicated report of a unique missense mutation in *UCHL1*, another gene encoding an enzyme involved in proteasome-mediated degradation.[22]

Replication studies in other cohorts will be necessary to assess the exact role of *UBE2L3* in Parkinson's disease.

By opposition, the extended sequencing of *PICK1* in additional patients and control subjects reinforced the preliminary results obtained in stage 1. Although the overall rate of non-synonymous coding variants in patients versus controls is non-significant, the fact that the four mutations identified in five out of 500 patients were absent from the 856 control individuals suggests that rare coding variants of *PICK1* are associated with Parkinson's disease (Fisher's exact test, $P = 0.007$). Conversely, the observation of three non-synonymous coding variants in 856 control subjects not found in patients with Parkinson's disease was likely due to chance (Fisher's exact test, $P = 0.3$). Moreover, the locations of the mutations found in patients provided clues about the functional significance of the genetic data. The *PICK1* gene encodes a synaptic scaffolding protein that binds to a wide range of ion channels, neurotransmitter receptors and transporters, including AMPA receptor subunits GluR2/3.[23] The 415 amino acid polypeptide of PICK1 comprise a PDZ domain, a BAR domain and a C-terminal acidic region constituted of a stretch of glutamic and aspartic acid residues (**Figure 3.1D**).[24] Two of the four mutations carried by individuals with Parkinson's disease are predicted to disrupt the C-terminal acidic region and the tryptophan residue in position 412 (413 in rat) of PICK1 (E387_E388del and W412X). According to the observation that these motifs are found in known interacting partners of the ARP2/3 complex, detailed functional studies in cultured neurons have shown that PICK1 binds to F-actin and to the Arp2/3 complex and inhibits Arp2/3-mediated actin polymerization; a mechanism demonstrated to be necessary for the proper PICK1-dependant NMDA-induced AMPA receptor internalization.[25] Furthermore, although overexpression of PICK1 induced enhanced AMPA receptor endocytosis through the inhibition of the Arp2/3 complex, the PICK1^{W413A} mutant eliminated Arp2/3 binding, failed to inhibit actin polymerization and more importantly, blocked NMDA-induced AMPA receptor endocytosis by a dominant-negative effect. These functional data point towards disrupted AMPA receptor trafficking in Parkinson's disease, at least in the patient

carrying the W412X mutation and strongly suggest that this heterozygous nonsense mutation is pathogenic through a dominant-negative effect.

Four of the five patients carrying *PICK1* mutations are idiopathic sporadic Parkinson's disease cases with an age of onset ranging from 53 to 72 years (Table 3.4). Unfortunately, for the W412X carrier, familial history of Parkinson's disease was not available. The absence of reported familial history of Parkinson's disease in their first degree relatives suggests that these mutations may have incomplete penetrance. In the absence of additional family members with Parkinson's disease to perform segregation analysis and given the rarity of the mutations found in affected individuals, the issue is to define whether these mutations are pathogenic. A duplication of a glutamic acid residue (E388dup) in the C-terminal acidic region of PICK1 was found in a control individual, thus challenging the biological relevance of the E387_E388del mutation carried by a case with Parkinson's disease.

Altogether, our findings with the support of previous functional data indicate that mutations in *PICK1* constitute a rare predisposing factor for late-onset Parkinson's disease, which is consistent with the reported role of enhanced responsiveness to glutamate and associated excitotoxicity in the pathogenesis of Parkinson's disease.[26, 27] Dopaminergic neurons of the substantia nigra pars compacta whose the massive loss underlies Parkinson's disease display high concentration of glutamate receptors[28] and overactivity of excitatory glutamate neurons of the subthalamic nucleus that project to these dopaminergic neurons is thought to enhance this neurodegenerative process.[27]

Strikingly, parkin has recently been shown to decrease postsynaptic excitatory glutamatergic transmission whereas loss of parkin or expression of mutated forms of the protein render neurons vulnerable to excitotoxicity, through enhanced glutamatergic transmission and proliferation of excitatory synapses.[29] Given that PICK1 functionally interacts with parkin via a PDZ-mediated interaction,[30] PICK1 might be the missing

molecular link between pathogenic mutations of *Parkin* and neuronal vulnerability caused by aberrant glutamate neurotransmission.

Finally, our findings further support the emerging evidence that drugs modulating the activity of glutamate receptors may be promising to treat patients with Parkinson's disease.[31, 32] The NMDA receptor antagonist amantadine is already used in Parkinson's disease[33] and other glutamate receptor antagonists are in active development and testing.[34, 35]

3.6 Acknowledgements

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3.8 Tables and figures

Table 3.1. List of the eight selected genes encoding proteins functionally associated with parkin.

Gene	**Functional link with parkin**
BCL2-associated athanogene 5 (*BAG5*)	Inhibits parkin, involved in degeneration of dopaminergic neurons.[36]
Cyclin-dependent kinase 5 (*CDK5*)	Phosphorylates parkin,[37] regulates mitochondrial fission.[38]
Dynamin 1-like (*DNM1L*) Drp1	Promotes mitochondrial fission in parkin-deficient cells.[39]
Epidermal growth factor receptor pathway substrate 15 (*EPS15*)	Ubiquitinated by parkin, involved in EGF receptor trafficking.[40]
Protein interacting with PRKCA1 (*PICK1*)	Ubiquitinated by parkin,[30] involved in AMPA receptor trafficking.[41]
Ubiquitin-conjugating enzyme E2L 3 (*UBE2L3*) UbcH7	E2 ubiquitin-conjugating enzyme associated with parkin.[10]
Ubiquitin-conjugating enzyme E2L 6 (*UBE2L6*) UbcH8	E2 ubiquitin-conjugating enzyme associated with parkin.[10]
Ring finger protein 11 (*RNF11*)	E3 ubiquitin ligase and component of Lewy bodies.[42]

Table 3.2. Characteristics of the cohorts of patients used in stage 1 and 2.

Clinical data	**Stage 1**	**Stage 2**	
	FC cohort	**FC cohort**	**German cohort**
Unrelated individuals	95	212*	288
Male sex	70.5%	66%	51%
Ethnicity	Caucasian, French Canadian	Caucasian, French Canadian	Caucasian, German
Positive familial history (1st degree relative)	7.4%	12%	19.8%
Age of onset, mean (range)	54.5 (32-76)	56.8 (30-80)	49.1 (16-79)

Abbreviations. FC. French Canadian. *Including the 95 individuals from stage 1.

Table 3.3. Summary of coding variants identified.

Gene	Nucleotide	Amino acid	Type	dbSNP	htz/hmz	Freq (/95)	HWE
BAG5	c.75A>G	P25P	SC	rs7148456	htz/hmz	51/3	0.71
CDK5	c.246C>T	F82F	SC	-	htz	1	1.0
DNM1L	c.120A>C	S40S	SC	rs10844308	htz/hmz	29/3	0.98
DNM1L	c.252G>A	G84G	SC	rs2272238	htz/hmz	29/4	0.98
DNM1L	c.918A>G	T306T	SC	rs10844318	htz/hmz	28/4	0.89
DNM1L	c.966C>T	Y322Y	SC	-	htz	3	1.0
DNM1L	c.1470A>G	E490E	SC	-	htz	2	1.0
DNM1L	c.1715C>G	S572C	NSC	-	htz	1	1.0
DNM1L	c.1968C>T	L656L	SC	-	htz	9	1.0
DNM1L	c.2157A>G	A719A	SC	-	htz	1	1.0
EPS15	c.603G>T	V201V	SC	-	htz	2	1.0
EPS15	c.669A>C	A223A	SC	rs34704431	htz	7	1.0
EPS15	c.1313C>T	S438L	NSC	rs41292521	htz	5	1.0
EPS15	c.1329C>T	Y443Y	SC	rs1065754	htz/hmz	36/18	0.11
EPS15	c.2060C>T	T687M	NSC	-	htz	1	1.0
EPS15	c.2466A>G	I822M	NSC	rs17567	htz/hmz	36/4	0.79
PICK1	c.473G>A	R158Q	NSC	-	htz	1	1.0
PICK1	c.1158_1163del6	E387_E388del	IF del	-	htz	1	1.0
UBE2L3	c.358_359ins79	V120EfsX9	FS ins	-	htz	1	1.0

Abbreviations: SC. Synonymous coding variant. NSC. Non-synonymous coding variant. FS ins. Frameshift insertion. IF del. In-frame deletion. Htz/hmz. Changes found in a heterozygous state (htz), a homozygous state (hmz), or both (htz/hmz). Freq. Number of individuals carrying a given variant out of 95 individuals with PD. Hardy-Weinberg

equilibrium test (HWE) was performed using Haploview, version 4.1. Reference sequences used for the description of sequence variations: *BAG5* (NM_001015049), *CDK5* (NM_004935), *DNM1L* (NM_012062), *EPS15* (NM_001981), *PICK1* (*NM_001039583*), and *UBE2L3* (NM_198157). No coding variants were found in *UBE2L6* and *RNF11*.

Table 3.4. Individuals carrying non-synonymous coding changes in *PICK1*.

Individual	Nucleotide	Amino acid change	Sex	AOO	FH	Levodopa responsive	Polyphen[a]	SIFT[b]	Panther[c]	ESEfinder
1. Individuals with Parkinson's disease (n = 500)										
FC PD case	c.473G>A	R158Q	F	53	no	yes	Benign (1.2)	Tolerated (0.58)	-3.09	Affect ESE
FC PD case	c.1158_1163del6	E387_E388del	M	66	no	yes	n.a.	n.a.	n.a.	Affect ESE
FC PD case	c.554G>A	R185Q	F	72	no	yes	Benign (0.2)	Tolerated (0.38)	-1.47	Affect ESE
German PD case	c.554G>A	R185Q	F	58	no	yes				
German PD case	c.1236G>A	W412X	F	57	unknown	yes	n.a.	n.a.	n.a.	Affect ESE
2. Control subjects (n = 856)										
FC control	c.542C>T	S181L					Benign (0.32)	Tolerated (0.97)	-1.57	Affect ESE
FC control	c.1161_1163dup3	E388dup					n.a.	n.a.	n.a.	Affect ESE
German control	c.191T>G	V64G					Possibly damaging (1.89)	Affect protein function (0)	-3.94	Affect ESE

Abbreviations. F. female. M. male. AOO. Age of onset. FH. Reported familial history of Parkinson's disease in first degree relatives. Three bioinformatic prediction programs (Polyphen, SIFT and Panther) were used to predict the impact of amino acid substitutions on the structure and function of the protein. [a]The lower the score, the more benign the substitution is. [b]The higher the score, the more tolerated the change is. [c]A score of 0 to -3 is considered to be tolerated whereas a score from -3 to -10 is not tolerated. ESEfinder was used to predict the effect of variants on splice sites and/or exonic splicing enhancers (ESEs).

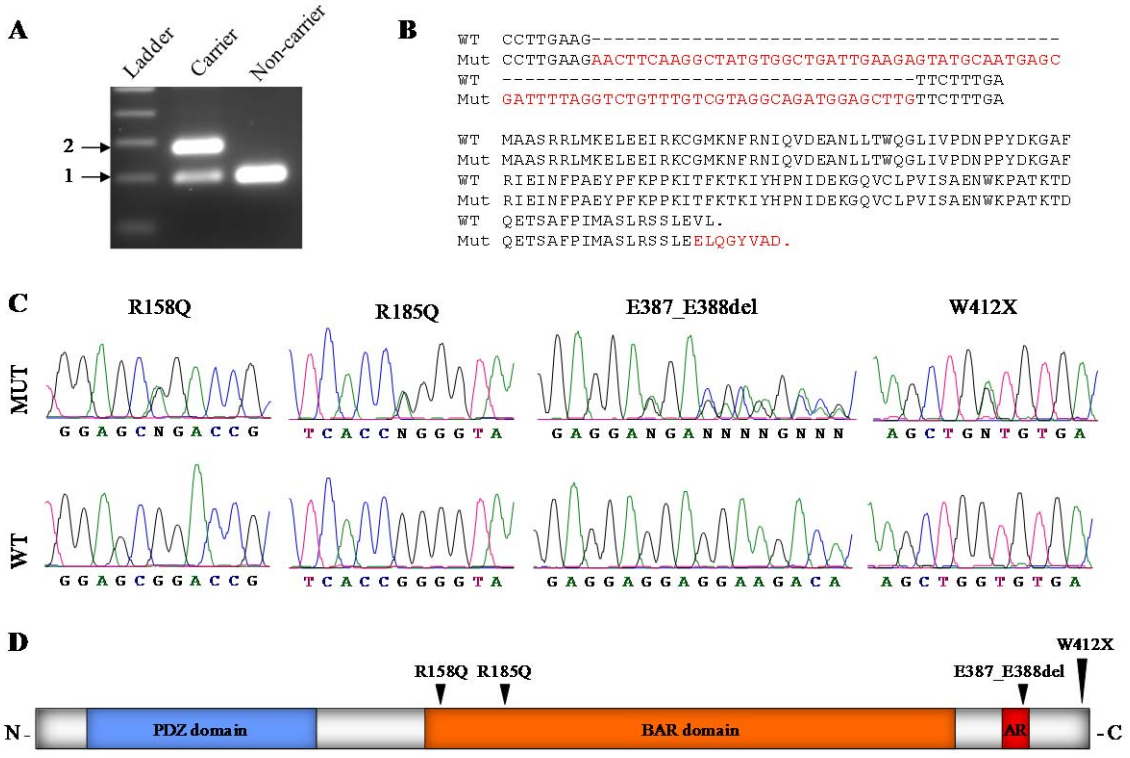

Figure 3.1. Mutations in *UBE2L3* and *PICK1*. (**A**) Polymerase chain reaction product of the patient carrying a heterozygous 79-bp insertion in *UBE2L3* (middle) compared to a non-carrier (right). The wild-type allele (203-bp) and the mutated allele (282-bp) are indicated by arrows 1 and 2, respectively. (**B**) Top: DNA sequences of the wild-type allele of *UBE2L3* (WT) and the 79-bp insertion in red (Mut). Bottom: Predicted effect of the *UBE2L3* frameshift insertion (V120EfsX9) on protein sequence (NP_937800.1). (**C**) Sequence traces of the four *PICK1* mutations identified in five individuals with Parkinson's

disease (MUT) and control sequence traces (WT). (**D**) Linear protein structure of PICK1 showing the locations of the PDZ and BAR domains and the acidic region (AR). Amino acid changes found in patients (R158Q, R185Q, E387_E388del and W412X) are indicated by arrowheads.

3.9 Supplementary material

Supplementary figure 3.1. Multi-species alignment for *PICK1*. Sequence cluster alignment was done by the Clustal W method. Non-synonymous coding variants found in patients are highlighted (yellow when they are conserved between humans and other species; green when they differ). The C-terminal stretch of glutamic and aspartic acid residues is highlighted in grey.

```
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MFADLDYDIEEDKLGIPTVPGKVTLQKDAQNLIGISIGGGAQYCPCLYIV 50 Macaca mulatta
MFADLDYDIEEDKLGIPTVPGKVTLQKDAQNLIGISIGGGAQYCPCLYIV 50 Canis familiaris
MFADLDYDIEEDKLGIPTVPGKVTLQKDAQNLIGISIGGGAQYCPCLYIV 50 Mus musculus
MFADLDYDIEEDKLGIPTVPGTVTLKKDSQNLIGISIGGGAQYCPCLYIV 50 Gallus gallus
MFTDMDYELEEDKLGIPTVPGTVTLKKDSQNLIGISIGGGAQFCPCLYIV 50 Danio rerio

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QVFDNTPAALDGTVAAGDEITGVNGRSIKGKTKVEVAKMIQEVKGEVTIH 100 Macaca mulatta
QVFDNTPAALDGTVAAGDEITGVNGRSIKGKTKVEVAKMIQEVKGEVTIH 100 Canis familiaris
QVFDNTPAALDGTVAAGDEITGVNGKSIKGKTKVEVAKMIQEVKGEVTIH 100 Mus musculus
QVFDNTPAALDGTVAAGDEITGVNGKSVKGKTKVEVAKMIQMVKGEVTIH 100 Gallus gallus
QVFDNTAAALDGTLAAGDEITGVNGKPVKGKTKVEVAKMIQAVQGEVVIQ 100 Danio rerio

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YNKLQADPKQGMSLDIVLKKVKHRLVENMSSGTADALGLSRAILCNDGLV 150 Macaca mulatta
YNKLQADPKQGMSLDIVLKKVKHRLVENMSSGTADALGLSRAILCNDGLV 150 Canis familiaris
YNKLQADPKQGMSLDIVLKKVKHRLVENMSSGTADALGLSRAILCNDGLV 150 Mus musculus
YNKLQADPKQGKSLDIVLKKVKHRLVENMSSGTADALGLSRAILCNDGLV 150 Gallus gallus
YNKLQADPKQGKSLDIVLKKVKHRLVENMSSGTADALGLSRAILCNDGLV 150 Danio rerio

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KRLEELERTAELYKGMTEHTKNLLRAFYELSQTHRAFGDVFSVIGVREPQ 200 Macaca mulatta
KRLEELERTAELYKGMTEHTKNLLRAFYELSQTHRAFGDVFSVIGVREPQ 200 Canis familiaris
KRLEELERTAELYKGMTEHTKNLLRAFYELSQTHRAFGDVFSVIGVREPQ 200 Mus musculus
KRLEELERTAELYKGLTEHTKSLLRAFFELSQTHRAFGDVFSVIGVREPQ 200 Gallus gallus
KRLEELEKTAELYKGLMEHTKRLLRAFYELSQTHRAFGDVFSVIGVREPQ 200 Danio rerio

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PAASEAFVKFADAHRSIEKFGIRLLKTIKPMLTDLNTYLNKAIPDTRLTI 250 Macaca mulatta
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PAASEAFVKFADAHRSIEKFGIRLLKTIKPMLTDLNTYLNKAIPDTRLTI 250 Canis familiaris
PAASEAFVKFADAHRSIEKFGIRLLKTIKPMLTDLNTYLNKAIPDTRLTI 250 Mus musculus
PAASEAFVKFADAHRNIEKFGIHLLKTIKPMLTDLNTYLNKAIPDTRLTI 250 Gallus gallus
AAASEAFVKFAEAHRNMEKFGIQLLKTIKPMLHDLNTYLHKAIPDTKLTI 250 Danio rerio

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KKYLDVKFEYLSYCLKVKEMDDEEYSCIALGEPLYRVSTGNYEYRLILRC 300 Macaca mulatta
KKYLDVKFEYLSYCLKVKEMDDEEYSCIALGEPLYRVSTGNYEYRLILRC 300 Canis familiaris
KKYLDVKFEYLSYCLKVKEMDDEEYSCIALGEPLYRVSTGNYEYRLILRC 300 Mus musculus
KKYLDVKFEYLSYCLKVKEMDDEEYSCIALGEPLYRVSTGNYEYRLILRC 300 Gallus gallus
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RQEARARFSQMRKDVLEKMELLDQKHVQDIVFQLQRLVSTMSKYYNDCYA 350 Macaca mulatta
RQEARARFSQMRKDVLEKMELLDQKHVQDIVFQLQRFVSTMSKYYNDCYA 350 Canis familiaris
RQEARARFSQMRKDVLEKMELLDQKHVQDIVFQLQRFVSTMSKYYNDCYA 350 Mus musculus
RQEARTRFAKMRKDVLEKIELLDQKHVQDIVFQLQRFVSTMSKYYDDCYA 350 Gallus gallus
RQEARARFAKMRKDVLEKIELLDQKHVQDIVFQLQRFVSGMSHYYDDCYA 350 Danio rerio

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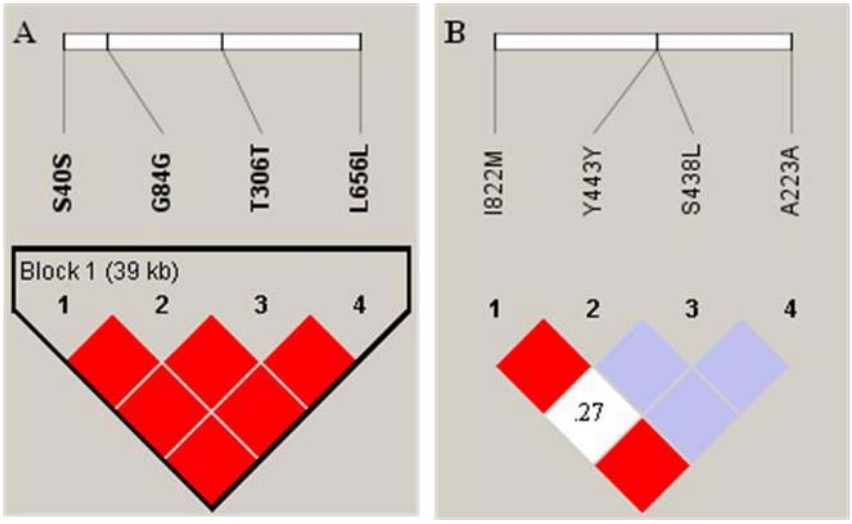
VLRDADVFPIEVDLAHTTLAYGLNQEEFTDGEEEEEE--EDTAAGEPSRD 398 Macaca mulatta
VLRDADVFPIEVDLAHTTLAYGLSQDEFTDGEDEEDEDDEDTATGEPSRD 400 Canis familiaris
VLQDADVFPIEVDLAHTTLAYGPNQGSFTDGEEEDEEE-EDGAAREVSKD 399 Mus musculus
VLRDADVFPIEVDLARTTLNYG-QKDTYTDGAEEEEG-------GGSERE 392 Gallus gallus
VLKDADVFPIEVDLSRTMINYSGQSLTYEDEDEEETSR-----AQNQQQE 395 Danio rerio

TRGAAGPLDKGGSWCDS- 415 Homo sapiens
TRGAAGPLDKGGSWCDS- 415 Macaca mulatta
ARGAAGPLDKGGSWCDS- 417 Canis familiaris
ACGATGPTDKGGSWCDS- 416 Mus musculus
SSGKED--ANGEKLIDDA 408 Gallus gallus
ENGAEKLIDDQ------- 406 Danio rerio
```

Supplementary figure 3.2. Pairwise linkage disequilibrium diagrams for the common SNPs (minor allele frequency > 0.025) found in *DNM1L* (**A**) and *EPS15* (**B**). Pairwise linkage disequilibrium was measured as D' and calculated using Haploview, version 4.1. Red squares indicate D' values of 1 (i.e. complete linkage disequilibrium). Blue squares indicate inability to calculate D' values due to too large D' confidence intervals (0.04-0.99).



Chapitre 4 : La dynamique mitochondriale dans la maladie de Parkinson : séquençage du gène *MFN1*

Présentation de l'article

Tel que mentionné en introduction, il est maintenant clair qu'un dysfonctionnement des mitochondries joue un rôle dans la MP. Si on sait de longue date qu'une inhibition du complexe I de la chaîne respiratoire est impliquée dans la pathogénèse de la MP *(232)*, la plus récente hypothèse pour expliquer la dégénérescence des neurones dopaminergiques propose une perturbation de la dynamique mitochondriale *(190)*. Les mitochondries sont des organelles particulièrement dynamiques qui fusionnent et se divisent constamment, se renouvellent et migrent en fonction des changements environnementaux et des besoins des cellules *(233)*. Ce mécanisme joue notamment un rôle clé au niveau de la survie cellulaire *(234)* et est primordial pour les neurones en raison de leurs importants besoins en énergie et de la nécessité de transporter les mitochondries jusqu'aux synapses. D'ailleurs, plusieurs gènes codant pour des protéines impliquées dans le processus de fusion/fission mitochondriale contiennent des mutations responsables de maladies neurologiques développementales ou dégénératives *(235-237)*. Chez les mammifères, la fusion mitochondriale est contrôlée par trois GTPases, soit les mitofusins (MFN) 1 et 2, situées sur la membrane externe, et OPA1 qui est localisée sur la membrane interne. La fission est assurée par la protéine Drp1 *(233)*.

Un déséquilibre du processus de fusion/fission mitochondriale pourrait être associé à la MP pour plusieurs raisons. La fusion assure le maintien de l'ADN mitochondrial en limitant le nombre de mitochondries n'ayant pas de nucléoïde après des cycles de division *(238)*. La complémentation entre les mitochondries fusionnées permet de restaurer la morphologie et la fonction des mitochondries ayant subi des dommages à leur ADN *(239)*. Une augmentation de la fragmentation mitochondriale pourrait donc être impliquée dans la pathogénèse de la MP sachant que cette dernière a été associée à des dommages à l'ADN mitochondrial *(164, 165, 240)*. Il est même possible que le taux plus élevé de mutations observé dans l'ADN mitochondrial de patients parkinsoniens soit une conséquence d'un déséquilibre de la dynamique mitochondriale combiné à une augmentation du stress oxydatif. De plus, L'exposition de neurones primaires ou de neuroblastes en culture à des neurotoxines qui provoquent la mort des neurones dopaminergiques comme la roténone, la

6-hydroxydopamine ou le MPP+ provoque une fragmentation mitochondriale *(229, 241, 242)*. Enfin, des travaux sur parkin et PINK1 chez la drosophile ont démontré que ceux-ci régulent la dynamique mitochondriale en augmentant la fission et/ou en diminuant la fusion *(224)*. Le rôle de ces deux protéines au niveau de la régulation de la dynamique mitochondriale a aussi été observé dans les cellules humaines. Par contre, l'effet serait à l'opposé de ce qui a été proposé chez la drosophile, car la perte de *Parkin* ou de *PINK1* provoque une fragmentation mitochondriale *(225, 243)* et les fibroblastes de patients parkinsoniens porteurs de mutations dans *PINK1* présentent un taux plus élevé de mitochondries fragmentées *(243)*. À contrario, un autre étude a montré que les fibroblastes de patients avec des mutations dans *Parkin* présentent des mitochondries anormalement longues *(244)*, ce qui concorde avec le phénotype observé chez les mouches sans *Parkin (174)*. Même si plusieurs de ces études se contredisent et nécessitent des recherches plus poussées, elles s'accordent toutes sur le fait que certains gènes responsables de la MP régulent la dynamique mitochondriale et que ce mécanisme pourrait être impliqué dans sa pathogénèse, comme le suggèrent les nombreux indices provenant des analyses fonctionnelles des facteurs de prédisposition environnementaux.

À la lumière de ces observations et toujours dans une logique de séquençage de gènes candidats, nous avons analysé le gène *MFN1* chez des patients parkinsoniens. Ce gène code pour mitofusin 1, l'une des protéines responsables du processus de fusion des mitochondries *(245)*. L'objectif était d'évaluer si des mutations dans *MFN1* pourraient être associées à la MP.

Contribution des auteurs

JBR : conception de l'étude, acquisition et analyse des résultats génétiques et rédaction de l'article.

KG : acquisition et analyse des résultats fonctionnels et rédaction de l'article.

JS : acquisition et analyse des résultats génétiques.

SA et **MK** : soutien technique pour les études fonctionnelles.

IB : génotypage des microsatellites.

SC, **EP**, **MP**, **NJ**, **VS**, **ML**, **PP**, **SD**, **CK**, **RHM** et **ND** : recrutement des patients.

MPD : supervision des analyses statistiques.

EAF : idée originale de séquencer *MFN1*, supervision du projet et rédaction de l'article.

PD et **GAR** : supervision du projet et rédaction de l'article.

MFN1 mutations in individuals with Parkinson's disease

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Manuscrit en préparation.

4.1 Abstract

Dysfunctions in mitochondrial dynamics have been recently linked to Parkinson's disease (PD). Here we report non-synonymous coding mutations including a nonsense change and a rare founder missense mutation of *MFN1* in idiopathic sporadic PD patients. The *MFN1* gene encodes mitofusin 1, which is one of the core components of the mitochondrial fusion/fission machinery. These findings further support the involvement of mitochondrial dynamics in the pathogenesis of PD.

4.2 Text

Increasing evidence suggest that mitochondrial dysfunction on account of fusion and fission imbalance is involved in several neurodegenerative disorders and Parkinson disease (PD) is now believed to be one of them[1,2]. Hypotheses supporting such a dysfunction initially stemmed from observations of enhanced mitochondrial fission in neuroblasts, primary cortical neurons and differentiated dopaminergic neurons treated with neurotoxins that were commonly used to create models of PD[3-5]. The subsequent identification of two PD causative genes, *PINK1* and *parkin*, further supported this course of event since their products participate in mitochondrial fusion/fission regulatory events[6,7]. The mitochondrial fusion/fission machinery is controlled by four core components: fusion is mediated by dynamin-related GTPases mitofusin (MFN) 1 and 2, and Optic Atrophy 1 (Opa1), while fission is essentially regulated by dynamin-related protein 1 (Drp1)[8]. Strikingly, mutations in *MFN2* and *OPA1* have been observed to cause two distinct autosomal dominant neurodegenerative disorders, namely Charcot-Marie-Tooth disease-2A2 (CMT2A2)[9] and optic atrophy type 1 (OPA1)[10], respectively. A *de novo* dominant-negative mutation in *DNM1L*, the gene encoding Drp1, was found in a sporadic case of fatal encephalopathy[11]. No disease-associated mutations have thus far been reported for *MFN1*.

Evidence supporting that MFN1 overexpression protects primary cortical neurons from nitric oxide or rotenone-induced mitochondrial fission and cell death[3] has led us speculate about a possible role of MFN1 in PD. To investigate this premise, we screened French Canadian (FC) PD patients from Quebec province for the presence of *MFN1* mutations. *MFN1* is positioned on chromosome 3q26.32, which is not a region previously linked to PD, and consists of 18 exons (17 coding). We first sequenced the entire coding region of *MFN1* in 212 unrelated FC individuals with PD (**Supplementary Table 4.1**) and 475 FC control subjects, including 190 age-matched and 285 general population controls. Following sequences traces analysis, we identified one heterozygous nonsense mutation (R640X) and three distinct non-synonymous coding variants (R226Q, Q315R, and R630G) in eight of these PD cases (**Fig. 4.1**, **Supplementary Fig. 4.1**, and **Table 4.1**). Three distinct non-synonymous coding variants (I48L, R226Q, and R630G) were found in four control individuals; thus indicating *MFN1* appears to have a low degree of sequence variability (Table 4.1). The R226Q, Q315R, R630G and R640X changes were observed in two, three, two and one FC individuals with PD, respectively. Although the R226Q and the R630G substitutions were also found in control individuals, which suggest these particular variants are likely not associated with PD, the two other variants found in PD patients (Q315R and R640X) were absent from the 475 controls cohort (950 total chromosomes). The 3.8% of non-synonymous coding variants (including the nonsense) observed amongst PD cases of FC origin is significantly higher than what can be observed in control individuals (0.8%) (Fisher's exact test, $P = 0.01$).

