Les pesticides et la MP

Un des chapitres précédents fait état du lien trouvé entre l'injection fortuite de 1-methyl-4-phényl-1,2,3,6-tétrahydropyridine (MPTP) et le déclenchement précoce d'un syndrome parkinsonien. Étant donnée l'homologie structurelle entre le paraquat et le MPTP, ces grands titres médiatiques ont incité les chercheurs à mener des recherches sur le lien causal entre l'exposition à des pesticides et le déclenchement de la MP (Lewin, 1985).

Les pesticides

Définition réglementaire

Le terme « pesticide » couvre deux grandes catégories de produits : les biocides et les produits phytopharmaceutiques. Les biocides sont « destinés à détruire, repousser ou rendre inoffensifs les organismes nuisibles, à en prévenir l'action ou à les combattre de toute autre manière, par une action chimique ou biologique », alors que les produits phytopharmaceutiques sont définis comme les « produits, sous la forme dans laquelle ils sont livrés à l'utilisateur, composés de substances actives, phytoprotecteurs ou synergistes, ou en contenant, et destinés à (…) protéger les végétaux » (définitions du ministère de l'agriculture).

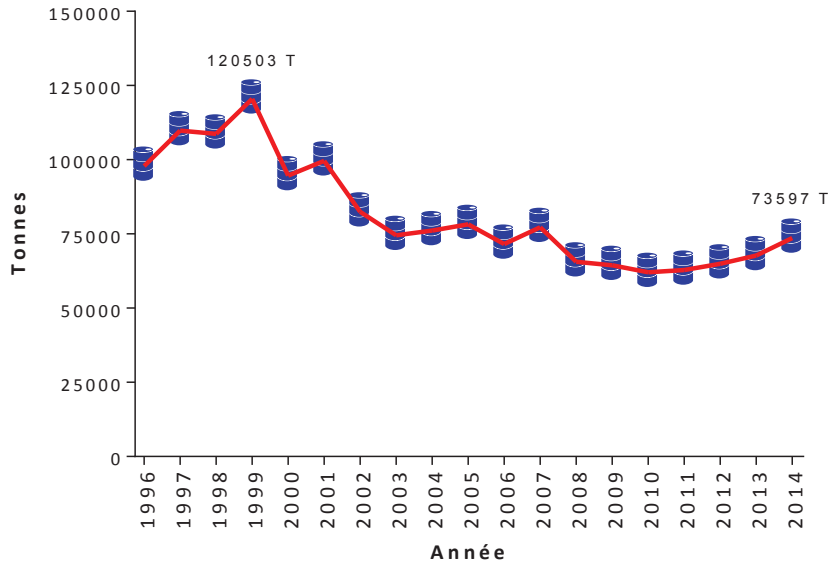


Figure 32: Données sur les quantités de pesticides vendues en France de 1996 à 2014. Sources UIPP et Banque nationale des ventes pour les distributeurs.

Les biocides comportent donc deux sous-catégories : les produits antiparasitaires (par exemple les insecticides, acaricides ou rodonticides) et les produits de protection, servant à traiter des surfaces telles que le bois ou des textiles. Les biocides sont réglementés par la directive CE 2012/528. Les produits phytopharmaceutiques sont réglementés par la directive européenne CE1107/2009 et regroupent par exemple les insecticides, herbicides, fongicides, molluscicides ou nématicides.

Usage des pesticides en France

La France possède la plus grande surface agricole d'Europe avec 28,98 millions d'hectares, devant l'Espagne et l'Allemagne. En termes d'achat de substances actives, la France était au 2eme rang européen en 2013 avec 66 659 tonnes, après l'Espagne (69 587 tonnes) et devant l'Italie (49 011 tonnes). L'évolution de la distribution de pesticides en France montre une forte baisse depuis la fin des années 1990, passant de 120 503 tonnes en 1999 à 62 065 t en 2010, soit une baisse de 48% en 10 ans. Depuis 2010, la distribution de pesticides est de nouveau en hausse, avec un poids total de pesticides vendus de 73 597 tonnes en 2014, soit une hausse de presque 20% en 4 ans, indiquée sur la Figure 32.

L'utilisation des pesticides est générale et permet la pérennité des infrastructures et le maintien de la salubrité dans un but de santé publique. Par exemple, en France l'un des plus gros utilisateurs de pesticides est la SNCF, dans le but d'entretenir et de pérenniser les voies ferroviaires et les ouvrages d'art. En 2012 la SNCF a utilisé 150 tonnes de pesticides pour l'entretien de ses 70 000 km du réseau ferré national. (Source SNCF Réseau, actualité du 10/09/2013).

Les pesticides ont été et sont toujours au cœur de plusieurs affaires sanitaires, notamment avec le scandale du chloredécone aux Antilles Françaises en 1993, ou avec les œufs contaminés au fipronil de l'été 2017. Ainsi des résidus de pesticides à l'état de traces sont continuellement retrouvés sur des aliments destinés à la consommation.

Les modalités d'exposition aux pesticides

L'exposition professionnelle

L'exposition professionnelle aux pesticides est la plus grande modalité d'exposition en France. Celle-ci touche plus de 5 millions de personnes selon la mutuelle sociale agricoles.

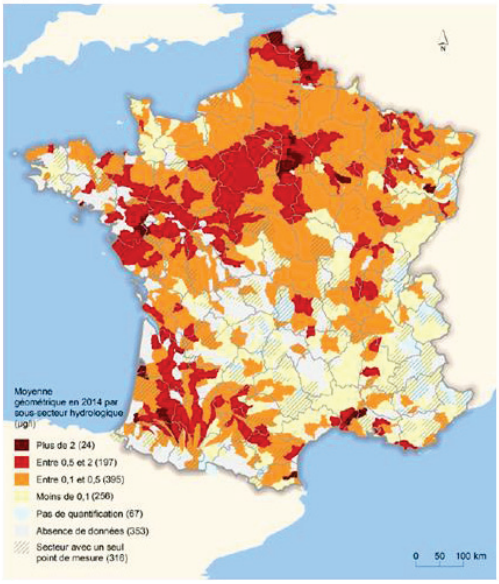
L'exposition professionnelle peut se séparer en 3 phases. La première concerne l'achat, le transport et le stockage. Dans cette modalité d'exposition le manutentionnaire, ou son véhicule, peut être mis en contact avec des substances chimiques vendues sous forme concentrée. Les emballages de substance chimiques, stockées sous forme de sacs ou de bidons, peuvent être altérées et mener à des fuites. La seconde phase d'exposition concerne la préparation, l'épandage et le nettoyage des appareils servant à leur application. En effet, tout pesticide nécessite la manipulation d'un produit commercial et ceci peut mener à des accidents impliquant là aussi une forte concentration de produit. Lors de l'épandage, deux facteurs sont à prendre en compte : la durée d'intervention et la dissémination en continu du pesticide proche de l'opérateur, celui-ci pouvant être mis en contact avec la substance appliquée sur diverses surfaces contaminées par le produit. La phase de nettoyage comporte aussi un risque de contamination, notamment par les projections d'eau ou par l'évacuation des eaux de lavage. La troisième phase d'exposition concerne la réentrée dans les cultures, celle-ci présente là encore une possibilité de mettre en contact l'opérateur avec les substances actives pulvérisées (Belsey *et al.*, 2011).

L'évaluation de l'exposition professionnelle dépend d'une multitude de paramètres propres à chaque type de cultures traitées, la surface de ces cultures, des équipements de protection individuelle portés, de l'expérience de l'opérateur ou encore de la machine de pulvérisation utilisée (Tielemans *et al.*, 2007). Cependant aucune variable globale ne permet de quantifier avec précision l'exposition d'un opérateur, de plus, aucune donnée ne peut définir une moyenne d'exposition générale à des substances chimiques.

L'exposition de la population générale

L'exposition de la population générale aux pesticides se fait principalement par l'alimentation, due à l'ingestion d'aliments ou de boisson présentant des résidus de pesticides (Oates et Cohen, 2011).

A



B

Échantillons	Échantillons contenant des résidus quantifiables	Échantillons non-conformes
Fruits	69,50%	0,9 % (essentiellement poires et fraises)
Légumes	36,60%	1,8 % (principalement céleris en branches, endives et manioc)
Légumineuses	44,20%	2,60%
Graines et fruits oléagineux	28,60%	3,60%
Thé, café, infusion	54,50%	9,10%
Épices	33,30%	1 échantillon
Céréales	60,80%	2 échantillons

Figure 33 : Les pesticides et la France. **A**. Infographie de ministère de l'agriculture concernant la contamination des eaux de surface par des résidus de pesticides. **B**. Étude sur l'alimentation totale EAT menée par l'ANSES étudiant les résidus de pesticides sur l'alimentation et leur respect des normes sanitaires.

À titre d'exemple, la présence de pesticides en France sous forme de traces est généralisée. Selon le commissariat général au développement durable, 91 % des points de contrôle en des eaux de surface (lacs et rivières) présentent des traces de pesticides (Figure 33 A). Le captage de ces eaux de surface puis leur traitement pour l'alimentation du réseau de distribution mène à une distribution de ces pesticides par l'eau de ville. Cependant le code de la santé publique (article R.1321-2) fixe la concentration de pesticide maximale 0.5 µg/L et la concentration maximale pour chaque pesticide à 0.1 µg/L. Au cours de l'année 2014, 3,86 millions d'habitants (soit 6,0 % de la population française), ont été alimentés par de l'eau du robinet au moins une fois non-conforme, dépassant les limites légales en termes de concentration en pesticides. (Bilan de la qualité de l'eau au robinet du consommateur vis-à-vis des pesticides en 2014, Ministère de la santé). Cette exposition par l'eau de distribution touche donc plusieurs millions de personnes en France.

L'autre aspect de l'exposition générale des populations se présente par l'ingestion de fruits et légumes contenant des traces de pesticides (Barnat *et al.*, 2010). Dans son l'étude sur l'alimentation totale (EAT, 2011), l'Agence nationale de sécurité sanitaire, de l'alimentation, de l'environnement et du travail (ANSES) présente un rapport d'expertise concernant la présence de résidus de pesticides dans 194 aliments de consommation courante. 283 produits phytosanitaires ont été choisis sur des critères d'enjeux dans l'évaluation des risques pour différentes substances ou par la description de l'évolution dans le temps des expositions. Sur les 283 substances actives recherchées, 62 sont classées prioritaires en termes de surveillance dans l'exposition alimentaire, dans le cadre des travaux de l'Observatoire des résidus de pesticides. Les résultats montrent une absence de risque d'exposition chronique pour 96% des substances évaluées. Subséquemment, 4% des substances évaluées sont donc possiblement contributrices à l'exposition générale de la population. Ces substances sont : Dithiocarbamates, Ethoprophos, Carbofuran, Diazinon, Méthamidophos, Disulfoton, Dieldrine, Endrine, Heptachlore (Bechaux *et al.*, 2013). Dans cette étude, la substance présentant le plus grand risque de dépassement des limites réglementaires est le diméthoate, autorisé avant l'étude en tant qu'insecticide pour le traitement des vignes, des cultures fruitières et légumières. Depuis les conclusions de l'étude, ce pesticide a été interdit en France, en 2016.

Une autre voie d'exposition possible de la population générale est la riveraineté avec des exploitations agricoles par la dissémination aérienne de résidus de pesticides. Plusieurs études ont montré la présence de résidus de pesticides dans les poussières présentes à l'intérieurs des habitations riveraines de champs, et ce jusqu'à plus d'un kilomètre de distance par rapport au champ (Quiros-Alcala *et al.*, 2011).

Ces trois exemples d'exposition alimentaire à des pesticides, par l'eau de distribution, par l'alimentation et dans les poussières à l'intérieur des domiciles montrent qu'il existe une exposition chronique et à faible dose de toute la population française à des produits phytosanitaires.

Liens entre pesticides et MP

Après plus de trois décennies de recherche concentrant la toxicité potentielle des pesticides et leurs liens avec la MP, beaucoup d'études épidémiologiques s'accordent sur le lien causal entre l'exposition à des pesticides et le déclenchement précoce de la MP (Elbaz *et al.*, 2009; Firestone *et al.*, 2005; James et Hall, 2015; Tanner, 2010; van der Mark *et al.*, 2012; van der Mark *et al.*, 2014; Vanacore *et al.*, 2002). D'ailleurs, depuis 2012, la MP est reconnue comme maladie d'exposition professionnelle par la mutuelle sociale agricole.

Parmi les pesticides incriminés, deux pesticides principaux ont été liés épidémiologiquement avec le déclenchement précoce de la MP : la roténone et le paraquat (Spivey, 2011; Tanner *et al.*, 2011). Seuls ces deux pesticides seront développés dans cette thèse.

Roténone

La roténone est un pesticide naturel isolé dans plusieurs espèces végétales, telle que la *Paraderris elliptica* en Asie ou le bois poison en Afrique, vu sur la figure 34.

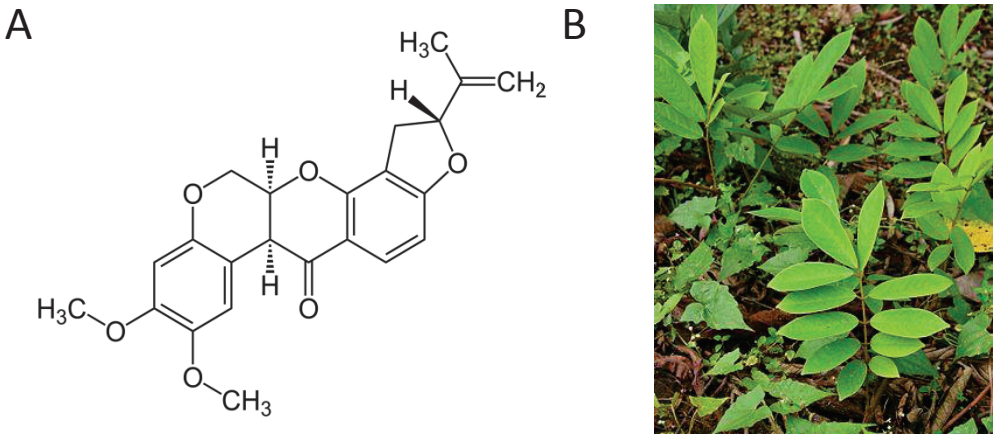


Figure 34 : La roténone. **A**. Structure chimique de la roténone. **B**. La plante *Paraderris elliptica*, espèce dont peut être extraite la roténone.

La roténone est un insecticide naturel, autorisé en agriculture biologique jusqu'en avril 2008 date à laquelle la commission européenne demande à tous les états membres de retirer les AMM des formulations contenant cette substance. En France, une dérogation d'usage pour l'agriculture biologique a persisté jusqu'en avril 2011, date de l'interdiction

totale de la roténone en France. La cause de l'interdiction de la roténone est essentiellement due à ses effets sur l'homme, notamment concernant l'altération de la fertilité, sa toxicité pour l'embryon durant la grossesse et sa mise en cause dans la maladie de Parkinson.

Depuis son interdiction, il est peu probable que la roténone soit encore un contributeur majeur dans la pathogenèse des cas idiopathiques de la MP. Cependant, les mécanismes de la toxicité de cette molécule sont assez bien définis et l'étude de ceux-ci a permis de mieux comprendre certains mécanismes impliqués dans la perte de neurones dopaminergiques. Ceci montre l'intérêt des études utilisant des pesticides interdits mais considérés comme référentiels, d'une part pour caractériser des modèles d'exposition et d'autre part pour étudier les mécanismes de déclenchement de la maladie. Par ses propriétés particulièrement hydrophobes, la roténone à la faculté de traverser facilement la BHE (Hernandez-Romero *et al.*, 2012; Murakami *et al.*, 2015), cet insecticide peut donc exercer son action neurotoxique directement dans les structures cérébrales.

Paraquat

Le paraquat est un herbicide de synthèse non sélectif ayant longtemps été l'herbicide le plus utilisé dans le monde (Yu *et al.*, 2007). Il est encore utilisé dans plus de 100 pays et son utilisation alternative est en plein essor dans certains pays où des végétaux ont développé une résistance au glyphosate, notamment aux États-Unis (Sammons et Gaines, 2014).

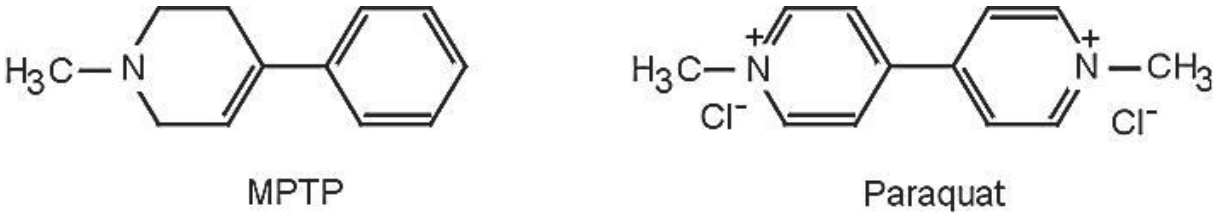


Figure 35 : Structure du MPTP et du paraquat. Les deux molécules partagent une certaine homologie

Le paraquat (N, N'-diméthyl-4-4'-bipyridium), aussi appelé dichlorure de paraquat ou méthyl viologène, est un composé extrêmement soluble (620 g/L), ce qui rend son utilisation

en recherche particulièrement aisée. C'est une molécule analogue au MPTP, comme il est visible sur la figure 35, et il a été associé étiologiquement au déclenchement de la MP par des études épidémiologiques (Firestone *et al.*, 2005; Lee *et al.*, 2012; Tanner *et al.*, 2011).