The nonsense mutation (R640X) provided an additionally persuasive clue about the potential functional significance of our genetic data. This mutation is indeed predicted to truncate the C-terminal region of MFN1 (Fig. 4.1a) which contains a heptad repeat region 2 (HR2) known to participate in the formation of an antiparallel coiled coil domain mediating the tethering of mitochondria before outer membrane fusion[12].

In order to test if our *MFN1* findings in the FC population could also explain PD in other groups of PD patients, we subsequently screened for mutations the entire coding region of *MFN1* in two additional populations: North American from the United States (US) and German. The US collection contained 94 unrelated PD cases, and the German collection 288 unrelated PD cases (Supplementary Table 4.1). We uncovered two of the previously identified missense changes (R226Q and Q315R) in three sporadic US PD cases (Table 4.1). Subsequent sequencing of *MFN1* in 96 matched control subjects from the US revealed the presence of the R226Q change in a control individual. The observation of the Q315R variant in one US patient further supports the likelihood of its causative nature as it was absent from the control individuals of this group. The R226Q change, which was also observed in the cohort of US control individuals, conversely confirmed it is likely a polymorphism (Table 4.1). The *MFN1* investigation of the German PD cases only identified the R226Q substitution in one patient, indicating that mutations in *MFN1* are unlikely to be a significant cause of PD in this population. Even though the R226Q and R630G changes were observed in control individuals, it may still constitute a risk factor for PD, according to their apparent but non-significant overrepresentation in FC patients with PD compared to control individuals. Due to the rarity of these variants, large-scale studies will be necessary to verify this possibility.

The observation of the Q315R change in four out of 306 FC and US PD cases and its absence in 571 ethnically matched controls strongly suggests that this particular variant is a rare predisposing factor for PD (Fisher's exact test, $P = 0.015$). In an attempt to assess whether the Q315R substitution is a founder or a recurrent mutation, we genotyped nine microsatellites and 30 single nucleotide polymorphisms (SNPs) that are spanned over a 3.3 Mb region surrounding *MFN1* in the four Q315R mutation carriers (three FC and one US patients) and in 47 FC controls (94 chromosomes). We also verified their parents' surnames (as well as grandparents' surnames for FC individuals) to ensure that none of these PD patients were closely related. Haplotype analysis using PHASE software v2.1[13] indicated

that the four Q315R carriers share a 33-marker haplotype that spans a 2.5 Mb region bounded by the D3S3037 and D3S3603 markers (**Table 4.2**). The Q315R carrier from the US resides in Massachusetts and it is unfortunately impossible to confirm whether he has FC ancestors, as suggested by haplotype analysis. However it is estimated that between 1840 and 1930 approximately 900,000 FC from Quebec migrated to the US, in particularly to Massachusetts region because of its geographical proximity. According to the 2000 US census (http://www.census.gov), about 2.5 million Americans claimed to have FC ancestors. Furthermore, founder mutations specific to FC have been found in Americans[14]. These data support an identity-by-descent for the Q315R mutation rather than recurrent mutation events. As suggested by the fact that *MFN1* mutations appear to be significantly overrepresented in FC individuals with PD and given that three Q315R carriers are FC, we hypothesize that this mutation originated from the FC population. The overrepresentation of this mutation in FC PD patients is likely due to the particular genetic demography of Quebec. Indeed, FC mainly descend from a small number of original immigrants and the population rapidly expanded, which gave rise to what is now recognized as a founder population[15]. Population specific mutations are well documented in PD as illustrated by *LRRK2* mutations; the R1441G founder mutation has almost exclusively been found in the Basque founder population[16], and the G2019S mutation is particularly prevalent in Ashkenazi Jews and North African Arabs PD patients[17,18]. Both mutations were previously reported to be absent in PD patients of FC origin[19,20].

Similarly to heterozygous *MFN2* mutations in CMT2A2, our genetic data suggest that a dominant negative mechanism or haploinsufficiency of *MFN1* predispose to PD[9]. MFN1 and MFN2 were both described to share some redundant functions. Indeed, they can form homotypic and heterotypic complexes and both type of complexes do mediate mitochondrial fusion[21]. However, they do display tissue-specific differences in expression levels, and both are required for normal mitochondrial morphology, indicating that they also possess distinct functions[21,22]. Despite several years of investigation, the exact pathogenic mechanism of *MFN2* mutations underlying CMT2A2 remains unclear[23]. Due to

complementation between MFN1 and 2, the negative impact of CMT2A2 alleles on mitochondrial fusion was only observed in double MFN-null cells[24]. Furthermore, mitochondrial fusion was not found to be disrupted in fibroblasts of CMT2A2 patients with *MFN2* mutations[25], suggesting these mutations may either disrupt other functions of MFN2 yet to be determined or may be pathogenic only in specific neuronal populations or in particular conditions. This may also be the case for *MFN1* mutations, which would be consistent with the fact that the etiology of PD likely involves subtle changes that eventually appear to be pathogenic in specific vulnerable neuronal subpopulations in aging individuals.

Although the two PD causative genes *PINK1* and *parkin* have been shown to promote fission and/or inhibit fusion of mitochondria in *Drosophila*[26], the opposite observation resulted from functional studies in HeLa cells, primary mouse neurons and human neuroblastoma cells[27,28]. In the latter, overexpression of MFN2, OPA1 or a dominant-negative mutant of Drp1 rescued the mitochondrial fragmentation phenotype triggered by parkin or PINK1 deficiency[28]. Unfortunately, the effect of MFN1 overexpression was not assessed. Fibroblasts derived from patients with *PINK1* mutations also displayed mitochondrial fragmentation[27]. Altogether and combined with our genetic findings, these observations point towards a significant role for imbalanced mitochondrial fusion and fission in the pathogenesis of PD. Nonetheless, further studies will be necessary to define the exact functional relationship between parkin, PINK1 and the dynamin-related GTPases that regulate mitochondrial dynamics, including MFN1.

Strikingly, none of the five affected individuals carrying Q315R or R640X mutations reported familial history of PD (Table 4.1). Clinical features were consistent with a typical L-dopa responsive PD with an age of onset ranging from 46 to 69. Although one may question the reliability of self-reported family history, the rate of positive family history in our PD cohort does not differ from other reports (Supplementary Table 4.1). Chronic administration to rats of the widely used pesticide rotenone inhibits mitochondrial

complex I activity, triggers selective nigrostriatal dopaminergic degeneration, induces the formation of Lewy body-like inclusions and produces a locomotor phenotype resembling PD[29]. Interestingly, by promoting mitochondrial fusion, MFN1 was alleged to protect neurons from mitochondrial fragmentation induced by parkinsonian neurotoxins, including rotenone[3]. Thus, *MFN1* mutations may have incomplete penetrance and predispose to PD in combination with exposure to neurotoxins that constitute risk factors for PD such as pesticides. This will need to be checked in large cohorts of carriers of *MFN1* mutations. Overall, the R640X nonsense mutation and the Q315R founder mutation identified in *MFN1* indicate that mutations in this gene constitute a rare predisposing factor for late-onset PD. Consistently with recent hypotheses; it confirms the central role of mitochondrial dynamics in the pathogenesis of the disorder. Finally, it further emphasizes the value of inbred populations in genetic studies of PD. Replication studies in other populations will be necessary to assess the worldwide rate of PD-causative mutations of *MFN1*.

4.3 Acknowledgements

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4.5 Tables and figures

Table 4.1. Individuals carrying non-synonymous coding changes in *MFN1*.

Cohort	Coding DNA	Amino acid	Status	Sex	Onset	FH	Polyphen[a]	SIFT[b]	Panther[c]	ESEfinder
212 French Canadian PD patients	c.677G>A	R226Q	PD patient	M	70	No	Possibly damaging (1.54)	Affect protein function (0.01)	-2.50	No effect
	c.677G>A	R226Q	PD patient	M	57	No				
	c.944A>G	Q315R	PD patient	F	69	No	Benign (0.62)	Tolerated (0.37)	-1.88	Affect ESE
	c.944A>G	Q315R	PD patient	M	46	No				
	c.944A>G	Q315R	PD patient	M	65	No				
	c.1888A>G	R630G	PD patient	M	69	No	Probably damaging (2.63)	Affect protein function (0)	-2.11	No effect
	c.1888A>G	R630G	PD patient	M	62	No				
	c.1918C>T	R640X	PD patient	M	52	No	n.a.	n.a.	n.a.	No effect
475 French Canadian controls	c.142A>C	I48L	Control				Benign (0.78)	Tolerated (0.24)	n.a.	No effect
	c.677G>A	R226Q	Control							
	c.1888A>G	R630G	Control							
	c.1888A>G	R630G	Control							
94 PD patients United States	c.677G>A	R226Q	PD patient	M	63	unknown				
	c.677G>A	R226Q	PD patient	F	57	No				
	c.944A>G	Q315R	PD patient	M	61	No				
96 controls United States	c.677G>A	R226Q	Control							
288 German PD patients	c.677G>A	R226Q	Patient	F	30	Yes				

Abbreviations. Coding DNA. Description of sequence variations in the coding DNA reference sequence of *MFN1* (NM_033540). Amino acid. Description of sequence variants on protein level. M. Male. F. Female. FH. Reported familial history of PD in first degree relatives. Onset. Age of onset of PD. Three bioinformatic prediction programs (Polyphen, SIFT and Panther) were used to predict the impact of amino acid substitutions on the structure and function of the protein. [a]The lower the score, the more benign the substitution is. [b]The higher the score, the more tolerated the change is. [c]A score of 0 to -3 is considered to be tolerated whereas a score from -3 to -10 is not tolerated. ESEfinder was used to predict the effect of variants on splice sites and/or exonic splicing enhancers (ESEs).

Table 4.2. Haplotype sharing for PD patients carrying the Q315R mutation.

Marker	Position (bp)	FC1	FC2	FC3	US1	Freq (%)
D3S2412	178547255	206	194	194	194/206	194= 44.7
D3S3511	178878254	264/260	264/262	264	264	264 = 17
D3S3715	178895021	137/139	137/143	137	137	137 = 19.2
D3S3037	178924191	213/193	213	213	217	213 = 37.2
D3S3730	180029295	142	142	142	142/150	142 = 13.8
rs2677760	180385966	C	C	C	C	C = 43.6
rs1607237	180432999	C	C	C	C	C = 37.2
rs7645550	180451336	C	C	C	C	C = 57.5
rs1170672	180451354	T	T	T	T	T = 91.5
rs1468924	180465671	G	G	G	G	G = 61.7
rs1861803	180471395	C	C	C	C	C = 39.4
rs1861804	180471536	C	C	C	C	C = 52.1
rs6443638	180492858	C	C	C	C	C = 42.6
rs6790163	180495241	T	T	T	T	T = 41.5
rs16848314	180505230	T	T	T	T	T = 79.8
rs6794192	180510515	T	T	T	T	T = 27.2
rs6762399	180545409	T	T	T	T	T = 44.7
rs2287312	180552870	T	T	T	T	T = 71.3
rs9822116	180557317	G	G	G	G	G = 43.6
c.944A>G	**180568554**	**G**	**G**	**G**	**G**	
rs2111534	180573474	C	C	C	C	C = 42.2
rs13098637	180575516	T	T	T	T	T = 80.8
rs9865666	180575691	C	C	C	C	C = 28.7
rs10049286	180589549	G	G	G	G	G = 70.2
rs3853153	180589721	G	G	G	G	G = 29.4
rs13083369	180591619	G	G	G	G	G = 73.4
rs17292964	180600365	G	G	G	G	G = 72.8
rs6443645	180603389	G	G	G	G	G = 28.3
rs3961211	180608098	A	A	A	A	A = 28.7
rs7618348	180627441	C	C	C	C	C = 42.6
rs754574	180660557	G	G	G	G	G = 76.6
rs7428403	180663639	G	G/A	G	G/A	G = 69.2
rs1362644	180680976	C	C	C	C	C = 27.7
rs2339802	180711066	T	T	T	T	T = 44.7
rs1345445	180733426	C	C	C	C	C = 52.1
rs4855089	180742423	G	G	G	G	G = 64.9
D3S3699	180779739	250	250	250	250	250 = 35.1
D3S3565	180964590	158	158	158	158	158 = 56.4
D3S3603	181397570	132/144	134	132	132	132 = 67
D3S3662	181883281	189/191	189/191	189/191	189/171	189 = 60.6

Abbreviations: FC. French Canadian patient. US. Patient from the United States. Freq. Frequency of the shared allele (derived from the genotyping of 47 FC controls). The haplotype shared by mutation carriers is indicated by dark gray shading. Light gray shading

indicates incomplete allele sharing. Both alleles are shown when phase is uncertain according to the default phase confidence threshold (p = 90%). Confidence probabilities associated with each phase call are shown in **Supplementary Table 4.2**. The Q315R mutation is indicated in bold. Microsatellite allele calls were determined according to the Centre d'Étude du Polymorphisme Humain (CEPH) database (http://www.cephb.fr).

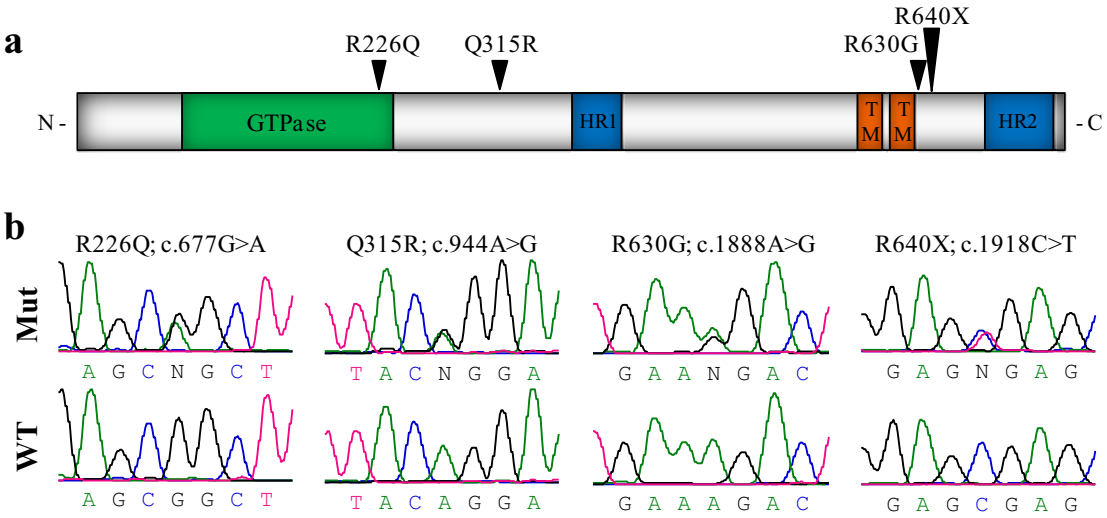

Figure 4.1. *MFN1* mutations in individuals with PD. **(a)** Linear protein structure of MFN1 indicating the known functional domains of the protein, i.e. the GTPase domain (green), the two heptad repeats HR1 and 2 (blue), and the two transmembrane domains (TM, orange). Amino acid changes found in PD cases are shown above the protein with arrowheads indicating their position. **(b)** Sequence traces of non-synonymous coding variants in *MFN1* found in individuals with PD.

4.6 Supplementary material

4.6.1 Supplementary tables and figures

Supplementary table 4.1. Characteristics of the PD cohorts recruited.

Clinical data	**Cohorts**		
	FC	**US**	**German**
Unrelated individuals	212	94	288
Male sex	66%	73%	51%
Ethnicity	Caucasian, French Canadian	Caucasian, North American	Caucasian, German
Positive familial history (1st degree relative)	12%	34%	19.8%
Age of onset, mean (range)	56.8 (30-80)	56.7 (32-83)	49.1 (16-79)

Abbreviations: FC. French Canadian. US. United States. German cohort: Percentage of individuals with familial history of PD in first degree relatives was based on 125 individuals. These data were unavailable in 163 PD cases. Age of onset was available in 179 individuals.

Supplementary table 4.2. Confidence probabilities associated with each phase call.

Marker	FC1		FC2		FC3		US1	
D3S2412	206	=	194	=	194	=	194/206	0.51
D3S3511	264/260	0.51	264/262	0.54	264	=	264	=
D3S3715	137/139	0.52	137/143	0.54	137	=	137	=
D3S3037	213/193	0.52	213	=	213	=	217	=
D3S3730	142	0.90	142	0.98	142	0.99	142/150	0.84
rs2677760	C	=	C	1.00	C	1.00	C	=
rs1607237	C	=	C	1.00	C	1.00	C	=
rs7645550	C	=	C	=	C	1.00	C	=
rs1170672	T	=	T	=	T	=	T	0.96
rs1468924	G	0.99	G	=	G	1.00	G	=
rs1861803	C	0.99	C	1.00	C	1.00	C	0.98
rs1861804	C	0.99	C	1.00	C	1.00	C	0.98
rs6443638	C	0.99	C	1.00	C	1.00	C	0.98
rs6790163	T	0.99	T	1.00	T	1.00	T	0.98
rs16848314	T	=	T	=	T	=	T	=
rs6794192	T	1.00	T	=	T	1.00	T	=
rs6762399	T	1.00	T	=	T	1.00	T	=
rs2287312	T	1.00	T	=	T	1.00	T	=
rs9822116	G	1.00	G	=	G	1.00	G	=
rs2111534	C	1.00	C	=	C	1.00	C	=
rs13098637	T	=	T	=	T	=	T	=
rs9865666	C	1.00	C	=	C	1.00	C	=
rs10049286	G	1.00	G	=	G	1.00	G	=
rs3853153	G	1.00	G	=	G	1.00	G	=
rs13083369	G	=	G	=	G	=	G	=
rs17292964	G	=	G	=	G	=	G	=
rs6443645	G	1.00	G	=	G	1.00	G	=
rs3961211	A	1.00	A	=	A	1.00	A	=
rs7618348	C	1.00	C	=	C	1.00	C	=
rs754574	G	=	G	=	G	0.99	G	=
rs7428403	G	=	G/A	0.89	G	0.99	G/A	0.57
rs1362644	C	=	C	0.93	C	0.99	C	0.95
rs2339802	T	=	T	=	T	0.99	T	0.97
rs1345445	C	=	C	=	C	0.98	C	0.97
rs4855089	G	=	G	0.93	G	=	G	=
D3S3699	250	=	250	0.93	250	=	250	0.95
D3S3565	158	=	158	=	158	=	158	=
D3S3603	132/144	0.53	134	=	132	=	132	=
D3S3662	189/191	0.51	189/191	0.52	189/191	0.50	189/171	0.54

For each patient, the left and right columns indicate the shared haplotype reconstructed by the PHASE software and the confidence probabilities associated with each phase call, respectively. Phase known positions are indicated by "=". Both alleles are shown when phase probability is < 90%.