Pour ce qui est de la réglementation de ce produit, il est interdit en Europe et en France depuis 2007, par l'avis publié dans le journal officiel du 4 Aout 2007, retirant l'autorisation de mise sur le marché des produits phytopharmaceutiques contenant du paraquat (R BIX (AMM n° 8700169)). Alors que l'Europe a banni ce pesticide, celui-ci est encore utilisé dans plus d'une centaine de pays de par le monde, notamment aux États Unis, où son usage est non seulement encore autorisé, mais en dix ans son utilisation a été multipliée par 4, atteignant plus de 3000 tonnes en 2015. (Article du 21 décembre 2016 publié dans le New York Times https://www.nytimes.com/2016/12/20/business/paraquat-weed-killer-pesticide.html?_r=0)

D'autres pays ont banni le paraquat car un des usages détournés de ce pesticide est en tant qu'agent de suicide, une simple gorgée de ce pesticide suffit à déclencher un décès par fibrose pulmonaire en 20 jours (Xie *et al.*, 2012). Par exemple, la Corée du Sud a connu un déclin du nombre de suicide de 10% l'année suivant l'interdiction du paraquat (Myung *et al.*, 2015).

Mécanismes de toxicité du Paraquat

Suite aux études épidémiologiques liant le paraquat à la MP, des équipes ont cherché à comprendre par quel(s) mécanisme(s) biologiques(s) cette substance pouvait être neurotoxique.

Le paraquat est très toxique pour le système respiratoire et est faiblement métabolisé lors d'une ingestion car il est retrouvé sous sa forme initiale dans les urines (Tanner *et al.*, 2011). Des études se sont intéressées sur le devenir du paraquat une fois injecté chez la souris, ainsi le paraquat peut traverser la BHE et atteint le cerveau (Corasaniti *et al.*, 1998). Ceci pourrait être un élément déterminant du lien existant entre le paraquat et la MP car, en plus d'atteindre le cerveau, il se produit une bioaccumulation du paraquat

dans cet organe. Dans le cerveau, sa demi-vie est de 4 semaines, suivant une seule injection i.p. à la concentration de 10 mg/kg (Prasad *et al.*, 2007).

Les mécanismes de toxicité du paraquat impliqueraient 3 voies : le métabolisme de la dopamine, le stress oxydatif et la formation d'agrégats d'α-syn.

Le paraquat et la dopamine

La bioaccumulation du paraquat dans le cerveau pourrait impliquer un transport actif par les canaux sodiques afin de traverser la BHE (McCormack et Di Monte, 2003). Le paraquat cible spécifiquement les neurones dopaminergiques et induit une perte neuronale. Dans des modèles murins, il est montré que l'administration de paraquat par voie intrapéritonéale induit une dégénérescence spécifique des neurones dopaminergiques (Kachroo *et al.*, 2010; Li *et al.*, 2005). Cette perte neuronale pourrait être due à l'implication d'un transporteur de la dopamine. En effet, dans un modèle murin, l'utilisation d'un inhibiteur sélectif du transport de la dopamine réduit l'absorption du paraquat (Shimizu *et al.*, 2003), et le blocage du transporteur classique de la dopamine (DAT, Dopamine Transporter), protège spécifiquement dans un modèle cellulaire (Yang et Tiffany-Castiglioni, 2005). Cependant, le lien entre le paraquat et la perte de neurones dopaminergiques est contestée. En effet, une équipe financée par Syngenta, firme commercialisatrice des mélanges comportant du paraquat, contredit les précédentes études sur les effets du paraquat et montre dans une étude que les neurones dopaminergiques ne sont pas touchés lors de l'exposition de souris par voie alimentaire au paraquat (Breckenridge *et al.*, 2013; Minnema *et al.*, 2014).

Une hypothèse intéressante et assez peu reprise dans les recherches récentes serait que la toxicité spécifique du paraquat envers les neurones dopaminergiques pourrait être due à l'homologie structurelle existant entre la dopamine et le paraquat. En effet, cette homologie structurelle entre le paraquat et le MPTP a déjà été évoquée, mais l'élément intéressant est que ces deux molécules s'apparentent aussi à la dopamine, sans être parfaitement homologues (Jakowec et Petzinger, 2004; Lewin, 1985), comme il est vu sur la Figure 36. Ainsi, lorsque le paraquat est ingéré et qu'il est transporté par voie systémique

jusqu'au cerveau où il traverse la BHE, le paraquat pourrait-être capté par les récepteur à la dopamine, ce qui expliquerait sa toxicité sélective envers ce type de neurones.

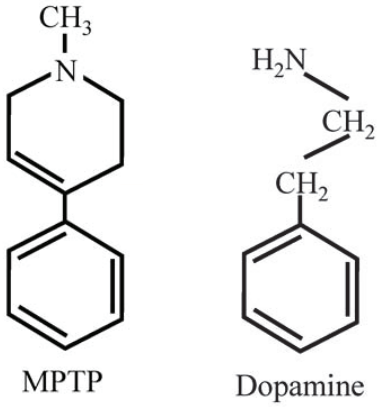


Figure 36 : Comparaison structurelle entre le MPTP et la dopamine, adapté de (Jakowec et Petzinger, 2004)

Stress oxydatif

Le paraquat serait toxique par l'induction d'un stress oxydatif dans la cellule par la création de dérivés réactifs de l'oxygène (ROS, reactive oxygen species), réaction décrite dans la figure 37. Par l'oxydoréduction du paraquat, l'ion paraquat^{2+} capte un électron réducteur par la chaîne respiratoire mitochondriale et créé un anion superoxyde $O^{2-\bullet}$, un ROS. Ceci permet au paraquat de se régénérer, et peut ainsi recommencer ce cycle, créant ainsi des ROS de façon continue (Mohammadi-Bardbori et Ghazi-Khansari, 2008).

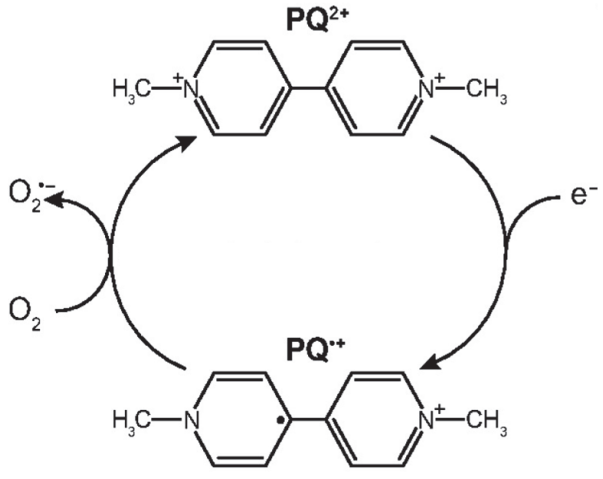


Figure 37 : Voie de création des ROS par le paraquat, induisant un stress oxydatif généré en continue dans la cellule. Adapté de (Cocheme et Murphy, 2008).

Ce stress oxydatif ainsi créé interfère avec le transport d'électrons dans la mitochondrie, particulièrement dans la SN, ceci inhibe le Complexe 1 de la chaîne respiratoire mitochondriale, ainsi montré dans des modèles *in vitro* (Cocheme et Murphy, 2008; Richardson *et al.*, 2005). Cependant, l'inhibition du Complexe 1 par le paraquat pourrait ne pas être la cause des pertes de neurones dopaminergiques, comme il a été montré par Choi et collègues dans un modèle transgénique de souris déficiente en une protéine nécessaire à la fonction du Complexe I, où le paraquat induit quand-même une perte de neurones dopaminergiques dans ces souris (Choi *et al.*, 2008).

Formation d'agrégats d'a-synucléine

L'autre aspect relevant des effets du paraquat sur les neurones est la formation d'agrégats d'α-syn, cependant les mécanismes réels d'induction de l'expression de l'α-syn sont encore assez peu connus. Plusieurs études ont montré *in-vivo* que l'administration de paraquat pouvait induire une synucléinopathie, notamment dans le striatum de souris C57Bl/6 après injection systémique (Wills *et al.*, 2012). Dans le modèle murin Thy1, surexprimant l'α-syn humaine, l'injection systémique de paraquat induit une augmentation de l'agrégation de la protéine et la résistance à la protéinase K (Fernagut *et al.*, 2007). Aussi, toujours dans un modèle *in-vivo*, il est montré que le paraquat mène à la formation d'agrégats contenant de l'α-syn dans la substance noire, parallèle à l'augmentation (réversible) de la concentration cellulaire en α-syn (Manning-Bog *et al.*, 2002). Un élément original est rapporté par Uversky et collègues où il montre *in vitro* que le paraquat accélère la formation de fibrille (Uversky *et al.*, 2001). Cependant il n'y aurait pas de fixation directe entre le paraquat et l'α-syn, comme l'indique une étude *in* vitro portant sur de l'α-syn recombinante, indiquant que les effets du paraquat dans la MP ne seraient pas dus à une interaction directe avec α-syn, renforçant l'idée que son rôle est limité à l'inhibition du Complexe I mitochondrial (Maturana *et al.*, 2015).

Pour résumer ces trois aspects de la toxicité du paraquat, depuis son entrée dans l'organisme jusqu'à l'induction d'un stress oxydatif dans la mitochondrie, les mécanismes associés à cette toxicité semblent être élucidés, cependant le lien entre le paraquat et l'augmentation de l'α-syn *in-vivo* et *in-vitro* reste encore à être clarifié. Le paraquat est donc un oxydant fort pour l'organisme. Le fait que cette molécule soit elle aussi considérée

comme une molécule de référence dans les études sur la MP justifie son utilisation pour étudier les mécanismes d'initiation de la pathologie.

Contexte et objectifs du doctorat

La maladie de Parkinson (MP) est la deuxième maladie neurodégénérative la plus fréquente en France, après la maladie d'Alzheimer. Cette maladie se traduit par des tremblements incontrôlables et continus, symptôme le plus visible de la maladie. D'un point de vue biologique, la MP appartient à la famille des synucléines, comportant la MP, la DCL et la MSA. Ces maladies ont le point commun d'être causées par l'α-syn, protéine neuronale dont les fonctions précises restent encore à être déterminées distinctement. Cette protéine fait partie intégrante du contexte de la thèse présentée ici. La MP, les autres synucléinopathies et une majorité des maladies neurodégénératives se caractérisent par une longue phase latente exempte de symptômes moteurs. Pour les synucléinopathie, durant cette période l'α-syn est au centre des processus pathologiques.

La MP possède une évolution lente, dont les premiers symptômes moteurs caractéristiques apparaissent tardivement, une à deux décennies après l'initiation potentielle de la maladie. Particulièrement, dans cette maladie des pertes de neurones dopaminergiques sont observées dans la substance noire, une aire cérébrale impliquée dans l'inhibition du mouvement. On estime donc que les premiers symptômes moteurs apparaissent lorsque plus de 70 % de cette zone cérébrale est détruite, à un stade où le cerveau est ainsi particulièrement touché. Des pesticides ont été montrés comme pouvant mener spécifiquement à la perte de neurones dopaminergiques et ces substances ont été liées épidémiologiquement au déclenchement de la MP. Ainsi, le paraquat et la roténone ont été montrés comme pouvant être liés au déclenchement de syndromes parkinsonien chez des agriculteurs utilisant ces pesticides. Depuis, cet effet neurotoxique a été attesté par beaucoup d'études faisant de ces deux substances des pesticides de référence dans l'étude des synucléinopathies. Cependant, les effets précis de ces pesticides sur le compartiment nerveux présent dans l'intestin sont encore peu étudiés.

Le titre initial du projet de thèse financé par le dispositif ARC 3 de la région Auvergne Rhône Alpes est « *Étude de l'implication du système nerveux périphérique dans le déclenchement d'une α-synucléinopathie centrale : approche comparée d'exposition alimentaire à des pesticides dans différents modèles murins de maladie de Parkinson* ». Les objectifs de cette thèse sont donc d'explorer les effets de l'exposition par voie orale au paraquat, herbicide considéré comme une molécule de référence dans l'étude des synucléinopathies, dans différents modèles murins, sauvages et transgéniques. Particulièrement, l'effet du paraquat sera investigué dans deux systèmes nerveux : le SNC et le système nerveux présent dans l'intestin, désigné comme SNE. Aussi, dans ce travail, seule la voie d'administration orale est utilisée à une dose relativement faible pour se rapprocher le plus possible des conditions d'absorption de pesticides par l'homme.

Deux objectifs majeurs lient ces travaux de thèse avec les thématiques de l'Agence Nationale de la Santé et de Sécurité Sanitaire, Alimentation Environnement Travail (ANSES) sont identifiés ici. Le premier est un objectif scientifique où les thématiques abordées permettent d'investiguer les effets de l'ingestion d'un pesticide considéré comme neurotoxique sur l'α-syn présente dans le SNE et dans le SNC. Le deuxième objectif est un objectif de santé publique, ayant pour but à plus long terme de créer une base méthodologique appliquée à l'évaluation de substances chimiques.

Ce projet s'articule autour de 3 parties :

1. **La mise en place d'un modèle d'exposition aux pesticides par voie orale chez la souris.**

La première partie de la thèse a porté sur le choix et la mise en place d'un modèle d'exposition orale de souris à un pesticide, le paraquat. Ainsi, deux modes d'administration ont été testés à différentes doses : le gavage intragastrique et l'administration par l'eau de boisson. Les résultats acquis ont mené au choix de l'administration du pesticide par l'eau de boisson dans la suite des expérimentations, en se basant sur la faible mortalité et l'excellente tolérance de cette voie d'administration.

2. **L'étude histologique de l'induction de l'α-synucléine phosphorylée dans l'intestin de souris transgéniques exposées au paraquat.**

Fort de la sélection de l'administration du paraquat par l'eau de boisson, le projet de thèse s'est poursuivi par l'étude par immunohistochimie de coupes de cerveaux et d'intestins de souris sauvages et transgéniques présentant une synucléinopathie spontanée. Des données précédentes montrent que ces souris transgéniques présentent des dépôts d'α-syn phosphorylée à partir de 3,5 mois d'âge dans le SNE et dans le cerveau simultanément (Bencsik *et al*., 2014). Nous avons donc exposé des souris TgM83 pendant 6 à 8 semaines au paraquat par l'eau de boisson et des souris C57Bl/6 pendant 6 semaines. L'étude des intestins et des cerveaux des souris TgM83 montre que l'exposition au paraquat accélère l'apparition de cette synucléinopathie par la détection de la protéine phosphorylée dans le système nerveux entérique de ces souris dès 3 mois d'âge, cependant cette accélération n'est pas constatée dans le cerveau aux temps étudiés. Dans la lignée C57Bl/6, les 6 semaines d'exposition au pesticide par voie orale n'engendrent pas l'apparition de dépôts d'α-syn phosphorylée dans le compartiment nerveux de l'intestin cependant une gliose réactionnelle est attestée par la surexpression de la GFAP dans ces intestins.

3. L'étude biochimique des modulations des niveaux d'α-synucléine induites par l'exposition orale au paraquat chez la souris sauvage et transgénique.

Pour aller plus loin dans la compréhension des modifications biologiques induites par l'exposition à un pesticide, la thèse s'est poursuivie par l'étude topographique, région par région, des niveaux d'expression de l'α-syn en conditions physiologiques et en conditions de stress oxydatif induit par l'exposition au paraquat, tant dans les aires cérébrales que dans l'intestin. Ainsi, la mise au point des techniques biochimiques d'extraction et d'analyse de l'α-synucléine totale à partir d'échantillons intestinaux a permis de montrer l'augmentation globale des niveaux d'α-synucléine tant dans le système nerveux entérique que dans le système nerveux central suivant une exposition au paraquat.

L'ensemble des résultats originaux présentés dans cette thèse s'inscrit dans le but d'avoir une meilleure compréhension de l'effet d'un pesticide, le paraquat, sur différents système nerveux par une approche originale d'exposition orale, plus proche des modalités d'exposition de la population générale. Aussi, en lien avec les missions du laboratoire ANSES de Lyon, la mise en place d'un modèle d'exposition à des produits phytosanitaires pourrait servir de base à l'évaluation des risques toxicologiques pour d'autres substances chimiques, en lien avec les synucléinopathies.

Résultats expérimentaux

Dans cette partie 3 productions scientifiques seront détaillées :

Poster présenté au Xème congrès de la société des neurosciences

1 *Neurodegenerative model in mice induced by chronic oral exposure to pesticide: comparative study between drinking water and intra-gastric administration*

Article 1 : Publié dans Journal of Neuropathology & Experimental Neurology

2 *Oral Exposure to Paraquat Triggers Earlier Expression of Phosphorylated α-Synuclein in the Enteric Nervous System of A53T Mutant Human α-Synuclein Transgenic Mice*

Article 2 : Soumis dans le journal Neurotoxicology

3 *A widespread increase of α-synuclein expression in the brain and intestine of mice chronically exposed to paraquat through drinking water*

1 Neurodegenerative model in mice induced by chronic oral exposure to pesticide: comparative study between drinking water and intra-gastric administration

Nicolas Naudet, Emilie Antier, Damien Gaillard, Eric Morignat, Latifa Lakhdar, Christel Vanbesien, Anna Bencsik

Résumé des données présentées par poster au XIème congrès de la Société Française des Neurosciences à Montpellier en mai 2015.

Dans la première partie du travail, les expérimentations ont porté sur la détermination de la « meilleure » voie d'exposition orale et chronique au paraquat, comprenant une excellente tolérance et un respect du bien-être animal. Ces résultats apparaissent uniquement dans un poster présenté au XIème congrès de la société des neurosciences.

Ainsi, pour déterminer cette voie d'exposition orale, deux modes d'administration ont été testées :

Le gavage intragastrique

Le gavage intragastrique correspond à l'administration d'une quantité précise d'une solution de pesticide directement dans l'estomac de la souris à l'aide d'une sonde de gavage. Cette technique est lourde, tant pour l'opérateur que pour la souris. En effet, ce mode d'administration est astreignant et très lourd sur le plan éthique car il impose une manutention quotidienne de tous les animaux des lots exposés, mais aussi des lots contrôles, étant eux aussi soumis à l'administration d'eau par voie intragastrique. Ceci représente pour l'opérateur une charge de travail particulièrement conséquente nécessitant un savoir-faire et une précision dans l'exécution du gavage. Aussi, cette méthode invasive induit un stress conséquent à l'animal. Outre le fait que cette voie soit contraignante, elle présente l'avantage de proposer un parfait contrôle de la dose quotidienne administrée à la

souris, cependant cette dose est distribuée de façon relativement « aigue » tous les jours, les souris n'ayant qu'une seule prise de pesticide quotidienne.

La dilution du paraquat dans l'eau de boisson

L'administration du paraquat par la dilution de celui-ci dans l'eau de boisson est une voie d'exposition simple, adaptée à ce pesticide car il est extrêmement soluble. Les souris consomment l'eau additionnée de la substance sélectionnée. Contrairement au gavage intragastrique, ce mode d'administration n'est pas astreignant car il ne nécessite aucune manutention de la souris. De ce fait la souris ne subit aucun stress lié à la manutention et au gavage. L'un des points négatifs de ce mode d'administration concerne la dose ingérée par la souris. En effet, cette dose de pesticide ne peut être qu'estimée, en multipliant la quantité théorique d'abreuvement par la concentration présente dans l'eau de boisson. L'avantage de cette voie d'administration est la répartition de la dose sur la journée, à l'inverse du gavage intra gastrique.

Dans une première série d'expérimentations, les deux voies d'administration ont été testées sur plusieurs lots de 6 souris. Le gavage intragastrique a été testé à 3 doses différentes : 5, 10 et 20 mg/kg/jour pendant 6 semaines alors que l'exposition par eau de boisson a été testée à la dose de 10mg/kg/jour, correspondant à une concentration de 50 µg/mL dans l'eau d'abreuvement, pendant 6 semaines. Un suivi quotidien des souris exposées a été assuré, comprenant l'observation de l'état général des souris ainsi que la pesée. Aussi, des tests Rotarod ont été réalisés de façon hebdomadaire afin de voir si des déficits moteurs sont induits par l'exposition orale au paraquat.

Les résultats de ces tests préliminaires indiquent que l'administration par l'eau de boisson est parfaitement tolérée, avec un poids stable et aucune perte d'individus. A l'inverse, l'administration par gavage mène à plusieurs éléments problématiques dans les deux plus fortes doses testées dans notre étude. Le premier élément étant une perte de poids d'environ 3 à 5 grammes des souris exposées dans les premières semaines d'exposition. Le deuxième élément étant une mortalité concernant 33 % des individus aux deux plus fortes doses, dans les premières semaines d'exposition. Seule la dose la plus faible, à savoir 5 mg/kg, n'engendre aucune perte de poids et aucune mortalité. Les analyses par test Rotarod ne montrent aucune différence entre les lots exposés et les lots témoins

pour les deux modalités d'exposition testées ici, indiquant qu'il n'y a aucune altération motrice induite par le paraquat aux doses et aux temps étudiés.

Dans cette présentation, des tests étudiant la TH et la GFAP dans les cerveaux de ces souris ont été amorcés par la quantification numérique de marquages immunohistochimiques avec des anticorps anti-TH et anti-GFAP. Cependant ces marquages portaient seulement sur une coupe de substance noire, alors que l'analyse rigoureuse de la quantité de TH dans un cerveau se fait par une analyse stéréologique, exhaustive de toute la SNpc. Ces résultats ne sont pas pris en comptes dans la thèse présentée ici.

En conclusion, à doses équivalentes de 10 mg/kg/jour, l'administration du pesticide par l'eau de boisson est parfaitement tolérée, alors que l'administration par gavage intra-gastrique mène à des pertes d'individus, couplé à une administration du pesticide affectant considérablement le bien-être animal.

NEURODEGENERATIVE MODEL IN MICE INDUCED BY CHRONIC ORAL EXPOSURE TO PESTICIDE: COMPARATIVE STUDY BETWEEN DRINKING WATER AND INTRA-GASTRIC ADMINISTRATION

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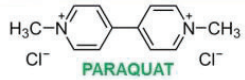
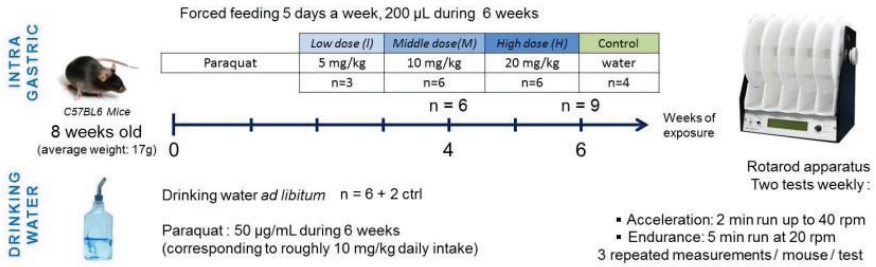
INTRODUCTION

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease after Alzheimer's and constitutes a true burden for the aging population. Most cases are sporadic and a multifactorial etiology involving genetic and environmental factors is acknowledged. Among environmental factors, pesticides were identified as being related to PD cases in particular after occupational exposure, but it remains poorly understood when and how pesticides could participate to the pathogenesis of PD. Rodent models of pesticide exposure by oral route mimic well the development of the disease which can be followed by the progressive expression of the main hallmark of PD, alpha-synuclein (αSYN) aggregates within the nervous system as well as other markers such as neuroinflammation through reactive gliosis or neurodegeneration through dopaminergic loss.
In that context, the aim of this pilot study is to compare intra-gastric and drinking water exposure to pesticide on the induction of neurodegenerative processes in the central nervous system in order to prepare longer experiments programmed to study the impact of chronic pesticide exposure.

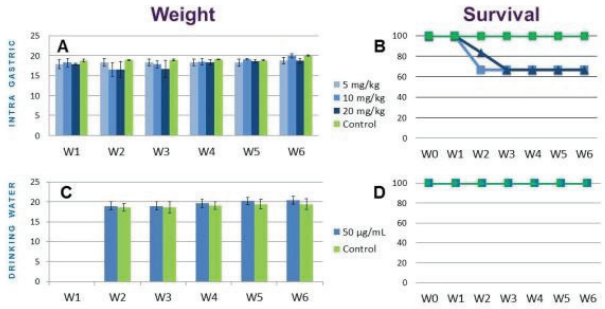
MATERIALS & METHODS



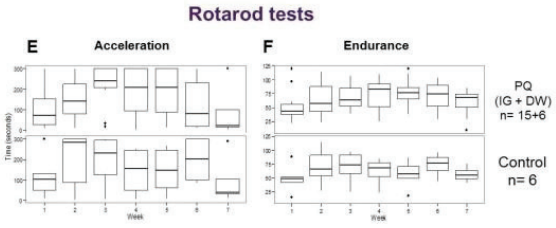
Most widely non-selective herbicide used in the world. Soluble and stable in water, with great persistence in soil and water table. May contaminate fruits, vegetables and tap water. Forbidden in France since 2007. Acute intoxication leads to respiratory distress, kidney failure and death. Chronic intoxication lead to bioaccumulation in several organs (brain, kidney, liver, lung) and lung fibrosis. May pass through blood brain barrier. Neurototoxic effects: oxidative stress in mitochondria of dopaminergic (DA) neurons, leads to cell death. PQ is associated to PD (epidemiological studies in farm workers). PQ used in rodent as models to explore mechanisms of PD triggering, DA neurons depletion, αSYN aggregation (PD-like lesion) within the brain. (Prasad et al., 2009, Berry et al., 2010, Mandel et al., 2011).



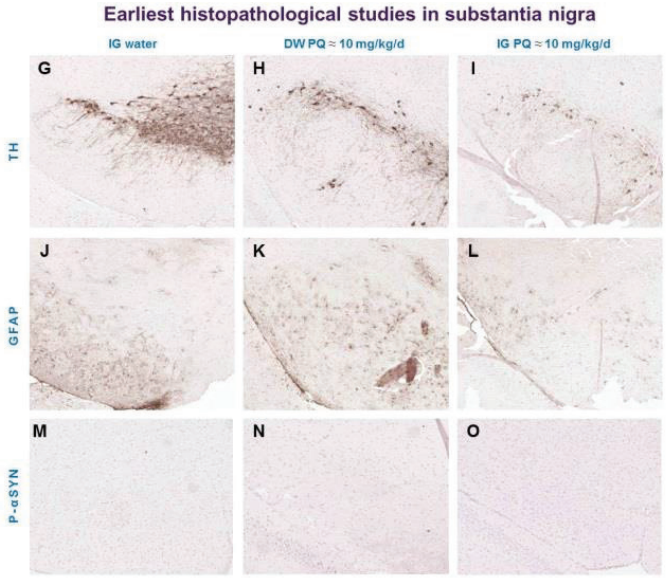
RESULTS



Effect of chronic paraquat oral intake on mice weight and survival. Loss of weight within the first weeks of exposure leading sometimes to death (B). Appears to be dose dependent. No loss in drinking water group. (**A-C**): mean of the weight of all mice, daily weighted.(**B-D**): Survival curves, expressed in percentage of surviving mice.



Rotarod tests are performed to detect locomotor deficiency: Due to low number of mice (pilot study) all PQ exposed mice were grouped (Intra gastric (IG) + drinking water (DW)), as for controls. Data reveal high heterogeneity inter and intra groups during the time dependent study. No statistical differences between exposed and controls could be observed. (**E**) Acceleration test, (**F**) Endurance test.



Preliminary results reveal apparent DA neuron loss and reactive gliosis in Substantia nigra (SN) after six weeks of exposure. Tyrosine hydroxylase (TH), Glial Fibrilary Acid Protein (GFAP) and Phosphorylated Alpha-Synuclein (P-αSYN) immunohistochemistry staining. PQ exposure appears to affect DA neurons, induce reactive gliosis, for both ways of exposure, in the SN. No αSYN phosphorylation. (**G-J-M**) control mouse. (**H-K-N**) mouse exposed to PQ through drinking water (50 µg/mL). (**I-L-O**) mouse exposed to PQ by intra-gastric route (10 mg/kg/d).

CONCLUSION

The first observations indicate a better tolerance of PQ administration through drinking water. Compared to intragastric exposure with a comparable dose of exposure, PQ administration through drinking water allows normal gain of weight and avoid mortality. Rotarod tests reveal high individual variability whatever the group and after 6 weeks of chronic exposure to PQ no significant locomotor defects could be detected. First histopathological data show a possible early dopaminergic neurons loss in the SN in addition to reactive gliosis in mice exposed during 6 weeks to PQ, by intra-gastric route and through drinking water as well. Also, these observations will be comforted with precise quantification of TH signals in order to study a possible dose dependant effect on DA loss. Still the pilot study already show the possibility to use drinking water as way of PQ administration in longer studies programmed to mimic more complete neurodegenerative process.

References : *Prasad K. et al., journal of experimental neurology, 2009. Berry C. et al., Cell death and differenciation, 2010. Mandel JS. et al., Regulatory Toxicology and Pharmacology, 2011.*
Acknowledgements : Authors are grateful to Région Rhône Alpes for the PhD granting of N. Naudet.

Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail ANSES Lyon, Unité Maladies Neurodégénératives

2 Article N°1 : Oral Exposure to Paraquat Triggers Earlier Expression of Phosphorylated α-Synuclein in the Enteric Nervous System of A53T Mutant Human α-Synuclein Transgenic Mice

Nicolas Naudet, Emilie Antier, Damien Gaillard, Eric Morignat, Latifa Lakhdar, Thierry Baron et Anna Bencsik

Résumé des résultats publiés dans Journal of Neuropathology and Experimental Neurology (JNEN) – Publié le 12 octobre 2017

Naudet et al., 2017 – doi : 10.1093/jnen/nlx092

Ce premier article de thèse a pour but d'étudier les effets du paraquat administré par voie orale dans deux compartiments nerveux distincts : le cerveau et l'intestin. Dans cet article, deux lignées murines ont été exposées au paraquat, la souris TgM83 et la souris sauvages C57Bl/6.

Ainsi dans cet article, ayant choisi l'exposition au paraquat par l'eau de boisson, des souris jeunes, âgées de 8 semaines d'âge, ont été exposées par l'eau de boisson à ce pesticide à la dose de 10 mg/kg/jour pendant 6, 7 ou 8 semaines.

La souris TgM83 est une souris transgénique exprimant la protéine α-syn humaine mutée au niveau du résidu 53 sur la protéine où une alanine devient une thréonine (A53T), mutation retrouvée dans les cas familiaux de la MP. La protéine exprimée dans ces souris comporte donc une mutation au niveau de la deuxième hélice α présente dans la structure de la protéine.

Les souris de la lignée TgM83 développent spontanément une synucléinopathie dont les symptômes moteurs sont caractérisés, dans cette lignée murine, par l'apparition à partir de 8 mois d'une rigidité du train arrière associée à une démarche présentant des membres inférieurs « fuyants » lors des déplacements. Une publication précédente émanant de notre

laboratoire a permis de montrer que cette pathologie se traduit par l'apparition de la forme phosphorylée de l'α-syn en sérine 129 dans le cerveau. La cinétique d'apparition de cette forme phosphorylée est particulière. En effet, entre 3,5 et 4 mois d'âge, des inclusions nucléaires de synucléine sont détectable dans le cerveau, entre 6 et 7 mois d'âge, ces inclusions deviennent cytoplasmiques. Cette étude a aussi permis de montrer que ces souris développent aussi une synucléinopathie dans le système nerveux entérique, plus précisément dans les plexus myentériques, détectable entre 3,5 et 4 mois d'âge. Cet article montre l'apparition parallèle et simultanée de la synucléinopathie spontanée dans le SNE et le SNC de cette lignée transgénique. (Bencsik *et al.*, 2014).

Pour les deux lignées observées, l'exposition par l'eau de boisson au paraquat est parfaitement tolérée et aucune perte majeure dans les effectifs de chaque lot n'est observée. Les souris suivent une prise de poids constante, n'indiquant aucunes différences entre les souris exposées et les souris contrôles. Aussi, les tests locomoteurs de Rotarod n'indiquent pas de différences sur les compétences locomotrices entre les souris exposées et les témoins.

L'exposition au paraquat pendant 6 semaines par voie orale chez la souris C57Bl/6 permet d'observer plusieurs éléments. (1) Il n'y a pas de protéines d'α-syn phosphorylée en S129 détectable dans le système nerveux entérique de ces souris aux temps d'exposition considérés, (2) Une augmentation significative de la GFAP remarquée dans ces plexus myentériques. Ceci montre que dans cette lignée, l'exposition orale au paraquat induit une gliose réactionnelle dans les intestins mais n'induit pas de synucléinopathie aux temps d'exposition considérés.

Dans le modèle TgM83, l'exposition par voie orale au paraquat mène à l'induction précoce de la synucléinopathie spontanée dans les intestins de cette souris, visible par l'apparition de l'α-syn phosphorylée S129 dans les plexus myentériques dès 6 semaines d'exposition au paraquat, correspondant à un âge de la souris de 3 mois. Cette expression est constante et persiste à la 7éme et 8éme semaine. Dans les contrôles, les plexus myentériques ne sont pas marqués par l'α-syn phosphorylée S129, comme il est visible dans les résultats de cet article. Cependant, dans des données non publiées nous remarquons l'apparition de l'α-syn phosphorylée S129 dans les contrôles à la 9ème semaine d'exposition,

correspondant à un âge de 17 semaines soit environ 4 mois (résultats non exposés dans cet article). Dans cette lignée murine aussi, une surexpression de la GFAP est également identifiée dès 6 semaines d'exposition au paraquat. Il est à noter que l'analyse en parallèle des cerveaux de ces souris indique l'absence d'α-syn phosphorylée aux temps d'exposition considérés, tant de la SNpc que dans le tronc cérébral.

En résumé ces résultats montrent l'accélération d'une pathologie de l'α-syn dans le système nerveux entérique de souris transgéniques exposées au paraquat. Ceci représente un modèle d'exposition à un pesticide attestant du pouvoir d'accélération d'une synucléinopathie de la substance considérée.

J Neuropathol Exp Neurol
Vol. 0, No. 0, 2017, pp. 1–12
doi: 10.1093/jnen/nlx092

OXFORD

ORIGINAL ARTICLE

Oral Exposure to Paraquat Triggers Earlier Expression of Phosphorylated α-Synuclein in the Enteric Nervous System of A53T Mutant Human α-Synuclein Transgenic Mice

Nicolas Naudet, MSc, Emilie Antier, BS, Damien Gaillard, BS, Eric Morignat, MSc, Latifa Lakhdar, PhD, Thierry Baron, DVM, PhD, HDR, and Anna Bencsik, PhD, HDR

Abstract

The misfolded α-synuclein protein, phosphorylated at serine 129 (pSer129 α-syn), is the hallmark of Parkinson disease (PD). Detected also in the enteric nervous system (ENS), it supports the recent theory that PD could start in the gut, rather than the brain. In a previous study, using a transgenic mouse model of human synucleinopathies expressing the A53T mutant α-synuclein (TgM83), in which a neurodegenerative process associated with α-synuclein occurs spontaneously in the brain, we have shown earlier onset of pSer129 α-syn in the ENS. Here, we used this model to study the impact of paraquat (PQ) a neurotoxic herbicide incriminated in PD in agricultural workers) on the enteric pSer129 α-syn expression in young mice. Orally delivered in the drinking water at 10 mg/kg/day for 6–8 weeks, the impact of PQ was measured in a time-dependent manner on weight, locomotor abilities, pSer129 α-syn, and glial fibrillary acidic protein (GFAP) expression levels in the ENS. Remarkably, pSer129 α-syn was detected in ENS earlier under PQ oral exposure and enteric GFAP expression was also increased. These findings bring additional support to the theory that neurotoxic agents such as PQ initiate idiopathic PD after oral delivery.

Key Words: α-Synuclein, A53T, Chronic oral exposure, Enteric nervous system, Paraquat, Parkinson disease.