Supplementary figure 4.1. Multi-species alignment for *MFN1*. Sequence cluster alignment was done by the Clustal W method. Missense variants found in PD patients are highlighted (yellow when they are conserved between humans and other species; green when they differ). The nonsense mutation is highlighted in red.

```
MAEP-VSPLKHFVLAKKAITAIFDQLLEFVTEGSHFVEATYKNPELDRIA   49 MFN1 Homo sapiens
MAET-VSPLKHFVLAKKAITAIFDQLLEFVTEGSHFVEATYKNPELDRIA   49 MFN1 Macaca mulatta
MAET-VSPLKHFVLAKKAITAIFGQLLEFVTEGSHFVEATYRNPELDRIA   49 MFN1 Gallus gallus
MAET-VSPLKHFVLAKKAITAIFGQLLEFVTEGSHFVEATYRNPELDRIA   49 MFN1 Mus musculus
MAEAAVSPLKHFVLAKKTITAIFEQLLDYVTEGAAFVEATYRNPELEHVA   50 MFN1 Rattus norvegicus
MEQLDPSPLKRFVLAKKKISEVFEQLLVYVQEGCEFVKETCANESLENIA   50 MFN1 Xenopus tropicalis
MEEASVSPLKHFVLAKKSITSIFDQLVEYVSEGVTFVEETFQNPELKNVS   50 MFN1 Xenopus laevis
MEEASVSPLKHFVLAKKAITSIFDQLVEYVSEGVTFVEATFQNPELNNVS   50 MFN1 Danio rerio

TEDDLVEMQGYKDKLSIIGEVLSRRHMKVAFFGRTSSGKSSVINAMLWDK   99  MFN1 Homo sapiens
TENDLVEMQGYKDKLSIIGEVLSRRHMKVAFFGRTSSGKSSVINAMLWDK   99  MFN1 Macaca mulatta
SEDDLVEIQGYRNKLAVIGEVLSRRHMKVAFFGRTSSGKSSVINAMLWDK   99  MFN1 Gallus gallus
TEDDLVEIQGYRNKLAVIGEVLSRRHMKVAFFGRTSSGKSSVINAMLWDK   99  MFN1 Mus musculus
TEDELAEIQAYKKKLAVIGEVLSRRHMKVAFFGRTSSGKSSVINAMLWDK  100 MFN1 Rattus norvegicus
NKDQLDEIESYTSKLSIIQEVLARRHMKVAFFGRTSNGKSTVINAMLRDR  100 MFN1 Xenopus tropicalis
TEDDVNKVKASKTKLAGIGEVLARRNMKVAFFGRTSSGKSTVINSMLWDK  100 MFN1 Xenopus laevis
TEDDLKKIQASKTKLAGIGEVLARRNMKVAFFGRTSSGKSTVINSMLWDK  100 MFN1 Danio rerio

VLPSGIGHITNCFLSVEGTDGDKAYLMTEGSDEKKSVKTVNQLAHALHMD  149 MFN1 Homo sapiens
VLPSGIGHITNCFLSVEGTDGDKAYLMTEGSDEKKSVKTVNQLAHALHMD  149 MFN1 Macaca mulatta
VLPSGIGHTTNCFLSVEGTDGDKAYLMTEGSDEKKSVKTVNQLAHALHMD  149 MFN1 Gallus gallus
VLPSGIGHTTNCFLSVEGTDGDKAYLMTEGSDEKKSVKTVNQLAHALHMD  149 MFN1 Mus musculus
VLPSGIGHTTNCFLSVEGTDGDKAYLMTEGSDEKKSVKTVNQLAHALHMD  150 MFN1 Rattus norvegicus
VLPSGIGHTTNCFLSVEGTDEDKAFLKTEGSEEEKSIKTVNQLAHALHMD  150 MFN1 Xenopus tropicalis
VLPSGIGHTTNCFLSVEGTEGDKAYLMTEGSEEKKSVKTVNQLAHALHMD  150 MFN1 Xenopus laevis
VLPSGIGHTTNCFLSVEGTDGDKAYLMTESSEEKKSVKTVNQLAHALHMD  150 MFN1 Danio rerio

KDLKAGCLVRVFWPKAKCALLRDDLVLVDSPGTDVTTELDSWIDKFCLDA  199 MFN1 Homo sapiens
KDLKAGCLVRVFWPKAKCALLRDDLVLVDSPGTDVTTELDSWIDKFCLDA  199 MFN1 Macaca mulatta
KDLKAGCLVHVFWPKAKCALLRDDLVLVDSPGTDVTTELDIWIDKFCLDA  199 MFN1 Gallus gallus
KDLKAGCLVHVFWPKAKCALLRDDLVLVDSPGTDVTTELDIWIDKFCLDA  199 MFN1 Mus musculus
KDLKAGCLVHVFWPKSKCALLRDDLVLVDSPGTDVTTELDSWIDKFCLDA  200 MFN1 Rattus norvegicus
ESLDAGCLVKVFWPKTKCALLRDDLVLVDSPGTDVTTELDSWIDKFCLDA  200 MFN1 Xenopus tropicalis
KDLGAGCLVHVFWPKAKCALLRDDLVLVDSPGTDVTTELDSWIDKFCLDA  200 MFN1 Xenopus laevis
KDLGAGCLVHVFWPKAKCALLRDDLVLVDSPGTDVTTELDSWIDKFCLDA  200 MFN1 Danio rerio

DVFVLVANSESTLMNTEKHFFHKVNERLSKPNIFILNNRWDASASEPEYM  249 MFN1 Homo sapiens
DVFVLVANSESTLMNTEKHFFHKVNERLSKPNIFILNNRWDASASEPEYM  249 MFN1 Macaca mulatta
DVFVLVANSESTLMNTEKHFFHKVNERLSKPNIFILNNRWDASASEPEYM  249 MFN1 Gallus gallus
DVFVLVANSESTLMNTEKHFFHKVNERLSKPNIFILNNRWDASASEPEYM  249 MFN1 Mus musculus
DVFVLVANSESTLMNTEKHFFHKVNERLSKPNIFILNNRWDASASEPEYM  250 MFN1 Rattus norvegicus
DVFVLVANSESTLMNTEKHFFHKVNEKLSKPNIFVLNNRWDASAAEPEYM  250 MFN1 Xenopus tropicalis
DVFVLVANSESTLMNTEKHFFYKVNEKLSKPNIFILNNRWDASASEPEYM  250 MFN1 Xenopus laevis
DVFVLVANSESTLMNTEKHFFHKVNEKLSKPNIFILNNRWDASASEPEYM  250 MFN1 Danio rerio

EDVRRQHMERCLHFLVEELKVVNALEAQNRIFFVSAKEVLSARKQKAQGM  299 MFN1 Homo sapiens
EDVRRQHMERCLHFLVEELKVVNALEAQNRIFFVSAKEVLSARKQKAQGM  299 MFN1 Macaca mulatta
EDVRRQHMERCLHFLVEELKVVSPSEARNRIFFVSAKEVLNSRKHKAQGM  299 MFN1 Gallus gallus
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EDVRRQHMERCLHFLVEELKVVSPLEARNRIFFVSAKEVLNSRMNKAQGM  299 MFN1 Mus musculus
EDVRRQHMERCLTFLVDELKVIDPVEARNRIFFVSAKEVLSARRQKAQGM  300 MFN1 Rattus norvegicus
EDVRKQHTDRCVNFLVEELKVVDRAQAPNRIFFVSAKEVLNSRMQRAQ--  298 MFN1 Xenopus tropicalis
EDVRKQHMERCQSFLVDELKVVDSLEAQKRIFFVSAKEVLNARMHKAQGM  300 MFN1 Xenopus laevis
EDVRKQHTERCQSFLVDELKVVDSSEAQKRIFFVSAREVLNARMYKAQGM  300 MFN1 Danio rerio

PESGVALAEGFHARLQEFQNFEQIFEECISQSAVKTKFEQHTIRAKQILA  349 MFN1 Homo sapiens
PESGGALAEGFQARLQEFQNFEQIFEECISQSAVKTKFEQHTIRAKQILA  349 MFN1 Macaca mulatta
PEGGGALAEGFQARLQEFQNFEQTFEECISQSAVKTKFEQHTIRAKQILD  349 MFN1 Gallus gallus
PEGGGALAEGFQARLQEFQNFEQTFEECISQSAVKTKFEQHTIRAKQILD  349 MFN1 Mus musculus
PESGGALAEGFQTRFQEFQNFEQIFEECISQSAVKTKFEQHTIRAKQIID  350 MFN1 Rattus norvegicus
---GGALAEGFQDRLKEFQNFERSFEECISHSAVKTKFEQHTIRAKQITE  345 MFN1 Xenopus tropicalis
PEAGAALAEGFHTRFLEFQKFEKLFEECISQSAVKTKFEQHTITAKQITD  350 MFN1 Xenopus laevis
PEAGAALAEGFHTRFLEFQKFEKLFEECISQSAVKTKFEQHTITAKQITD  350 MFN1 Danio rerio

TVKNIMDSVNLAAEDKRHYSVEEREDQIDRLDFIRNQMNLLTLDVKKKIK  399 MFN1 Homo sapiens
TVKNIMDSINLAAEEKRHYSVEEREDQIDRLDFIRNQMNLLTLDAKKKIK  399 MFN1 Macaca mulatta
TVKNILDSVNVAAAEKRVYSMEEREDQIDRLDFIRNQMNLLTLDVKKKIK  399 MFN1 Gallus gallus
TVKNILDSVNVAAAEKRVYSMEEREDQIDRLDFIRNQMNLLTMDVKKKIK  399 MFN1 Mus musculus
TEKNIMDAINVAAAEKRVLSMEEREDQKDRLDFVRNQLNILTEDTKEKIK  400 MFN1 Rattus norvegicus
TVKDIMDQINITAAEKRVVSLEEREYLIDRLDFVRNQLNLLIQDIKKKIK  395 MFN1 Xenopus tropicalis
TVKAIMDAINIKAADKRVRCMVERENQNDRLEYVKTQMLILTEEIEKRIL  400 MFN1 Xenopus laevis
TVKAIMDAINIKAADKRVRCMVERENQNDRLEYVQTQLLILTEEIERRIL  400 MFN1 Danio rerio

EVTEEVANKVSCAMTDEICRLSVLVDEFCSEFHPNPDVLKIYKSELNKHI  449 MFN1 Homo sapiens
EVTEEVANKVSCAMTDEICRLSVLVDEFCSEFHPNPDVLKIYKSELNKHI  449 MFN1 Macaca mulatta
EVTEEVANKVSCAMTDEICRLSVLVDEFCSEFHPTPSVLKVYKSELNKHI  449 MFN1 Gallus gallus
EVTEEVANKVSCAMTDEICRLSVLVDEFCSEFHPTPSVLKVYKSELNKHI  449 MFN1 Mus musculus
RVTEEVENKVSSAMTDEICRLSVLVDEFYSDFHPSPQVLKLYKTELNKHI  450 MFN1 Rattus norvegicus
AITEEVENKVSNAMAEEICRLSVIIDEFRSDFHPSPQMLKMYKSDLLSHI  445 MFN1 Xenopus tropicalis
HITKEVNEQVSSAMMDEIYHLSLLVDKFDAEFHPSLSVLKLYKNELNLHI  450 MFN1 Xenopus laevis
HITKEVNEQVSNAMTDEIHHLSLLVDKFDGEFHPSLSVLKLYKNELNTHI  450 MFN1 Danio rerio

EDGMGRNLADRCTDEVNALVLQTQQEIIENLKPLLPAGIQDKLHTLIPCK  499 MFN1 Homo sapiens
EDGMGRNLADRCTDEVNALMLQSQQEIIENLKPLLPAGIQDKLHTLIPCQ  499 MFN1 Macaca mulatta
EDGMGRNLADRCTNEVNASILQSQQEIIENLKPLLPAGIQNKLHTLIPCK  499 MFN1 Gallus gallus
EDGMGRNLADRCTSEVNASILQSQQEIIENLKPLLPAGIQNKLHTLIPCK  499 MFN1 Mus musculus
EDGLGKNLADRCSSEVNESMHQSQQEIIENLKPLLPSGAQNQLHLLIPCR  500 MFN1 Rattus norvegicus
EQGMGKNLAFRCSDAVNASVQCSQQDMIECLKPLLPSAAQTQLHMLIPSR  495 MFN1 Xenopus tropicalis
ENGMGRKLADRCSNAVNMSIQKAQEDIIDKLMPLLPPSVQDQTTISLPCK  500 MFN1 Xenopus laevis
ENGMGRKLANRCSDAVNSSIQRAQEDIIEKLLPLLPPSVQDQTVVSLPCK  500 MFN1 Danio rerio

KFDLSYNLNYHKLCSDFQEDIVFRFSLGWSSLVHRFLGPRNAQRVLLGLS  549 MFN1 Homo sapiens
KFDLSYNLNYHKLCSDFQEDIVFHFSLGWSSLVHRFLGPRNAQRVLLGLS  549 MFN1 Macaca mulatta
KFDLSYDLNCHKLCSDFQEDIVFRFSLGWSSLVHRFLGSTNAQRVLLGLS  549 MFN1 Gallus gallus
KFDLSYDLNCHKLCSDFQEDIVFRFSLGWSSLVHRFLGSTNAQRVLLGLS  549 MFN1 Mus musculus
KFDLSYDLNCHSLCADFQEDIMFHFSLGWTSLVNRFLGPKHAQRVLLGLA  550 MFN1 Rattus norvegicus
KFELSYDLNCATLCSDFQENIEFQFSLGWSALVHRFLGPVNAKRALM-LV  544 MFN1 Xenopus tropicalis
KFDLSYNINCEKLCSDFQEDVEFRFSFGLTSLANRFLG-CKTSHLLLGFA  549 MFN1 Xenopus laevis
TFDLSYNINCENLCCDFQEDIEFRFSFGLMALANRFLG-CKNSHLLMGFA  549 MFN1 Danio rerio

EPIFQLPRSLASTPTAPTTP-------ATPDNASQEELMITLVTGLASVT  592 MFN1 Homo sapiens
EPIFQLPRSLASTPSAPTTP-------ATPDNASQEELMITLVTGLASLT  592 MFN1 Macaca mulatta
EPIFQVPRSLASTPTAPSNP-------AAPDNAAQEELMITLITGLASLT  592 MFN1 Gallus gallus
EPIFQVPRSLASTPTAPSNP-------AAPDNAAQEELMITLITGLASLT  592 MFN1 Mus musculus
DPNLQIPRPLATTPSAASLPA------ATPENTSPDDFMVPLVMSLASLT  594 MFN1 Rattus norvegicus
DQNLQLTAPTTPTAPAVVQSQNSRALGQNQASVTQEELMLTMVNNLASVT  594 MFN1 Xenopus tropicalis
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EPMFQIPRPVS-TPSATGNP------VLPPQPASQEELMLSVMSGLASLT   592 MFN1 Xenopus laevis
KPIFQIPQPVT-TPSATVNP------ILPPQPATPEELMLSVVSGLASLT   592 MFN1 Danio rerio

SRTSMGIIIVGGVIWKTIGWKLLSVSLTMYGALYLYERLSWTTHAKERAF   642 MFN1 Homo sapiens
SRTSMGIIIVGGVIWKTIGWKLISVSLTMYGALYLYERLSWTTHAKERAF   642 MFN1 Macaca mulatta
SRTSMGIIVVGGVIWKTVGWKLISVTLSMYGALYLYERLTWTTRAKERAF   642 MFN1 Gallus gallus
SRTSMGIIVVGGVIWKTVGWKLISVTLSMYGALYLYERLTWTTRAKERAF   642 MFN1 Mus musculus
SRTSMSIIVVGGVIWKTVGWKLISLSLSMYGLLYLYERLTWTTKAKERAF   644 MFN1 Rattus norvegicus
SRTSMSVIIVGGVVWRTVGWRLIALSMSMYGLLYLYERLTWTTKAKERAL   644 MFN1 Xenopus tropicalis
SRGSMSVIIVGGVIWRTVGWRLISGMMLAYGGLYLYERLTWTTRAKERAF   642 MFN1 Xenopus laevis
SRGSMGVLIVGGVVWRTVGWRLITGMLLTYGSLYVYERLTWTTRAKERTF   642 MFN1 Danio rerio

KQQFVNYATEKLRMIVSSTSANCSHQVKQQIATTFARLCQQVDITQKQLE   692 MFN1 Homo sapiens
KQQFVNYATEKLKMIVSSTSANCSHQVKQKMATTFARLCQQVDITQKQLE   692 MFN1 Macaca mulatta
KQQFVNYATEKLQMIVSFTSANCSHQVQQEMATTFARLCQQVDVTQKHLE   692 MFN1 Gallus gallus
KQQFVNYATEKLQMIVKFTSANCSHQVQQEMATTFARLCQQVDVTQKHLE   692 MFN1 Mus musculus
KQQFVNYATEKLQMIVSLTSANCSHQVQQEMATTFARLCQQVDVTQKNLE   694 MFN1 Rattus norvegicus
KKQFVDYATEKLQMIISFTSSNCSHQVQQEIASTFARLCQQVDITQKDLE   694 MFN1 Xenopus tropicalis
KKQFVNYAAQKLKLIVSFTSANCSQQVQKELDNTFKRLCQQVDVTERDLA   692 MFN1 Xenopus laevis
KRQFVNYAAQKLKMIVSFTSANCSQQVNKELDNTFKRLHQQVEVTVRDLT   692 MFN1 Danio rerio

EEIARLPKEIDQLEKIQNNSKLLRNKAVQLENELENFTKQFLPSSNEES    741 MFN1 Homo sapiens
EEIARLPKEIDQLEKIQNNSKLLRNKAVQLENELENFAKQFLPSSNEES    741 MFN1 Macaca mulatta
EEIARLSKEIDQLEKIQNNSKLLRNKAVQLESELENFSKQFLHPSSGES    741 MFN1 Gallus gallus
EEIARLSKEIDQLEKIQNNSKLLRNKAVQLERELENFSKQFLHPSSGES    741 MFN1 Mus musculus
KEIARLSKEIDQLENIQSHSKQLRNKAIHLENELDRFTKHFLQKSK---    740 MFN1 Rattus norvegicus
SDIQRLTDKIQKLETVQNRSKVLRHKATALEMQLEDFSSQYLRLQP---    740 MFN1 Xenopus tropicalis
ESIDKLSEEIHQLEQIQSNSKALRNRASRIEGELRNFAKSFLPQNK---    738 MFN1 Xenopus laevis
ESIDKLSEEIHQLEQIQSNSKVLRNRASRIEGELRSFSKSFLPHNI---    738 MFN1 Danio rerio
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4.6.2 Supplementary methods

Patient and control sample collection

Neurologists specialized in PD collected and diagnosed all cases using Ward and Gibb criteria for PD[1]. Informed written consent was obtained from each individual prior to being enrolled in the study. All procedures were approved by the local ethics committees on human experimentation. Blood samples were obtained and DNA was extracted from peripheral blood using standard methods. Five hundred seventy-one unrelated ethnically-matched control subjects were also used in this study, including 475 French Canadian (FC) individuals (285 general population and 190 age-matched control subjects) and 96 age-matched American population controls. Age-matched control subjects underwent the same neurological examination as the patients.

Amplicon design and gene screening

Primers were designed using the ExonPrimer program from the UCSC genome browser to amplify fragments covering coding exons plus at least 50-bp into introns before and after each exon. The coding region of all 17 coding exons of MFN1 (accession number NM_033540) was sequenced in each patient and control. PCR products were sequenced at the Genome Quebec Centre for Innovation according to standard procedures. Variants identified were confirmed by a second PCR amplification followed by forward and reverse resequencing.

Statistical analysis, genotyping and haplotype analysis

Fisher's exact test was performed with the use of the 2BY2 program from the UTIL package (by Jurg Ott). Microsatellite genotyping was performed by PCR amplification with fluorescently labeled primers. ABI 3730 DNA Analyzer and GeneMapper software v4.0 (Applied Biosystems) were used to call alleles. CEPH Reference samples 1331-01 and

1332-12 were genotyped to determine the size of each allele. SNP genotyping was performed by PCR amplification and direct sequencing. The PHASE software v2.1 (http://www.stat.washington.edu/stephens/software.html), which implements an algorithm based on Bayesian inference, was used to infer haplotypes[2].

References

1. Ward, C.D. & Gibb, W.R. Research diagnostic criteria for Parkinson's disease. Adv Neurol 53, 245-9 (1990).

2. Stephens, M. & Scheet, P. Accounting for decay of linkage disequilibrium in haplotype inference and missing-data imputation. Am J Hum Genet 76, 449-62 (2005).

Chapitre 5 : Discussion

5.1 Rôle des études génétiques dans la maladie de Parkinson

Il apparaît aujourd'hui clair que la MP est très hétérogène sur le plan génétique. D'aucuns seraient en droit de questionner la réelle utilité de trouver des mutations qui ne concernent qu'un nombre restreint de cas. En effet, l'application de ces découvertes au niveau épidémiologique est limitée par le petit nombre de porteurs de mutations ; la pénétrance incomplète et la rareté des mutations compliquent le diagnostic génétique présymptomatique *(246)* et le développement de stratégies thérapeutiques ciblant spécifiquement l'impact négatif de ces mutations peut sembler peu pertinent si ces traitements ne sont finalement efficaces que chez une infime minorité de patients.

Il faut cependant souligner que la recherche sur la MP poursuit deux buts fondamentaux : identifier des éléments présymptomatiques ayant un bon pouvoir prédictif et développer des traitements neuroprotecteurs. Ces deux objectifs passent nécessairement par une meilleure compréhension de la pathogénèse de la MP au niveau moléculaire et il a été clairement établi que la recherche génétique participe de manière significative à ce processus. Même si les mutations identifiées au cours de la dernière décennie ne concernent qu'un petit nombre de cas parkinsoniens, ces percées génétiques sont en grande partie responsables des avancées majeures dans la compréhension des mécanismes moléculaires à l'origine de la MP *(152)*. L'exemple le plus flagrant est le gène *SNCA* qui code pour l'alpha-synucléine. Des mutations ponctuelles de ce gène n'expliquent qu'une poignée de cas à travers le monde, mais ces observations ont mené à la découverte que l'alpha-synucléine est la composante principale des corps de Lewy *(148)*. De plus, les différents gènes identifiés et les études moléculaires subséquentes ont corroboré l'hypothèse proposée par l'analyse fonctionnelle du MPTP ; soit qu'un dysfonctionnement mitochondrial serait au cœur de la pathogénèse de la MP *(167, 191)*. Ces découvertes ont aussi mené au développement de modèles animaux déficients pour ces gènes, à partir desquels il est aujourd'hui possible de tester de nouvelles approches thérapeutiques *(143)*.

L'autre argument qui questionne la pertinence des études génétiques sur la MP souligne le fait que les formes héréditaires rares ne sont pas représentatives de la majorité

des cas parkinsoniens. Cependant les données des études d'association du génome entier suggèrent que des polymorphismes fréquents de *LRRK2*, *SNCA* et *MAPT*, en influençant les niveaux d'expression de ces gènes, pourraient également prédisposer aux formes sporadiques à début tardif de la MP *(132, 133)*. De plus, depuis la découverte de mutations de *LRRK2* et de *GBA* dans un nombre significatif de patients sporadiques *(80, 113)*, le domaine commence à réaliser que les gènes à l'origine identifiés dans des grandes familles parkinsoniennes peuvent également contenir des mutations rares à pénétrance incomplète à l'origine de formes sporadiques.

L'identification de mutations dans *LRRK2* et *GBA* a été grandement facilitée par l'étude de populations fondatrices et/ou relativement homogènes *(55, 78, 79, 112)* et il est maintenant clairement établi que la fréquence des mutations pathogéniques responsables de la MP varie d'une population à l'autre. Cette dernière observation s'applique aussi aux polymorphismes associés à la MP. En effet, les deux plus récentes études d'association du génome entier ont démontré que des polymorphismes dans le gène *MAPT* prédisposent à la MP dans les populations d'origine européenne mais pas dans la population japonaise *(132, 133)*. Nous estimons donc que l'étude d'une population fondatrice comme les CF du Québec pourrait s'avérer particulièrement efficace pour identifier de nouvelles variations génétiques prédisposant à la MP. Il est en effet possible de découvrir des mutations qui sont spécifiques ou surreprésentées dans cette population comme nous l'avons vu avec la mutation Q315R du gène *MFN1* (chapitre 4). De plus, dans le cas de mutations rares, le principal défi est d'établir clairement leur association avec la MP en dépit du nombre restreint de patients porteurs de ces mutations. L'étude d'une population fondatrice comme les CF constitue là aussi un avantage certain car une surreprésentation de variations génétiques rares chez les patients parkinsoniens risque moins d'être due à un mauvais appariement entre les cas et le contrôles, et ce en raison de la relative homogénéité génétique de cette population.

5.2 Le gène *LRRK2*

Nos travaux sur *LRRK2* suggèrent que des mutations de ce gène ne constituent pas une cause fréquente de la MP dans la population CF du Québec. Ces résultats ont été corroborés par une étude indépendante publiée simultanément dans le même journal *(247)*. Néanmoins, aucune de ces deux études n'a fait un séquençage exhaustif de la région codante du gène et toutes deux étaient basées sur un nombre relativement restreint de patients. La présence des mutations de *LRRK2* chez un nombre marginal de patients CF demeure donc possible et même probable, sachant que de telles mutations ont été identifiées dans les populations française et nord-américaine blanche *(80)*. Pour vérifier cette hypothèse, un plus grand nombre de patients CF et le séquençage exhaustif du gène seront nécessaires. L'absence de mutations dans notre cohorte est probablement due au fait que la plupart de nos patients présentent des formes sporadiques de la MP alors que les mutations de *LRRK2* sont plus souvent retrouvées dans des formes familiales *(80)*. La découverte de mutations, même rares, nous permettrait d'étudier leur impact sur la fonction de la protéine. Il semble que ces mutations augmentent l'activité kinase de LRRK2. Elles prédisposeraient donc à la MP par un effet dominant plutôt qu'en raison d'une haploinsuffisance *(74, 218, 248)*.

La découverte de mutations fréquentes dans certaines populations, à l'image de la mutation G2019S chez les juifs ashkénazes et autour de la Méditerranée, ouvre un large champ d'études et devrait permettre d'identifier des facteurs de prédisposition génétiques ou environnementaux en étudiant des cohortes d'individus porteurs de ces mutations. Ces recherches pourraient mener à l'identification d'éléments présymptomatiques et de facteurs aggravants ou protecteurs, ce qui aiderait au développement de nouvelles approches thérapeutiques et à la mise en place de mesures de prévention chez les porteurs présymptomatiques. Malheureusement, l'absence de telles mutations dans *LRRK2* chez les CF ne nous permettra pas de faire ce genre d'études.

5.3 Séquençage de gènes candidats

Ce projet de recherche était basé sur l'hypothèse que des mutations dans des gènes impliqués dans les mécanismes moléculaires sous-tendant la MP pourraient expliquer un certain nombre de cas parkinsoniens. Notre cohorte était composée de patients non-reliés présentant majoritairement une forme sporadique à âge de début tardif de la MP. Nous anticipions que les gènes prédisposant à la MP présenteraient des taux de mutations codantes plus élevés chez les patients que chez les contrôles. Le séquençage de 25 gènes candidats chez des patients parkinsoniens CF et le suivi plus approfondi, chez des patients et des contrôles additionnels, des gènes présentant des mutations potentiellement pathogéniques ont mené à l'identification de mutations rares prédisposant probablement à la MP dans les gènes *MFN1* et *PICK1*.

5.3.1 Le gène *PICK1*

Le séquençage du gène *PICK1* chez 500 patients parkinsoniens CF et allemands a mené à l'identification de mutations absentes dans les contrôles de même origine ethnique dans 1% de nos patients. Individuellement, aucune de ces mutations n'est significativement surreprésentée dans notre cohorte de patients. Parmi ces mutations, la question centrale est donc de différencier les changements probablement pathogéniques des variations observées chez des patients mais non-associées à la MP. Cette question est d'autant plus cruciale que quelques variations codantes ont aussi été trouvées dans des sujets contrôles. Par un heureux hasard, il s'est avéré que la mutation tronquante W412X touche un acide aminé essentiel à l'interaction entre PICK1 et le complexe Arp2/3 *(249)*. Dans les neurones, la mutation du tryptophane situé à la position 412 abroge l'interaction entre PICK1 et Arp2/3, et bloque l'internalisation des récepteurs AMPA par un effet dominant négatif, ce qui suggère fortement que la mutation W412X prédispose à la MP. Ce résultat est d'autant plus intéressant que l'excitotoxicité du glutamate a été associée à la MP *(250, 251)* et que des

antagonistes des récepteurs du glutamate sont utilisés pour traiter certains patients parkinsoniens *(252, 253)*. Les deux mutations faux-sens R158Q et R185Q dans le domaine BAR pourraient affecter la capacité de PICK1 à interagir avec les lipides de la membrane plasmique ou les vésicules synaptiques, et perturber le processus d'internalisation des récepteurs AMPA. Cette hypothèse devra être validée par des études fonctionnelles. La mutation E387_E388del qui enlève deux acides glutamiques à la région acide en C-terminal de PICK1 est plus problématique car un contrôle est porteur d'une duplication d'un acide glutamique au même endroit (E388dup). Il est toujours possible qu'une perte de deux acides aminés dans ce motif soit pathogénique alors que l'ajout d'un acide aminé n'ait pas d'effet biologique. Cependant, cette hypothèse demeure largement spéculative et devra être vérifiée au niveau fonctionnel. La mutation faux-sens V64G trouvée uniquement chez un contrôle est située dans le domaine PDZ de PICK1, mais le résidu valine à cette position n'est pas conservé dans les autres protéines humaines contenant un domaine PDZ similaire à celui de PICK1 *(254)*. Enfin, le remplacement de la sérine en position 181 par une leucine chez un sujet contrôle n'a probablement pas d'effet biologique significatif car une leucine est retrouvée à cette position d'après l'alignement des séquences des protéines contenant des domaines BAR *(255)*.

La protéine parkin régule négativement la transmission du glutamate, probablement pour limiter les effets excitotoxiques de ce neurotransmetteur. Une surexpression de parkin au niveau post-synaptique cause une diminution du nombre de synapses excitatrices alors que l'expression de formes mutées de la protéine augmente leur prolifération par un effet dominant-négatif *(223)*. Les autres molécules impliquées dans ce mécanisme sont encore inconnues. Sachant que PICK1 est mono-ubiquitinylée par parkin et que ce processus régule l'activité de certains canaux ioniques *(231)*, il serait intéressant de vérifier si cette modification post-traductionnelle régule aussi l'internalisation des récepteurs glutamatergiques orchestrée par PICK1 et si le phénotype des formes mutantes de parkin sur la transmission du glutamate pourrait être renversé par la surexpression de PICK1. L'observation d'un effet dominant-négatif pour une mutation de *Parkin* est surprenante car

les mutations de ce gène qui prédisposent à la MP sont récessives et causent une perte de fonction de la protéine. Il est possible que cet effet soit dû à une incapacité de parkin à réguler PICK1 de manière convenable.

La plupart des études fonctionnelles de PICK1 se sont concentrées sur son rôle dans la régulation des récepteurs du glutamate. Un groupe de recherche a cependant démontré que PICK1 peut s'associer aux mitochondries et que l'ancrage de la protéine kinase C alpha (PKCalpha) à ces organelles dépend de son interaction directe avec PICK1 *(256)*. De plus, PICK1 aurait des propriétés anti-apoptotiques en permettant à PKCalpha de phosphoryler le facteur anti-apoptotique Bcl-2 *(257)*. Sachant qu'un dysfonctionnement du complexe I mitochondrial rend les cellules plus vulnérables aux propriétés excitotoxiques du glutamate *(258)*, cette observation intrigante pourrait être un des mécanismes moléculaires qui expliqueraient pourquoi des mutations dans PICK1 prédisposent à la MP. De ce point de vue, PICK1 serait une protéine qui protège les neurones en promouvant l'internalisation des récepteurs glutamatergiques tout en inhibant l'apoptose mitochondrie-dépendante. Des mutations de *PICK1* perturbant ces deux fonctions rendraient alors les neurones plus vulnérables à l'excitotoxicité du glutamate et provoqueraient une augmentation de la mort neuronale par apoptose.

5.3.2 Le gène *MFN1*

D'après les données découlant du séquençage de contrôles, le gène *MFN1* contient intrinsèquement un nombre faible, mais plus élevé que *PICK1*, de variations codantes. Il est possible que cela soit dû à la complémentation qui existe entre MFN1 et MFN2 *(259)*. Nous avons néanmoins observé un taux de mutations significativement plus élevé chez les patients parkinsoniens que chez les sujets contrôles CF. Cependant, parmi les quatre mutations identifiées dans huit patients CF, seulement deux d'entre elles (Q315R et R640X) n'ont pas été trouvées dans notre cohorte de contrôles. La mutation faux-sens Q315R est présente chez quatre patients parkinsoniens dont un américain et une analyse

d'haplotypes a démontré que c'est une mutation fondatrice. Prises ensembles, ces données suggèrent fortement que cette mutation est associée à la MP. Par contre, aucun indice fonctionnel n'est encore disponible pour vérifier si son caractère pathogénique est dû à une haploinsuffisance ou à un effet dominant. La même question se pose pour la mutation R640X. À la lumière de nos données, il est également impossible de déterminer si les autres variations codantes dans *MFN1* prédisposent à la MP. En effet, même si nous avons observé un taux significativement plus élevé de mutations chez les patients que chez les contrôles, l'association individuelle entre les mutations faux-sens R226Q et R630G et la MP n'est pas significative. Si ces substitutions prédisposent à la MP, elles ont un effet modeste et leur rareté fait en sorte que des cohortes de patients et de contrôles beaucoup plus importantes vont être nécessaires pour les associer de manière définitive à la MP. D'un point de vue génétique, ce type de variations est particulièrement difficile à valider *(136)*.

Pour expliquer le fait qu'aucun des patients porteurs des mutations Q315R et R640X ne présente d'histoire familiale de MP, nous avons émis l'hypothèse que ces variations ont une pénétrance incomplète et pourraient induire la MP lorsque combinées à d'autres facteurs de risque comme des facteurs environnementaux. Cette idée s'appuie notamment sur le fait que les neurones traités avec diverses neurotoxines associées à la MP présentent une fragmentation mitochondriale accrue *(229, 241, 242)*. De plus, la surexpression de MFN1 protège ces neurones contre les effets néfastes de la roténone *(241)*, une molécule présente dans la composition de nombreux pesticides qui inhibe spécifiquement l'activité du complexe I mitochondrial. Chez le rat, l'administration chronique de roténone provoque une dégénérescence hautement sélective des neurones dopaminergiques de la SNpc ; et ce, malgré le fait que l'activité du complexe I est réduite dans tout le cerveau. Elle induit aussi la formation de corps de Lewy et mène à un phénotype ressemblant à la MP, caractérisé par une bradykinésie, de la rigidité et des problèmes de posture *(260)*. Un autre aspect de ces travaux est particulièrement intéressant : la vulnérabilité des rats à la roténone varie beaucoup d'une souche ou d'un animal à l'autre, mais cette variabilité est fortement réduite par l'utilisation de lignées syngéniques

(*inbred*). Cela suggère que d'importants déterminants génétiques modulent la susceptibilité à une inhibition du complexe I mitonchondrial et nourrit l'hypothèse voulant que la MP résulte d'une combinaison de facteurs génétiques et environnementaux.