INTRODUCTION

Parkinson disease (PD) is the second most frequent neurodegenerative disease in the world after Alzheimer disease. In Europe, PD crude prevalence rate ranges between 65.6 per 100 000 and 12 500 per 100 000 (1). PD belongs to the so called alpha-synuclein (α-syn) aggregation diseases, also named synucleinopathies, including at least 3 diseases: PD, dementia with Lewy bodies and multiple system atrophy (2). These diseases are characterized by a neurodegeneration associated with accumulation of a pathological form of α-syn and propagated in cerebral structures, such as Lewy neurites and bodies, neuronal inclusions for PD or dementia with Lewy bodies, and glial cytoplasmic inclusions for multiple system atrophy (3, 4). Lewy bodies are composed mostly of α-syn and ubiquitin (5–7). Phosphorylation at serine 129 (pSer129 α-syn) and oligomerization of α-syn are recognized as key events in PD pathogenesis (8, 9). The distribution of Lewy inclusions in the brain has been associated with PD staging (10–12). The temporal progression of the disease revealed by these studies suggests that the pathology could initiate other than in the brain, such as the digestive tract and olfactory bulb, possibly as a result of oral or inhalation exposure to xenobiotic agents carrying neurotoxicity. This hypothesis, called Braak's theory, was reinforced by several descriptions of pathological α-syn deposits in various peripheral nervous tissues, particularly in the enteric nervous system (ENS) of advanced as well as of preclinical PD cases (13, 14). Among the suspected xenobiotics, agricultural chemicals were identified by several epidemiological studies (15–17). Tanner et al reported a list of pesticides that present a higher correlation with the appearance of PD (18). Among them, pesticides inhibiting the mitochondrial Complex I and increasing oxidative stress appear to be more prone to induce sporadic PD upon exposure. Amid suspected products, rotenone, maneb and paraquat (PQ) have been studied experimentally and confirmed their neurotoxic effect in accordance with typical PD features (19, 20). PQ has a structure similar to that of MPP^+ (deriving from 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridin [MPTP]), a well-known toxic molecule that can cause permanent Parkinsonian syndrome in humans. Also known as 1, 1′-dimethyl-4, 4′-bipyridinium, PQ is a quaternary ammonium nonselective herbicide progressively banned from the market in several countries but is still widely used in many countries all over the world. PQ was shown to be linked with early PD onset and its neurotoxicity has been assessed by several studies (16, 21).

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This study was partly supported by ANSES; however, there is no grant number associated with this funding source. Nicolas Naudet is supported by a doctoral grant from the Academic Research Community 3—Région Auvergne—Rhône Alpes.

The authors have no duality or conflicts of interest to declare.

Supplementary Data can be found at http://www.jnen.oxfordjournals.org.

When it comes to linking PD initiation with oral exposure to pesticides there is a paucity of published documentation. One study has shown the link between mice oral exposure to pesticide (i.e. rotenone) with pathological staging of PD, supporting Braak's hypothesis of the peripheral initiation of the disease (22). Very few studies are using oral exposure to study the impact of pesticides on PD and, to the best of our knowledge, there have been no studies investigating the impact of oral exposure to pesticides on the α-syn pathology within the ENS, with the exception of the work published by Pan-Montojo et al (22). However, studies using other routes of administration of pesticides (e.g. intravenous or intraperitoneal routes) represent the majority of the available research. In 2007, Prasad's team showed that PQ can accumulate in the mouse brain after oral administration in a dose-dependent manner. Systemic injection at the dose of 10 mg/kg showed that PQ persists in the ventral midbrain of mice for a prolonged time compared with other organs, with a half-life of ~1 month, indicating that PQ crosses the blood–brain barrier and persists within the brain (23, 24).

With the above considerations, we set up the first study to investigate the potential neurotoxic effects of PQ, administrated orally, on the ENS in mutant TgHuA53T transgenic mice overexpressing human α-syn, with the A53T mutation along with murine α-syn (TgM83 lineage) (25). In this mouse model, the pathological α-syn (pSer129 α-syn) appears spontaneously in the brain and ENS several months before the symptoms appear (26). The pSer129 α-syn is detectable clearly from the age of 6 months in the brain, and as early as 4 months in the ENS (26); therefore, we used young mice (2 months-old) exposed chronically for a period of 2 months. Using immunohistochemical (IHC) techniques, we analyzed the expression of the pSer129 α-syn in ENS of PQ-exposed mice compared with controls.

MATERIALS AND METHODS

Animals and Experimental Design

We used a transgenic mouse model of human synucleinopathy, TgM83 (HuA53T α-syn transgenic mice), that overexpresses A53T mutant human α-syn under a prion promoter (B6; C3H-Tg(SNCA)83Vle/J) (25). Groups of 23 (exposed) and 11 (control) transgenic mice were composed of young (2 months-old, 19–23 g) homozygous TgM83 male and female mice (produced in Anses Lyon approved animal facilities, PFEA). A wild type control group of 12 young, female C57Bl/6 mice (8 weeks-old, #18–22 g body weight) expressing only murine α-syn (Janvier, Le Genest-Saint-Isle, France) was used for PQ exposure (n = 6) and control tap water exposure (n = 6). Mice were maintained in the PFEA with a controlled temperature and hygrometry, 12-hour day/night cycle and were fed ad libitum. All the experimental procedures were conducted in compliance with procedures agreed by ANSES/ENVA/UPEC ethic committee (N°14-004).

PQ (methyl viologen dichloride hydrate, ref. 856177, Sigma–Aldrich, St. Louis, MO) was administered to TgM83 mice by drinking water (50 µg/mL), ad libitum, for 6, 7, or 8 weeks (n = 7–8/time point; Fig. 1A) and to C57Bl/6 mice by drinking water (50 µg/mL), ad libitum, for 6 weeks (n = 6; Fig. 1B); approximate daily PQ intake is roughly equivalent to 10 mg/kg bw/day taking account an average daily water intake estimated to be ~1.5 mL/10 g body weight/day; http://web.jhu.edu/animalcare/procedures/mouse.html). Because PQ is stable in water for more than 14 days at 54 °C (http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Evaluation04/paraquat.pdf) and sensitive to UV actions, a fresh PQ solution was prepared every 14 days and kept at 4 °C in glass bottle covered with aluminum sheet. The solution was used as drinking water that was changed twice a week. Weights were monitored weekly. After initial training, basal locomotor performances for each mouse were recorded prior to pesticide exposure by performing 2 rotarod tests weekly: endurance (3 × 5 minutes at 20 RPM) and acceleration (2 minutes at 4–40 RPM). The mean latency to fall was recorded and analyzed. At each end-point, mice were killed by 200 µL of pentobarbital (54.7 mg/mL) i.p. injection. Intestines were collected from the distal stomach to the distal colon, opened and rinsed in a bath of 0.1 M PBS, and then rolled in spiral shape directly within an inclusion cassette with the upper section at the center, using a method adapted from Moolenbeek et al (27) (Supplementary Data Fig. S1A). Once post-fixed for at least 48 hours in a buffered 10% formalin solution, samples were embedded in paraffin following a routine protocol. Five-micrometer sections of guts were collected on adhesive slides (Surgipath X-tra adhesive, Leica, Wetzlar, Germany).

IHC Detection of pSer129 α-Synuclein and GFAP in ENS

Identification of the pSer129 α-syn (1/500 ab 51253, AbCam, Cambridge, UK) as a marker of α-synucleinopathy, and the detection of glial fibrillary acidic protein (GFAP) (1/500, ab7260, AbCam, Cambridge, UK) as a marker of a reactive gliosis in the ENS was accomplished by IHC. Importantly, if several antiphosphorylated α-synuclein antibodies successfully detect phosphorylated form in the CNS of mouse ([P-syn/81 A] [ab184674], [EP1536Y], and [ab 51253]), only one allows its detection in the ENS (ab 51253) (Supplementary Data Fig. S2). The IHC procedure applied was similar to that described previously (26). Briefly, successive slides of gut sections were used to reveal each of the markers. Biotinylated secondary antibodies used at 1/200 (antiRabbit ref. 4010-05 and antimouse ref. 1010-05, Cliniscience, Nanterre, France), combined with avidin biotin complex (Vectastain ABC HRP Kit, Vector Laboratories, Burlingame, CA) and 3, 3′-diaminobenzidine peroxidase intensified with nickel (DAB Peroxidase (HRP) Substrate Kit, Vector Laboratories), revealed the specific staining that appeared as dark brown deposits (Supplementary Data Fig. S1C–E). Finally, the histological organization of each tissue sample was visualized using aqueous hematoxylin staining.

To characterize the type of enteric neurons that sustain pSer129 α-synuclein expression, a double-labeling experiment was conducted, as described previously (28), using either rabbit polyclonal anti tyrosine hydroxylase (TH) antibodies (1:200, Abcam) or mouse monoclonal anticholine acetyltransfer-

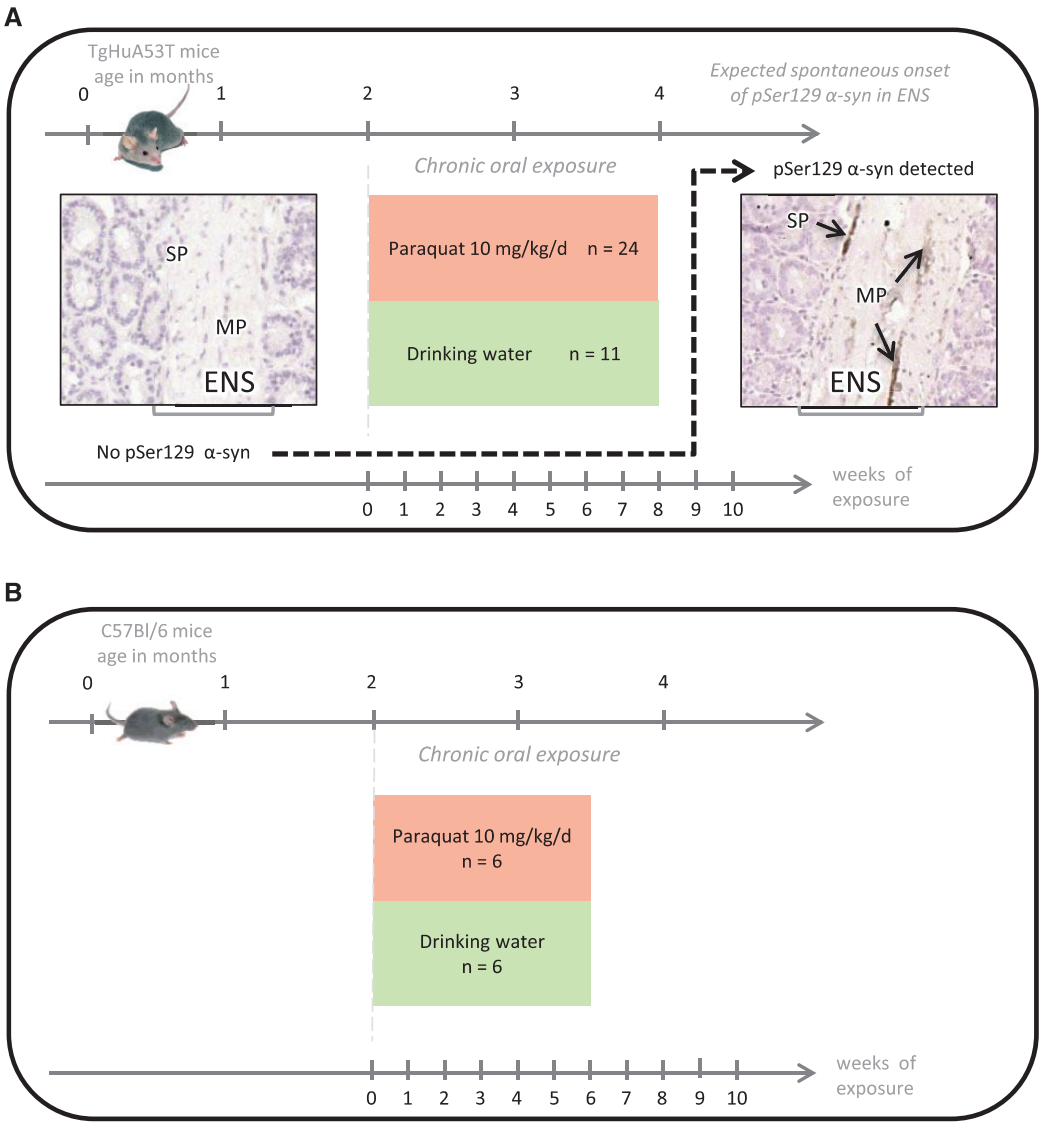

FIGURE 1. (A) Experimental design of the chronic paraquat (PQ) oral exposure experiments in TgM83 in order to study the impact on pSer129 α-synuclein expression in the ENS. PQ was delivered in the drinking water (50 μg/mL, ad libitum) for 6, 7, and 8 weeks (n = 23) compared with control mice (n = 11) exposed to only tap water as drinking water. The endpoints were selected with the knowledge of absence pSer129 α-synuclein expression in the ENS expected in young mice of control groups. This is illustrated in the left panel by a microphotograph of an intestine section, compared with obvious expression in each animal from the age of 4 months. The right panel shows the pSer129 α-synuclein expression detected by IHC in the ENS, which appeared as dark brown deposits in neurons of the muscular plexus). **(B)** Experimental design of the chronic PQ oral exposure experiments in the control wild type group (C57Bl/6). PQ was delivered in the drinking water (50 μg/mL, ad libitum) for 6 weeks (n = 6) compared with control mice (n = 6) exposed to only tap water as drinking water.

ase (ChAT) antibodies (1: 200, Chemicon, Temecula, CA). Gut samples were first labeled with pSer129 α-synuclein antibodies following the same protocol as above. Then, after DAB-$NiCl_2$ staining, the slides were thoroughly rinsed in 0.1 M PBS prior to a 30-minute incubation in a blocking reagent (Roche-Boehringer, Meylan, France) solution at RT. Subsequently, tissue samples were incubated with antiTH or antiChAT antibodies overnight at RT. Again, the avidin–biotin complex system was used to detect antibody binding sites, but peroxidase activity was detected using AEC (3 amino-9 ethyl-carbazol) that produces red precipitates. Finally, the histological organization of each tissue sample was visualized using aqueous hematoxylin staining.

Quantitative Study of pSer129 α-Synuclein and GFAP Expression in ENS

Observations and image captures (10× objective) were performed on an Olympus BX51 microscope (Olympus, Tokyo, Japan) coupled to an image analysis station (Explora

Nova, La Rochelle, France). The sample preparation (Swiss roll method) allowed complete access of the entire gut from the duodenum to the end of the colon using only 1 slide. To cover a representative area of ENS, 10 (GFAP) to 15 (pSer129 α-syn) images were randomly collected in the largest part available, the jejunum (Supplementary Data Fig. S1B). A classical scoring system of phosphorylated α-syn and GFAP markers in each mouse and among the 10–15 images per mouse was refined to a semiquantitative approach. The surrounding background for each image to be analyzed was subtracted systematically and automatically. Densities of phosphorylated α-syn and GFAP in the ENS of the jejunum were assessed using Morpho Strider software (Explora Nova, La Rochelle, France) by measuring the staining inside the region of interest (ROI), the ENS for the gut (Supplementary Data Fig. S1F–I). The density of staining was calculated by the formula:

$$\textit{Density of staining} = \frac{\text{Surface stained by antibody of interest}}{\text{Surface of the ROI}} \times 100$$

Results are expressed as means of density of pSer129 α-syn or GFAP staining within the nervous plexus.

Statistical Analyses

Survival estimates were computed using the Kaplan Meier method and survival distributions were compared between groups using the log rank test. Comparison of the weight between exposed and control mice was done the same way. The Welch *t*-test was used to compare the latency between control and PQ groups every week for acceleration and endurance. Statistical analyses for quantification of density of staining of pSer129 α-syn and GFAP were done comparing exposed to control groups using the one way ANOVA test, after having verified the homoscedasticity with the Levene test. Statistical analyses were made with R software (R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org).

RESULTS

Chronic Oral Exposure to PQ up to 8 Weeks Had No Significant Effect on Mouse Weight, Survival, and Locomotor Performances

PQ oral exposure was from 6 to 8 weeks on 3.5–4-month-old TgM83 mice. At this age, transgenic mice do not yet show PD-like pathology (paralysis) that appears rather from 8 months of age; however, as shown in our previous study, pSer129 α-syn starts to be detectable at the age of 4 months in the ENS and 6 months in the brain (26). Weight and survival of young TgM83 mice indicated no significant differences between PQ-exposed mice and controls (Supplementary Data Fig. S3A, B). The 2 locomotor tests (endurance and acceleration) performed weekly on mice using rotarod apparatus showed no significant impairment of mice locomotor skills along PQ oral exposure (Supplementary Data Fig. S3C, D). In the wild type control group, PQ oral exposure performed during 6 weeks on C57Bl6 mice did not trigger weight or survival differences with the nonexposed group. There was no significant alteration either in the locomotor performances measured by the rotarod tests (data not shown).

Oral Exposure to PQ Does Not Induce pSer129 α-Synuclein Expression in the ENS of C57Bl/6 Mice

The phosphorylated S129 α-syn was not observable in any of the C57BL6 mice exposed to PQ for 6 weeks (Fig. 2A). However, the response of the glial compartment of the ENS investigated through the level of GFAP expression appeared significantly ($p < 0.0001$) increased (Fig. 2B) compared with the controls. This suggests the presence of a reactive gliosis in the ENS compartment of PQ-exposed mice.

Oral Exposure to PQ Leads to Earlier pSer129 α-Synuclein Expression in the ENS of TgM83 Mice

The expression of pSer129 α-syn revealed by IHC was quantified in several portions of the jejunum both visually and numerically. In control groups ($n = 4$) exposed to normal drinking water, pSer129 α-syn appeared to be faintly detectable (~4/15 images faintly stained per positive mouse) in the ENS of some mice (3/11 animals), and within a few neurons of the ENS, mostly in the myenteric plexus. At 8 weeks, the expression of pSer129 α-syn appeared more clearly in 2 out of 4 mice as revealed by the higher density detected using quantitative analysis (Fig. 3A). This could be interpreted as the first signs of local accumulation expected to appear more clearly in older mice (26). Strikingly, in the PQ-treated group, pSer129 α-syn was clearly detectable in 19/23 mice, and as early as from 6 weeks of exposure in 7/8 mice (Fig. 3A, B). Quantitative analyses showed that pSer129 α-syn immunolabeling was significantly higher ($p < 0.001$) in PQ-exposed mice and these differences in the levels of expression appeared similar after 7 and 8 weeks of exposure.