D'autres évidences supportent l'idée que MFN1 joue un rôle protecteur en réponse à des stress environnementaux. Il a notamment été démontré que les cellules exposées à divers facteurs de stress (irradiation aux ultraviolets, administration d'actinomycine D, cycloheximide et autres) répondent à ces stimuli en augmentant de façon drastique le processus de fusion mitochondriale, ce qui provoque la formation de réseaux de mitochondries fortement allongées et interconnectées *(261)*. Dans les fibroblastes embryonnaires de souris, ce mécanisme qui a été appelé « hyper-fusion mitochondriale induite par le stress » requiert MFN1 mais pas MFN2. En effet, le phénomène d'hyper-fusion n'est pas observable pour les cellules MFN1 -/- exposées à un stress alors qu'il a lieu dans les cellules MFN2 -/- *(261)*. Ces données démontrent clairement qu'en dépit d'une certaine complémentation, MFN1 et MFN2 peuvent jouer des rôles distincts. De plus, cela suggère qu'une des fonctions spécifiques à MFN1 est de protéger les cellules contre des stress environnementaux en fusionnant les mitochondries, et ce afin de limiter les dommages subits à ces organelles. Sachant que nos données génétiques suggèrent que des mutations dans *MFN1* sont associées à des formes sporadiques de la MP à âge de début tardif, cette fonction est aussi cohérente avec l'hypothèse écogénétique voulant que l'apparition sporadique de la MP soit due à une combinaison de facteurs de prédisposition génétiques et environnementaux.

Pour valider nos résultats sur le gène *MFN1* et confirmer les différentes hypothèses qui en découlent, des études génétiques indépendantes de *MFN1* dans diverses populations et des études fonctionnelles détaillées devront être menées. Par exemple, il serait intéressant de vérifier si les mutations de *MFN1* arrivent à renverser le phénomène de fragmentation mitochondriale provoqué par la roténone comme le fait la surexpression de la protéine

MFN1 sauvage et si ces mutations perturbent l'hyper-fusion mitochondriale induite par le stress.

5.3.3 Avantages et limites de notre approche

Le principal point fort de la méthode mise en œuvre pour déterminer si des mutations de *PICK1* et *MFN1* prédisposent à la MP réside dans le fait que la totalité de la région codante des deux gènes a été séquencée dans un grand nombre de patients et de contrôles. Contrairement à la plupart des études génétiques qui se limitent au séquençage de patients et se contentent de vérifier la présence de mutations spécifiques dans des contrôles appariés, notre approche systématique permettait d'avoir une vue d'ensemble de la variabilité de la séquence codante des gènes étudiés et d'identifier sans biais méthodologique des mutations potentiellement pathogéniques.

L'originalité de nos travaux réside dans le fait d'avoir directement séquencé des gènes candidats dans une cohorte de patients correspondant à la réalité de la MP, c'est-à-dire un groupe de cas principalement constitué de formes sporadiques à âge de début tardif. Il faut en effet savoir que la plupart des groupes de recherche en génétique sur la MP utilisent des cas familiaux pour identifier des mutations rares à forte pénétrance et valident leurs résultats dans des cohortes élargies de cas-témoins. Cette méthode augmente les chances de trouver des mutations car l'agrégation familiale élimine la possibilité que ces formes de MP soient d'origine non-génétique. De plus les mutations identifiées peuvent être validées en vérifiant leur ségrégation dans les familles concernées. À l'exception de *GBA (112)*, tous les gènes identifiés à ce jour dans la MP l'ont été par cette approche. Par contre, cela diminue fortement les chances de trouver des mutations prédisposant exclusivement aux formes sporadiques qui représentent la grande majorité des patients vus en clinique.

Au cours des dernières années, l'idée voulant que des polymorphismes communs soient à l'origine des formes sporadiques de la MP a joui d'une grande popularité. Les

diverses études d'association du génome entier ont obtenu des résultats mitigés et toutes démontrent assez clairement que des polymorphismes fréquents ne peuvent expliquer à eux seuls la présence de formes sporadiques de la MP (section 1.5). Cette approche est particulièrement limitative car elle nécessite un nombre gigantesque de patients et de contrôles et elle n'identifie généralement pas directement les variations génétiques responsables de la susceptibilité à la maladie, mais se limite plutôt à trouver des polymorphismes en déséquilibre de liaison avec ces facteurs de susceptibilité.

En plus de tous les arguments mentionnés dans la section 1.9.2 pour appuyer l'hypothèse voulant que des mutations rares dans un grand nombre de gènes prédisposent à la MP, le séquençage de gènes candidats était un moyen original, relativement simple et direct pour identifier des mutations pathogéniques. La principale limite de notre étude réside dans le petit nombre de gènes analysés. Le projet de recherche idéal aurait probablement été de séquencer de manière systématique tous les partenaires d'interaction connus de parkin, LRRK2, alpha-synucléine, PINK1, DJ-1, MAPT et autres. Cela aurait potentiellement mené à l'identification de certains mécanismes moléculaires plus susceptibles que d'autres de contenir des mutations pathogéniques et nous aurait permis de tirer certaines conclusions quant au mécanismes particulièrement critiques dans la MP.

Si notre approche a mené à l'identification de mutations potentiellement pathogéniques dans *MFN1* et *PICK1*, nous ne pouvons pas conclure de manière définitive que des mutations rares dans les autres gènes que nous avons examinés ne prédisposent pas à la MP. En d'autres mots, le fait de ne pas avoir trouvé de mutations dans le gène *RNF11* ne veut pas dire qu'il ne sera jamais associé à la MP. Notre cohorte de départ (95 patients CF) était trop petite pour exclure de manière définitive la présence de mutations pathogéniques ayant une fréquence allélique autour de 1%.

Comme nous l'avons vu aux chapitres 3 et 4, le principal avantage de séquencer un petit nombre de gènes réside dans la relative simplicité de l'interprétation des résultats. Néanmoins, contrairement aux nouvelles techniques de séquençage à grande échelle, ce

type d'étude ne permet pas d'avoir une vue d'ensemble des variations génétiques à l'échelle du génome des patients. Notre approche n'a donc pas la capacité de conclure à une hérédité de type monogénique (une seule mutation pathogénique à relativement forte pénétrance) ou polygénique de la forme sporadique de la MP (une addition de plusieurs variations individuellement associées à un risque faible de développer la MP).

La rareté des mutations identifiées dans *MFN1* et *PICK1* et limitative pour conclure de manière définitive et non équivoque que ces mutations sont pathogéniques. Ces données devront passer le test obligatoire de tout nouveau résultat génétique, soit la réplication de ces observations par d'autres laboratoires de recherche. En plus d'être rares, ces mutations sont retrouvées dans des cas sporadiques, ce qui élimine toute possibilité de faire des études de ségrégation pour vérifier que ces variations sont bel et bien associées à la MP au sein des familles concernées. Le peu de données fonctionnelles pour appuyer les résultats génétiques constitue également un facteur limitatif important. Pour MFN1 comme pour PICK1, les études fonctionnelles des mutations sont en cours. Il faut cependant noter que des mutations prédisposant à des formes sporadiques ont à priori une pénétrance incomplète, ce qui veut dire que ce sont par nature des changements subtils qui n'ont pas un effet biologique évident. De plus, la MP apparaît généralement après de nombreuses années de vie, ce qui suggère que les mutations pathogéniques la sous-tendant n'affectent pas des mécanismes moléculaires critiques pour les cellules avant un certain âge et qu'elles ont donc probablement un effet biologique subtil ou relié à des circonstances particulières.

5.4 Problèmes non résolus et pistes de solutions

5.4.1 Approches possibles pour découvrir de nouveaux gènes

Les données disponibles sur la génétique de la MP démontrent qu'un large éventail de variations génétiques peut en être la cause : 1) des mutations généralement rares fortement pénétrantes à l'origine de formes familiales de la maladie (*SNCA*, *Parkin*,

LRRK2, *PINK1*, *DJ-1*, *GBA*) *(55-60, 112)*; 2) des mutations généralement rares à pénétrance modérée ou *de novo* dans des cas sporadiques (*SNCA*, *LRRK2*, *GBA*, *PICK1*, *MFN1*) *(69, 80, 81, 113)*; et 3) des polymorphismes à fréquence élevée dans la population générale mais qui sont surreprésentés ou sous-représentés chez les patients parkinsoniens (*SNCA*, *LRRK2*, *MAPT*, *PARK16*) *(132, 133)*. En dépit des percées significatives dans le domaine, la majorité des facteurs de prédisposition génétiques demeure inconnue à ce jour. Diverses approches ont été utilisées afin d'identifier ces variations génétiques.

L'analyse de grandes familles permet de trouver la première catégorie de mutations. Cette approche est bien maîtrisée et devrait mener à la découverte de nouvelles mutations à l'origine de formes familiales. Son efficacité n'est plus à démontrer, d'autant plus qu'il s'est avéré que la plupart des gènes identifiés contiennent aussi des mutations moins pénétrantes chez des cas sporadiques et des polymorphismes associés à la MP. Par contre, cette méthode est limitée par le nombre restreint de familles suffisamment grandes, d'un point de vue statistique, pour faire des études de liaison du génome entier.

Les études d'association du génome entier ont certes identifié des polymorphismes associés à la MP mais ont par la même occasion montré leurs limites pour ce type de maladie. Il est toujours possible de faire de nouvelles études dans des cohortes encore plus grandes de patients et de contrôles, mais cette stratégie ne servira qu'à identifier de nouveaux polymorphismes ayant un rôle encore plus marginal dans la pathogénèse de la MP. Par contre, ce type d'étude pourrait être utile pour répondre à des questions spécifiques, comme identifier des variations génétiques modifiant le risque de développer la MP chez les porteurs de la mutation G2019S de *LRRK2*, détecter des polymorphismes associés à des sous-phénotypes précis dans des études n'incluant que des cas, ou trouver des polymorphismes associés à un risque plus élevé de développer la MP en fonction de l'exposition aux pesticides ou à d'autres facteurs de risque environnementaux.

L'approche la moins étudiée car la plus récente est le séquençage génomique *(262, 263)*. À l'image de l'engouement suscité il y a quelques années par la mise au point des

études d'association du génome entier, Cette technologie disponible depuis peu est porteuse de beaucoup d'espoirs *(264)*. Par contre, comme pour les études d'association du génome entier, il est important de bien encadrer ce type d'étude pour augmenter les chances de trouver des mutations pathogéniques parmi des milliers de variations génétiques. Une approche possible serait de séquencer la totalité des exons du génome (ou exome) *(265)* dans des formes familiales de la MP et de valider les variations trouvées en vérifiant leur ségrégation au sein de ces familles et en séquençant les gènes présentant des mutations potentiellement pathogéniques dans des cohortes élargies de patients et de contrôles.

À moins de séquencer un grand nombre de patients, cette méthode risque d'être moins efficace pour les cas sporadiques car le principal défi sera d'identifier, parmi un nombre impressionnant de mutations potentiellement dommageables, laquelle (ou lesquelles) est la variation responsable de la MP pour chacun des cas séquencés. Étant donné l'hétérogénéité génétique qui sous-tend la MP, il est possible que 50 cas sporadiques soient dus à autant de mutations pathogéniques différentes, rendant ainsi la détection de telles mutations presque impossible. Nos résultats et les difficultés rencontrées pour interpréter individuellement les mutations dans *MFN1* et *PICK1* ne sont qu'un avant-goût des défis auxquels feront face les équipes de recherche qui tenteront d'identifier des mutations pathogéniques rares prédisposant aux formes sporadiques par du séquençage à grande échelle.

Une approche probablement plus appropriée pour trouver des mutations prédisposant aux formes sporadiques serait d'appliquer la méthode de séquençage de gènes candidats présentée dans cette thèse à un beaucoup plus grand nombre de gènes. Le désavantage de cette méthode réside dans le fait qu'elle ne constitue par un criblage systématique de tous les gènes du génome. Néanmoins, son moindre coût par rapport au séquençage de l'exome permettrait d'analyser un beaucoup plus grand nombre de patients et de contrôles. Étant donné l'hétérogénéité génétique de la MP, plus la cohorte de patients

utilisée sera importante, plus il sera aisé d'identifier des taux de mutations significativement plus élevés dans les patients que dans les sujets contrôles.

Le séquençage à grande échelle constitue probablement l'approche la plus prometteuse pour identifier de nouvelles mutations prédisposant aux formes familiales ou sporadiques de la MP et devrait permettre de clarifier le mode de transmission des formes sporadiques, à condition qu'un grand nombre de cas soient séquencés.

5.4.2 Génétique de la maladie de Parkinson dans la population québécoise

Nous disposons actuellement de très peu de données sur la génétique de la MP dans la population CF du Québec. De meilleures connaissances dans ce domaine pourraient s'avérer utiles, surtout si des mutations fondatrices y sont présentes. Pour étudier la question en détail, différentes mesures devraient être prises. Nous devrions tout d'abord continuer le recrutement de patients et de sujets contrôles afin d'élargir notre cohorte et séquencer tous les gènes connus pour avoir une idée précise de la proportion de patients présentant des mutations pathogéniques dans *SNCA*, *Parkin*, *LRRK2*, *PINK1*, *DJ-1* et *GBA*. Cela permettrait aussi de déterminer si les patients CF sont porteurs de mutations spécifiques ou surreprésentées dans cette population. Les polymorphismes associés à la MP d'après les deux plus récentes études d'association du génome entier *(132, 133)* devraient être génotypés dans la totalité de notre cohorte pour déterminer leur contribution à la susceptibilité à la MP chez les CF. Par la suite, un projet de séquençage à grande échelle dont les modalités restent à déterminer chez des patients ne présentant pas de mutations connues pourrait être mis en œuvre. À terme, l'objectif est d'avoir une vue d'ensemble des variations génétiques prédisposant à la MP et d'appliquer ces connaissances au niveau clinique dans le but d'améliorer le sort des patients.

5.4.3 Pathogénèse moléculaire

Malgré des progrès évidents dans la compréhension de l'étiologie de la MP, de nombreuses questions restent sans réponse. Le principal problème des études fonctionnelles des gènes sous-tendant la MP vient du fait que ces derniers codent pour des protéines aux fonctions multiples. Le défi est de déterminer parmi cet éventail de fonctions diverses quelles sont les mécanismes biochimiques les plus à même de contribuer à la mort des neurones dopaminergiques. L'identification de nouveaux gènes combinée aux études fonctionnelles des protéines qu'ils encodent devrait nous permettre de dresser un tableau plus clair des mécanismes pathogéniques sous-jacents. L'hypothèse jouissant du plus grand nombre d'indices tangibles propose un dysfonctionnement des mitochondries comme acteur principal de la neurodégénérescence dopaminergique. De nombreuses observations, dont le fait que les cinq gènes au cœur de la MP jouent un rôle au niveau des mitochondries (voir section 1.7.3) convergent vers cette idée.

Les mécanismes pathogéniques proposés sont une diminution de l'activité du complexe I et le stress oxydatif qui en résulte *(191)*, des mutations dans l'ADN mitochondrial *(165)* et un déséquilibre de la dynamique de ces organelles *(190)*. Il est important de noter qu'un lien de cause à effet existe entre ces trois éléments clés. Notamment, la production de radicaux libres par la chaîne respiratoire rend l'ADN mitochondrial plus susceptible à l'apparition de mutations *(266)* et la fusion restaure la fonction des mitochondries ayant subi des dommages à leur ADN *(239)*. De plus, les inhibiteurs du complexe I associés à la MP comme la roténone et le MPP+ induisent également une fragmentation mitochondriale *(241)*. Enfin, les mitochondries synaptiques présentent une morphologie ponctiforme et sont plus sensibles à une inhibition du complexe I que les mitochondries non-synaptiques qui sont généralement plus allongées *(267)*.

Ces différentes perturbations induiraient la mort des neurones dopaminergiques par un mécanisme apoptotique dépendant des mitochondries *(167, 268)*, un processus régulé par les protéines aux effets pro ou anti-apototiques de la famille Bcl-2 *(269)*. Les fonctions de MFN1 et PICK1 pourraient offrir des indices supplémentaires afin d'expliquer les mécanismes moléculaires à l'origine de la dégénérescence neuronale dans la MP : par exemple, l'homologue du facteur anti-apoptotique Bcl-2 chez le nématode promeut la fusion des mitochondries par un mécanisme dépendant des homologues de MFN1, 2 et OPA1 *(270)* alors que PICK1 serait responsable de la phosphorylation de Bcl-2 par PKCalpha (voir section 5.3.1). Ces données sont très fragmentaires mais elles suggèrent que PICK1 et MFN1 régulent le processus d'apoptose dépendant des mitochondries.

5.4.4 Dégénérescence sélective des neurones dopaminergiques

Diverses hypothèses ont été émises pour tenter de comprendre les raisons de la dégénérescence hautement sélective des neurones dopaminergiques de la SNpc dans la MP. Encore une fois, les mitochondries pourraient fournir certains éléments de réponses. Il est possible que ces neurones soient plus vulnérables à une inhibition du complexe I, à un déséquilibre de la dynamique mitochondriale et à l'excitotoxicité du glutamate.

Il a été suggéré que cette sous-population neuronale serait intrinsèquement plus vulnérable que d'autres neurones à une inhibition des différents complexes de la chaîne respiratoire *(271)*. Par exemple, comment expliquer que l'administration de roténone chez le rat provoque une diminution de l'activité du complexe I mitochondrial dans tout le cerveau mais que la dégénérescence neuronale qui en découle n'affecte que les neurones dopaminergiques nigrostriataux *(260)* ? Des travaux récents pourraient fournir une explication à cette observation. Il a en effet été démontré chez la souris que les neurones dopaminergiques de la SNpc présentent une masse de mitochondries moindre que les sous-populations neuronales non dopaminergiques de la SN ou les neurones dopaminergiques de l'aire tegmentale ventrale *(272)*. Par contre, les neurones dopaminergiques du noyau

interfasciculaire présentent une masse mitochondriale comparable à ceux de la SNpc, indiquant que ce facteur ne peut expliquer à lui seul la dégénérescence sélective caractéristique de la MP.

Encore chez la souris, les neurones dopaminergiques seraient trois fois plus sensibles que les neurones GABAergiques ou cholinergiques aux effets toxiques combinés de l'exposition à la roténone et au glutamate *(273)*. L'excitotoxicité du glutamate est due à la dépolarisation prolongée des neurones qui induit un influx important de Ca^{2+} *(274)*. Ce dernier est séquestré dans le réticulum endoplasmique et les mitochondries. Si l'influx de Ca^{2+} dans les mitochondries participe à la production d'ATP, des concentrations trop élevés compromettent ce processus et induisent une dépolarisation des mitochondries, ce qui augmente le stress oxydatif et l'apopotose *(275, 276)*. En altérant les niveaux d'ATP et/ou en provoquant un déséquilibre de l'homéostasie du Ca^{2+}, un dysfonctionnement du complexe I mitochondrial pourrait rendre les neurones plus vulnérables à l'excitotoxicité du glutamate *(258)*. Les neurones dopaminergiques de la SNpc seraient aussi particulièrement vulnérables au phénomène d'excitotoxicité car ils contiennent des concentrations élevées de récepteurs du glutamate *(277)*. De plus ils sont stimulés par les neurones glutamatergiques du noyau sous-thalamique, ce qui pourrait les rendre plus vulnérables à une inhibition du complexe I, à un déséquilibre de la dynamique mitochondriale, ou à un débalancement de l'homéostasie du Ca^{2+} *(258)*. Il a d'ailleurs été suggéré que l'hyperactivité des neurones excitateurs du noyau sous-thalamique pourrait participer au processus neurodégénératif de la MP *(251)*.

Les neurones dopaminergiques de la SNpc sont entre autres caractérisés par de longs et fins axones *(278)*, ce qui suggère qu'ils pourraient être particulièrement sensibles à une bonne régulation de la dynamique mitochondriale, essentielle au transport de ces organelles *(190)*. De plus, les mitochondries synaptiques, qui sont caractérisées par une morphologie ponctiforme, sont plus vulnérables que les mitochondries non-synaptiques aux effets néfastes d'une surcharge de Ca^{2+} *(279)* et à une inhibition du complexe I *(267, 280)*.

Il est également important de noter que l'exposition au NMDA, par le biais de la production d'oxyde nitrique, provoque une augmentation de la fission mitochondriale *(241)*. Ces données sont particulièrement intéressantes car elles soulignent une fois de plus le lien qui existe entre la régulation de la dynamique mitochondriale, l'activité du complexe I et l'excitotoxicité du glutamate.

En résumé, les neurones dopaminergiques de la SNpc sont stimulés en permanence par des neurones glutamatergiques excitateurs et sont caractérisés par un nombre peu élevé de mitochondries et par des axones relativement longs. Cette conjoncture de facteurs les rendrait plus vulnérables à un dysfonctionnement du processus de production d'énergie, à un déséquilibre de la dynamique mitochondriale, à l'excitotoxicité du glutamate ou à un déséquilibre de l'homéostasie du Ca^{2+}. Comme nous venons de le voir, de nombreux liens de cause à effet existent entre ces différents éléments (**Figure 1**). Ces anomalies pourraient provoquer la mort des neurones dopaminergiques nigrostriataux par un mécanisme d'apoptose dépendant des mitochondries. Il est possible que MFN1 et PICK1 jouent un rôle clé dans ce processus neurodégénératif ; en affectant la dynamique mitochondriale dans le cas des mutations de *MFN1*, et en augmentant l'excitotoxicité du glutamate pour les mutations de *PICK1*.

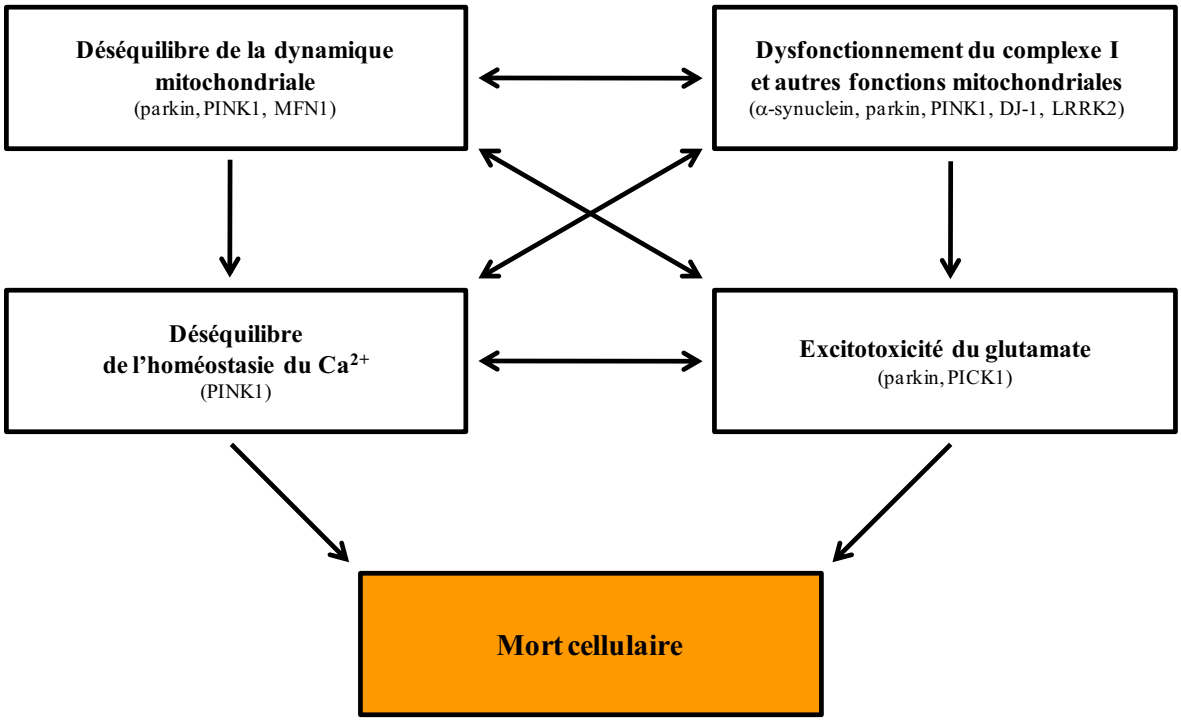

Figure 1. Mécanisme proposé pour expliquer la mort sélective des neurones dopaminergiques de la SNpc. Les gènes mutés prédisposant à la MP ayant un rôle hypothétique ou avéré dans chacun de ces mécanismes moléculaires sont indiqués entre parenthèses.

5.5 Conclusion

Depuis la description clinique de la « paralysie agitante » au début du XIXème siècle, des progrès majeurs ont été réalisés dans la compréhension et le traitement de la MP. Néanmoins, de nombreuses questions restent sans réponse et aucune approche thérapeutique capable de renverser l'effet irréversible de la neurodégénérescence n'est encore disponible. Au cours de la dernière décennie, des percées significatives en génétique humaine ont mené à l'identification de plusieurs gènes responsables du développement de la MP. La caractérisation des produits de ces gènes avance à grands pas et permet

aujourd'hui de mieux discerner les acteurs de sa pathogénèse moléculaire. Les travaux sur des cultures cellulaires ou des modèles animaux allant de la levure au singe ont démontré que la plupart des gènes responsables de la MP interagissent dans des voies moléculaires communes. En outre, ces modèles sont d'excellents outils pour tester de nouvelles approches thérapeutiques en phase préclinique. Aujourd'hui, bien que confrontée à de multiples défis, la nouvelle génération de stratégies thérapeutiques est porteuse de nombreux espoirs et le développement de traitements neuroprotecteurs a quitté le domaine de la science-fiction. Par contre, le vieillissement de la population et l'augmentation de l'espérance de vie laissent présager une forte augmentation de la prévalence des maladies neurodégénératives comme la MP. La grande diversité des types de variations génétiques prédisposant à la MP met en évidence la nécessité de continuer à utiliser des approches variées pour identifier de nouveaux gènes causatifs ou des facteurs de susceptibilité génétiques. La mise au point de nouvelles technologies de séquençage génomique est également porteuse d'espoir et devrait accélérer de manière significative l'élucidation des facteurs de prédisposition génétiques encore inconnus. Le données présentées dans cette thèse suggèrent que cette approche pourrait être efficace pour expliquer les formes sporadiques de la MP mais illustrent en même temps le défi que représente la validation des mutations rares à pénétrance incomplète. L'étude d'une maladie aussi complexe que la MP requiert l'utilisation d'outils variés et une approche pluridisciplinaire (clinique, génétique des populations, génétique humaine, biologie moléculaire, pharmacologie, etc.). Une collaboration accrue entre ces différents domaines serait souhaitable afin d'appliquer le plus rapidement possible les données générées par la recherche fondamentale à la prévention et au traitement de cette maladie qui reste à ce jour incurable.

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Annexe : informations supplémentaires

Curriculum vitae

Scolarité

Diplôme	Discipline	Établissement	Pays	Inscription	Obtention
Doctorat	Biologie moléculaire	Université de Montréal	Canada	05/2006	05/2010
Maîtrise	Biologie moléculaire	Université de Montréal	Canada	09/2004	05/2006
Baccalauréat	Sciences Biologiques	Université de Montréal	Canada	09/2001	09/2004
Baccalauréat français (DEC)	Section Scientifique	Collège Stanislas, Montréal	Canada	09/1999	06/2001

Bourses et prix obtenus par voie de concours

Bourse ou prix	Montant/année	Durée
Bourse d'excellence – Catégorie Doctorat (CRCHUM)	1 000$	1 an (2006-2007)
Bourse d'études supérieures du Canada - Bourse au doctorat (IRSC)	35 000$	3 ans (2006-2009)
Bourse de maîtrise (CRCHUM)	2 000$	1 an (2005-2006)

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Rivière JB *et al.* Genetic analysis of a large French Canadian Tourette Syndrome family. European Human Genetics Conference. June 2008. Barcelona, Spain.

Rivière JB *et al.* Linkage analysis in five French Canadian families with Essential Tremor. 60th Annual Meeting of the American Academy of Neurology. April 2008. Chicago, Illinois. U.S.A

Rivière JB *et al.* Large-scale screening of X-linked synaptic genes in Tourette Syndrome cases. 57th Annual Meeting of the American Society of Human Genetics. October 2007. San Diego, California. U.S.A

Rivière JB *et al.* Association study of the GABAA cluster on 15q11-13 with Tourette Syndrome in the French Canadian population. European Human Genetics Conference. June 2007. Nice, France.

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Mutations in *DCC* Cause Congenital Mirror Movements

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Mirror movements (MM) are contralateral involuntary movements that mirror voluntary ones. MM are occasionally found in young children, but persistence beyond age 10 is unusual except in certain disorders of nervous system crossing such as Klippel-Feil and Kallmann syndrome. The study of individuals with MM can provide important insights into the mechanisms of contralateral innervation.

We recently described a large four-generation French Canadian (FC) family with isolated congenital MM (CMM) (*1*). Pedigree analysis suggested autosomal dominant inheritance with incomplete penetrance. We conducted a genome-wide linkage analysis and identified a single significant locus on chromosome 18q21.2. Haplotype analysis indicated that all affected individuals share a common risk haplotype (figs. S1 and S2). The region spans 2.5 Mb and contains three known genes, including *DCC* (deleted in colorectal carcinoma).

Sequencing of the 29 coding exons of *DCC* revealed a guanine to adenine substitution at the first intronic nucleotide after exon 6, in the splice donor consensus sequence of the exon (c.1140+1G>A, Fig. 1A left). The mutation segregated with the risk haplotype and was not found in 760 unrelated Caucasian controls, including 512 FC. Copy number variation analysis in 315 FC controls did not reveal any structural variants encompassing *DCC* exons.

To assess the effect of this mutation on splicing, we extracted RNA from immortalized lymphoblast cells derived from four mutation carriers and one noncarrier relative and performed reverse transcription polymerase chain reaction (RT-PCR). Amplification of the cDNA between exons 4 and 7 and subsequent gel electrophoresis identified a 408–base pair (bp) fragment corresponding to the predicted amplicon size found in all individuals and a 253-bp fragment found only in affected individuals (Fig. 1B). Sequence analysis confirmed that the 253-bp fragment corresponds to an aberrant transcript lacking exon 6. Abnormal skipping of exon 6 leads to a frameshift mutation from amino acid 329 and the introduction of a stop codon 15 amino acids further down the new reading frame (p.V329GfsX15). This mutation is predicted to result in a truncated DCC protein lacking most of its functional domains. This mutant DCC does not bind netrin (Fig. 1, C and D, and fig. S3).

To confirm the role of *DCC* in CMM, we sequenced *DCC* in a previously described Iranian family with CMM (*2*). We found a guanine insertion in exon 3 (Fig. 1A right) segregating with the disorder (fig. S4) that results in a frameshift also predicted to result in a truncated DCC protein [c.571dupG; p.V191GfsX35 (Fig. 1D)]. This mutation was absent in 538 unrelated control individuals.

DCC is a receptor for netrin-1, a diffusible extracellular protein that helps guide axons of the developing nervous system across the body's midline (*3*). The fourth and fifth fibronectin type III repeats in DCC are responsible for its binding to netrin-1 (*4*). Mice with homozygous null *DCC* mutations have severe defects of commissural development in the brain and spinal cord, with absent corpus callosum and decreased number and misrouting of commissural axons (*5*). "Kanga" mice have deletions of *DCC* exon 29 and exhibit mirror-type movements that result in a distinctive hopping gait (*6*). The latter mutant also shows defects in the crossing of corticospinal tracts and persistence of ipsilateral corticospinal tracts in hindbrain and spinal cord. This is consistent with human neurophysiological studies in which unilateral stimulation of the motor cortex in patients with MM evokes, in contrast to normal participants, a bilateral response (*7*) (fig. S5), indicating a misdirected ipsilateral corticospinal connection.

We propose that the *DCC* mutations in individuals with CMM cause a reduction in gene dosage and less robust midline guidance, which may lead to a partial failure of axonal fiber crossing and development of an abnormal ipsilateral connection. Our genetic findings confirm that *DCC* has a central role in the development of human nervous system lateralization.

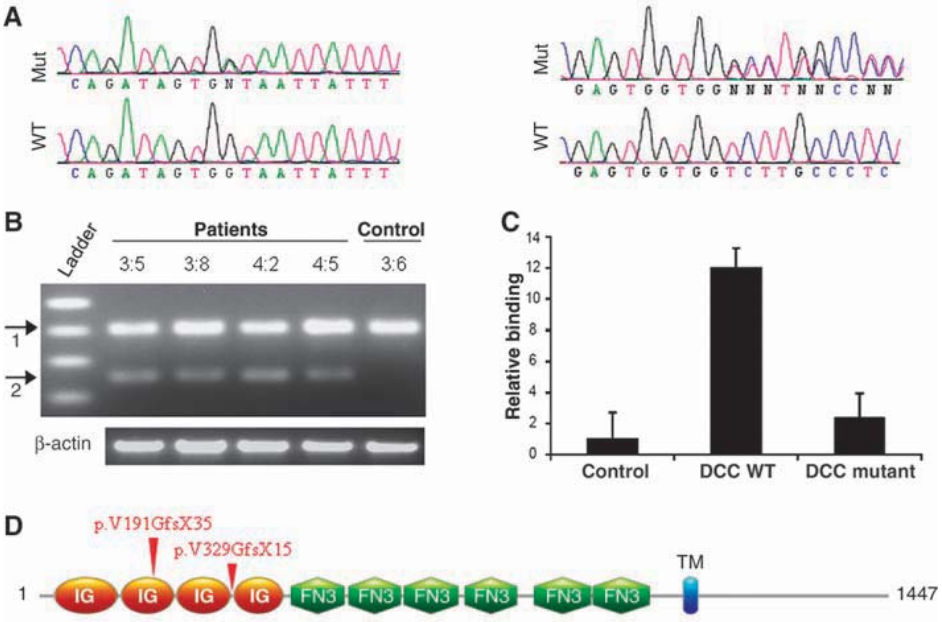

Fig. 1. *DCC* mutations in individuals with CMM. (**A**) *DCC* sequence data showing c.1140+1G>A mutation in the FC family (left) and c.571dupG mutation in the Iranian family (right). The traces show the mutant (Mut) and wild-type (WT) sequences. (**B**) Electrophoresis of RT-PCR products derived from FC patient mRNA. Shown are the normal 408-bp fragment (arrow 1) and the aberrant 253-bp (arrow 2) fragment. β-actin was coamplified as an internal control. (**C**) Mutant DCC from the FC family does not bind netrin. COS7 cells were transfected with a control plasmid, a plasmid expressing wild-type (WT) DCC (IB07), or a plasmid expressing mutant DCC (HZ29). (**D**) Schematic of the DCC protein, showing the four extracellular immunoglobulin-like C2-type domains (IG), the six extracellular fibronectin type 3 domains (FN3), and the transmembrane domain (TM). Arrowheads above the protein show the positions of the frameshift mutations.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/328/5978/592/DC1
Materials and Methods
Figs. S1 to S5
Table S1
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Genome-Wide TDT Analysis in French-Canadian Families with Tourette Syndrome

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Tourette syndrome (TS) is a complex neurodevelopmental disorder with an estimated prevalence of 1%[1] that is characterized by motor and vocal tics as well as psychiatric comorbidities, such as obsessive-compulsive disorder (OCD) and attention deficit hyperactivity disorder (ADHD).[2-4] Despite a strong genetic contribution,[5,6] no common variants have been clearly associated with the disorder, possibly because of allelic and non-allelic genetic heterogeneity. Several candidate genes involved in dopaminergic neurotransmission have been analyzed based on the observation that neuroleptics are used to treat TS patients. Positive association results between TS and some of these genes have been reported.[7-9] However, because other studies failed to replicate these results, the role played by dopaminergic in TS remains unclear.[10-12] Founder populations are known to offer several advantages for the gene-mapping of complex traits, as the reduced genetic heterogeneity of population isolates is thought to simplify the genetic background of complex traits and higher frequencies of specific mutations inherited from a common ancestor may be observed. Moreover, even if the number of implicated genes is not decreased, the allelic heterogeneity is decreased and the presence of a common haplotype that segregates among patients is more probable than within a heterogeneous population.[13] For instance, the G2019S substitution in the *LRRK2* gene accounts for 20–40% of North-African Arab and Ashkenazi patients with Parkinson disease.[14,15] In the French-Canadian (FC) population, founder mutations in the *BRCA1* and *BRCA2* genes were identified in 40% of patients from families with a high risk of breast and ovarian cancer.[16] For several reasons, the FC population of Quebec displays all the characteristics of a population isolate. An estimated 2,600 pioneers who settled in Quebec before 1680 account for two thirds of the modern FC gene pool and a vast majority of FC people have, as ancestors, approximately 7,000 individuals who immigrated to Quebec before 1760. Given that these founders rarely mixed with other immigrants over three centuries and that there was a sustained demographic growth in the FC population, the ~6 million FC individuals currently living in Quebec inherited most of their genes from a relatively small pool of founders.[17]

Based on a cohort of 217 FC trios (father, mother and proband) presenting with TS, the goal of this study was to assess the presence of frequent and highly penetrant alleles predisposing to TS in the FC population. Affected individuals and their relatives were recruited through the Montreal General Hospital and the Sainte-Justine Hospital (Montreal). Experienced clinicians performed diagnostic evaluations of TS, chronic tics and ADHD using the Diagnostic and Statistical Manual of Mental Disorders-IV criteria. Obsessive-compulsive disorder was evaluated using the Yale–Brown Obsessive-Compulsive Scale.[18] We obtained approval from the ethics committee of our institution, as well as informed consent from all participants. The DNA was extracted from whole blood using standard procedures. For the purpose of the family-based genome-wide association analysis, we selected 95 trios among the 217 nuclear families recruited. To avoid the inclusion of sporadic cases, we selected only patients with a positive familial history of TS, tics or comorbid psychiatric disorders. Among the 95 selected TS cases, 73 were male, 31 presented with OCD and 53 received a diagnosis of ADHD. The DNA samples from the probands and their parents were sent to the deCODE genotyping service (http://www.decode.com/ genotyping). Five hundred fifty-one highly polymorphic microsatellites covering the 22 autosomes and the X chromosome with an average marker density of 8 centimorgans were genotyped using standard methods. The successful genotyping rate was 95.8% and Mendelian inconsistencies were systematically removed. We performed a family-based transmission disequilibrium test (TDT) using the Family-Based Association Test (FBAT) program, version 2.0.2C.[19,20] The TDT determines whether an excess or a lack of transmission of alleles to the affected offspring occur. Multiallelic tests of association were performed for each marker. The additive genetic model was applied, as it often performs best, even when the genetic model is not additive.[21] The significance level was set at 0.00009 after Bonferroni correction for multiple testing. Employing the TDT Power Calculator software, we assessed the statistical power of the TDT from 95 trios[22]. Table 1 displays the number of trios

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Table 1: Computation of the statistical power: Number of trios necessary to achieve 90% power in the TDT

		Linkage disequilibrium coefficient			
		1.0	0.8	0.6	0.4
Frequency of the disease susceptibility allele	0.4	50	81	145	325
	0.2	66	105	187	420
	0.1	87	138	247	552
	0.05	126	198	353	789

The frequency of marker allele segregating with the disease susceptibility allele is 0.4, and the recombination fraction between the marker locus and the disease susceptibility locus is 0. Genotype penetrances for *AA* (homozygotes for the susceptibility allele), *Aa* (heterozygotes for the susceptibility allele), and *aa* (non-carriers of the susceptibility allele) are 0.98, 0.28 and 0.01, respectively.

estimated to be necessary to achieve 90% statistical power with a significance level of 0.00009, according to variable disease susceptibility allele frequencies and degrees of linkage disequilibrium between a marker and a disease susceptibility locus. Although none of the markers reached the significance level for association, we obtained *P* values < 0.005 for four markers (D7S2485, D13S271, D15S1016 and D19S605; Figure and Table 2). None of the markers are located in any of the chromosomal loci proposed to be linked to TS by the Tourette Syndrome Association International Consortium for Genetics.[23,24] To attempt to replicate our findings, the four markers were genotyped in the remaining 122 FC trios from our initial cohort of 217 FC trios with TS. Unfortunately, the analysis of the genotype data from the 217 FC trios revealed that none of the markers remained associated with TS (Table 2).

Our data suggest that common and highly penetrant founder mutations are unlikely to predispose to TS in the FC population. Our inability to replicate the association when the whole TS cohort was included in the analysis suggests that the peaks observed in the initial analysis were likely false-positive signals, which is strengthened by the fact that no single marker reached the genome-wide significance threshold. One may argue that the lack of replication of the association in the whole cohort may stem from the fact that we selected the 95 trios based on positive familial history of TS, and that the 122 remaining trios may comprise a significant proportion of non-genetic cases of TS. This is unlikely, as the vast majority of the cases in our cohort have first-degree relatives with TS, tics, ADHD or OCD, which indicates that sporadic cases with TS are the exception, rather than the rule. Our results suggest that, even in the case of a founder population (such as the FC), the genetically complex nature of TS renders this type of analysis powerless; the lack of significant association results in the analysis presented here may be explained by the complex inheritance pattern of TS and its comorbidities[25], which have a great negative impact on family-based studies. We are aware that our study presents several limitations, which include the relatively small number of families and the low marker density of the genome-wide scan. As presented in Table 1, statistical power computation indicates that our sample size was probably large enough to detect an allele segregating with a founder mutation carried by 20 to 40% of patients, but the study was most likely underpowered for founder mutation frequencies of 10% or less, unless the marker allele was in perfect linkage disequilibrium with a founder mutation. A high-density single nucleotide polymorphism genome-wide association study using a large cohort of patients may be necessary to verify whether TS originates from the combined effect of several common and low penetrant alleles. It would also

Table 2: TDT analysis (FBAT v.2.0.2C) of the four markers exhibiting a *P* value < 0.005, and association results in the 217 FC TS trios

Marker	Chr	Position (bp)	95 trios *P* value	217 trios *P* value
D7S2485	7q21.11	83,997,436	0.000595	0.451994
D13S271	13q31.1	84,278,764	0.003924	0.325128
D15S1016	15q21.3	51,320,121	0.000341	0.234887
D19S605	19q13.42	60,443,625	0.003598	0.163923

"Chr" refers to the chromosome band. The position of markers is in base pairs (bp) and was derived from the publicly available human genome assembly (UCSC Genome Browser).

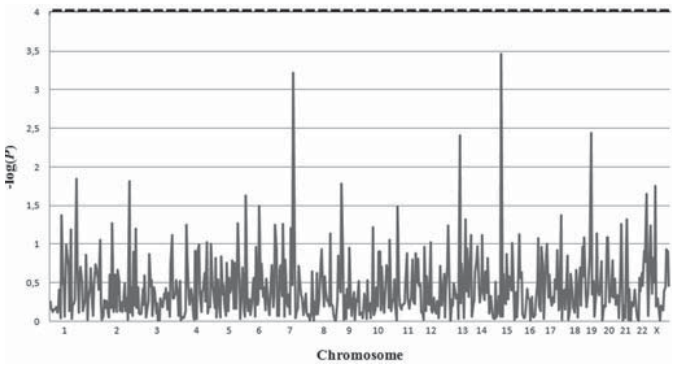

Figure: *Genome-wide TDT analysis using FBAT (v.2.0.2C) in 95 FC TS trios. TDT analysis of the 551 microsatellite markers is represented by –log P values. The dotted line indicates the genome-wide significance level, which was set at 0.00009 after correction for multiple testing.*

be feasible to use ADHD and OCD diagnoses as covariates, or to analyze subgroups of TS patients separately, based on their comorbidities. However, this approach would necessitate large cohorts of patients and would reduce the statistical power because of multi-test adjustments. If the genetic model for TS is that many cases are caused by individually rare, but highly penetrant mutations, this approach will not be successful, and a whole-genome resequencing analysis or large-scale screening of candidate genes will be warranted. Finally, this study illustrates the difficulty in identifying susceptibility genes for neuropsychiatric disorders and emphasizes the necessity to develop new gene-mapping approaches for these complex disorders.

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ORIGINAL CONTRIBUTION

Association of Intronic Variants of the *BTBD9* Gene With Tourette Syndrome

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Objective: To test the association between Tourette syndrome (TS) and genetic variants in genomic loci *MEIS1*, *MAP2K5/LBXCOR1*, and *BTBD9*, for which genome-wide association studies in restless legs syndrome and periodic limb movements during sleep revealed common risk variants.

Design: Case-control association study.

Setting: Movement disorder clinic in Montreal.

Subjects: We typed 14 single-nucleotide polymorphisms spanning the 3 genomic loci in 298 TS trios, 322 TS cases (including 298 probands from the cohort of TS trios), and 290 control subjects.

Main Outcome Measures: Clinical diagnosis of TS, obsessive-compulsive disorder, and attention-deficit disorder.

Results: The study provided 3 single-nucleotide polymorphisms within *BTBD9* associated with TS (χ^2=8.02 [P=.005] for rs9357271), with the risk alleles for restless legs syndrome and periodic limb movements during sleep overrepresented in the TS cohort. We stratified our group of patients with TS according to presence or absence of obsessive-compulsive disorder and/or attention-deficit disorder and found that variants in *BTBD9* were strongly associated with TS without obsessive-compulsive disorder (χ^2=12.95 [$P<.001$] for rs9357271). Furthermore, allele frequency of rs9357271 inversely correlated with severity of obsessive-compulsive disorder as measured by the Yale-Brown Obsessive Compulsive Scale score.

Conclusion: Variants in *BTBD9* that predispose to restless legs syndrome and periodic limb movements during sleep are also associated with TS, particularly TS without obsessive-compulsive disorder.

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RESTLESS LEGS SYNDROME (RLS) is a neurological condition characterized by a recurrent urge to move the legs, with or without an uncomfortable sensation.[1,2] Periodic limb movements during sleep (PLMS), which consist of repetitive and stereotyped movements of the legs, are frequently found in patients with RLS.[3,4] Tourette syndrome (TS) is a neurodevelopmental disorder characterized by motor and vocal tics,[5] often accompanied by attention-deficit disorder (ADD) with or without hyperactivity and obsessive-compulsive disorder (OCD).[6,7]

Although RLS and TS are distinct movement disorders, they share some common features, and a high prevalence of RLS has been reported in TS.[8,9] In addition, RLS and TS have been linked to dysfunction in frontostriatal circuits, and both are responsive to modification of dopamine neurotransmission.[10,11] Sensations relieved by movement are also found in TS cases; patients frequently describe urges or sensations preceding tics.[12] Although it is still unclear whether TS has a sensory component, this question is receiving great attention because sensory phenomena may help to identify more homogeneous subgroups of TS and OCD cases.[13]

Recently, a genome-wide association study identified the following 3 loci associated with RLS: *MEIS1* (OMIM 601739), *BTBD9* (OMIM 611237), and *MAP2K5/LBXCOR1* (OMIM 602520/611273).[14] Simultaneously, another genome-wide association study uncovered an association between variants in *BTBD9* and PLMS.[15]

To assess the role of these genes in TS, we analyzed 14 single-nucleotide polymorphisms (SNPs) spanning the 3 loci and reported to be associated with RLS.[14] We performed a case-control and a family-based association study and found that the predisposing alleles for RLS in the *BTBD9* gene are associated with TS, and particularly TS without psychiatric comorbidities.

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Group Information: The Montreal Tourette Study Group members are listed at the end of this article.

METHODS

SAMPLE COLLECTION

Two hundred ninety-eight French Canadian TS trios (father, mother, and proband) were re-

Table 1. Selected SNPs and Association Results in RLS From Previous Studies

SNP Information						Association Results in RLS	
Chromosome	Gene	SNP No.	dbSNP ID	Base Pair Position	Alleles, Major/Minor	Risk Allele[a]	MAF, Case/Control
2p	*MEIS1*	1	rs12469063	66617812	A/G	G	0.36/0.24
2p	*MEIS1*	2	rs2300478	66634957	T/G	G	0.37/0.24
6p	*BTBD9*	3	rs9394492	38440588	C/T	C	0.29/0.35
6p	*BTBD9*	4	rs4714156	38469090	C/T	C	0.16/0.24
6p	*BTBD9*	5	rs9296249	38473819	T/C	T	0.16/0.24
6p	*BTBD9*	6	rs9357271	38473851	T/C	T	0.16/0.24
6p	*BTBD9*	7	rs3923809	38548948	A/G	A	0.19/0.34
15q	*MAP2K5*	8	rs12593813	65823906	G/A	G	0.26/0.33
15q	*MAP2K5*	9	rs11635424	65824632	G/A	G	0.26/0.33
15q	*MAP2K5*	10	rs884202	65841442	A/G	A	0.25/0.33
15q	*MAP2K5*	11	rs4489954	65859129	G/T	G	0.24/0.31
15q	*MAP2K5*	12	rs3784709	65859329	C/T	C	0.25/0.32
15q	*MAP2K5*	13	rs1026732	65882139	G/A	G	0.25/0.33
15q	*MAP2K5*	14	rs6494696	65890260	G/C	G	0.25/0.33

Abbreviations: dbSNP, single-nucleotide polymorphism (SNP) database; ID, identification; MAF, minor allele frequency; RLS, restless legs syndrome.
[a] Indicates overrepresented allele in patients with RLS.[14,15]

Table 2. Case-Control Association Results

SNP No.	dbSNP ID	Genotyping Success Rate, %	HWE *P* Value	Alleles, Major/Minor	Risk Allele	MAF, Case/Control	χ^2 Value	*P* Value[a]
1	rs12469063	99.7	.93	A/G	G	0.26/0.23	1.19	.28
2	rs2300478	98.5	.30	T/G	G	0.26/0.25	0.16	.68
3	rs9394492	99.2	.94	C/T	C	0.31/0.35	1.79	.18
4	rs4714156	99.0	.64	C/T	C	0.17/0.23	7.25	**.007**
5	rs9296249	99.2	.91	T/C	T	0.16/0.22	6.72	**.01**
6	rs9357271	99.2	.78	T/C	T	0.17/0.23	8.02	**.005**
7	rs3923809	99.2	.89	A/G	A	0.29/0.30	0.26	.61
8	rs12593813	99.8	.13	G/A	G	0.34/0.33	0.18	.67
9	rs11635424	98.7	.14	G/A	G	0.34/0.33	0.07	.79
10	rs884202	99.5	.29	A/G	A	0.34/0.32	0.21	.65
11	rs4489954	98.7	.61	G/T	G	0.32/0.30	0.67	.41
12	rs3784709	99.8	.28	C/T	C	0.33/0.32	0.14	.71
13	rs1026732	99.3	.28	G/A	G	0.34/0.32	0.16	.69
14	rs6494696	98.8	.28	G/C	G	0.34/0.32	0.21	.64

Abbreviations: dbSNP, single-nucleotide polymorphism (SNP) database; HWE, Hardy-Weinberg equilibrium test; ID, identification; MAF, minor allele frequency.
[a] Indicates uncorrected. In the Bonferroni correction for multiple testing, the significance level was set to .0127, as explained in the "Statistical Analyses" subsection of the "Methods" section. Boldface *P* values are less than the significance threshold.

cruited through the Montreal General Hospital, Montreal, Quebec, Canada, and Sainte Justine Hospital for the purpose of family-based association studies. Exclusion criteria were (1) the inability to provide a consent form, (2) a history of another neurological disorder, and (3) the presence of tics induced by drugs or other factors. Ethics committee approval and informed consent were obtained. Experienced clinicians performed diagnostic evaluations of TS, chronic tics, OCD, and ADD during direct family interviews. Tourette syndrome, chronic tics, and ADD were diagnosed using criteria of the *Diagnostic and Statistical Manual of Mental Disorders* (Fourth Edition).[16] Obsessive-compulsive disorder was evaluated using the Yale-Brown Obsessive Compulsive Scale (YBOCS)[17] and classified as absent or present (<16 vs ≥ 16 points). Our cohort of TS cases consists of 298 unrelated probands originating from the collection of trios and 24 unrelated additional cases, for a total of 322 TS cases. Two hundred ninety unrelated healthy, ethnically matched individuals were used as control subjects for this group of patients. Genomic DNA was extracted from whole blood following standard methods.

SNP GENOTYPING

We selected 14 SNPs found to be associated with RLS or PLMS in the 2 genome-wide association studies (**Table 1**).[14,15] The SNPs were genotyped using a polymerase chain reaction system (TaqMan SNP genotyping assay with the 7900 fast real-time system; Applied Biosystems, Foster City, California) and commercially available software (SDS, version 2.2.2; Applied Biosystems) for allele calling.

STATISTICAL ANALYSES

Case-control association analysis, a transmission disequilibrium test, a Hardy-Weinberg equilibrium test, and pairwise linkage disequilibrium calculations were performed using Haploview, version 4.1.[18] Haplotype blocks were defined using the method of Gabriel et al[19] and implemented in Haploview. Single-factor analysis of variance was performed on YBOCS total scores

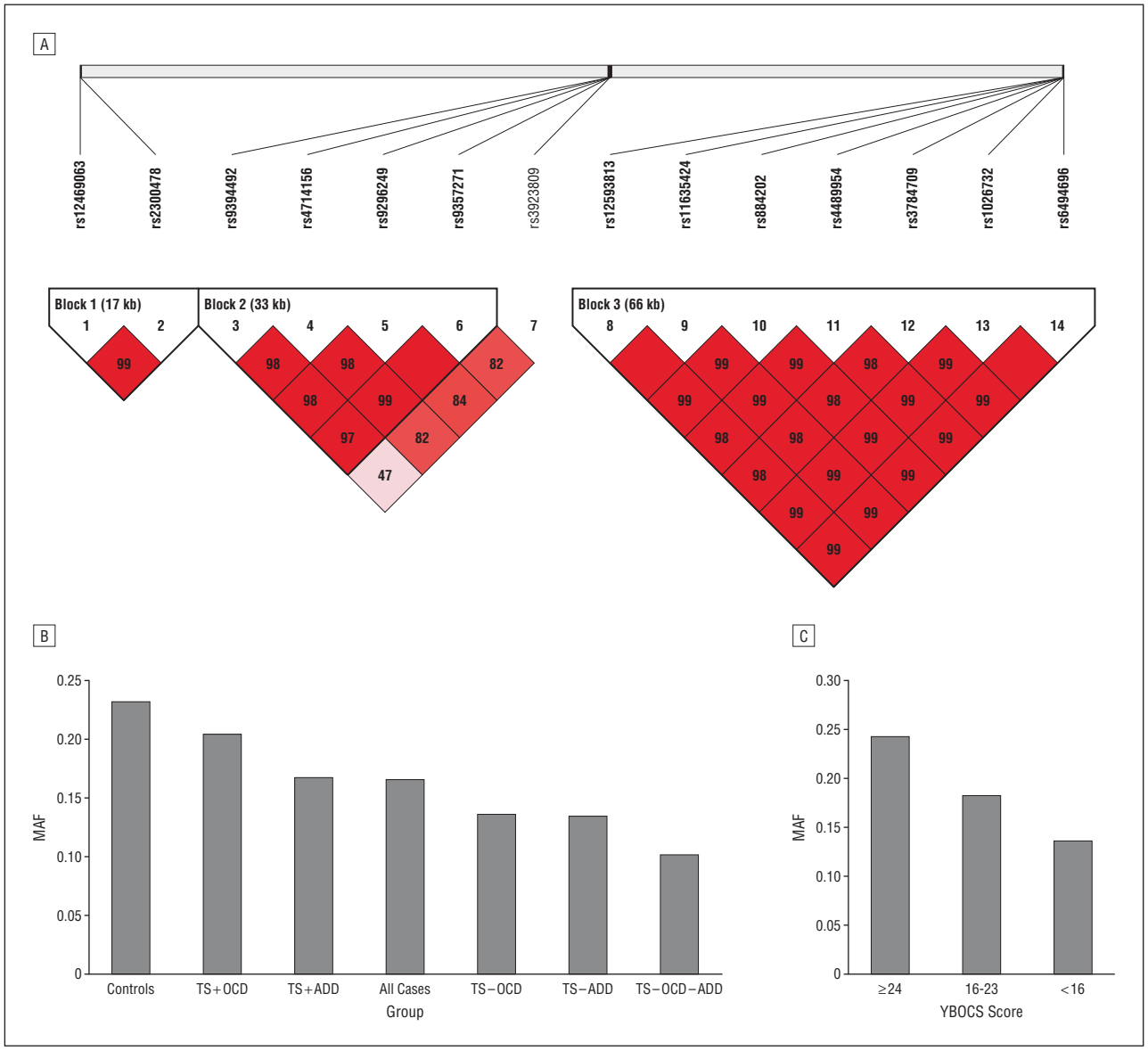

Figure. Findings in patients with Tourette syndrome (TS) with and without psychiatric comorbidities. A, Pairwise linkage disequilibrium diagrams for the 3 loci (*MEIS1*, *BTBD9*, and *MAP2K5/LBXCOR1*) with risk variance for restless legs syndrome. Pairwise linkage disequilibrium was measured as D′ and calculated from our case-control cohort, in Haploview, using the methods of Gabriel et al.[19] The white-to-red gradient reflects lower to higher linkage disequilibrium values, respectively; kb indicates kilobase. Single-nucleotide polymorphisms (SNPs) in bold listed across the top indicate haplotype blocks. These SNP alleles show strong linkage disequilibrium among themselves, and there is little evidence of recombination. B, Graphical representation of minor allele frequencies (MAFs) of rs9357271 in control subjects and in subgroups of patients, derived from the MAF values in Table 2. From left to right on the x-axis, subgroups of patients are aligned from highest to lowest MAF. C, Graphical representation of MAFs of rs9357271 in subgroups of patients classified according to Yale-Brown Obsessive Compulsive Scale (YBOCS) scores as having moderate obsessive-compulsive disorder (OCD) (YBOCS score of 16-23; MAF, 0.18; 64 individuals) and severe OCD (YBOCS score of ≥24; MAF, 0.24; 39 individuals). The TS−OCD subgroup is shown on the right (YBOCS score of <16; MAF, 0.14; 188 individuals). ADD indicates attention-deficit disorder with or without hyperactivity.

based on genotype subgroups obtained for rs9357271 (T/T, T/C, and C/C). Because of the strong linkage disequilibrium between the SNPs, a simple Bonferroni correction for multiple testing would have resulted in an overly conservative test. The association results from the 14 SNPs were therefore adjusted for multiple testing by using the method of Li and Ji[20] for the effective number of independent tests, and the significance level was set to $.0127 = 1 - [(1 - 0.05)^{0.25}]$. We further corrected for multiple testing using the Bonferroni method for the case-control analysis of rs9357271 in different TS subgroups. The significance level was set to $.00254 = .0127/n$, with $n = 5$ tests (TS+OCD, TS−OCD, TS+ADD, TS−ADD, and TS−OCD−ADD).

RESULTS

The genotyping success rate of the 14 SNPs ranged from 98.5% to 99.8%. As another quality control, Hardy-Weinberg equilibrium tests were performed, and the results revealed no significant deviation from this equilibrium (**Table 2**). Pairwise linkage disequilibrium measured by global D′ indicated that the markers spanning *MEIS1*, *BTBD9*, and *MAP2K5/LBXCOR1* are in strong linkage disequilibrium at each locus (**Figure**, A). Minor allele frequencies (MAFs) from our control group

(Table 2) were very similar to MAFs found in the control sample of the RLS study (Table 1).[14]

CASE-CONTROL ASSOCIATION RESULTS

Minor allele frequencies of the SNPs within *MEIS1* and *MAP2K5/LBXCOR1* did not significantly differ between TS cases and controls (SNPs 1 and 2 and SNPs 8-14) (Table 2). However, MAFs of rs4714156, rs9296249, and rs9357271, all located within intron 7 of *BTBD9*, were significantly lower in TS cases than in controls (0.17 vs 0.23 [P=.007], 0.16 vs 0.22 [P=.01], and 0.17 vs 0.23 [P=.005], respectively), with rs9357271 being the most strongly associated SNP (Table 2).

We divided our cohort of patients according to the presence or absence of ADD and OCD to compare the MAFs of the most significantly associated SNP (rs9357271) in cases with (TS + OCD and TS + ADD) and without (TS – OCD, TS – ADD, and TS – OCD – ADD) the 2 most prevalent psychiatric comorbidities observed in TS. The graphical representation of MAF (Figure, B) illustrates the important variation in allele frequency of rs9357271 between subgroups of patients with TS, which are aligned from highest to lowest MAF. The number of cases, MAF, and association results of each of the 5 TS subgroups are presented in **Table 3**. Minor allele frequencies determined for the TS + ADD subgroup were comparable to those found in the TS cases and the RLS cohort (0.17, 0.17, and 0.16, respectively) (Tables 1, 2, and 3), whereas the TS – OCD, TS – ADD, and TS – OCD – ADD subgroups yielded lower MAFs (0.14, 0.14, and 0.10, respectively) (Table 3). The TS + OCD subgroup presented the highest MAF (0.20) (Table 3). Concerning the association results, only the TS – OCD and TS – OCD – ADD subgroups reached the significance level of .00254 after Bonferroni correction for multiple testing.

The total YBOCS score is a measure of severity for patients with OCD. Patients with scores ranging from 16 to 23 are considered to have moderate OCD, and patients with scores of 24 or higher are severely affected. As shown in the Figure, B, and Table 3, TS cases with a YBOCS score of 16 or higher (TS + OCD) have a higher MAF than do TS cases with a YBOCS score of less than 16 (TS – OCD) (0.20 vs 0.14). The TS + OCD subgroup (103 patients) was then subdivided according to severity into moderate OCD (YBOCS score 16-23; 64 individuals) and severe OCD (YBOCS score ≥24; 39 individuals) (Figure, C). There was a significant difference in total YBOCS scores among the 3 genotype subgroups of rs9357271 (T/T, T/C, and C/C) (1-way analysis of variance, F_2=3.38 [P=.04]).

Table 3. Clinical Information and Association Results for rs9357271 in Subgroups of Patients With TS