Oral Exposure to PQ Leads to GFAP Expression in the ENS of TgM83 Mice

To complete this observation, the response of the glial compartment of the ENS was studied through the level of GFAP expression. The control group revealed no obvious GFAP staining in the jejunum sections whatever the time point considered. After 6 weeks of exposure, in the PQ-exposed group, GFAP level of expression was significantly ($p < 0.001$) increased compared with control group, and was similarly increased after 7 weeks of exposure; after 8 weeks of PQ exposure, GFAP expression was the highest (2.5× compared with the level reached after 6 weeks of exposure) suggesting a progressive reactive gliosis (Fig. 4A, B).

Phosphorylated α-Synuclein Is Mainly Expressed by ENS Neurons

The GFAP and pSer129 α-synuclein immunostainings on successive gut sections (Fig. 5G, H) clearly showed that neuronal cells rather than glial cells express abnormal α-syn.

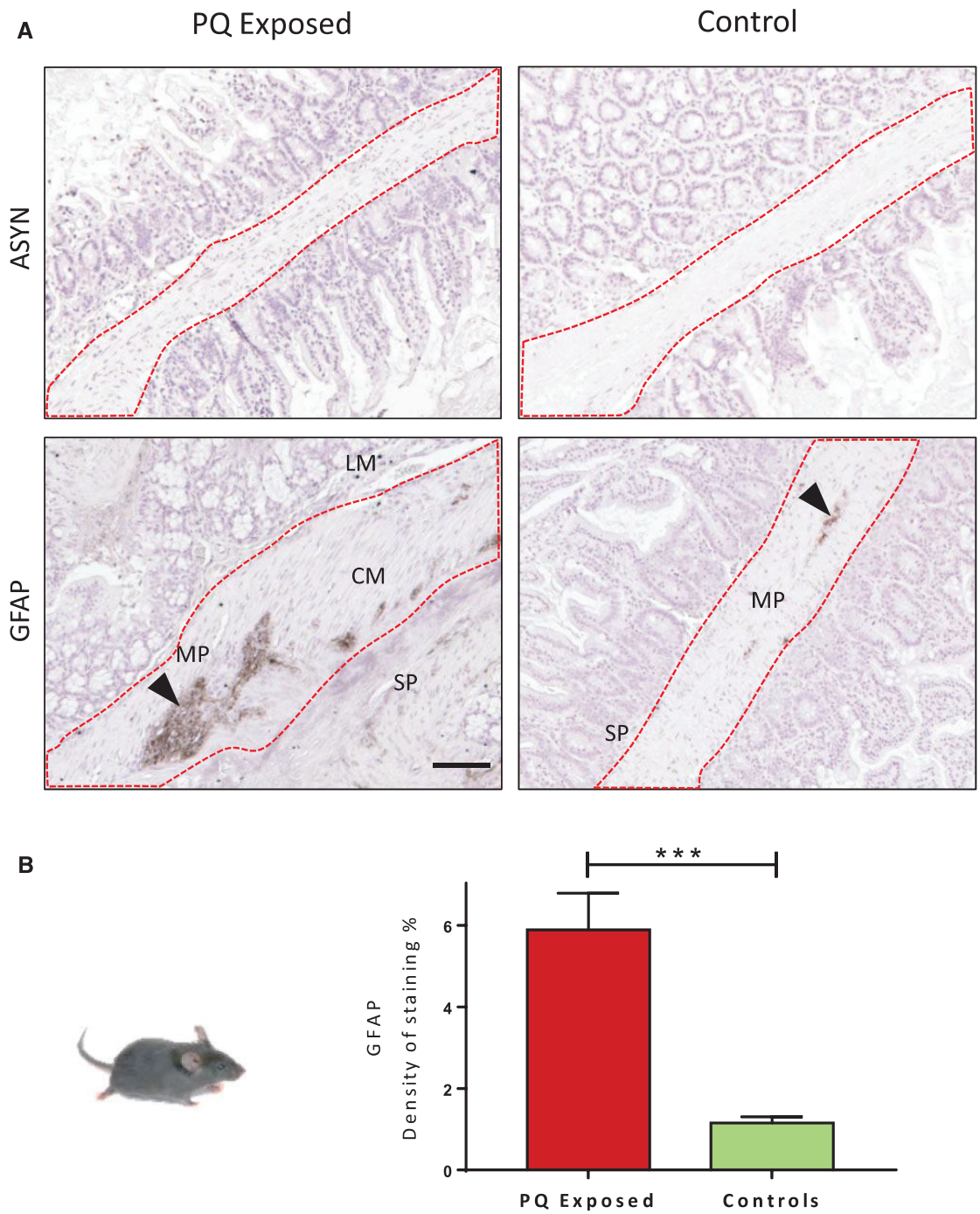

FIGURE 2. pSer129 α-syn IHC analyses in the ENS of wild type control group, C57Bl/6 mice exposed to paraquat (PQ) by drinking water or not (control). **(A)** Absence of pSer129 α-syn in the ENS of wild type mice, whereas a significant increase of GFAP (dark brown staining, arrow) is clearly seen in exposed animals. **(B)** Numeric quantification of GFAP density of staining in PQ-exposed and control mice. Data were analyzed by unpaired *t*-test ****$p < 0.0001$. SP, submucosal plexus; MP, myenteric plexus; CM, circular muscle; LM, longitudinal muscle. Scale bar: 100 μm.

However, a computer-assisted superposition of the labeling obtained on 2 successive slides suggested that some glial cells may accumulate a pathological form of α-syn (Fig. 5 inset G, H). In the PQ-exposed mice, cholinergic and catecholaminergic neurons most likely hosted the pSer129 α-synuclein. Indeed, in terminal stage TgM83 mice, double labeling using a specific antiChAT antibody revealed that cholinergic neurons of the myenteric plexus were accumulating

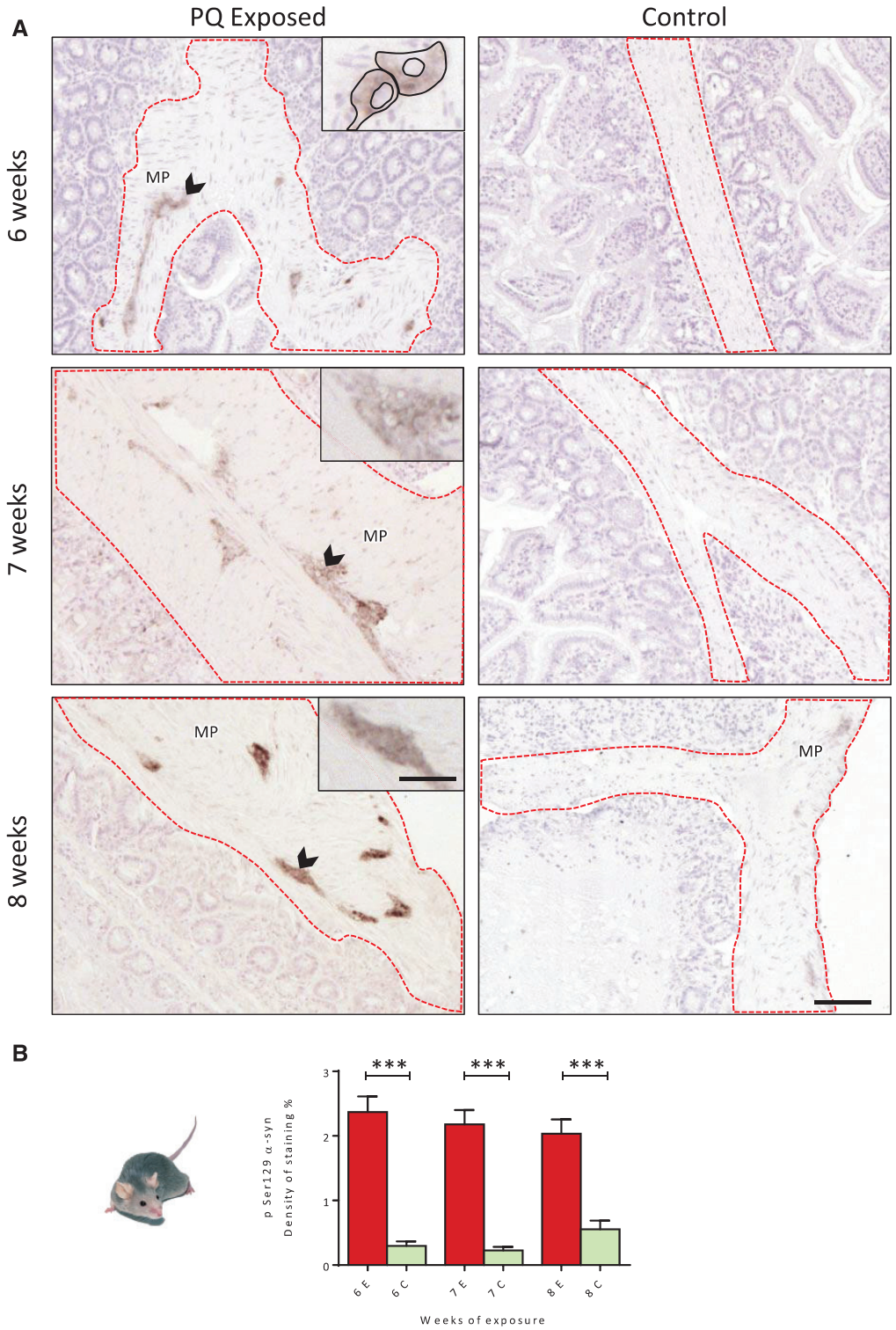

FIGURE 3. (A) Immunohistochemical detection of pSer129 α-syn (dark brown deposits) in the ENS of TgM83 mice exposed to PQ by drinking water up to 8 weeks at 10 mg/kg/day. **(B)** Time-dependent evolution of density of staining of pSer129 α-syn expressed in the ENS. PQ orally delivered was associated with a significant pSer129 α-syn increase as early as 6 weeks after the beginning of exposure. One way ANOVA test was used to analyze this data set; ***$p < 0.001$. E, Exposed; C, Control. Scale bars: Images 100 μm—insets 270 μm.

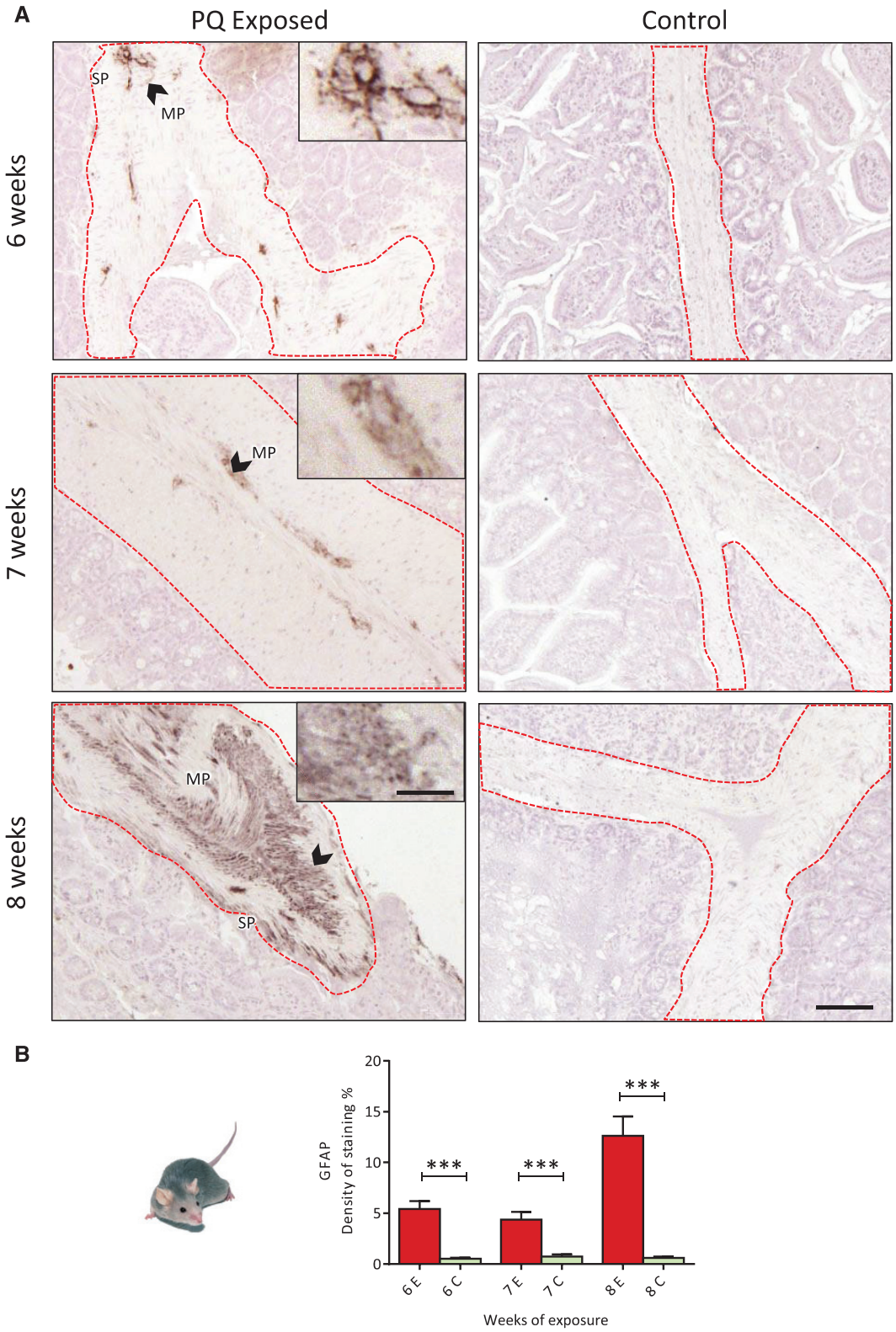

FIGURE 4. (A) Immunohistochemical detection of GFAP (dark brown deposits) in the ENS of TgM83 mice exposed to PQ by drinking water up to 8 weeks at 10 mg/kg/day. **(B)** Time-dependent evolution of density of staining of GFAP expressed in the ENS. PQ orally delivered was associated with a significant increase of GFAP expression in TgM83 mice ENS was observed (up to 5-fold after 8 weeks of exposure). One way ANOVA test was used to analyze this data set; ***$p < 0.001$. E, Exposed; C, Control. Scale bars: Images 100 μm—insets 270 μm.

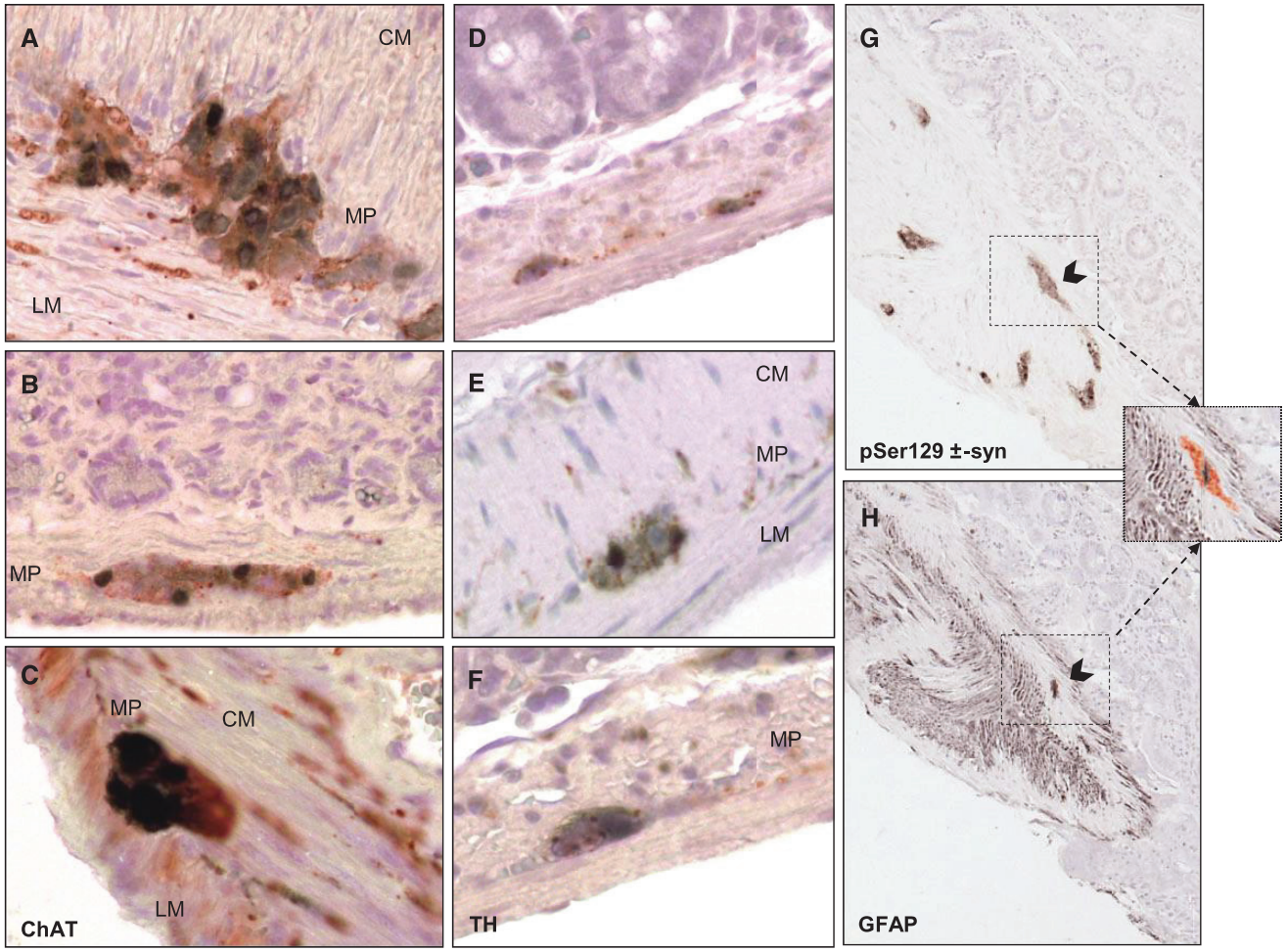

FIGURE 5. Cholinergic and catecholaminergic neurons of the enteric nervous system express phosphorylated α-synuclein. **(A)** Double immunolabeling shows that pSer129 α-synuclein (diffuse staining black-stained areas) colocalized with ChAT-immunoreactive neurons (red-stained areas) in the myenteric plexus. **(B)** Similarly, spheroid-(Lewy-bodies) like inclusions of pSer129 α-synuclein also colocalized with ChAT-immunoreactive neurons. **(C)** At higher magnification, large inclusions of pSer129 α-synuclein are detected within the soma of cholinergic neurons, but less so in their processes. (SP, submucosal plexus; MP, myenteric plexus; CM, circular muscle; LM, longitudinal muscle). **(D–F)** Double labeling shows that pSer129 α-synuclein expression (black-stained areas) in the submucosal plexus can be associated with TH-immunoreactive cells (red-staining) as illustrated in the myenteric plexus **(E, F)**. Phosphorylated α-Syn **(G)** and GFAP **(H)** immunostainings driven on successive gut sections clearly show that neuronal cells rather than glial cells express abnormal α-syn. However, some enteric glial cells may be able to host phosphorylated α-Syn as shown by the computer assisted superposition of these images (inset).

pSer129 α-synuclein within their cell bodies, and apparently less so in their neuronal processes (Fig. 5A–C). Not all aggregates of pSer129 α-synuclein colocalized with ChAT-labeled cell bodies. Although myenteric neurons are not frequently reported to be positively labeled with TH antibody, the possibility that catecholaminergic neurons may also accumulate pSer129 α-synuclein in the ENS was examined. The TH staining was less intense compared with ChAT staining in terms of number of labeled cells; some myenteric as well as submucosal neurons were immunoreactive for TH (Fig. 5D–F), and in the myenteric plexus, double labeling revealed that TH-positive cell bodies hosted pSer129 α-synuclein aggregates (Fig. 5E, F).

Eight-Week Oral Exposure to PQ Does Not Lead to Earlier pSer129 α-Synuclein Expression in the Brain of TgM83 Mice

To investigate the possible impact of PQ on brain expression of phosphorylated α-Syn, the brainstem and other brain regions were carefully analyzed. Compared with a positive control brain, no induction of phosphorylated α-Syn was seen, either in the dorsal nucleus of vagus nerve or elsewhere in the brain (Fig. 6), whatever the conditions studied, suggesting no effect of PQ administration on central expression of pathological α-Syn after 6 weeks of consecutive exposure through drinking water.

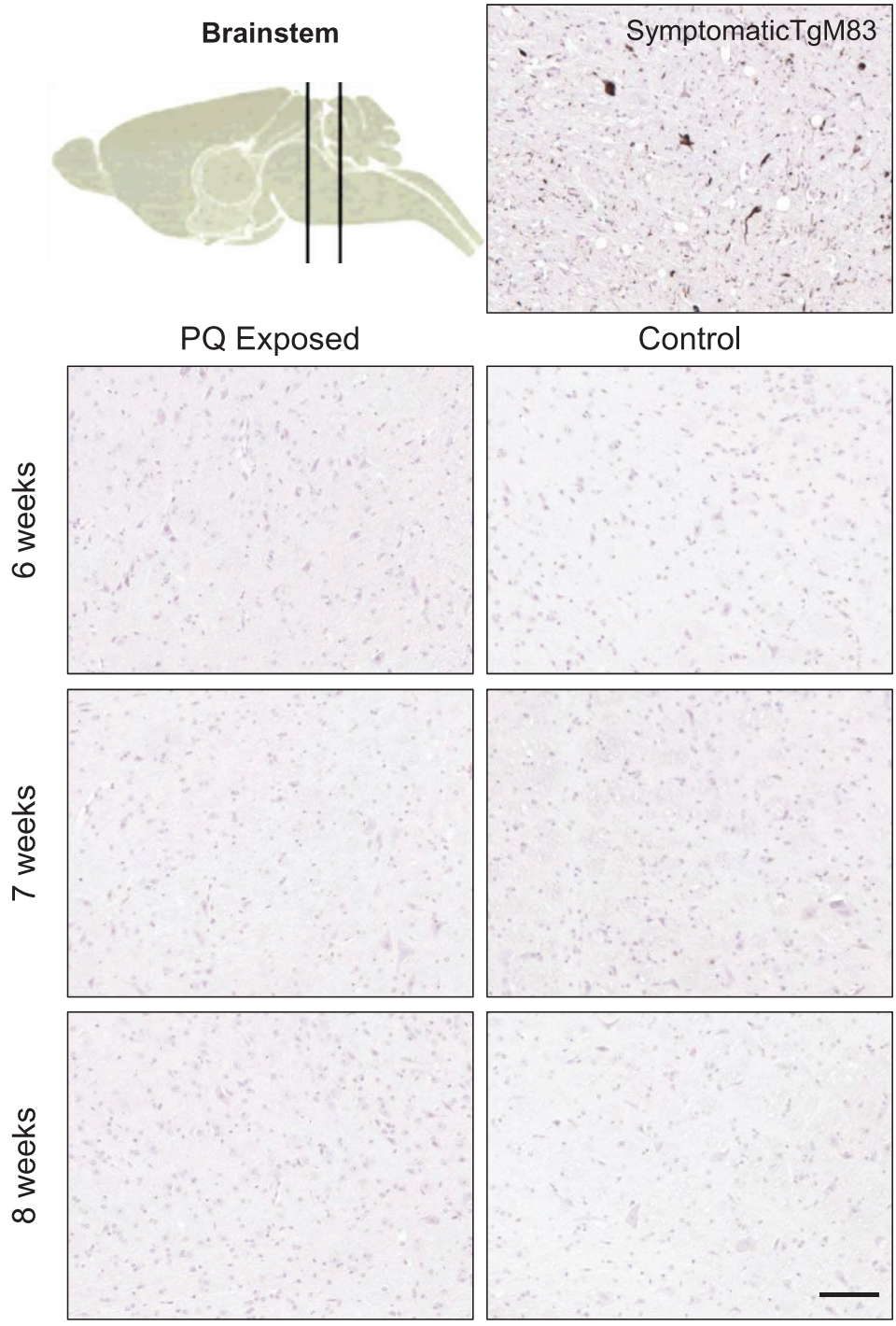

FIGURE 6. Absence of pSer129 α-syn in the brainstem of TgHuA53T mice after 6–8 weeks of oral exposure to PQ. A brain of 1.5-year-old TgHuA53T mouse was used as positive control. At the final stage of the disease, many neurons were accumulating pSer129 α-syn in the brainstem. Scale bar: 25 μm.

DISCUSSION

PQ dichloride, a widely used herbicide all over the world, is one of the pesticides that has been recognized as a possible factor in the pathogenesis of PD (18). Still, its neurotoxicity, far from being completely understood, has been mainly studied experimentally in central nervous system cells and not in peripheral nervous system cells, despite the possibility of interaction of PQ with the peripheral nervous system, notably in the case of oral exposure. The present original in vivo experiment using chronic PQ exposure by oral route

was designed in order to explore the latest hypothesis on early onset of PD in peripheral nervous compartments (29–31). PQ can reproduce some of the features of PD in animals, specifically a loss of dopaminergic neurons and an increase of α-syn aggregation in the brain (32, 33). Also, PQ is known to induce PD-like symptomatology in mice (34) but nothing has been reported on the effects of PQ in the ENS. In addition, the oral route is the less investigated, and as already underlined by Prasad et al in earlier studies, the manner of exposure appears to be critical (23). For the oral route, intragastric administration of PQ at 10 mg/kg/day in wild type mice was reported to be efficient but also triggered some deaths among mice (23, 35). In our experiment, we used the same daily dose, but PQ was delivered chronically using drinking water in order to mimic in a more realistic way what could happen if someone is orally exposed to that kind of environmental xenobiotic. Thus, the present study explored in a relevant way the question of how PQ administrated through drinking water may trigger early adverse effects on the ENS.

Our results show that up to 6 weeks of chronic exposure at 10 mg/kg/day through drinking water, PQ is well tolerated by the C57Bl6 mice, as indicated by absence of loss of weight and of mortality. Rotarod tests show no motor deficiencies suggesting no impact of dietary exposure to PQ on the brain. In the ENS present in the digestive tract, we report the absence of phosphorylated α-syn expression in the wild type mice exposed to PQ after 6 weeks; however, this observation cannot be considered as a proof of absence of neurotoxicity of PQ on the ENS of wild type mice since PQ exposure leads to reactive gliosis, displayed by the significant overexpression of GFAP. Still the duration of exposure appears not long enough to induce any pSer129 α-syn expression. This first observation in wild type mice could not be related to previous studies as there is no other study focusing on the effect of PQ in the ENS. In contrast, the results are different in the transgenic mice: at 8 weeks of chronic exposure at 10 mg/kg/day through drinking water, PQ is well tolerated by the transgenic mice, as indicated by absence of loss of weight and of mortality. Rotarod tests show no motor deficiencies, suggesting there is no effect of dietary exposure to PQ on the brain. In the meantime, the IHC analyses of the intestines revealed remarkable effects on pSer129 α-syn and GFAP expression. Indeed, pSer129 α-syn appeared to be detectable faintly in the ENS of control group and in few animals only. This shows the beginning of the spontaneous accumulation of the phosphorylated protein, observed in this model more clearly from 4 months of age (26). In contrast, in the PQ-exposed group, pSer129 α-syn was clearly detected in >80% of the mice, with strong expression levels, revealing a major impact seen after only 6 weeks of exposure and signifying an acceleration of the pathological process associated to this mouse model. Most probably cholinergic neurons harbor these pSer129 α-syn aggregates in accordance to the double labeling observed in the ENS of transgenic mice showing advanced disease. However, the Lewy bodies or inclusion bodies observed in old symptomatic TgM83 mice were not observed in the younger animals exposed to PQ. It does not mean that Lewy bodies could not be induced by PQ exposure at higher dose or longer time of exposure.

In contrast to these observations in the ENS, the brains of these mice were all devoid of any phosphorylated α-Syn. This indicates that the neurotoxic effect of PQ is expressed earlier in the ENS compared with the CNS and suggests that the dose of PQ and/or duration of the study were not sufficient to trigger an induction of pSer129 α-syn in the CNS compartment too. The difference between ENS and CNS could be explained by the fact that enteric neurons are more directly exposed to the ingested PQ. The intensity of the staining observed at this first experimental time point available suggests that expression of pSer129 α-syn may have possibly started even before. This first observation in TgM83 mice could not be related to previous studies as, to the best of our knowledge, there is no other study focusing on the effect of PQ in the ENS. An article published by Pan-Montojo et al studied the effect of rotenone orally administered to C57Bl/6 mice for 8 or 16 weeks in the ENS (22). Among other results, they showed that rotenone oral exposure by IG route leads to release and accumulation of pSer129 α-syn in the ENS. Therefore, using a different pesticides related to PD, our study adds experimental arguments in favor of PD initiation within this compartment and demonstrates a reason to focus on the ENS along with other toxin exposure. Altogether, our data suggest that the transgenic model could be more sensitive to PQ compared with wild type mice, but at this dose and quite short duration of exposure it is not sufficient to trigger pathological α-syn expression in the brain.

To complete this observation, we also introduced the study of the enteric glial cells. The contrast between control and exposed guts was even more obvious for the glial marker GFAP. The overexpression of GFAP, revealing a reactive gliosis when the phosphorylated α-syn is detectable, suggests a possible cooperation between enteric glial cells and neurons accumulating pathological α-syn. However, the likelihood that some glial cells may directly accumulate a pathological form of α-syn cannot be excluded, as suggested in some cases. This possibility is known and described in the brain, notably in specific forms of human alpha-synucleinopathies, such as multiple system atrophy, characterized by glial cytoplasmic inclusions. Reactive gliosis has been reported in the intestines of patients with inflammatory bowel disease (36, 37). Further data collected in cultured enteric glial cells support the link between enteric glial cells and inflammation; notably, these in vitro experiments report that pro-inflammatory cytokines such as tumor necrosis alpha increase the expression levels of GFAP (38) and that once reactive, enteric glia can secrete interleukin-6 (39). However, because there is not as much knowledge on the involvement of glial cells in the ENS compared with CNS, it is difficult to better interpret the link between the overexpression of GFAP and pathological α-syn expression observed in our model. An enteric glial reaction was suggested to occur in PD patients as shown by an upregulation of GFAP mRNA in colon biopsies (40) and was confirmed by increased GFAP protein levels in both mucosal and submucosal enteric glial cells (41). Indeed, even within the brain, the complexity of the role of astroglial cells in the initiation and progression of PD is far from being understood (42). This could, however, indicate that, to a greater extent, the inflammatory pathway may be activated with the pathological α-syn

presence, supporting the hypothesis that inflammation is involved in α-syn misfolding process. Interestingly, a recent study reported that inflammation may be even more severe in A53T mutant context (43); further studies will be necessary, starting with a more precise following of the kinetic response of both markers pSer129 α-syn and GFAP in the ENS under a xenobiotic stressor. Additionally, a deeper characterization of the pathological form of α-syn, using markers of other forms of α-syn, should be done, and in these longer studies, appearance of inclusion bodies should be combined with analysis of other markers such as ubiquitin and p62.

It is also known that PQ is weakly metabolized by the brain compared with other organs, as shown by local PQ accumulation in different brain areas (23). Therefore, it would be important to assess whether any PQ accumulation would also be possible in the ENS. Indeed, this could probably play an important role in the local response to such a chronic xenobiotic exposure. Thus, an immediate goal of the next study might be the precise quantification of PQ in the ENS compared with the brain. This would allow for exploration of the link between local PQ quantity and pathological α-synuclein accumulation and also to precisely determine the daily doses that could be associated with a deleterious effect on α-synuclein expression in the ENS. In the meantime, our study suggests that new studies need to be performed after longer exposures, resulting in higher cumulated doses to identify possible effects on pSer129 α-synuclein appearance within the brain, studies that should help to better delineate the hypothetical role of the ENS in the initiation of PD after chronic xenobiotic exposure.

Conclusions

Our study demonstrates that orally delivered, PQ is able to induce a neurotoxic response in the ENS of A53T mutant human α-synuclein transgenic mice, as shown by earlier and clear expression of pathological α-syn in this compartment combined to reactive gliosis induction, signifying an acceleration of the pathological process associated to this mouse model. This could be explained by the fact that enteric neurons are more directly exposed to the environmental xenobiotics ingested. Our study parallels Braak's theory in highlighting the ENS as a possible initiation site for α-synucleinopathy-related pathologies such as PD.

Finally, we show that the TgM83 transgenic mouse line may be a sensitive model addressing environmental stressors such as PQ, and is relevant for the study of how oral ingestion of such neurotoxic agents may initiate the accumulation of phosphorylated α-synuclein, the hallmark of PD, in the ENS. By showing an acceleration/exacerbation of the pathology within the ENS, where α-syn is the molecular target, this model should be a valuable tool for studying early events triggered by the other pesticides suspected to be linked to PD.