Clinical information				
Subgroup	No. (%)	MAF	χ^2 Value	*P* Value[a]
Controls	290 (100.0)	0.23	. . .	. . .
TS + OCD	103/291 (35.4)	0.20	0.61	.43
TS – OCD	188/291 (64.6)	0.14	12.95	**<.001**
TS + ADD	174/264 (65.9)	0.17	5.28	.02
TS – ADD	90/264 (34.1)	0.14	7.42	.006
TS – OCD – ADD	60/252 (23.8)	0.10	10.01	**.002**

Abbreviations: ADD, attention-deficit disorder with or without hyperactivity; ellipses, comparison group; MAF, minor allele frequency; OCD, obsessive-compulsive disorder; TS, Tourette syndrome.
[a] Indicates uncorrected. After Bonferroni correction for multiple testing, the significance level was set to .00254, as explained in the "Statistical Analyses" subsection of the "Methods" section. Boldface *P* values are less than the significance threshold. The OCD diagnosis, ADD diagnosis, and OCD and/or ADD diagnoses were undetermined in 31, 58, and 70 patients, respectively.

FAMILY-BASED ASSOCIATION RESULTS

As demonstrated in the case-control analysis, SNPs in *MEIS1* and *MAP2K5/LBXCOR1* were not found to be associated with TS. The major alleles of the 3 SNPs within *BTBD9*—rs4714156 (C), rs9296249 (T), and rs9357271 (T), which are the predisposing alleles in RLS—were found to be overtransmitted from parents to the probands with TS, although the *P* values were not significant after correction for multiple testing (**Table 4**). The

Table 4. Family-Based Association Results (Transmission Disequilibrium Test)

SNP No.	dbSNP ID	Alleles, Major/Minor	Risk Allele	T	NT	χ^2 Value	*P* Value[a]
1	rs12469063	A/G	G	117	123	0.15	.70
2	rs2300478	T/G	G	114	123	0.34	.56
3	rs9394492	C/T	C	132	115	1.17	.28
4	rs4714156	C/T	C	105	75	5.00	.03
5	rs9296249	T/C	T	104	77	4.03	.04
6	rs9357271	T/C	T	106	76	4.94	.03
7	rs3923809	A/G	A	133	121	0.57	.45
8	rs12593813	G/A	G	119	134	0.89	.35
9	rs11635424	G/A	G	115	127	0.60	.44
10	rs884202	A/G	A	120	134	0.77	.38
11	rs4489954	G/T	G	117	127	0.41	.52
12	rs3784709	C/T	C	123	134	0.47	.49
13	rs1026732	G/A	G	122	132	0.39	.53
14	rs6494696	G/C	G	121	133	0.57	.45

Abbreviations: dbSNP, single-nucleotide polymorphism (SNP) database; ID, identification; NT, nontransmitted; T, transmitted.
[a] Indicates uncorrected. In the Bonferroni correction for multiple testing, the significance level was set to .0127, as explained in the "Statistical Analyses" subsection of the "Methods" section.

observed overtransmission of the risk allele of rs9357271 in the family-based study mainly originated from the trios with probands who had TS–OCD (transmitted, 61; nontransmitted, 38) compared with the rest of the cohort (transmitted, 45; nontransmitted, 38) (data not shown).

COMMENT

By performing a case-control association study in the French Canadian population, we found a significant association between genetic polymorphisms within *BTBD9* and TS. The same overrepresented alleles in our TS cohort also predispose to RLS and PLMS.[14,15] Interestingly, *BTBD9* was the only gene significantly associated with RLS and PLMS in the 2 independent genome-wide association studies. This study provides evidence of a molecular genetic link between RLS and TS. However, this is not the first report of such common genetic variants accounting for the genetic predisposition of different clinical phenotypes, which may implicate common shared molecular pathways or impaired structural functions underlying different phenotypes. For instance, common variants in intron 5 of the *CDKAL1* gene have been associated with psoriasis, Crohn disease, and type 2 diabetes mellitus.[21-23]

The most significant SNP in *BTBD9* (rs9357271 [$P=.005$]) was further analyzed to verify the presence of more specific genotype-phenotype correlations in TS with and without OCD and ADD, which are the most prevalent TS comorbidities observed in clinical practice. Strikingly, MAFs of rs9357271 were found to be even lower in TS cases without OCD, ADD, or either of them than in the TS and RLS cohorts. However, only the TS–OCD (MAF, 0.14 [$P<.001$]) and the TS–OCD–ADD subgroups (MAF, 0.10 [$P=.002$]) reached the significance level for association after Bonferroni correction for multiple testing. We also found that the allele frequency of rs9357271 inversely correlated with OCD severity, as defined by the total YBOCS score, which further supports the association between variants in *BTBD9* and individuals with TS–OCD. Our results indicate that genetic polymorphisms within *BTBD9* may play a more significant role in the etiology of TS without psychiatric comorbidities (also termed *pure TS*) and particularly in TS without OCD. Bidirectional overlap between TS and OCD is well documented. Although TS + OCD is thought to constitute a form of TS rather than a form of OCD, patients with TS + OCD and those with TS–OCD present important phenotypic dissimilarities.[24-27] Thus, one may argue that genes underlying TS + OCD may at least partially differ from genes predisposing to TS alone. The fact that TS lies at the interface of neurology and psychiatry, together with the assumption that *BTBD9* predisposes to 3 distinct movement disorders (TS, RLS, and PLMS), led us to hypothesize that *BTBD9* is more specifically involved in the neurological component of TS and in the circuitry leading to abnormal movements than in the etiology of TS-related psychiatric behaviors.

We are aware that our study has several limits, including the relatively small number of cases and controls. However, the SNP MAFs found in our control group are very similar to the ones obtained in the original study.[14] The fact that we work with a population isolate (French Canadian samples) should also decrease the risk of case-control mismatch. Unfortunately, our clinical evaluation of TS does not include PLMS measurement, and it is still unknown whether PLMS is more frequently observed in patients with TS who have no psychiatric comorbidities. The described association between TS and *BTBD9* is unlikely to be explained by the RLS comorbidity in these patients with TS because RLS was found in only 10% of these cases.[8] The association between TS and *BTBD9* needs to be replicated in independent samples with the same phenotype and subphenotype classifications.

Although we observed an overtransmission of the *BTBD9* risk alleles from parents to probands with TS assessed by using a family-based association approach (with $P<.05$), this distortion in transmission did not reach the significance level for association after correction for multiple testing. We estimate that this lack of significant association is due to the complex inheritance pattern of TS and its comorbidities,[28-30] which have a greater negative effect on family-based studies than on case-control analyses.

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Correction

Error in Table. In the Neurological Review article titled "Progress and Challenges in RNA Interference Therapy for Huntington Disease" by Harper, published in the August 2009 issue of the *Archives* (2009;66[8]:933-938), an error occurred in the Table on page 935. In the "Species Specificity" column, the footnote symbol "c" in the row referencing the study by Machida et al should have been omitted.

Mutations in the nervous system–specific *HSN2* exon of *WNK1* cause hereditary sensory neuropathy type II

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Hereditary sensory and autonomic neuropathy type II (HSANII) is an early-onset autosomal recessive disorder characterized by loss of perception to pain, touch, and heat due to a loss of peripheral sensory nerves. Mutations in hereditary sensory neuropathy type II (*HSN2*), a single-exon ORF originally identified in affected families in Quebec and Newfoundland, Canada, were found to cause HSANII. We report here that *HSN2* is a nervous system–specific exon of the with-no-lysine(K)–1 (*WNK1*) gene. *WNK1* mutations have previously been reported to cause pseudohypoaldosteronism type II but have not been studied in the nervous system. Given the high degree of conservation of *WNK1* between mice and humans, we characterized the structure and expression patterns of this isoform in mice. Immunodetections indicated that this *Wnk1/Hsn2* isoform was expressed in sensory components of the peripheral nervous system and CNS associated with relaying sensory and nociceptive signals, including satellite cells, Schwann cells, and sensory neurons. We also demonstrate that the novel protein product of *Wnk1/Hsn2* was more abundant in sensory neurons than motor neurons. The characteristics of *WNK1/HSN2* point to a possible role for this gene in the peripheral sensory perception deficits characterizing HSANII.

Introduction

Hereditary sensory neuropathies form part of the inherited peripheral neuropathies that are subdivided into 3 categories, depending on the selective or predominant involvement of the motor or sensory peripheral nervous system (PNS) (1). The most common of these neuropathies affect both motor and sensory nerves. In the second category, only the peripheral motor nervous system is affected, and the neuropathy is classified as a distal hereditary motor neuropathy. Finally, there are neuropathies in which sensory dysfunction prevails, and these are referred to as hereditary sensory and autonomic neuropathies (HSANs). Hereditary sensory and autonomic neuropathy type II (HSANII; OMIM 201300) is an early-onset autosomal recessive disorder. It is characterized by loss of perception to pain, touch, and heat attributable to a partial loss of peripheral sensory nerves (2–4). In 2004, we reported mutations in the hereditary sensory neuropathy type II (*HSN2*) gene, a single-exon ORF identified in Quebec and Newfoundland families, as the cause of HSANII (5). Subsequently, 3 independent groups also reported causative *HSN2* mutations in unrelated populations (6–10).

In 2001, large intronic deletions in the with-no-lysine(K)–1 (*WNK1*) gene were reported to cause Gordon hyperkalemia-hypertension syndrome, also referred to as pseudohypoaldosteronism type II (PHAII; OMIM 145260) (11). PHAII is a dominant disorder, the main feature of which is hypertension (12, 13). Members of the WNK family contain a Ser/Thr catalytic domain similar to that of other kinases. However, one of their unique characteristics is that the well-conserved lysine residue of the active domain is instead a cysteine (14). In the case of WNK1, this kinase domain extends from the end of exon 1 to exon 4. WNK1 has an autoinhibitory domain of its kinase activity (15). WNK1 has been shown to interact with a number of cellular proteins (e.g., synaptotagmin-2, MEKK2/3, protein kinase B) (16–18). Early experiments using WNK1-specific antibodies demonstrated that WNK1 is not present in all cells; rather, it was mostly localized to the polarized epithelia of the liver and kidney (19). With the exception of a recent report in which neural precursor cells were used (20) and of one in which WNK1 expression was observed in the developing brain (21), very little has been reported about WNK1 in the nervous system. Given the symptoms of PHAII patients, WNK1 experiments conducted to this day were done with kidney or liver tissues or in cellular models derived from these. We now report that *HSN2*, which was initially believed to lie within intron 8 of *WNK1*, is a nervous system–specific exon of *WNK1* that we will refer here as the *WNK1/HSN2* isoform.

Results

Compound heterozygous mutation in WNK1 and HSN2 cause HSANII. A French family (Figure 1A) was referred to G.A. Rouleau's group in Montreal by L. Faivre for an *HSN2* genetic analysis to confirm the diagnosis of HSANII in an 18-year-old female. Clinically, she had classic HSANII symptoms (2–4). Examination of the patient revealed no dysautonomia or abnormal blood pressure. Vegetative tests elicited by cutaneous stimulation revealed normal sympathetic reflexes. Parasympathetic cardiac function was found to be normal during a forced breathing test. The only apparent autonomic dysfunction observed in the patient was excessive hand sweating.

Nonstandard abbreviations used: DRG, dorsal root ganglia; HSANII, hereditary sensory and autonomic neuropathy type II; HSN2, hereditary sensory neuropathy type II; PHAII, pseudohypoaldosteronism type II; PNS, peripheral nervous system; UTR, untranslated region; WNK1, with-no-lysine(K)–1.

Conflict of interest: The authors have declared that no conflict of interest exists.

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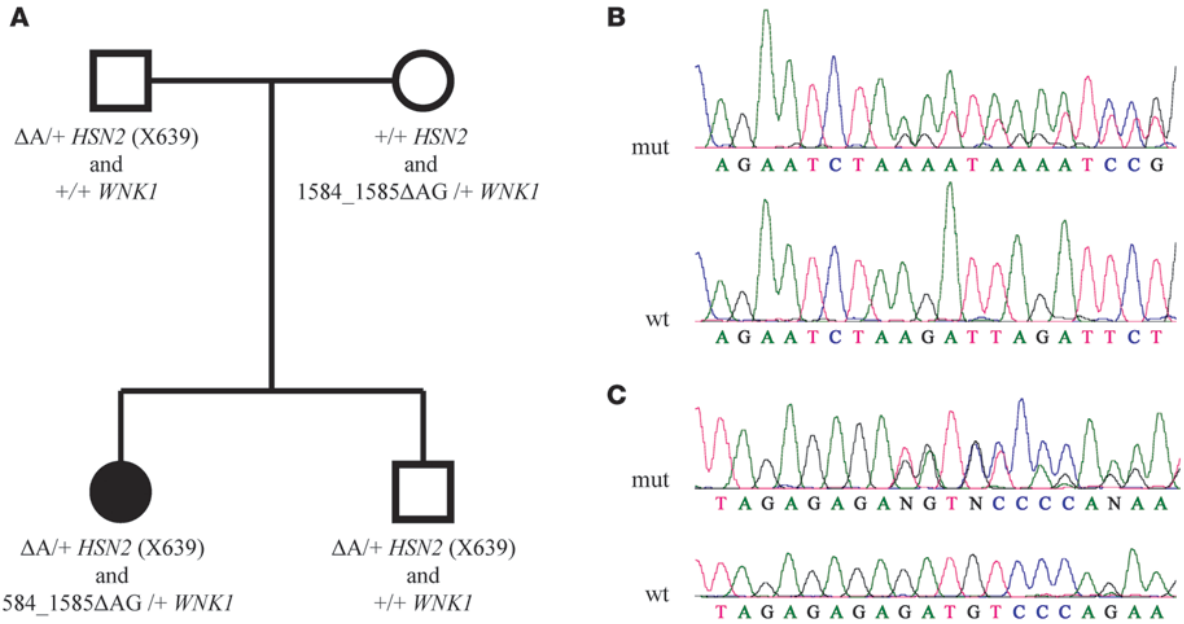

Figure 1
Mutations in the *WNK1*/*HSN2* gene. (**A**) Segregation of the 2 mutations identified in the nuclear family. The single affected individual has compound heterozygous mutations. Sequencing traces show the 1-bp deletion (639delA, Arg214fsX215) identified in *HSN2* (**B**) and the 2-bp deletion (1584_1585delAG, Asp531fsX547) identified in exon 6 of *WNK1* (**C**), with sequencing traces from a normal control. mut, mutated sequencing trace; wt, control sequencing trace.

DNA was obtained from the daughter, brother, and both parents (Figure 1A). Sequencing the *HSN2* ORF identified a heterozygous 1-bp deletion (639delA, Arg214fsX215) in the affected daughter, her asymptomatic brother, and the father (Figure 1B). Surprisingly, no other mutation could be found in *HSN2*. Despite the multiple studies (5–10) that authenticated the *HSN2* ORF as causative of HSANII and its apparent independence from *WNK1*, we decided to screen the entire coding sequence of *WNK1* and identified a second mutation (1584_1585delAG, Asp531fsX547) in exon 6 of *WNK1* (Figure 1C). This 2-bp deletion was inherited from the mother, absent in the unaffected brother, and predicted to result in a truncated protein at amino acid 547 of *WNK1*. The mother presented no abnormal blood pressure or symptoms that could be connected to HSANII. This should not be surprising, given that all *WNK1* mutations so far associated with hypertension were all large deletions in the gene's first intron that led to an overexpression of the gene (11). Given that both mother and daughter presented no obvious symptoms of abnormal blood pressure, it can be surmised that partial loss of WNK1 function has no blood pressure phenotype. The unexpected discovery of these compound heterozygous mutations in the affected daughter — one carried in *WNK1* and the other in HSN2 — led us to speculate that *HSN2* might be an alternative exon of *WNK1*, rather than an independent gene.

Nervous tissues express an HSN2 mRNA with a size similar to that of WNK1. Because the coding regions and regulatory elements of *WNK1* and *HSN2* are well conserved between mouse and human (86% identical), we used the mouse *Wnk1* orthologous gene to test our hypothesis. To detect the mRNA encoding the *Hsn2* sequence, a Northern blot of adult mouse tissues was probed with a 434-bp DNA fragment that recognized the putative 3′ coding region of *Hsn2*. A single band, with a size slightly greater than approximately 10 kb (Figure 2A, top), appeared in nervous system tissues exclusively. This band was predominant in the spinal cord, but it was also detected in the brain and dorsal root ganglia (DRG) of adult mice. At E13, the mouse embryo appears to express this mRNA in its body rather than its distal limb area and its nose area. The size of this mRNA was in accordance with observations made by 3 independent groups that detected an approximately 10.5-kb *WNK1* mRNA in the brain (14, 22, 23). A smaller kidney-specific isoform (~9.0 kb) in which exons 1–4 of *WNK1* are replaced by an alternative exon 4B has also been reported (22, 23). Our result suggests that *HSN2* is part of the *WNK1* mRNA, making it an unreported and novel nervous system–specific disease-causing isoform of *WNK1* (*WNK1/HSN2*). Past poly(A) Northern blot and RT-PCR investigation of *HSN2* failed to detect this species, because the probes and primers used were designed to recognize what were the 2 putative untranslated region (UTR) of *HSN2* (5), which now appear to be spliced out of the *WNK1/HSN2* mRNA. By comparison, *Wnk1* mRNAs that do not contain *Hsn2* sequences were detected using a *Wnk1*-specific probe in a broad range of neuronal and non-neuronal tissues (Supplemental Figure 4; supplemental material available online with this article; doi:10.1172/JCI34088DS1). Expression of *Wnk1* in DRG was previously recorded in the course of a gene expression microarray study (24).

RT-PCR shows that Hsn2 has flanking exons that are those of Wnk1. To further investigate whether *Hsn2* is an alternatively spliced exon of *Wnk1*, we performed RT-PCR reactions with primers flanking the region between the coding region of *Hsn2* and its neighboring *Wnk1* exons (Figure 2C, primers 1 and 3a for exon 8 to *Hsn2*; primers 4 and 5 for *Hsn2* to exon 10). When amplifications between *Wnk1* exon 8 and *Hsn2* were performed, a 160-bp band was visible in all neuronal tissues (Figure 2D, arrow 3) but absent from non-neuronal tissues. A very weak 160-bp band is visible in the kidney, but in light of subsequent immunodetections (see below), this may be due to contamination of the kidney sample with adrenal glands; moreover, the Northern

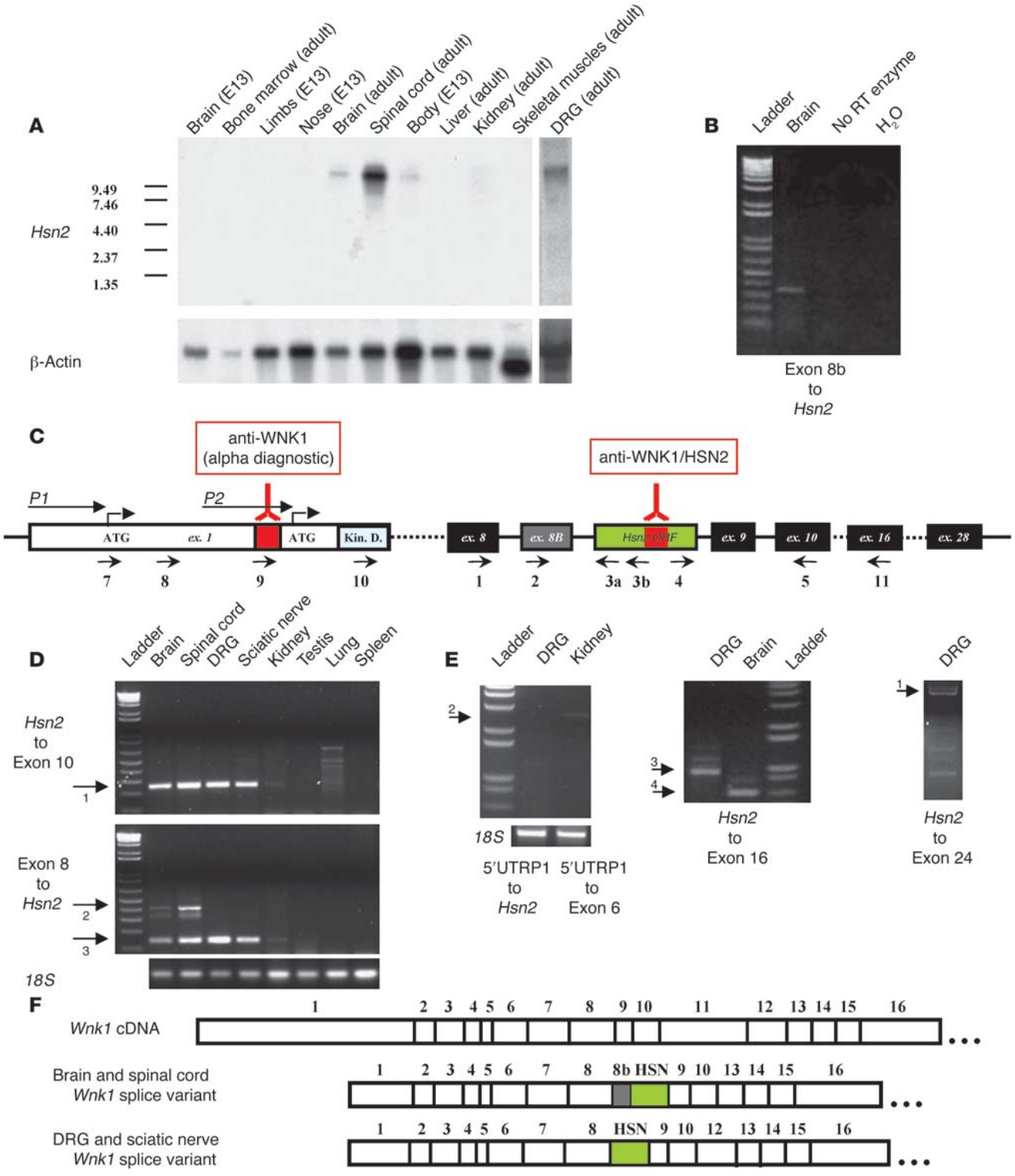
A
Brain (E13)
Bone marrow (adult)
Limbs (E13)
Nose (E13)
Brain (adult)
Spinal cord (adult)
Body (E13)
Liver (adult)
Kidney (adult)
Skeletal muscles (adult)
DRG (adult)
9.49
7.46
Hsn2
4.40
2.37
1.35
β-Actin
B
Ladder
Brain
No RT enzyme
H2O
Exon 8b
to
Hsn2
C
anti-WNK1
(alpha diagnostic)
anti-WNK1/HSN2
P1
P2
ATG
ex. 1
ATG
Kin. D.
ex. 8
ex. 8B
Hsn2 ORF
ex. 9
ex. 10
ex. 16
ex. 28
7
8
9
10
1
2
3a
3b
4
5
11
D
Ladder
Brain
Spinal cord
DRG
Sciatic nerve
Kidney
Testis
Lung
Spleen
Hsn2
to
Exon 10
1
Exon 8
to
Hsn2
2
3
18S
E
Ladder
DRG
Kidney
2
18S
5′UTRP1
to
Hsn2
5′UTRP1
to
Exon 6
DRG
Brain
Ladder
3
4
Hsn2
to
Exon 16
DRG
1
Hsn2
to
Exon 24
F
Wnk1 cDNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Brain and spinal cord
Wnk1 splice variant
1 2 3 4 5 6 7 8 8b HSN 9 10 13 14 15 16
DRG and sciatic nerve
Wnk1 splice variant
1 2 3 4 5 6 7 8 HSN 9 10 12 13 14 15 16

Figure 2
RNA expression analyses of *Hsn2* messenger. (**A**) Northern blotting of *Hsn2* mouse tissues. The membrane was hybridized with *Hsn2* probe (top) before it was stripped and rehybridized with a β-actin probe (bottom). (**B**) RT-PCR amplifications between putative exon 8B (primer 2) and *Hsn2* (primer 3b). (**C**) Diagram of *Wnk1* encompassing *Hsn2*. Below are the numbered primers (Supplemental Table 1). The positions of *Wnk1* promoters are indicated by black arrows, and the sites detected by anti-HSN2 and anti-WNK1 (Alpha Diagnostic International) are indicated in red. Kin. D., WNK1 kinase domain. (**D**) RT-PCR amplifications from brain, spinal cord, DRG, sciatic nerve, kidney, testis, lung, and spleen cDNA. Bottom: Amplifications between exon 8 (primer 1) and *Hsn2* (primer 3a). Top: Amplifications between *Hsn2* (primer 4) and exon 10 (primer 5). Arrows 1, 2, and 3 are, respectively, at 250, 420, and 160 bp. (**E**) RT-PCR amplifications between *Hsn2* and upstream and downstream region of *Wnk1*. Left: RT-PCR between a region upstream to the 5′UTR of P1 (primer 5′UTRP1) and *Hsn2* (primer 3a) in DRG. The same panel shows the amplification between the same 5′UTR of the P1 region and exon 6 in the kidney. Middle: Amplifications between *Hsn2* (primer 4) and exon 16 (primer 11). Arrows 1, 2, 3, and 4 are, respectively, at approximately 2.2 kb, 950 bp, 650 bp, and approximately 3.8 kb. Right: Amplifications between *Hsn2* (primer 4) and exon 24 (primer not shown in **B**). (**F**) Comparison of *Wnk1*/*Hsn2* isoforms with the most common isoform of *Wnk1*.

blot analysis (Figure 2A) established the absence of *Hsn2*-containing mRNA in this organ. This 160-bp band corresponded to the predicted size of a single poly(A) species that would contain both exon 8 of *Wnk1* and *Hsn2*; this was subsequently confirmed by the sequencing. The same amplifications of the region between *Wnk1* exon 8 and *Hsn2* also yielded an additional band of approximately 420 bp in the brain and spinal cord. Sequencing of this 420-bp fragment revealed the existence of an mRNA that contained exon 8 of *Wnk1*, a novel exon downstream of exon 8 (putative exon 8B in Figure 2, C and F) and the *Hsn2* exon. The splicing of this putative exon 8B in the brain *Wnk1* mRNA was confirmed by RT-PCR reactions with primers in *Hsn2* and the putative exon 8B (Figure 2C, primers 2 and 3b), which produced a band of 320 bp (Figure 2B). The putative exon 8B encodes an ORF of 86 amino acids, and this peptide sequence was used to identify orthologous exon 8B sequences from birds, amphibians, and fish (Figure 3A). When amplifications between *Wnk1* exon 10 and *Hsn2* were prepared (Figure 2C, primers 4 and 5), a single band of 250 bp corresponding to the predicted size of a single poly(A) transcript encoding *Wnk1*'s exons 10, 9, and *Hsn2* (Figure 2D, top) was visible, again only in neuronal tissues; this was confirmed by sequencing. More RT-PCR reactions were then performed to investigate which of the more distal exons of *Wnk1* are also parts of the *Wnk1/Hsn2* isoform. In order to investigate the exons upstream to *Hsn2*, separate reactions were performed using a primer located in *Hsn2* (Figure 2C, primer 3a) and a series of primers in *Wnk1* exon 1 (Figure 2C, primers 7–10). When analyzed on agarose gel, the reactions from DRG, using primers 3a and 7, yielded a single band of approximately 2.2 kb (data not shown), and the amplifications between primers 3a and 8, 9, or 10 yielded single bands as well (data not shown). *WNK1* transcripts have been reported to be initiated from 2 distinct promoters (P1 and P2) (25); to investigate which promoter was used for the transcription of *Wnk1/Hsn2*, we used RT-PCR (Figure 2E, arrow 2). The existence of multiple *Wnk1* promoters is an important point, and multiple approaches (RT-PCRs and Western immunodetections) were necessary to establish which promoter appears to be used for WNK1/HSN2 (see Discussion). To observe the exons downstream to *Hsn2*, reactions were performed using a primer in *Hsn2* and a primer in *Wnk1* exon 16 (Figure 2C, primers 4 and 11). When loaded on gel, these amplifications revealed an approximately 650-bp band from the brain tissues and an approximately 950-bp band from the DRG (Figure 2E, arrows 3 and 4). The subsequent sequencing of these 2 fragments showed that both the brain and DRG lacked exon 11 (462 bp) and that the brain additionally lacked exon 12 (285 bp) (Figure 2F); this alternative splicing confirmed previous reports in which exons 11 and 12 were shown to be skipped in some *Wnk1* mRNAs expressed in mouse tissues (13, 23). Further amplifications of the region between the last exons of *Wnk1* and *Hsn2* were, however, more difficult because of the large distance between these primers; nonetheless, a product corresponding to the expected mRNA size (3.8 kb) could be amplified from *Hsn2* to exon 24 of *Wnk1* (Figure 2E, arrow 1). A product was also amplified from *Hsn2* to *Wnk1* exon 25, but its intensity was very weak (data not shown).

Rapid amplification of cDNA end reactions from Hsn2. To corroborate the results of the various RT-PCR products, 5′ rapid amplification of cDNA ends (5′RACE) reactions were also initiated to characterize *Wnk1/Hsn2* isoforms (with or without putative exon 8B). DNA sequencing of the region upstream to *Hsn2* confirmed the presence of a highly conserved splice junction (Figure 3B), and the mRNA did contain *Wnk1* exons 2–8 (with the inclusion of exon 8B in some cases) and the *Hsn2* exon. The distance between *Wnk1* exon 1 and *Hsn2* was too large (~2.2 kb) for the 5′RACE reaction to proceed that far from *Hsn2*. DNA sequencing of the region amplified by primers in *Wnk1* exon 10 and *Hsn2* also revealed a splice junction highly conserved across species in the region 3′ to *Hsn2* (Figure 3B). No 3′RACE reactions were done because of the size of *Wnk1*, but the gene has been reported to have 2 alternative polyadenylation sites (25). The distance between *Hsn2* and these 2 polyadenylation sites is too large (~4 kb) for direct amplifications to be possible, and this portion of *Wnk1* does not permit an easy distinction of *Wnk1/Hsn2* and *Wnk1* transcripts by PCR. It has, however, been reported that the second site appears to be more abundantly used in tissues where *WNK1* expression is high, such as the brain (25).

Western blot detection of the Wnk1/Hsn2 isoform. In order to confirm that *Hsn2* is an exon of *Wnk1*, we prepared Western blots using whole protein lysates from adult mice, and we separately detected them with an antiserum specific to the C-terminal portion of HSN2 and a purified commercial antibody that recognizes the N terminus of WNK1 (Alpha Diagnostic International) (Figure 2C). Past reports examining WNK1 expression in mouse showed that its MW was slightly greater than 250 kDa (26, 27), and this was confirmed in every tissue detected here with anti-WNK1 (Figure 4, top). By comparison, the anti-HSN2 antiserum detected a band only in tissues of the nervous system, and the MW of this band was smaller (~230 kDa) (Figure 4, middle). The anti-WNK1 antibody failed to reveal any band at approximately 230 kDa in which WNK1/HSN2 was detected with the anti-HSN2 antiserum. To confirm the specificity of the signal detected with the anti-HSN2 antiserum, it was preincubated with its antigenic peptide prior to its use for Western blot and immunohistochemistry detections. This competition step was found to prevent the detection of the approximately 230-kDa band observed in the DRG (Supplemental Figure 1). *WNK1* was previously established to contain 2 distinct promoters (P1 and P2) (25) (Figure 2C), and 2 distinct bands could be observed (~230 and slightly greater than ~250 kDa) when other anti-WNK1 antibodies were used (R&D Systems and Kinasource; Supplemental Figure 2). Together, the detections of Western blots with alternative anti-WNK1 antibodies and the Western blot detection presented in Figure 4 suggest that

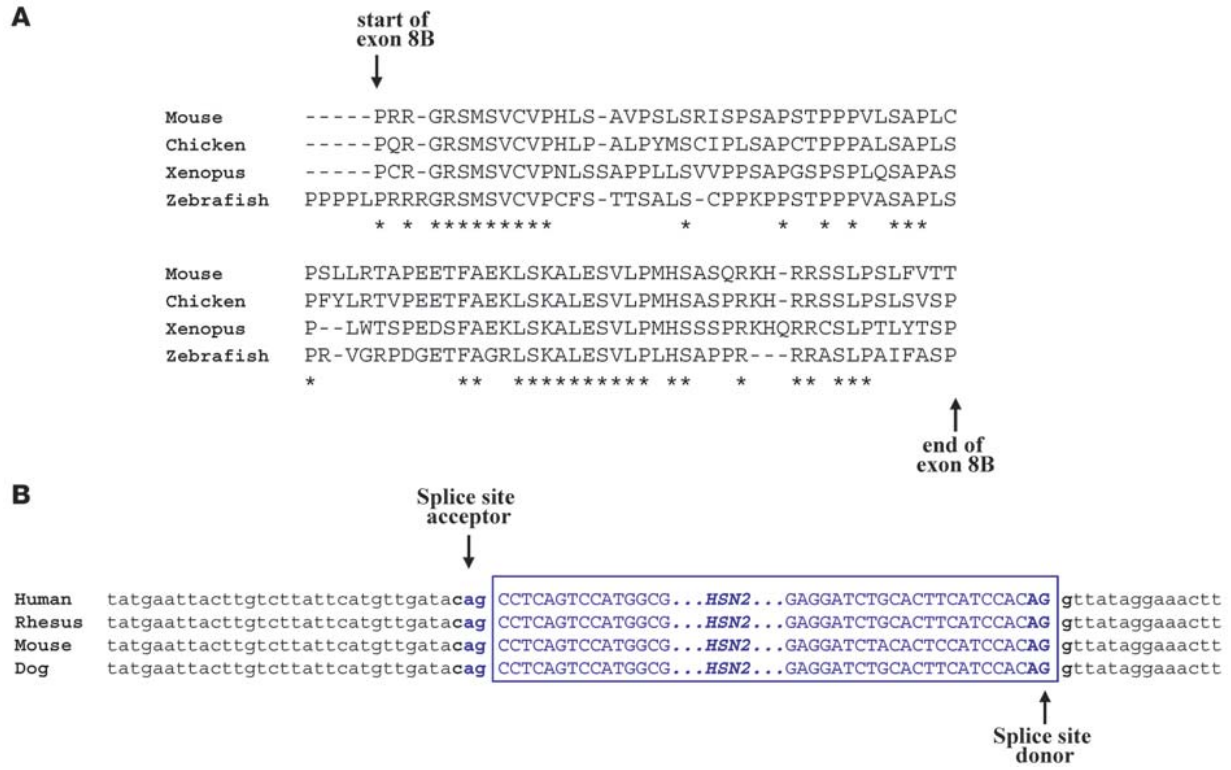

Figure 3

Flanking junction sites of the novel alternatively spliced *WNK1*/*HSN2* isoforms. (**A**) RACE revealed that exon 8B was specifically spliced in some transcripts from mouse brain and spinal cord and the amino acids encoded by this mouse putative exon 8B of the longer *Wnk1*/*Hsn2* isoform are highly conserved across species. A comparison of the amino acids encoded by the exon 8B ORF in the mouse was made with different species/taxa (chicken, *Xenopus*, and zebrafish), and greater than 95% residues are fully or highly conserved (an asterisk below the residues indicates those that are fully conserved across the different taxa/species). (**B**) The amino acid sequences of the splice acceptor and splice donor sites that flank the *HSN2* exon are highly conserved across species. The regions in blue represent the sequence flanking *HSN2*, whereas the regions indicated in black are spliced out during mRNA maturation. Splice acceptor and donor sites are in bold characters. The sequences presented were obtained from the UCSC Genome Bioinformatics Browser (http://genome.ucsc.edu).

it is most likely the second ATG (+640) that is used in the translation of WNK1/HSN2. The 2 alternative polyclonal anti-WNK1 antibodies (Kinasource and R&D Systems) recognize portions of the protein that are downstream to the second ATG, while the anti-WNK1 antibody from Alpha Diagnostic International recognizes a portion (TSKDRPVSQPSLVGSKE) of the protein (14) that is located between the first and the second ATG of the promoters P1 and P2.

Immunohistological investigation of WNK1/HSN2 distribution. While WNK1/HSN2 was observed in both the CNS and PNS, the DRG and sciatic nerves are the tissues with the highest expression (Figure 4). We therefore chose to perform immunohistochemistry to investigate which cells inside the DRG and sciatic nerve expressed WNK1/HSN2. In DRG, the signal was predominantly in the satellite cells that envelop sensory neurons (Figure 5, A and B, arrows), but low expression was also observed in the cell bodies of neurons (Figure 5, A and B, arrowheads). The DRG anti-HSN2 detections were overlaid with parallel detections made using a cocktail of anti–SMI-31 and anti–SMI-32 (neuronal markers). The identity of satellite cells was established with an antibody specific to the glutamine synthetase (28) (data not shown). In cross sections of the sciatic nerve, a strong WNK1/HSN2 signal was visible in the Schwann cells that surround axons (Figure 5D) and in a mosaic distribution of axons (Figure 5, C and D). The same neuron-specific antibodies were used to make parallel detection and overlay images. Given the distribution of WNK1/HSN2-positive axons in the sciatic nerve, which includes fibers from sensory and motor neurons, separate cross sections from both dorsal (Figure 5E) and ventral roots were prepared (Figure 5F). WNK1/HSN2 expression in the 2 roots revealed a striking difference, as the signal was substantially stronger in dorsal roots (almost exclusively containing sensory axons) than in ventral roots (almost exclusively containing motor axons). Given the weak expression of WNK1/HSN2 in the cell body of the DRG neurons, it generally appears that neurons express more WNK1/HSN2 in the axon than the cell body in vivo. To further investigate this, we prepared primary cultures of sensory neurons from DRG and detected these with anti-HSN2 and anti-WNK1 (Alpha Diagnostic International) antibodies. These detections were subsequently overlaid with detections made with the antibodies recognizing the neuronal markers mentioned above (Supplemental Figure 3, A–D). The results showed that in these primary neurons, WNK1/HSN2 is expressed in both the cell body and axons, while WNK1 is only expressed in the cell body and not in the axons. Moreover, immunohistochemistry detection of both DRG and sciatic nerve using anti-WNK1 showed that the protein was ubiquitously expressed in the neuronal somata of the DRG neurons but absent from axonal fibers of sciatic nerve (Supple-

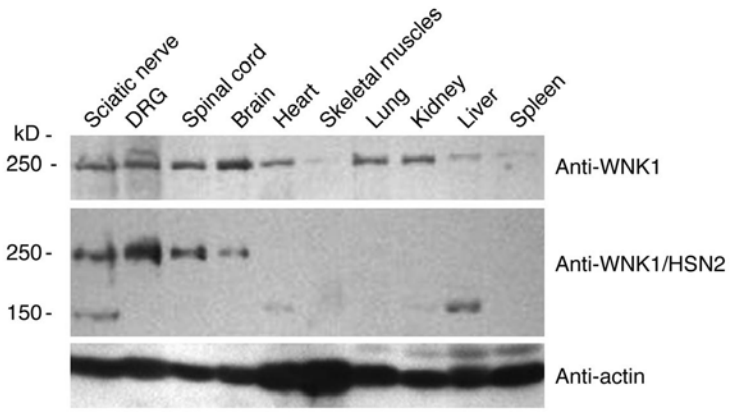

Figure 4
Separate Western immunodetections of WNK1 and WNK1/HSN2. Various mouse tissues from adult or E13 animals were loaded and detected with anti-WNK1 (Alpha Diagnostic International) (top) or with IgG purified anti-HSN2 antibody (middle). Expression of WNK1 was observed in all the lysates, and the expression of WNK1/HSN2 was limited to lysates from neuronal tissues. The membrane detected in the top panel was stripped and then detected with the second antibody. An anti-actin antibody was used to confirm that the loading protein was equal in different lanes (bottom).

mental Figure 3, E–H). The Schwann cells surrounding the fibers of the sciatic nerve express WNK1 (Supplemental Figure 3H, arrow) in addition to WNK1/HSN2 (Figure 5D).

The expression of WNK1/HSN2 in components of the CNS was also investigated by immunohistochemistry (Figure 6). Cross sections of adult mouse spinal cord were prepared and detected with the anti-HSN2 antiserum. In the spinal cord, we observed a strong signal in superficial layers (LI and LII) (Figure 6, A and B), which receive the neuronal projections that carry a variety of sensory information (including most nociceptive information from the PNS). The expression of WNK1/HSN2 also appeared in the fibers of the Lissauer tract (Figure 6B). The neuronal nature of fibers expressing WNK1/HSN2 in LI and LII was confirmed using the neuronal marker SMI (Figure 6B) and a detection using an anti-NeuN antibody (Figure 6D). The axon fibers of dorsolateral funiculus (DLF) and lateral funiculus (LF), which contain ascending sensory fibers, also expressed WNK1/HSN2 (Figure 6, A and B).

Discussion

At the time of the publication of the identification of *HSN2*, the most remarkable feature of this gene was its single-exon structure. The exon mapped within the intron on the same strand and in the same orientation as another gene, *WNK1* (5). While the characterization of the *HSN2* ORF was hampered the existence of an incomplete EST sequence, the ORF was nonetheless found to have three methionines in 5′ and an AATAAA poly(A) addition signal in 3′. While the *HSN2* ORF was novel, *WNK1* had been extensively studied in the context of tissues that are affected in PHAII patients. No symptom overlap was apparent between PHAII and HSANII.

We have now established that *HSN2* is as an alternatively spliced exon of *WNK1* and that this selectively occurs in nervous tissues. The *WNK1/HSN2* nervous system isoforms appear to include either *HSN2* alone or *HSN2* along with a novel exon (putative exon 8B). EST clones encompassing both *Wnk1* and *Hsn2* were previously observed in a mouse E10.5 cDNA library (I.M.A.G.E. 30862659 and 30862741). However, while sequencing these ESTs would have validated our observations, they are no longer available. Prior to this report, only 2 reports examined WNK1 expression in the nervous system (20, 21). In one of them, by Sun et al., neural precursor cells and the anti-WNK1 antibody from Alpha Diagnostic Inter-

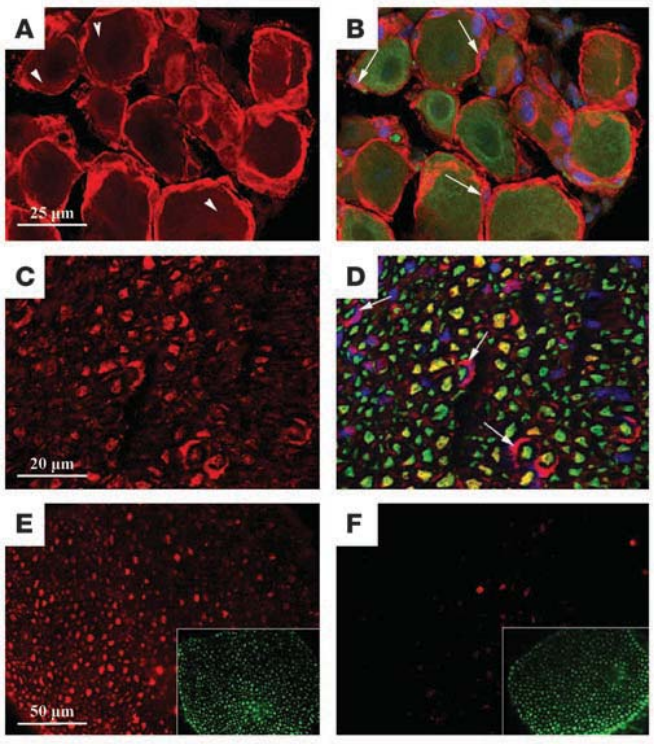

Figure 5
WNK1/HSN2 histological immunodetections. (**A**) Immunohistochemistry detection of adult mouse DRG (from L5 sections) with anti-HSN2 antiserum (red). A clear immunoreactive signal is visible in the satellite cells (arrows) and in some of the neuronal somata (arrowheads). (**B**) Overlaid images of the detections with anti-HSN2 (red in **A**), a mix of axonal markers (SMI-31/32 mix; green), and nuclear staining (TOTO-3 iodide; blue). Colocalization of the signals (yellow overlay) shows that WNK1/HSN2 is expressed in some of the axonal fiber and satellite cells, which surround the neuronal somata (arrows). (**C**) Adult mouse sciatic nerve cross sections detected with the anti-HSN2 antiserum (red) show the presence of the protein in a mosaic distribution of axons. (**D**) Overlaid images of the detection with anti-HSN2 (red in **C**), the axonal markers (green), and nuclear staining (blue) show that not all axonal fibers express WNK1/HSN2 (yellow) and that some do not express WNK1/HSN2 (green). Cross sections of dorsal roots through which sensory axons pass (**E**) and of ventral roots through which motor axons transit (**F**) were detected with anti-HSN2 (red) and the axonal marker (green in **E** and **F**, insets). The majority of motor neuron axonal fibers showed weak or no WNK1/HSN2 signal. In contrast, the HSN2 signal was strong in most of the axonal fibers of the sensory neurons in the dorsal roots. Original magnification of insets, ×400.

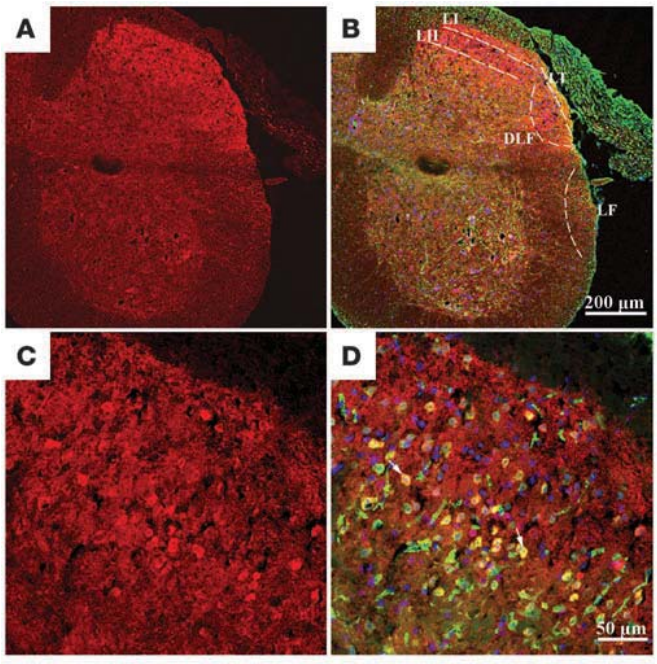

Figure 6
WNK1/HSN2 protein expression in the adult mouse CNS. (**A**) Low-magnification image of immunohistochemistry detections of adult mouse spinal cord cross section with anti-HSN2 antiserum. The anti-HSN2 antiserum gave a strong signal (red) in the superficial layers (LI and LII) of the dorsal horn, the dorsolateral funiculus (DLF), the lateral funiculus (LF), and the Lissauer tract (LT). (**B**) Overlaid images of the HSN2 signal (red in **A**), the signal from the anti–SMI-31/32 axonal marker (green), and the nuclear fluorescent labeling (blue). (**C**) Immunodetection of LI and LII with anti-HSN2 (red). (**D**) Overlaid images of the detection of HSN2 (red in **C**), NeuN (green), and the nuclear labeling (blue). Arrows indicate neurons where the colocalization is observed, confirming the presence of HSN2 protein in cells of this region.

national were used, and a protein said to have a MW of approximately 230 kDa instead of approximately 250 kDa as shown here was observed. It is possible that when Sun et al. identified WNK1, they referred to the antibody specification sheet (catalog WNK11; Alpha Diagnostic International), which indicates that the MW of WNK1 is 230 kDa. However, a number of independent groups reported the MW of WNK1 to be approximately 250 kDa (17, 26, 27, 29). Even though the neuron-specific isoforms (HSN2 alone or HSN2 with exon 8B) of WNK1 incorporate additional amino acids, their MW is nonetheless lower than that of the previously reported WNK1 long isoform, even considering the skipping of exon 11 in the DRG and of exons 11 and 12 in the brain. This lower MW of WNK1/HSN2 is likely attributable to the use of the second promoter (P2) of *WNK1* (25). When the P1 promoter is used, translation is initiated at the ATG (+1), and when the P2 promoter is used, translation is initiated at the more downstream ATG (+640). In both instances the kinase domain of WNK1 is retained, but the use of the second ATG makes the mRNA 642 bp shorter and the protein 214 amino acids smaller. An in vitro study has previously shown that both promoters (P1 and P2) are active and that the second ATG of the P2 promoter is actually sufficiently used to make this form the dominant one in the kidney (25). No examinations have thus far been made in CNS or PNS tissues, but past investigation of the region immediately upstream of the P2 promoter with DNA analysis software (TESS; http://www.cbil.upenn.edu/cgi-bin/tess/tess) identified transcription binding sites that are recognized by neuronal transcription factors (e.g., NF-ATp, HES-1) (21). Unfortunately our 5′RACE reactions did not proceed far enough to provide information about which of the two promoters was used for WNK1/HSN2 transcription. RT-PCR using a primer upstream of the 5′UTR of P1 and a primer in *Hsn2* were prepared with RNA from DRG; the same 5′UTR of P1 primer was used in separate reactions with a primer located in exon 6 of *Wnk1* to amplify kidney cDNA (Figure 2E, arrow 2). The first amplification gave no product (even though long-range PCR conditions had allowed us to amplify larger fragment such as the one between the *Hsn2* and *Wnk1* exon 24). The second amplification, however, produced a fragment of the expected size (~2.5 kb). This suggests that the transcription of *Wnk1/Hsn2* occurs through the second promoter. The comparison of different anti-WNK1 antibodies (Figure 4 and Supplemental Figure 2) also suggested that it is the second ATG that is used to initiate the translation of WNK1/HSN2.

The recessive mutations described in HSANII, here and in previous reports (5–10), all lead to truncations of the WNK1/HSN2 nervous system–specific protein. The disease-causing mutations in *WNK1* identified to date were large, heterozygous intronic deletions that increase the gene expression (11). This impact on the expression level in PHAII patients may explain the absence of hypertension in individuals affected with HSANII, as the expression of the *WNK1* isoform (in which the *HSN2* exon is not incorporated) should not be affected. It is hard to speculate whether some HSANII cases may later be found to have mutations in *WNK1* and not in the *HSN2* portion of *WNK1/HSN2*. However, genetic data presented here suggest that one mutation in the *HSN2* exon is sufficient to cause the HSANII phenotype when combined with a mutation in *WNK1* on the other allele. Moreover, homozygous mutations disrupting *WNK1* isoforms (without *HSN2*) may be lethal, which would explain why all loss-of-function mutations reported to date were located in the *HSN2* exon. A 2003 report supports this possibility, as homozygous mutations of the mouse *WNK1* were shown to be embryonically lethal (30).

While our results indicate that *WNK1/HSN2* is expressed in components of both the CNS and PNS, the HSANII symptoms indicate a defect in peripheral sensory perception. This is consistent with the disease phenotype in which expression of neuronal WNK1/HSN2 appears to be stronger in the sensory neurons than in motor neurons. The primary event of this disorder is liable to occur in sensory fibers, as a very high number of unmyelinated sensory fibers are observed in HSANII. In conjunction with the absence of motor deficit, this constitutes a distinctive pathological hallmark of the disease (31). The expression of WNK1/HSN2 in the outer edge of the DLF, which is where the Lissauer tract travels, may be very relevant for the development of HSANII symptoms, as cells from the Lissauer tract has been reported to be missing in familial, congenital, and universal insensitivity to pain (32, 33). These observations suggest that a more detailed investigation of WNK1/HSN2 expression in components of the posterior horn (e.g., the substantia gelatinosa, where the pain-sensing unmyelinated C fibers are located) (34) at different levels of the spinal cord is needed. Even though no strong evidence supports the hypothesis that myelinating Schwann cells are involved in HSANII, it may eventually be interesting to test whether Schwann cells normally proliferate in

the event of neuronal damage or degeneration. Biopsies of the sural nerve, which contains only sensory fibers, from an HSANII familial case study where multiple affected individuals were diagnosed showed not only a complete absence of myelination but also no evidence of Schwann cell proliferation (35). Based on our observation of WNK1/HSN2 expression in satellite and Schwann cells, it can be hypothesized that the symptoms worsen when these cells respond (28, 36) to the damage sensory neurons suffer because of mutant WNK1/HSN2 expression, although this is speculative. WNK1/HSN2 could act as molecular switch that initiates the proliferation of these cells. In other tissues, WNK1 was shown to activate ERK5 through a MEKK2/3-dependent mechanism, a signaling pathway involved in cell growth and proliferation (18). Though WNK1/HSN2 appears to be expressed predominantly in the PNS, it is also expressed in the CNS. The primary afferents of DRG that transmit sensory stimuli (among them nociceptive) signals are structures that enter the spinal cord through the dorsal root entry zone (DEZ). Once inside the spinal cord, these primary afferents make synapses with second-order neurons of laminae I, II (external; Figure 6A), V, and VI, and from there these fibers take different pathways to transmit HSANII-relevant sensory and nociceptive impulses to structures of the brain stem and diencephalons (37). The gray matter of the spinal cord appeared stained when we used anti-HSN2 and overlaid images of the anti-HSN2 detection and detection of the axonal markers SMI-31/32 (Figure 6), suggesting that any specific HSN2 staining in the gray matter (with the exception of the DEZ) comes mainly from axons, and not cell bodies. The expression of WNK1/HSN2 in the brain was not investigated, but a comprehensive analysis of this will eventually be informative and may help to better understand the disease. Given the strong expression of WNK1/HSN2 in the dorsal roots, it appears probable that the axons of the sciatic nerve expressing this protein are sensory. The difference in the level of WNK1/HSN2 expression observed in the neuronal somata of DRG (Figure 5A) and primary culture prepared from the same DRG (Supplemental Figure 3A) may be the consequence of the activation the neuronal pathway typical of cultured primary neurons. The mechanical, chemical, and new in vitro environment stresses these neurons undergo when the primary culture is established may also activate the expression of WNK1/HSN2. Together, the histological observations suggest that WNK1/HSN2 may have a critical role in the development of the pain-sensory pathways in both the CNS and PNS. WNK1 expression in the PNS during development has not yet been well investigated. Only a few observations made in the CNS were reported, and these showed that WNK1 expression could be seen in the granular layer and cerebellar Purkinje cells, and only weak staining could be observed in the molecular layer and white matter (21). The observation that WNK1 is primarily present in the cell body while WNK1/HSN2 is in the axon may suggest a role in sensory axon maintenance, which is compatible with the neuropathy seen in HSANII.

The sequences of *HSN2* and of the putative exon 8B have no known motif that suggests a particular function, and so how their insertion in *WNK1* affects this protein's function remains to be elucidated. The role of WNK1 in PHAII is partly thought to be attributable to its interaction with WNK4, with ion channels, and with cotransporters through which sodium absorption and potassium wasting is modulated (13). Under normal conditions, WNK1 participates in the regulation of a number of K^+ channels such as ROMK1 (38) and Na^+, K^+, $2Cl^-$, and Na^+Cl^- cotransporters such as NKCCs and NCCs (39). These interactions indicate that WNK1 plays an important role in the regulation of ionic transport across the plasma membrane. In the context of HSANII, it is important to note that the activity of a very relevant ion transporter, TRPV4, is regulated by WNK1 and WNK4 (40). TRPV4 is a vanilloid receptor involved in thermal and mechanical nociception (40). $TRPV4^{-/-}$ mice were found to exhibit hypoalgesic responses to pressures of the tail and acid applications and they have a delayed response to escape from hot temperature (41, 42). How the alternative splicing of *HSN2* and/or of the putative exon 8B to form a nervous system–specific *WNK1/HSN2* isoform affects the normal activity of WNK1 or its affinity for WNK4, TRPV4, or other pain receptors activity is unknown. At this point, it may only be postulated that WNK1/HSN2 is involved in the proper localization and/or ionic regulation of TRPV channels in the nervous system.

Our study of *Wnk1/Hsn2* stresses the importance of tissue-specific alternative splicing, as it shows that mutations in different splice variants may affect protein function differently and consequently lead to very different tissue-specific pathologies. The 9 laminopathies (including lipodystrophies, muscular dystrophies, and progeroid syndromes) that are caused by mutations of the *LMNA/C* gene are good examples of such a phenomenon (43, 44). Unlike the situation with *WNK1*, there is no association between the position or the exon where the mutation occurs and the tissue or system affected.

Methods

Mutation detection. After written informed consent was received from the family, blood samples were collected and DNA was extracted from peripheral blood lymphocytes using a standard protocol. All 28 exons of *WNK1* and the single predicted exon of *HSN2* were amplified by PCR using flanking intronic primers before they were sequenced with an ABI 3700 sequencer, according to the manufacturer's recommended protocol (Applied Biosystems).

RNA isolation and mRNA purification. All animal experiments were approved by the Institutional Committee for Animal Protection (CIPA) of the Centre Hospitalier de l'Université de Montréal. Total RNA was prepared from the different tissues of C57BL/6 adults and E13 embryos according to the method described by Chomczynski and Sacchi (45), and poly(A) RNA was subsequently purified using an Oligotex mRNA Midi Kit (QIAGEN).

Northern blot analyses. Purified poly(A) RNA (2 μg) from the different tissues (C57BL/6 adult and E13 embryo) was electrophoresed on a denaturing formamide/formaldehyde gel and transferred onto a Hybond nylon membrane (GE Healthcare). The membrane was hybridized with a PCR-amplified murine *Hsn2* fragment of 435 bp that recognized the 3′ region of the gene. This 435-bp probe was generated using the primers 5′-CATGCTCAAACACCAAGTTCTT-3′ and 5′-TGAAGCAGATAAGACCTGCTGA-3′ that cover the region found between position 768 and 1,203 bp past the 5′ *Hsn2* spliced-in sequence (Figure 3B). The membrane was also probed with β-actin DNA fragments. Probes were radiolabeled with [α-^{32}P]dCTP–labeled Random prime PCR fragment (Rediprime II Random Prime Labelling System; GE Biosciences). Before use, the fragments were sequenced to confirm their identity. Hybridization was performed according to the procedure previously described by Houle et al. (46). *Wnk1* Northern blotting was performed with an amplified DNA template using 5′-TGACATCGAAATCGGCAGAGGCT-3′ and 5′-GGGTACGGGTAGAATTAGCAGAAG-3′ primers. The probe spans from the end of exon 1 to exon 6 (850 bp).

Analysis of Wnk1 splicing events by RT-PCR. cDNA synthesis and PCR analysis have been described previously by Herblot et al. (47). Total RNA (1 μg) was used as template for first-strand DNA synthesis. Long-range PCR was performed using Long PCR Mix (Fermentas). For each amplification of a specific cDNA, 18S, was coamplified as internal control to correct for variations in loading. Oligonucleotide sequences are listed in Supplemental

Table 1. cDNAs were amplified for 35 cycles, and PCR conditions were: initial denaturation of 94°C for 3 minutes, then 35 cycles of 93°C for 10 seconds, 55°C for 30 seconds, and 68°C for 2 minutes and 45 seconds and then 1 cycle of 68°C for 10 minutes. The amplified fragments were analyzed by agarose gel electrophoresis, and when the sequence of specific bands was investigated, the band was then purified with the QIAEX kit (QIAGEN) and cloned in TOPO PCRII (Invitrogen). Sequence analysis was performed on the ABI 3700 sequencer at the Génome Québec Innovation Centre.

5′RACE assay. RNA was used in conjunction with the GeneRacer kit (Invitrogen) for the 5′RACE and 3′RACE assays. PCR products from the RACE reaction were analyzed on agarose gel and subcloned into TOPO-4 Vector (Invitrogen). Ninety-six clones were chosen, and 23 were sent for sequencing.

Ortholog identification. Orthologous sequences were identified by performing a tBLASTn search of GenBank genome sequences using the peptide sequence encoded by exon 8B of the mouse gene.

Antibody production and immunodetection. DNAstar software version 5.02 was used to examine protein sequence homology, and freely available software (ANTIGENIC; http://immunax.dfci.harvard.edu/Tools/antigenic.html) was used to explore the peptide sequence of HSN2 with good antigenicity. Following these analyses, the best candidate protein sequences were selected for the synthesis of peptides (Sheldon Biotechnology Centre, McGill University) to be used to prepare rabbit polyclonal antibodies at our facilities. An LSPQSVGLHCHLQPVT peptide corresponding to a sequence in the 3′ end of the *HSN2* exon was the one that allowed the preparation of the best antibody in terms of antigenicity and specificity. The peptides were injected with complete and subsequently with incomplete Freund's adjuvant in rabbit to produce an antiserum specific to HSN2 protein (McGill animal facility). Either crude serum (1:1,000) or IgG purified antibody (1:5,000; Montage; Millipore) was used for the Western blot (7.5%) and histological immunodetections. C57BL/6 adult mouse tissues were prepared in SUB lysis buffer (8 M urea, 0.5% SDS, 200 mM β-mercaptoethanol) and resolved by SDS-PAGE. Anti-WNK1 antibody (Alpha Diagnostic International) was used at 1:1,000. The anti-WNK1 made to recognize the kinase domain (Kinasource) was used at 1 μg/ml, and the anti-WNK1 from R&D Systems was used at 0.5 μg/ml. TOTO-3 iodide (Molecular Probes; Invitrogen) was used to generate nuclear staining.

Immunohistochemistry was performed as described previously (48). A cocktail of anti–SMI-31 and anti–SMI-32 (SMI Monoclonals; Covance) was used at 1:1,000 as an axonal marker. Neuronal marker NeuN antibody (Upstate) was used at 1:150. Alexa Fluor 555 secondary anti-rabbit and Alexa Fluor 488 secondary anti-mouse antibodies (Molecular Probes; Invitrogen), respectively, were used (1:1,000) to visualize rabbit and mouse primary antibodies. Our observations were carried out using a Leica TCS SP5 broadband confocal microscope. The system was equipped with the AOBS (acousto-optical beam splitter) for optimal beam splitting. Control immunodetections were made using the preimmunized serum obtained from rabbits, which subsequently yielded the anti-HSN2 antiserum, after their exposure to the HSN2 antigenic peptide, and these did not show a specific signal in either immunohistochemistry or Western blots.

For competition experiments, anti-HSN2 antiserum was incubated with 5-fold excess of its antigenic peptide overnight at 4°C. The same amounts of protein lysates from adult mouse DRG were loaded on a SDS-PAGE and transferred on PVDF membrane. One strip was incubated with anti-HSN2 antiserum alone, and a second strip incubated with the anti-HSN2/peptide mix overnight at 4°C.

Primary cultures of DRG sensory neurons. Adult mouse sensory neuronal cultures were established essentially as described by Seilheimer et al. (49) with some modifications. DRG were dissected from adult mouse C57BL/6 and incubated with 10 mg/ml of collagenase D (Roche) for 45 minutes at 37°C. Trypsin (0.25%) was added at the end of the collagenase D treatment for 30 minutes at 37°C. The tissues were washed once with cold Neurobasal medium (Invitrogen) plus 2% inactivated goat serum and then triturated in warm Neurobasal medium plus serum using fire-polished Pasteur pipettes. One milliliter of the cell suspension was overlaid on 1 ml of 35% Percoll in saline (Pharmacia; Amersham Biosciences) and centrifuged at 10°C at 285 *g* for 15 minutes. The cell pellet, which includes sensory neurons, was washed in 5 ml of fresh medium and resuspended in fresh warm medium with 50 ng/ml of nerve growth factor and plated on pol-D-lysine/laminin–coated coverslips. They were cultured for 48 hours prior to immunocytochemistry. Immunostaining was performed essentially as described previously (50).

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A Mutation in the *HSN2* Gene Causes Sensory Neuropathy Type II in a Lebanese Family

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Hereditary sensory and autonomic neuropathy (HSAN) type II is an autosomal recessive disorder clinically characterized by distal and proximal sensory loss that is caused by the reduction or absence of peripheral sensory nerves. Recently, a novel gene called *HSN2* has been found to be the cause of HSAN type II in five families from Newfoundland and Quebec. Screening of this gene in an HSAN type II Lebanese family showed a 1bp deletion mutation found in a homozygous state in all affected individuals. This novel mutation supports the hypothesis that *HSN2* is the causative gene for HSAN type II.

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The hereditary sensory and autonomic neuropathies (HSANs) are characterized by loss of pain sensation in combination with other sensory and/or autonomic abnormalities.[1] The sensory and autonomic dysfunctions found in these disorders are thought to result from degeneration and/or abnormal development of peripheral nerves.[1,2] The HSANs are clinically classified into five distinct neurological disorders that differ by the prevalence of sensory or motor neuropathies (Table). HSAN type I is caused by autosomal dominant mutations in the *SPTLC1* gene[3,4] in some families, but the disorder is genetically heterogeneous, and there are at least two loci.[5] HSAN types III and IV are caused by autosomal recessive mutations located in the *IKBKAP* gene[6,7] and the *TRKA/NGF* receptor,[8] respectively. A mutation in the gene *NGFB* causes an HSAN type V–like disorder.[9]

HSAN type II is characterized by an early age of onset, typically presenting in the first 2 decades of life. Other characteristics include distal and proximal sensory loss, dysfunction of the autonomic nervous system, loss of tendon reflexes, presence of various mutilations, and slow progression of the disease with time.[10–12] Another subtype of HSAN type II that occurs without progression has also been described previously.[13]

Recently, Lafrenière and colleagues[14] identified a novel gene named *HSN2,* which causes HSAN type II. The gene, found at 12p13.33, is a single-exon gene located within intron 8 of the *PRKWNK1* gene and is transcribed from the same strand as the *PRKWNK1* gene. The HSN2 protein is predicted to have 434 amino acids[14] and at present has no known function. Lafrenière and colleagues studied five families that are from the two known population clusters of this disease, Quebec and Newfoundland. While there are many reports of single families with similar clinical features, the existence of this disease outside these clusters remains uncertain. To partly answer this question and to confirm the role of the *HSN2* gene in HSAN type II, we screened this gene in a Lebanese family affected with the HSAN type II phenotype.