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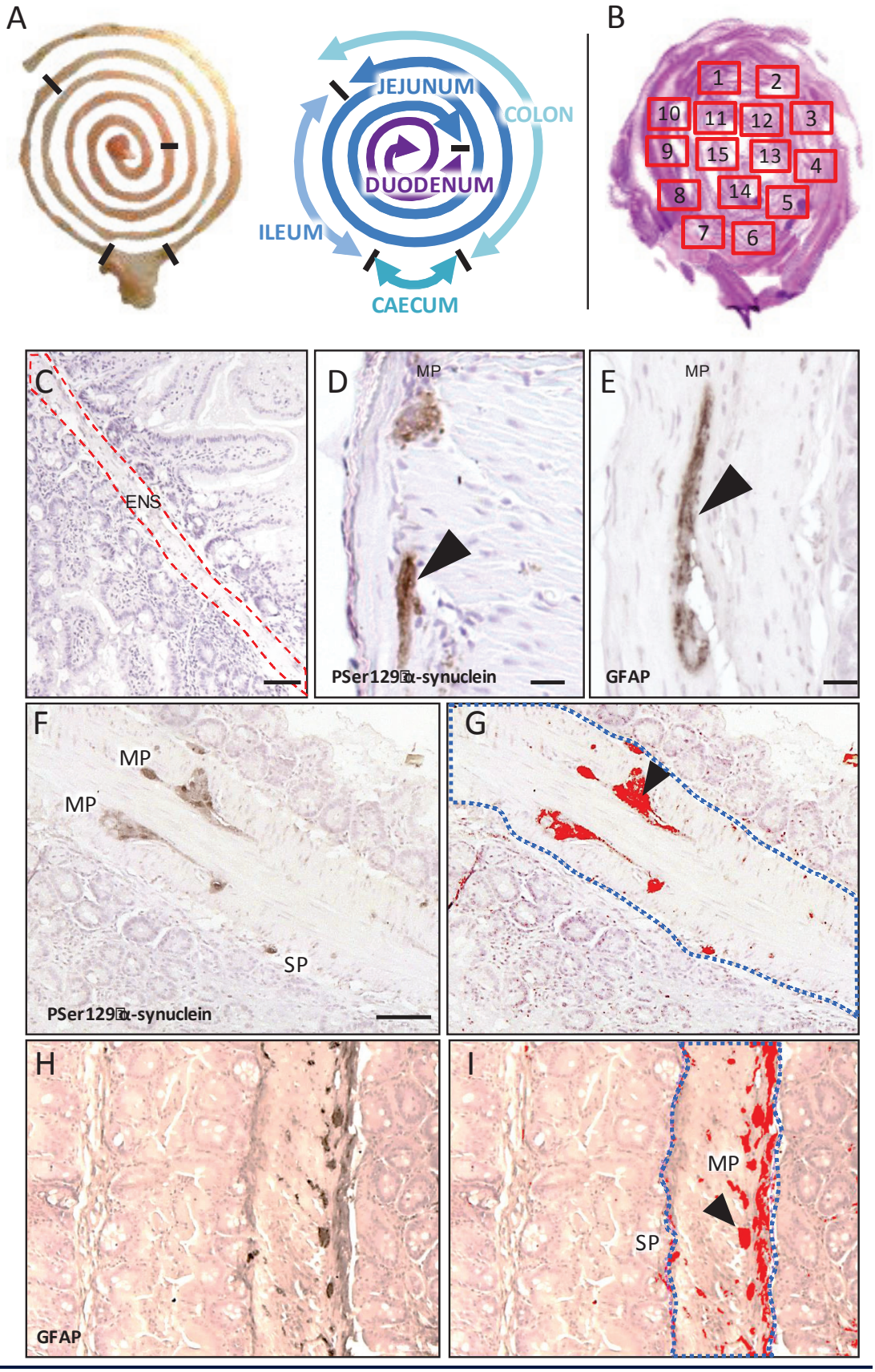
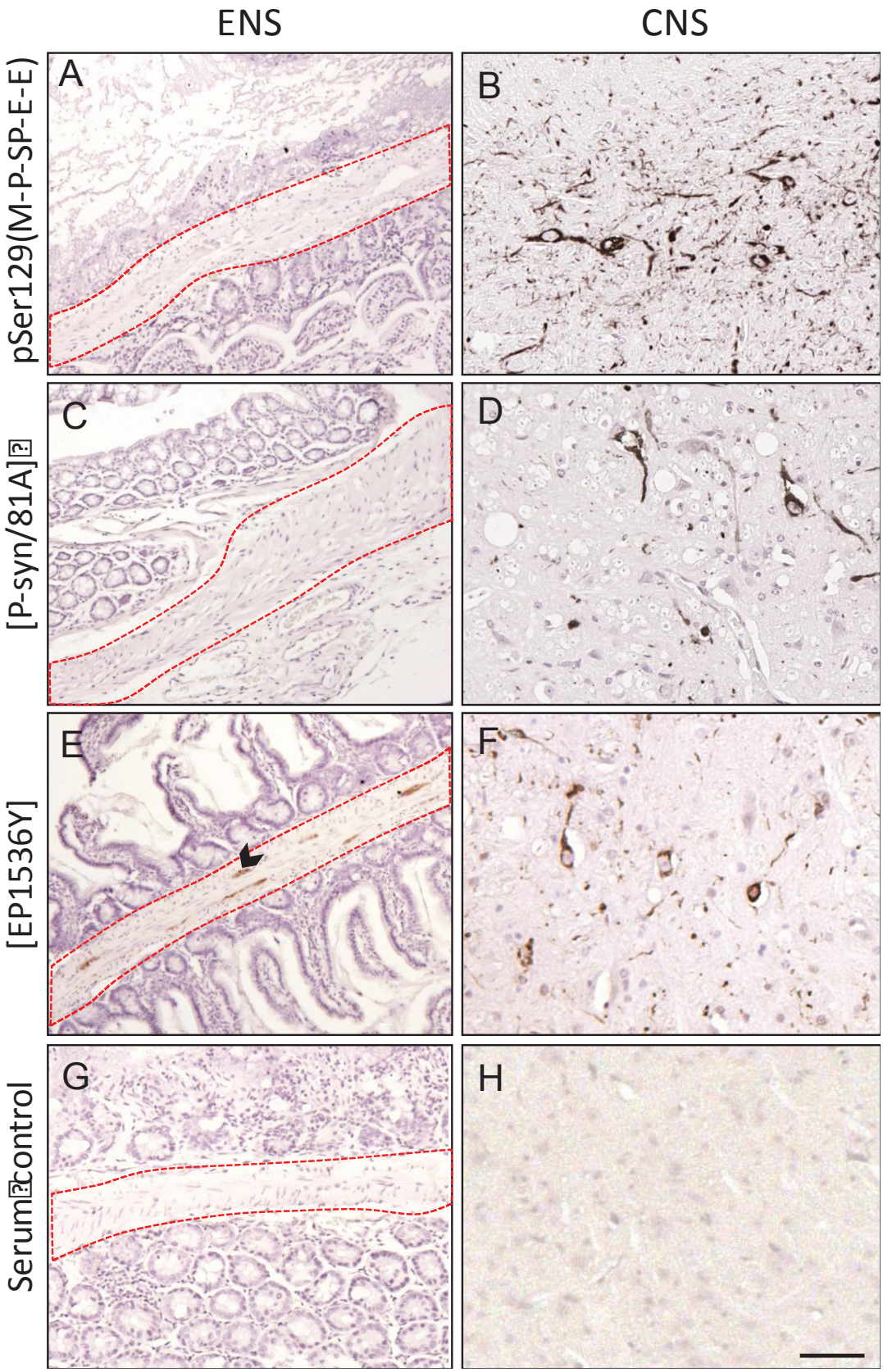
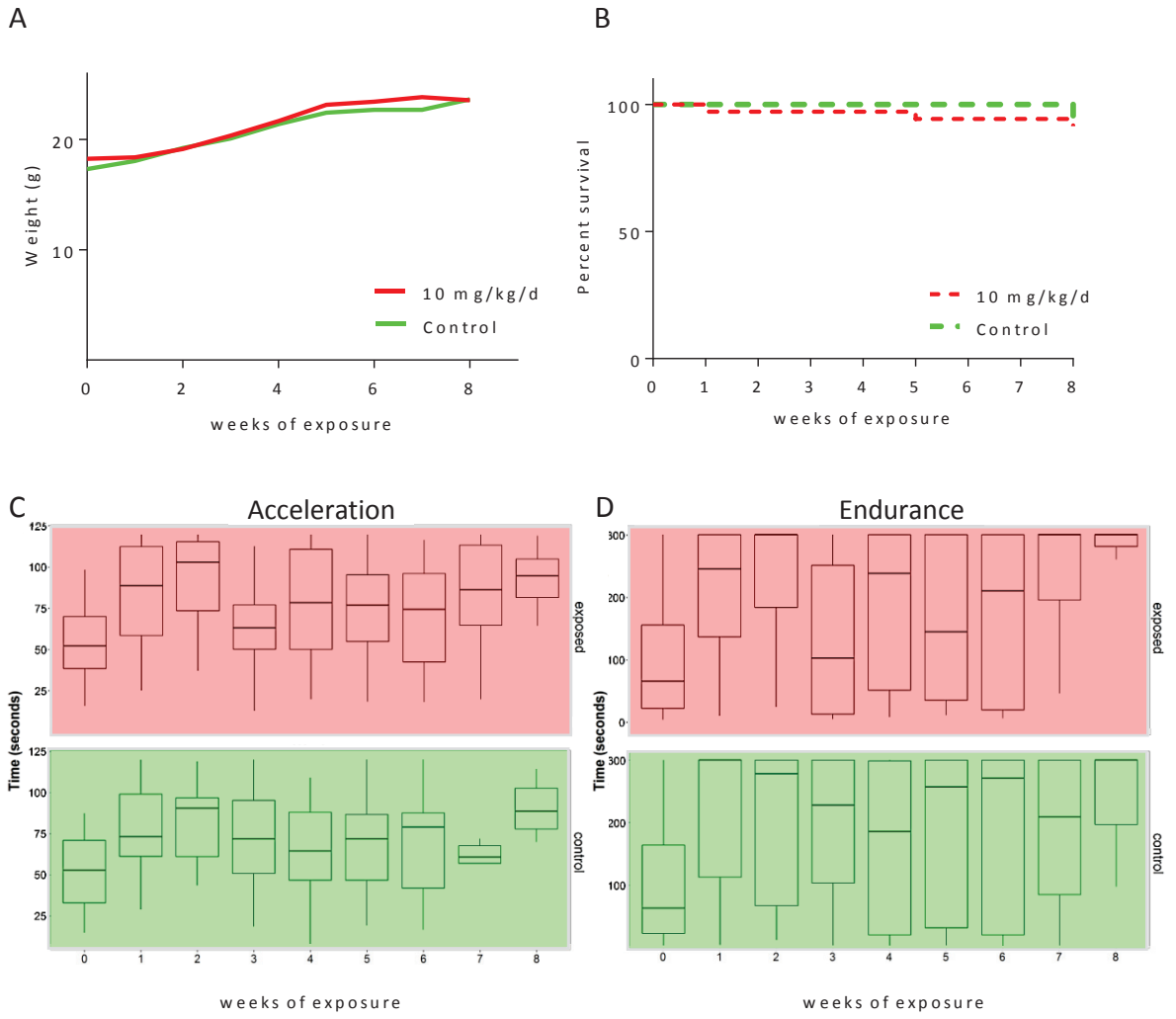
Supplemental Data Figure 1



Supplemental Data Figure 2



Supplemental Data Figure 3



3 Article N°2 : A widespread increase of a-synuclein expression in the brain and intestine of mice chronically exposed to paraquat through drinking water

Nicolas Naudet, Eric Morignat, Jérémy Verchère, Latifa Lakhdar, Dominique Bétemps, Anna Bencsik, Thierry Baron

Résumé des résultats soumis à publication dans le journal Neurotoxicology

Naudet et al., 2017 – soumis le 05 septembre 2017

Après avoir montré l'accélération d'une synucléinopathie lors de l'exposition par voie orale au paraquat par des techniques histologiques dans un premier article publié, les expérimentations suivantes ont porté sur l'étude biochimique de l'α-syn. Les résultats de cette étude sont soumis à publication dans le journal Neurotoxicology.

L'un des premiers obstacles concernant l'étude biochimique des niveaux d'α-syn dans l'intestin a été la mise en place d'une technique d'homogénéisation des échantillons intestinaux. Après plusieurs essais de dissections fines et d'homogénéisation d'intestins de souris à l'aide d'un ribolyseur, un protocole permettant d'obtenir un échantillon fluide et homogène a été définit. Ainsi, les intestins sont nettoyés, séparés en 7 portions comprenant le duodénum, le jéjunum (3 portions), l'iléon, le caecum et le colon, puis broyés à l'aide d'un ribolyseur en appliquant 3 cycles consécutifs de 50 secondes à 6000 tours minutes. Ceci permet d'obtenir une consistance fluide, facilement pipettable.

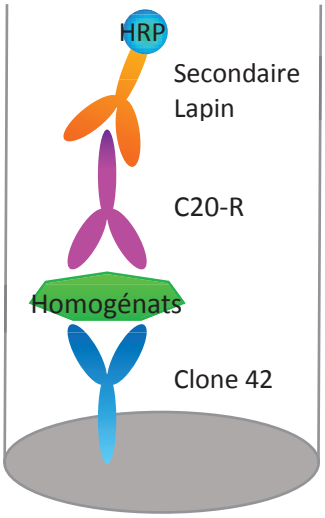


Figure 38 : Représentation schématique du format de test ELISA adapté de Emmanouillidou et al. 2011. Capture de la protéine d'intérêt avec l'anticorps Clone 42 et détection de celle-ci avec l'anticorps C20-R. Détection de ce complexe avec anticorps un secondaire dirigé contre les anticorps lapins, couplé à la peroxydase de Raifort (HRP).

Un format de tests ELISA détectant simultanément les formes d'α-syn non-phosphorylées et phosphorylées a été utilisé dans nos expérimentations (Emmanouilidou *et al.*, 2011). Ce format de test ELISA est décrit comme pouvant être utilisé pour la détection de l'α-syn avec une sensibilité et une spécificité élevée. Dans ce format, la capture l'α-syn se fait avec l'anticorps Clone 42 (BD bioscience). Cet anticorps reconnaît un épitope conservé dans l'α-syn humaine et de rongeur, il est dirigé contre la séquence 91-99 de la protéine et reconnait donc les formes humaines et murines de l'α-syn, mais ne reconnait ni la β-syn, ni la γ-syn. La détection de l'α-syn capturée se fait avec l'anticorps polyclonal C20R (Santa Cruz), dirigé contre un épitope inconnu situé dans la partie C-terminale de la protéine, lui aussi reconnaissant les formes humaines et murines. Ainsi sur la base des anticorps sélectionnés, ce format peut détecter les formes physiologiques et phosphorylées de la protéine, formes de l'α-syn décrites comme « totales » dans la suite de ce document. Il est à noter que ce format ne permet pas de discriminer une souris pathologique d'une souris saine, étant donné son affinité pour toutes les formes d'α-syn cellulaires non tronquées sur leur domaine C-terminal. Aussi, dans des travaux préliminaires à cet article, nous avons noté que, dans les concentrations utilisées pour étudier les échantillons cérébraux et intestinaux de la lignée TgM83 (à savoir 1/5000ème pour le cerveau et 1/2000ème pour l'intestin), le test ELISA présenté ici ne détecte que la forme humaine surexprimée dans cette souris. En effet, aux mêmes concentrations, des échantillons provenant de la lignée C57Bl/6 ne présente

aucune détection de la protéine, cette lignée étant le fond génétique associé à la lignée TgM83.

Le premier objectif de cet article a été d'étudier la distribution physiologique de l'α-syn dans le SNC et dans le SNE des deux lignées murines présentées. Nos portions d'intérêt sont, dans le cerveau, les bulbes olfactifs, le cortex, le striatum, l'hippocampe, le mésencéphale, le cervelet et le tronc cérébral et dans l'intestin, les zones d'intérêt sont le duodénum, les trois portions du jéjunum, l'iléon, le caecum et le colon.

Pour la lignée C57Bl/6, la distribution physiologique de l'α-syn dans le cerveau suit un gradient rostrocaudal, les bulbes olfactifs, le cortex et le striatum ayant une expression relativement égale, l'hippocampe ayant l'expression la plus forte. Les structures à l'arrière du cerveau, à savoir le mésencéphale, le cervelet et le tronc cérébral ayant une expression environ 20 fois plus faibles que les structures rostrales. Les intestins de ces souris présentent aussi une distribution de l'α-syn sous un gradient rostrocaudal. Le duodénum ayant une faible concentration en α-syn, le duodenum relativement plus, l'iléon encore plus forte et le caecum et le colon ayant les plus fortes concentrations en α-syn totale. Pour la lignée TgM83, les niveaux d'α-syn cérébrales présentent une expression relativement homogène, avec une expression plus forte dans l'hippocampe et le cervelet. Dans l'intestin, il est aussi vu un gradient rostrocaudal, mais à une concentration bien plus élevées que dans la lignée C57Bl/6.

Le second objectif de cet article est d'étudier les modulations des niveaux de cette α-syn « totale », en condition de stress xénobiotique induit par un pesticide, lors de l'exposition par voie orale au paraquat. Des souris jeune de 8 semaines d'âge ont ainsi été exposées au paraquat par l'eau de boisson pendant 9 semaines. Les cerveaux et intestins de ces souris ont été récoltés ont été analysés par ELISA, avec le même format utilisé pour étudié la distribution physiologique de l'α-syn, et par western blots, pour prouver la spécificité de chaque anticorps.

D'une façon marquante, l'exposition au paraquat induit une augmentation générale des niveaux de l'α-syn totale dans les deux compartiments nerveux étudiés et pour les deux lignées murines choisies ici. Pour la lignée C57Bl/6, toutes les zones du cerveau

sélectionnées présentent une augmentation significative de l'α-syn. Ces analyses par tests ELISA montrent que l'exposition chronique au paraquat dans l'eau de boisson pendant 9 semaines augmente les taux d'α-syn dans toutes les régions du cerveau examinées chez les souris transgéniques (Figure 2A de l'article n°2) et M83 transgéniques (Figure 3A de l'article n°2). Malgré des concentrations beaucoup plus faibles dans le mésencéphale, le tronc cérébral et le cervelet dans la lignée C57Bl/6, les taux d'α-syn mesurés en utilisant des dilutions d'homogénats cérébraux plus faibles (à savoir 1/50ème) montrent également une augmentation dans ces régions cérébrales pour les souris exposées. Les niveaux d'α-syn montrent également une augmentation au niveau du caecum et du côlon pour les souris sauvage (Figure 2B de l'article n°2) et transgéniques (Figure 3B de l'article n°2). Dans les trois sections de proximales de l'intestin, situées dans l'intestin grêle (duodénum, jéjunum et iléon), les taux d'α-syn ne montrent pas de différences significatives entre les souris exposées et non exposées, ce qui pourrait être lié au très faible niveau d'expression de la protéine dans ces zones pour les deux lignées de souris. Ces résultats ont été analysés en utilisant le test statistique de Mann-Whitney comparant chaque section exposée à la section appariée du groupe non exposé.

Des analyses par western blots ont été menées en parallèles pour prouver la spécificité de notre anticorps de capture et de notre anticorps de détection. Ces tests ont été concluants et montrent la spécificité de chaque anticorps.

Pour aller plus loin dans notre analyse, nous avons transféré ces résultats à un statisticien afin qu'il modélise l'effet du paraquat sur les systèmes nerveux central et entérique par une analyse multifactorielle. Ce type d'analyse permet d'étudier un ensemble d'individus décrits par un ensemble de variables afin de représenter les données par deux graphiques : la représentation dans un plan cartésien sur l'axe séparant le mieux les groupes d'individus et la représentation par cercle de corrélation où les données pointant dans la même direction sont corrélées. Cette analyse permet de consolider l'interprétation des données obtenues par ELISA car elle montre une séparation nette entre les groupes exposés et les témoins. Une analyse de « *clustering* » ans *a priori*, regroupant les individus par « *clusters* » montre que pour les souris C57Bl/6 l'exposition au paraquat par voie orale a un effet clair sur les souris, seule une souris (1/8) exposée se retrouve dans le cluster des souris

témoins. Pour les souris TgM83, deux souris (2/7) exposées se retrouvent dans le cluster des témoins. Ces résultats confirment que le paraquat induit une augmentation généralisée des niveaux d'α-syn tant dans le système nerveux central que dans l'intestin, plus précisément dans le caecum et le colon.