Materials and Methods

Clinical Presentation

Genetic analysis was based on a consanguineous multigenerational family (Fig 1), in which the parents of Patient 1 are first-degree cousins. A brother of the father (Patient 2), a sister of the mother (Patient 3), and another related individual (Patient 4) have also been diagnosed as HSAN type II patients. Diagnosis of the disease was made based on clinical criteria described in the literature.[10–12] The disorder began during the first decade of life in all patients. Patients suffer from loss of sensation and insensitivity to pain, leading to ulcerative lesions on the feet, bacterial infections, and amputations of the limbs. A nerve biopsy showed axons with a degenerated myelin substance in the peripheral nervous system.

After receiving written informed consent, we collected blood samples from the Lebanese family. DNA was extracted from peripheral blood lymphocytes by standard methods.

Linkage Analysis and Mutation Detection

To determine whether the family was linked to the 12p13.33 locus, where the HSN2 gene maps, we first genotyped it with six different markers: D12S352, D12S341, D12S94, D12S91, D12S389, and D12S1587 (see Fig 1). The haplotypes were constructed by using standard methods to identify the specific marker alleles segregating with each transmitted chromosome. The single exon of the *HSN2* gene was then amplified by polymerase chain reaction with three pairs of

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Table. Summarization and Characterization of the HSAN Disorders

HSAN Type	Transmission	Locus/Gene	Onset	Symptoms	Reference No.
I	AD	*SPTLCI*	Adult	Sensory, autonomic and reflex loss Severe mutilation and shortening of the feet Slow progression	3, 4
I b	AD	3p22-p24	Adult	Rare subtype of HSAN type I associated with cough and gastroesophageal reflux	16
II	AR	*HSN2*	Early	Sensory and tendon reflex loss Mutilation of hands + feet; skin ulcers Autonomic: mild abnormality or asymptomatic Slow progression	14
III	AR	*IKBKAP*	Birth	Ashkenazic Jewish descent Diminished lacrimation, defective thermoregulation, orthostatic hypotension, fixed pupils, excessive sweating, loss of pain, and temperature sensation, absent reflexes Death in 50% <30 to 40 years	6, 7
IV	AR	*TRKA/NGF* receptor	Birth	Recurrent episodes of unexplained fever, generalized anhidrosis, insensitivity to pain and temperature. Self-mutilating behavior and mental retardation	8
V	?	1q23.1	Early	Congenital loss of pain sensation in limbs Normal large fiber sensation, strength, and tendon reflexes Intact light touch and vibratory sensation	9, 17
V-like	AR	*NGFB*	Early	Severe loss of pain perception and impaired heat perception	9

AD = autosomal dominant; AR = autosomal recessive.

primers covering the entire coding sequence, in all family members. Each polymerase chain reaction product was sequenced on an ABI 3700 sequencer according to the manufacturer's recommended protocol (Applied Biosystems, Foster City, CA).

Results

As shown in Figure 1, a segregating haplotype was found for markers at the 12p13.33 locus. The four patients share the same haplotype in a homozygous state, suggesting the presence of a recessive mutation in the region. A significant maximum log-of-odds score of 3.11 at a θ value of 0 was calculated using the MLINK program of the LINKAGE package, version 5.1,[15] demonstrating that this family is linked to the *HSN2* locus. To calculate the log-of-odds scores, we used an autosomal recessive model, with a disease allele frequency of 0.007, a penetrance of 0.975, and a phenocopy rate of 0.000003, in accordance to the values used by Lafrenière and colleagues.[14] Two consanguineous loops were broken at individuals 18 and 24.

Sequencing of the *HSN2* gene showed a homozygous deletion of a cytosine (c.947delC) predicted to cause a frameshift, which would lead to the introduction of a stop codon at amino acid 317 (Fig 2). This mutation was found in a homozygous state in all four affected individuals and in a heterozygous state in the parents of Patient 1.

Discussion

Our results support the hypothesis that HSAN type II is caused by loss of function of the *HSN2* gene. The new truncating mutation found in this study removes the last 117 amino acids of the protein and is located just three bases after the C943T (Q315X) nonsense mutation reported in the previous study.[14] Furthermore, all four mutations found to date are similar in that they are all predicted to be deleterious mutations leading to truncation of the protein.

By finding a mutation in a Lebanese family, we have confirmed that HSAN type II exists outside of the two previously described disease clusters, Newfoundland and Quebec. However, other studies of HSAN type II cases in other populations are needed to determine the extent of genetic homogeneity of the disease and prevalence in other populations.

The nerve biopsy sample of one of the patients showed segmental demyelination and loss of axons in the peripheral nervous system, which is characteristic of HSAN type II.[10,12] The disorder results from ongoing degeneration rather than a problem of maldevelopment. Whereas the function of the HSN2 protein remains to be determined, we speculate that it may play a role in the development or maintenance of the Schwann cells surrounding certain types of axons. Alternatively, the HSN2 protein may constitute an im-

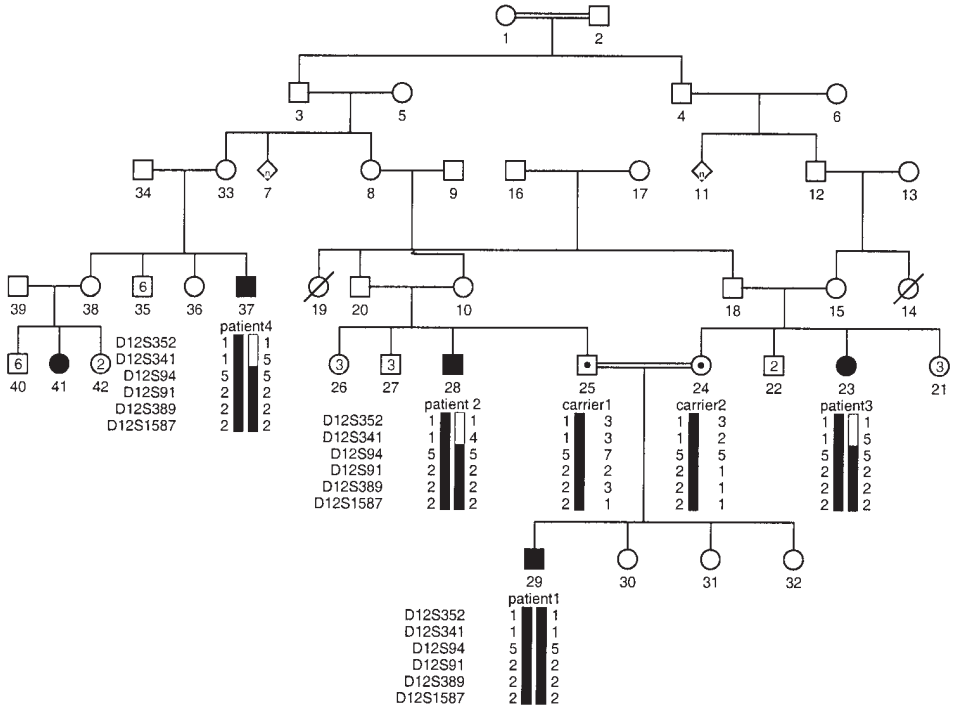

Fig 1. Lebanese family structure and segregation of the disease haplotype (black bars).

portant factor secreted by Schwann cells that is needed for development and/or survival of certain types of axons. In either scenario, loss of *HSN2* function would lead to an early onset progressive neuropathy affecting mainly certain populations of neurons with evidence of demyelination and axonal loss.

To further understand the genetic cause of HSAN type II, expression studies and confirmation of the role of the *HSN2* gene and product in human tissues will have to be undertaken.

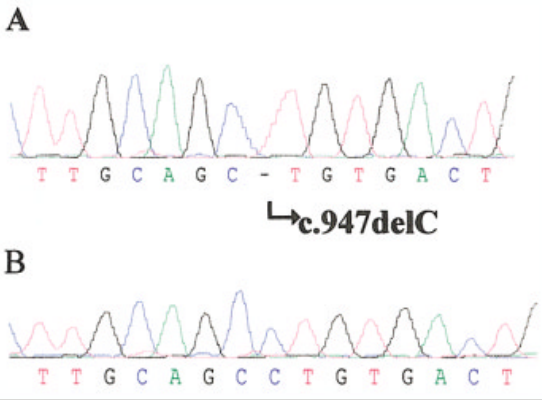

Fig 2. Sequence trace of the mutation found in the Lebanese family. (A) The c.947delC mutation was found in a homozygous state in all four patients, whereas the mutation was found in an heterozygous state in the parents of Patient 1 (data not shown). (B) A normal control sequence.

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Carbonic Anhydrase–Related Protein VIII: Autoantigen in Paraneoplastic Cerebellar Degeneration

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Serum of a patient with paraneoplastic cerebellar degeneration (PCD) and malignant melanoma had a unique reactivity with Purkinje cells that was not observed in serum of patients with PCD and other tumors, or with malignant melanoma without paraneoplastic syndromes. The screening of a human cerebellar complementary DNA expression library with the patient's serum resulted in the isolation of the *CA8* gene. *CA8* encodes carbonic anhydrase–related protein (CARP) VIII, preferentially expressed in Purkinje cells. The patient had intrathecal synthesis of CARP VIII antibodies. One of seven melanomas tested expressed CARP VIII. These data suggest CARP VIII may be an autoantigen involved in the pathogenesis of melanoma-associated PCD.

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Paraneoplastic cerebellar degeneration (PCD) is characterized by the selective damage of the Purkinje cells of the cerebellum.[1] The cause of the Purkinje cell loss is believed to be immune mediated.[2] Several antineuronal antibodies have been identified in PCD that are highly specific for particular tumors.[3–6] Most of these antibodies are probably not pathogenic because they recognize intracellular antigens.[7] However, these antibodies are considered surrogate markers of an immune reaction against relevant antigens that probably cause the Purkinje cell damage through the induction of antigen-specific cytotoxic T cells.[8] In this study, we characterize the antigen of a new anti–Purkinje cell antibody identified in a patient with PCD and melanoma.

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Distal truncation of KCC3 in non–French Canadian HMSN/ACC families

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ABSTRACT

Background: Hereditary motor and sensory neuropathy with agenesis of the corpus callosum (HMSN/ACC) is a severe and progressive autosomal recessive polyneuropathy. Mutations in the potassium-chloride cotransporter 3 gene (*KCC3*) were identified as responsible for HMSN/ACC in the French Canadian (FC) population. In the present study, the authors were interested in finding new mutations in non-FC populations, assessing the activity of mutant proteins and refining genotype-phenotype correlations.

Methods: The authors screened *KCC3* for mutations using direct sequencing in six non-FC HMSN/ACC families. They then assessed the functionality of the most common mutant protein using a flux assay in *Xenopus laevis* oocytes.

Results: The authors identified mutations in exon 22 of KCC3: a novel mutation (del + 2994-3003; E1015X) in one family, as well as a known mutation (3031C→T; R1011X) found in five unrelated families and associated with two different haplotypes. The function of the cotransporter was abolished, although a limited amount of mutant proteins were correctly localized at the membrane.

Conclusions: KCC3 mutations in exon 22 constitute a recurrent mutation site for hereditary motor and sensory neuropathy with agenesis of the corpus callosum (HMSN/ACC), regardless of ethnic origin, and are the most common cause of HMSN/ACC in the non–French Canadian (FC) families analyzed so far. Therefore, for genetic analysis, exon 22 screening should be prioritized in non-FC populations. Finally, the R1011X mutation leads to the abrogation of KCC3's function in *Xenopus laevis* oocytes, likely due to impaired transit of the cotransporter.

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GLOSSARY

ANOVA = analysis of variance; **CTD** = C-terminal domain; **FC** = French Canadian population; **HMSN/ACC** = hereditary motor and sensory neuropathy with agenesis of the corpus callosum; **KCC3** = potassium-chloride cotransporter 3; **mut** = mutated sequence; **NGS** = normal goat serum; **RVD** = regulatory volume decrease; **WT** = wild type sequence.

Hereditary motor and sensory neuropathy with agenesis of the corpus callosum (HMSN/ACC; OMIM #218000) is a severe progressive sensorimotor neuropathy associated with partial or complete agenesis of the corpus callosum, a delay in developmental milestones, a generalized hypotonia,[1] and the deterioration of both physical and mental abilities over time.[2]

Although rare worldwide, HMSN/ACC is found at the highest prevalence (1 in 2,117 live births) in the Charlevoix and Saguenay Lac St-Jean regions of the province of Quebec, where the carrier frequency is high (1 in 23 individuals).[3] The disease has also been

Supplemental data at www.neurology.org

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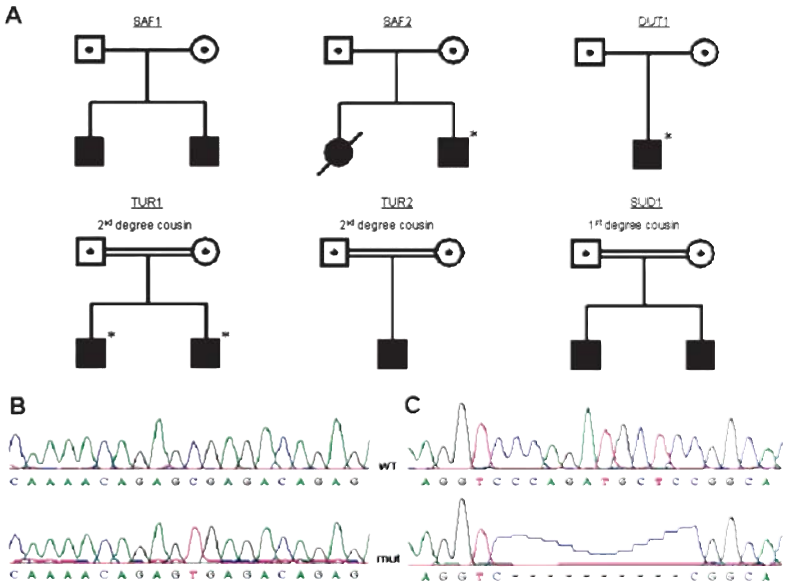
Figure 1 Hereditary motor and sensory neuropathy with agenesis of the corpus callosum pedigrees and mutations in *KCC3*



(A) SAF1 and SAF2 are Afrikaner families from South Africa. Family DUT1 originates from the Netherlands. Families TUR1 and TUR2 originate from Turkey. (B) All affected individuals of these five families are homozygous for the 3031C→T transition (R1011X). (C) Family SUD1 originates from Sudan; two affected individuals are homozygous for the del + 2994-3003 mutation (E1015X). WT = wild-type sequence; mut = mutated sequence. *Patients with epileptiform abnormalities on EEG.

reported in other countries.[4,5] The causative gene was shown to be *KCC3* (also known as *SLC12A6*), which codes for the potassium-chloride cotransporter 3 (KCC3).[6] The vast majority of HMSN/ACC mutations (8/9 mutations identified so far) are predicted to prematurely truncate the protein.[6,7]

KCC3 modulates the regulatory volume decrease (RVD) response in hypotonic conditions.[8] Therefore, the swollen axons observed in HMSN/ACC[1] may result from a systematic loss of KCC3 function, with harmful effects on nervous system integrity. However, most phenotypic aspects of the disease remain to be explained and little is currently known about structure-function relationships in this cotransporter. In this regard, naturally occurring mutations can help elucidate the pathogenesis of the syndrome and define KCC3 functional subdomains.

We report here the identification of a novel HMSN/ACC mutation (del + 2994-3003; E1015X) in a Sudanese family. We also provide the characterization of the most common HMSN/ACC mutation outside of French Canada (FC) (3031C→T; R1011X), originally detected in a Turkish family.[6] We identified this mutation in cases of Dutch, Afrikaner, and Turkish descent, found it associated with two different haplotypes, and demonstrated the resulting mutant protein's nonfunctionality in *Xenopus laevis* oocytes.

METHODS

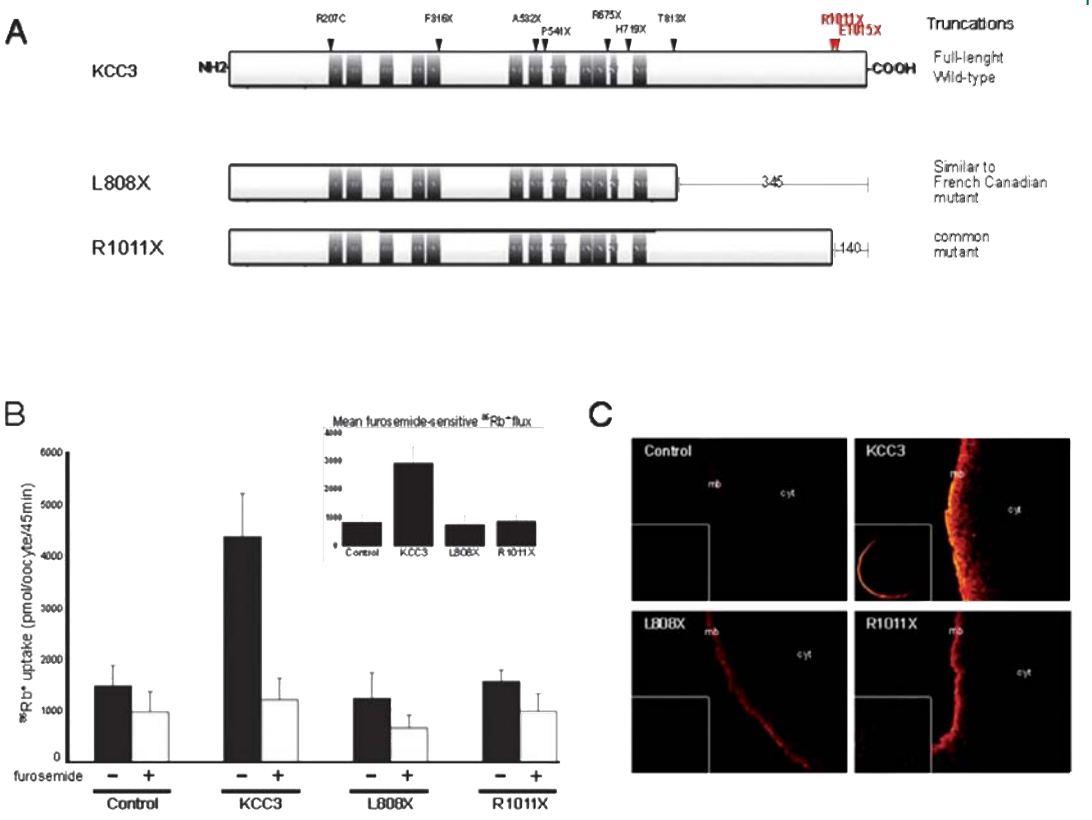
Clinical presentation and mutation screening of KCC3. Blood samples from six families (figure 1) were collected with informed consent: two families from Turkey, two from South Africa, one from Sudan, and one from The Netherlands. DNA was extracted from peripheral blood lymphocytes by standard methods. Clinical diagnosis of the disease was made by a neurologist (N.D.) according to the known basic features of HMSN/ACC. Detailed clinical features are presented in table E-1 on the *Neurology*® Web site at www.neurology.org. All 26 exons of *KCC3* were amplified by PCR with intronic primers and directly sequenced on an ABI 3700 sequencer, according to the manufacturer's recommended protocol (Applied Biosystems, Foster City, CA).

Functional assay. ***Plasmid constructs.*** The full-length KCC3a cDNA subcloned in a modified pGEMHE vector was used as the starting construct to allow expression of wild-type (wt) KCC3 in *X laevis* oocytes. Subsequently, the full-length KCC3 construct was digested by *Hind*III to produce a truncated L808X cDNA, which encodes a truncated transporter similar to the previously studied FC mutant protein (KCC3Q; T813X) bearing mutations in exon 18.[6] The HMSN/ACC 3031C→T nonsense mutation in exon 22 of *KCC3* was introduced by site-directed mutagenesis using a downstream primer (5′-GCTCTAGATCTGTCTCACTCTGTTTTGGA-3′) bearing the 3031C→T mutation to generate the R1011X mutant (see figure 2A for construct design).

Functional assay. The three pGEMHE templates were linearized and the cRNAs were transcribed in vitro using T7 RNA polymerase. The cotransporter activity was determined by assessing $^{86}Rb^+$ uptake in groups of 8 to 12 *X. laevis* oocytes injected with 46 nL of water with or without wt or mutant cRNA. The uptake experiment consisted of a 60-minute incubation period in a hypotonic medium (mM: 52 Na-cyclamate, 3.3 KCl, 0.74 $CaCl_2$, 0.82 $MgCl_2$, 10 HEPES/Tris, pH 7.4, with 10 μM ouabain), followed by a 45-minute incubation in an uptake medium (mM: 49 NaCl, 15 Na-cyclamate, 0.74 $CaCl_2$, 0.82 $MgCl_2$, 30 RbCl, 10 HEPES/Tris, pH 7.4, and 10 μM ouabain supplemented with 50 μCi of $^{86}Rb^+$-Cl^-). The KCC3-dependent uptake of $^{86}Rb^+$ was deduced by exposing groups of cRNA-injected oocytes to 1 mM furosemide.

Immunocytochemistry. Oocytes were fixed in ice-cold methanol containing 1% formaldehyde. Fixed oocytes were then incubated overnight in a 30% sucrose Barth's solution, embedded, and 10 μm thick cryosections of wt or mutant oocytes were placed on the same glass slide to allow comparison.[9] Nonspecific antibody binding was blocked in 5% normal goat serum (NGS) in PBS. Incubation with anti-KCC3 antibody raised against the N-terminus (1:500) in 3% NGS in PBS was performed overnight. Incubation with secondary antibody coupled to rhodamine (1:1,000) was performed and

Figure 2 Functional analysis of KCC3 mutants in *Xenopus laevis* oocytes



(A) Schematic representation of wild-type and truncated KCC3 constructs. The 12 transmembrane segments of KCC3 are represented in gray (I–XII). The L808X protein product is similar to the French Canadian mutant, and the R1011X protein represents the most common KCC3 mutant form. The number of amino acid residues deleted is indicated at the end of each mutant protein diagram. (B) Flux assay of full-length KCC3 (wt), L808X, and R1011X in *X laevis* oocytes. Representative measurements of $^{86}Rb^{+}$ uptake under hypotonic conditions in *X laevis* oocytes injected with the wild-type (KCC3) or mutant (L808X and R1011X) constructs. Total fluxes are indicated by black columns; the white columns correspond to the fluxes insensitive to 1 mM furosemide. The indicated SDs have been calculated for experimental groups of eight or more oocytes. Wild-type KCC3 activation mediated a significant transport, which was fully abolished by 1 mM furosemide. In contrast, neither of the mutant transporters was activated in the same conditions. Upper right corner, mean furosemide-sensitive fluxes calculated from 10 independent experiments. Note the furosemide-sensitive flux in control oocytes due to the activity of the endogenous *X laevis* KCC. (C) Immunofluorescence analysis of KCC3 localization at the membrane of *X laevis* oocytes. KCC3 immunostaining of 10 μm thick cryosections of (upper left) a water-injected oocyte as a control, (upper right) a wt KCC3-injected oocyte, (lower left) a L808X-injected oocyte, and (lower right) a R1011X-injected oocyte (each representative of 10 stainings, all shown at a 63× magnification). Lower left corners: corresponding 16× magnification of whole oocytes stained in red for KCC3. cyt indicates the cytoplasm, mb indicates the plasma membrane.

the sections were mounted for microscope observation.

Statistical analysis. Significance is defined as two-tailed $p < 0.05$, and the results are presented as means ± SD. The significance of the differences between oocyte groups was tested by one-way analysis of variance (ANOVA). A standard χ^2 calculation was performed to assess the association between the epileptiform EEG phenotype and the 3031C→T transition.

RESULTS **Mutation detection.** Sequencing of exon 22 of the *KCC3* gene in six HMSN/ACC families of non-FC origin revealed that the cytosine-to-thymine transition (3031C→T) previously identified in a single Turkish family[6] (Tur1, figure 1) was also present in four additional non-FC families. A total of eight cases from five distinct families of various origins (two Turkish families, two Afrikaner families from South Africa, and a Dutch family) were homozygous for this exon 22 mutation, which results in a premature stop codon at amino acid 1011 (R1011X). We also identified a novel 10-bp deletion within exon 22 of *KCC3* (del + 2994-3003), resulting in a premature termination of translation at amino acid 1015 (E1015X), in two affected siblings of Sudanese descent (figure 1). The direct sequencing of exon 22 also revealed known SNPs that permitted us to investigate the presence of a common haplotype among the five families sharing the 3031C→T mutation (table). Two different haplotypes were identified, one of which was shared by the Afrikaner and the Dutch cases;

Table Interfamily shared haplotypes in families with the 3031C→T transition

Identifier	bp	Alleles	Freq*	SAF1	SAF2	DUT1	TUR1	TUR2
rs347836	32415862	C/G	0.604	CC	C/G*	CC	GG	GG
Rs7164902	32338374	C/T	0.767	CC	CC	C/T†	CC	CC
Rs2290940	32331760	G/A	0.667	GG	GG	GG	GG	GG
Rs2290941	32331643	G/T	—	GG	GG	GG	GG	GG
Rs2705342	32331630	C/T	—	CC	CC	CC	CC	CC
Rs2705341	32331623	C/T	—	CC	CC	CC	CC	CC
Rs2290942	32331566	C/A	0.667	CC	CC	CC	CC	CC
Rs2615352	32331558	C/G	—	CC	CC	CC	CC	CC
Rs2244040	32331482	C/A	0.583	AA	AA	AA	CC	CC
Rs17236798	32330164	C/G	0.667	GG	GG	GG	GG	GG
Rs17817806	32317068	A/G	0.667	TT	TT	TT	TT	TT
Rs4577050	32316240	T/C	0.683	CC	CC	CC	TT	TT
c.3031C→T	32316212	C/T	—	TT	TT	TT	TT	TT
Rs16958873	32312880	A/G	0.917	AA	AA	A/G†	AA	AA

— Indicates unknown frequencies.
*Refers to the frequency of the first allele (determined in European samples).
†Phase undetermined.

the other haplotype was found in the Turkish patients.

Since the most common non-FC mutation observed in the *KCC3* gene targets exon 22, and since this truncating mutation only affects the distal part of the C-terminal domain (CTD) of KCC3 (thus being the least deleterious KCC3 mutation described so far), we investigated the functional consequences of distal CTD truncation in KCC3.

Functional studies. The effect of the distal C-terminal KCC3 mutation was evaluated by the measurement of R1011X activity (the mutant protein encoded by the 3031C→T mutation in exon 22); this measurement was then compared to the activity of full-length (wt) and truncated L808X (resulting from an exon 18 truncation mutation) KCC3 proteins. The function of the transporters was assessed by measuring the furosemide-sensitive uptake of $^{86}Rb^+$ in groups of swollen oocytes. Upon hypotonic activation, the activity of wild-type KCC3 was enhanced, as demonstrated by the significant increase in $^{86}Rb^+$ uptake (from 1,491 to 4,459 pmol/45 minutes/oocyte; $p < 0.0001$ ANOVA; figure 2B). Contrary to this observation, furosemide-sensitive $^{86}Rb^+$ uptakes for L808X and R1011X mutants were fully abolished ($p < 0.001$ ANOVA; figure 2B). Thus, a truncation restricted to a third of the CTD (R1011X) is sufficient to completely inactivate the transporter. These data suggest that the transporter requires the deleted amino acid residues for its appropriate activation; alternatively, this loss-of-function could be attributable to the absence of the transporter at the plasma membrane due to the faulty intracellular transit of the cotransporter.

To further investigate these possibilities, we assessed the membrane localization of KCC3 on oocyte sections with an antibody raised against the N-terminal domain of the protein. We identified a potential mutation-associated anomaly in KCC3's transit to the membrane, since L808X and R1011X signals were weaker than full-length KCC3 membrane signal; however, all three expressed proteins were successfully detected at the membrane (figure 2C). These results suggest that the mutant proteins are translocated to the membrane, where they are nonfunctional.

EEG. Four out of eight patients homozygous for the 3031C→T mutation presented epileptiform abnormalities on EEG or seizures, one patient had a normal EEG, and data were unavailable for three patients (table E-1). Comparatively, only 7 out of 83 patients (8.4%) presented an epileptiform EEG in a large cohort of FC HMSN/ACC patients, all homozygous for the founder 2436delG mutation (unpublished data). χ^2 analysis revealed an association between the 3031C→T mutation and the epileptiform EEG phenotype in the non-FC cases presented here, when compared to the frequency of this phenotype in our large cohort of FC patients ($\chi^2 = 22.08$, 1 df, $p < 0.001$).

DISCUSSION Here we report the identification of a common genetic variant in the *KCC3* gene causing HMSN/ACC outside of Quebec. Haplotype analysis demonstrated that the variant found in the Afrikaner and the Dutch families came from the same ancestor, but occurred independently from the variant identified in the Turkish patients. It is indeed not surprising to find the same haplotype in the Afrikaner and the Dutch patients, since Afrikaner ancestors were Dutch settlers. Our data strongly suggest that this mutation may be a recurrent cause of HMSN/ACC in these countries. Therefore, the screening of exon 22 should be prioritized for HMSN/ACC diagnostic and prenatal testing in non-FC populations. The lack of known consanguinity in the two Afrikaner families and in the Dutch family further suggests that this variant is probably more frequent in South Africa and in the Netherlands than in Turkey. Moreover, despite the total absence of descriptions of the occurrence of HMSN/ACC in the Afrikaner population in the literature, this disease may be more frequent in this population isolate than in other parts of the world, due to its relative genetic homogeneity.

It is interesting to note that three of the six families are consanguineous unions, and two of the remaining three families are part of a founder population. These data suggest that HMSN/ACC is a rare disease where high frequencies will only occur in founder populations and consanguineous unions.

The evidence for a link between seizure disorders and SLC12 protein family members (to which the KCC proteins belong) has been increasing in the recent years. For example, NKCC1 inhibition suppresses epileptiform activities in hippocampal sections,[10] whereas hypomorphic KCC2-deficiency in mice leads to an increased accumulation of Cl^- in neuronal cells, which facilitates the manifestation of seizures.[11,12] In addition, recent data suggest that anomalies in chloride cotransporter levels contribute to temporal lobe epilepsy.[13] Finally, mutations in the K^+ Cl^- cotransporter kazachoc, known as the only kcc in Drosophila, increase seizure susceptibility in the fly[14] and KCC3 null mice also display reduced seizure threshold.[15] Consistently with these models, we suggest that HMSN/ACC cases bearing the 3031C→T transition may have an increased risk to develop epileptiform EEG abnormalities. However, considering the small number of cases with 3031C→T transition presented here, we are aware that the statistical results presented here should be taken with caution. A larger sample will be needed to validate this association.

Even though all known nonsense mutations are predicted to abolish KCC3 function, the common exon 22 mutation and the exon 18 FC mutation may lead to distinct phenotypes, which could partly explain our preliminary observation of an increased frequency of epileptiform EEG results associated with the exon 22 3031C→T mutation. One explanation might stem from the nature of the truncation mutations, where differential inactivation or translocation patterns could be related to epileptiform abnormalities on EEG through a yet unknown mechanism.

We provide evidence that the mutations characterized here result in KCC3's inactivation. Given the fact that the vast majority of HMSN/ACC mutations (figure 2A) result in a premature stop codon, protein truncation is the recurrent mechanism leading to this disease. To date, evidence for the functional inactivation of KCC3 in HMSN/ACC stems from one single publication involving the exon 18 FC mutation.[1] However, the exon 18 mutation removes over three-quarters of the CTD of KCC3 and can only be found in the FC population. The results of the present genetic study suggest that exon 22 mutations are more widespread worldwide, causing HMSN/ACC in ethnically diverse populations. In addition, exon 22 mutations remove only a third of the CTD and constitute, so far, the least deleterious truncation of KCC3. We found that the loss of the distal third of the CTD (140 amino acids) was sufficient to deregulate and inactivate the cotransporter. Therefore, this 140 amino acid segment defines the smallest distal functional domain of KCC3 so far identified.

We also assessed this protein's proper localization at the plasma membrane. All mutant proteins were detectable at the membrane, suggesting that these mutant forms of KCC3 are, at least in part, still correctly processed to the membrane. Since the *X laevis* oocyte expression system is not adequate for quantification of protein levels, but is mainly used to assess functionality of the different protein species, further experiments are required to establish which mechanisms are involved in the pathology and phenotypic presentation of HMSN/ACC.

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