Notre étude donne de nouveaux éléments sur les effets de l'exposition orale et à faible dose au paraquat sur les niveaux α-syn. De surcroît, nous fournissons ici la base d'un modèle expérimental utile et facile à mettre en œuvre pour d'autres études sur les effets des pesticides en relation avec la protéine d'α-syn.

HIGHLIGHTS

- a-Syn is more abundant in the frontal brain regions of C57Bl/6 mice
- In the intestine, much higher levels of a-Syn are found in the large intestine
- Paraquat exposure induces a widespread increase of a-Syn levels in the brain
- Paraquat exposure increases a-Syn levels in the large intestine

A widespread increase of alpha-synuclein expression in the brain and intestine of mice chronically exposed to paraquat through drinking water

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Keywords: alpha synuclein, brain, intestine, Parkinson's disease, Lewy bodies dementia, pesticide

This manuscript has not been submitted elsewhere. All the authors agreed on this version of the manuscript and have contributed to it.

None of the authors have a commercial or other association that might pose a conflict of interest.

This work was supported by ANSES. Nicolas Naudet is supported by a doctoral grant from Région Auvergne-Rhône-Alpes.

ABSTRACT

Alpha-synuclein (a-Syn) is an abundant neuronal protein involved in some neurodegenerative diseases such as Parkinson's disease (PD) but the mechanisms of pathogenesis still remain to be elucidated. It has been shown that even a relatively modest increase of a-Syn levels is sufficient to trigger pathogenic processes of PD, as shown by familial cases of PD caused by duplication or triplication of the SNCA encoding gene. Other genetic abnormalities such as mutations on SNCA gene (A30P, E46K, A53T…) can increase susceptibility and trigger early PD. Here we exposed orally wild-type (C57Bl/6) or transgenic mice (M83) expressing A53T mutated human a-Syn to paraquat (10 mg/kg/day), a well-known herbicide linked to PD risk, to decipher the early molecular events in two nervous compartments. By a-Syn quantitation in the brain and intestine using an ELISA test, our study shows that a 9 weeks exposure to paraquat through drinking water increases a-Syn levels in the whole brain and in the large intestine, in both mouse lines. To go further in the analysis of our results, we set up a multifactorial statistical analysis that also showed a heterogeneous individual response to the pesticide. Our study shows that a chronic oral exposure to low doses of paraquat induces a widespread a-Syn increase, in both central and enteric nervous systems, possibly contributing to explain the increased PD risk reported for this pesticide.

INTRODUCTION

Alpha-synuclein (a-Syn) is a neuronal protein highly expressed at synaptic terminals involved in synaptic activities (Burre *et al.*, 2017). Its involvement in Parkinson's disease (PD) has first been discovered following the identification of cases associated with mutations in the coding sequence of the SNCA gene, leading to amino acid substitutions on the protein such as the A30P, E46K or A53T mutations (Kasten and Klein, 2013; Polymeropoulos *et al.*, 1997). Other rare genetic cases were associated with duplications or triplications of the SNCA gene, leading to constitutive overexpression of the protein in the host; triplication leads to an earlier onset of the clinical disease than duplication, clearly showing the relationship between the a-Syn expression level and PD risk (Chartier-Harlin *et al.*, 2004; Singleton *et al.*, 2003). Some other neurodegenerative diseases are associated to a-Syn accumulation, as in PD. Indeed, intracellular neuronal a-Syn inclusions (called Lewy bodies and Lewy neurites) are also seen in dementia with Lewy bodies (DLB), and glial cytoplasmic inclusions (GCI) in oligodendrocytes are seen in multiple system atrophy (MSA). Most cases of these diseases are considered as sporadic, while hereditary mutations leading to familial cases account for less than 10 percent of total cases of a-synucleinopathies (Klein and Westenberger, 2012).

The etiology of sporadic cases of synucleinopathies remains unknown but seemingly involves a combination of genetic factors and environmental factors. Most data regarding this question have been obtained in PD, certainly in relation with the relatively easy diagnosis of the disease based on its typical motor clinical signs (shaking, rigidity, slowness of movement, and difficulty with walking). This allowed a number of epidemiologic studies that consistently recognized exposure to pesticides as a possible risk factor of the disease (Ascherio and Schwarzschild, 2016; Elbaz *et al.*, 2016; Goldman, 2014). However few studies were able to identify precisely the pesticides that are specifically involved, such as paraquat and

rotenone, which have been related to PD risk in several epidemiological studies (Allen and Levy, 2013; Baltazar *et al.*, 2014; Goldman, 2014).

This led to a large number of experimental studies to decipher the molecular mechanisms that could induce specific neurodegeneration of the nigrostriatal system in wild-type or transgenic mice or in rats following exposure to a neurotoxic compound. Indeed most studies focused on the nigrostriatal system because dopaminergic neurons of the *substantia nigra* are especially vulnerable and heavily affected in PD when typical clinical motor signs occur. However it is now recognized that the disease is much more widespread and can be clinically associated with a number of other clinical signs, more or less specific, sometimes long ago before the recognition of the disease (Klingelhoefer and Reichmann, 2015; Visanji and Marras, 2015). This is notably the case of olfactory deficits and constipation, which have been related to the presence of Lewy bodies and Lewy neurites into the olfactory tract and in the enteric nervous system respectively (Aldecoa *et al.*, 2015; Saito *et al.*, 2016). The Braak's theory postulates that the disease, resulting from a-Syn aggregation spreading, could progress to and throughout the central nervous system from these early lesions, reminding the behavior of prions (Braak *et al.*, 2004; Hawkes *et al.*, 2007; Visanji *et al.*, 2013). Among experimental studies, a series of observations have been recently reported using chronic exposure to rotenone of wild-type C57Bl/6 mice by oral route at low dose. These studies showed that, although rotenone remained undetectable into the brain, nigrostriatal neurodegeneration and motor deficits could be observed after several months of exposure (Pan-Montojo *et al.*, 2010). The a-Syn aggregation observed in the nigrostriatal system was preceded by up-regulation and aggregation of a-Syn into the enteric nervous system from which it could propagate, notably *via* the vagal nerve.

Instead, in our study using oral exposure to paraquat through drinking water, we used the ability of paraquat to easily accumulate into the brain of mice after low doses long-term exposure (Prasad *et al.*, 2009). This could illustrate one of the ways through which the general population may be orally exposed to neurotoxic chemicals, as this can happen for multiple contaminants through the diet (Traore *et al.*, 2016). Our objective here was to examine to what extent the oral exposure to paraquat could modify the a-Syn levels, which we measured using an ELISA test throughout the brain and intestine of mice exposed orally to paraquat. We assessed these effects in C57Bl/6 wild-type mice and in a transgenic mouse model overexpressing a (A53T) mutated form of the a-Syn human protein. We showed that, in both models, 9 weeks of chronic paraquat exposure leads to a widespread increase of a-Syn in both brain and intestine.

METHODS

Animal care

C57Bl/6J mice were purchased from Janvier (Le Genest-Saint-Isle, France) and M83 transgenic mice were produced in Anses Lyon animal facilities. M83 mice (B6;C3H-Tg[SNCA]83Vle/J, RRID:MGI:3603036, The Jackson Laboratory, Bar Harbor, ME, USA) express the human A53T mutated a-Syn protein under the control of the prion promoter (Giasson *et al.*, 2002). C57Bl/6S mice (Envigo, Huntingdon, UK), presenting a chromosomal deletion of a-Syn gene (Specht and Schoepfer, 2001), were used as control to ensure specificity of our western blots and ELISAs. Mean weight of each mouse was about 20 g. Mice were housed in a room with controlled temperature, hygrometry and light (12h/12h light-dark cycle). Food and water were provided *ad libitum*. All experiments were conducted in Anses Lyon approved experimental facilities (No. C69 387 0801) following the EEC Directive 86/609/EEC

and French decree No. 2013-118 and in compliance with a specific experimental protocol agreed by ANSES/ENVA/UPEC ethic committee (referral N°16-059).

Paraquat administration

Mice (C57Bl/6 male, M83 male and female) were randomly distributed into exposed and control groups (n=5 to 7 per group). Paraquat dichloride (Methyl viologen dichloride hydrate #856177-1G, Sigma Aldrich, Saint Louis MI) was administered to mice through drinking water exposure by dissolving the pesticide directly into opaque water bottles, to protect the solution from light. As paraquat is stable in water at least 1 week, solutions were changed once a week (Prasad *et al.*, 2009). Doses of paraquat were calculated given the theoretical daily water intake of mice (1.5 mL/10 g body weight/day, http://web.jhu.edu/animalcare/procedures/mouse.html). Based on an average estimated water consumption of 4 mL/day per mouse, paraquat was added to the water at a final concentration of 50 µg/mL, this corresponds to a daily dose of paraquat of ~ 10 mg/kg/day per mouse. Paraquat exposure by drinking water does not provoke taste aversion as normal intake is observed. Mice were continuously exposed to paraquat, beginning at 8 weeks of age and for 9 consecutive weeks. They were then euthanized by intra-peritoneal injection of 200 µL pentobarbital (54,7mg/mL). Brains and intestines were collected. Intestines were opened and rinsed in a 0,1 M PBS to remove the feces, then were rolled with the top of the digestive tract (duodenum) at the center of the spiral. All tissues were stored at -80°C.

Protein extraction

Brains were dissected, separating the olfactory bulbs, cerebral cortex, hippocampus, striatum, cerebellum, mesencephalon and brainstem as reported previously (Betemps *et al.*, 2015). Intestines were cleaned again by scrapping carefully the remaining feces and separated in 7 sections: duodenum, jejunum (3 parts), ileum, caecum and colon. Each part

was carefully weighted and grinded in a grinding tube (Brains: #077533, Dutscher, Brumath, France, Intestines: #3551197, Biorad, Marnes-la-Coquette, France) by a 2x20 seconds at 5000 rpm for brains and 3x50 seconds at 5000 rpm for intestines using a ribolyser (Precellys 24 homogenizer, Bertin Instruments, France). High salt buffer (50 mM Tris–HCl, pH 7.5, 750 mM NaCl, 5 mM EDTA, 1% phosphatase and protease inhibitor cocktails) was then added to a concentration of 10% mass/volume. All these steps involving biological samples were performed in tubes maintained on ice and using ice cold material.

ELISA

A-Syn levels in each sample were measured using a sandwich ELISA. 96 well plates (MaxiSorp™ Nunc, Thermo Scientific) were coated with 0.125 ng/µl of clone 42 monoclonal antibody (BD Biosciences, ref 610787) in 50 mM Na_2CO_3/$NaHCO_3$ (pH9.6) with 100 µl per well overnight at 4°C. Plates were washed 5 times with 300 µl of phosphate-buffered saline supplemented with 0.05% Tween20 (PBST) using an automatic washer (Hydrospeed, Tecan Life Science, Switzerland). Unspecific binding sites were saturated by adding Superblock T20 PBS blocking buffer (Pierce), shaking for 1 h at 150 rpm at room temperature (RT). Plates were washed again with PBST and tissue homogenates were added to the well (M83 – brain: 1:5000 – intestines: 1:500; C57Bl/6 – brain: 1/500 – intestine: 1:10, unless indicated) and incubated for 2 h with 150 rpm shaking at RT. Exposed and unexposed samples of each brain or intestine portions were analyzed on the same microplates to ensure reliability. Plates were washed again and captured a-Syn was detected by C20-R antibody (Santa Cruz, La Jola, CA) at 1:10000 dilution in PBST + BSA 1% (Gibco, Thermo Fisher, Waltham, MA) for 1 h at 150 rpm at RT. Plates were washed and HRP tagged anti-rabbit antibody (Anti-HRP rabbit Clinisciences ref 4010-05, France) was applied to the wells at 1:2000 dilution in PBST + BSA 1% for 1 h at 150 rpm at RT. Plates were washed and 100 µL of 3,3',5,5'-

tetramethylbenzidine (TMB) reagent (ref T0440, Sigma Aldrich) was added to each well and incubated for 15 min with shaking at 150 rpm. Enzymatic reaction was stopped by adding 100 µL of HCl 1M. Optical densities (ODs) are read at 450 nm by a microplate reader (CLARIOstar, BMG Labtech, Ortenberg, Germany). OD values of blank wells, performed in parallel but without adding homogenates, were subtracted from OD values for each well.

Western Blot

Due to different a-Syn levels on each compartments, various volumes of tissues homogenates were used to performed western blots. For M83 mice, equivalent of 40 µg of each brain parts and equivalent of 200 µg of intestinal tissues were runned on gels. For C57Bl/6 mice, equivalent of 200 µg of brain region and 3mg of intestinal tissues were used for each part studied.

Proteins were separated after heat denaturation (5 min at 100°C) on 12% TGX Stain Free FastCast acrylamide gels (ref. 1610185, Biorad), then blotted onto PVDF membranes (0.45µm Immobilon-P, Millipore). After migration, gels were activated for 1 min on Chemidoc MP apparatus (BioRad) and imaging was done using ImageLab software (BioRad). A-Syn was cross-linked to membranes using 0.4% paraformaldehyde diluted in PBS for 30 minutes and unspecific binding sites were saturated by adding 5% milk for 1 h. A-Syn was detected by either clone 42 (1:2000) monoclonal or C20-R (1:10000) polyclonal antibodies, diluted in PBST overnight at +4°C, gently shaking at 15 RPM. Membranes were then incubated with stabilized goat anti rabbit HRP (Interchim #32460) or anti mouse HRP (#32430) (1:1000), incubated for 1 h at room temperature. HRP was then visualized with chemiluminescent substrate (Supersignal WestDura, ref 34076, Thermo).

Statistical analysis

ODs of all the samples measured by ELISA were analyzed by Mann-Whitney test, comparing the means of three repetitions.

Multiple factor analysis (MFA)

A Multiple Factor Analysis was achieved to summarize information resulting from two sets of data, one set being composed by ELISA measures of a-Syn levels in the seven regions of the brain and the other set composed by ELISA measures of a-Syn levels in the caecum and colon, as too low levels of a-Syn are detected into the small intestine. The principle of this method is to reduce multidimensional data to their principal components, based on the assumption that the studied variables are not independent of each other (Bécue-Bertaut and Pagès, 2008). Each mouse is represented in a space with factorial axes defined by the best linear combination of the active variables, i.e. observed variables. Factorial axes are constructed from active variables whereas the interpretation of results is aided by supplementary variables. The two groups of active variables were the 7 brain measures of a-Syn and the 2 intestine measures of a-Syn in caecum and colon. One supplementary variable was used: the status of each mouse (control or exposed). MFA was performed with the "FactoMineR" R package (R Development Core Team, 2010).

Hierarchical ascendant clustering (HAC)

Clustering of mice was investigated using the Euclidian distance between principle coordinates (Bécue-Bertaut and Pagès, 2008). Principal coordinates were determined in a subspace that ensured good quality representation of the data to limit noise, i.e. the number of factorial axes ensuring 95% of the total variance was considered. HAC was performed on the MFA principal coordinates. The generalized Ward's criterion was used as the aggregation criterion for HAC. It consists in aggregating two clusters in a way that minimizes intra-cluster

variance and maximizes inter-cluster variance. HAC was consolidated by a K-means performed on the centers of the HAC clusters. HAC and K-means were performed respectively with R packages “cluster” and “stats”.

RESULTS

Alpha-synuclein (a-Syn) distribution in the brain and intestine of wild-type and M83 transgenic mice

A-Syn levels were measured by ELISA in the different regions of the brain and intestine, using clone 42 monoclonal antibody to capture total a-Syn and C-20R polyclonal antibody as detection antibody (Figure 2 A-B and 3 A-B). These antibodies are recognizing the 91-96 epitope and C-terminal part of the protein respectively. In the experimental conditions used, only the human a-Syn, which is expressed under the control of the prion promoter, is detected in M83 transgenic mice, as the endogenous levels of murine a-Syn are too low to be detected (data not shown). The a-Syn levels measured in both unexposed and paraquat exposed mice showed large individual variations for each brain and intestine regions examined. The general picture of a-Syn distribution throughout the brain and intestine is also illustrated by western blotting using C-20R or clone 42 detection in Figure 1. We checked that loads of proteins were equivalent among the diverse samples in the different Western blot lanes using stain-free gels (Supplemental figure 1). The specificity of a-Syn recognition in both ELISA and Western blot was confirmed by examination of brain and intestine samples derived from C57Bl/6 mice which do not express murine a-Syn (Specht and Schoepfer, 2001).

In the brain of wild type C57Bl/6, a-Syn is more abundant in the frontal regions including the olfactory bulbs, cerebral cortex, hippocampus and striatum, compared to the

mesencephalon, cerebellum and brainstem. In M83 mice, the a-Syn levels in all brain regions are roughly comparable. In the intestine, a-Syn is much more abundant in the caecum and colon in both mouse models (Figure 1, 2B and 3B), and a-Syn levels are lower in the small intestine, even in M83 transgenic mice.

Oral exposure to paraquat increases a-Syn levels in the brain and in the intestine of both wild-type and transgenic mice

ELISA analyses showed that chronic exposure to paraquat through drinking water during 9 weeks increased a-Syn levels in all the brain regions examined, in both wild-type (Figure 2A) and M83 transgenic (Figure 3A) mice. Despite much lower levels into the mesencephalon, brain stem and cerebellum of C5Bl/6 mice, a-Syn levels, measured at lower dilutions of the brain homogenates (1/50), were also increased in these brain regions of exposed mice (supplemental Figure 2). A-Syn levels also showed an increase in the caecum and colon of both wild-type (Figure 2B) and M83 transgenic (Figure 3B) mice. In the three sections of the small intestine (duodenum, jejunum and ileum), differences between exposed and unexposed mice were not statistically significant, which could be related to the very low a-Syn expression in these sections in both mouse lines. Statistical analysis using Mann-Whitney test comparing each exposed and unexposed section are detailed on supplemental data 3.

To go further in the interpretation of our experiments, we performed a multiple factor analysis (MFA), an approach that may underline correlations and clustering in some groups of individuals described by a set of variables (quantitative and / or qualitative). Figure 2C shows correlation circles in C57Bl/6 mice illustrating the consistent response to paraquat exposure. A-Syn levels in all the different regions of the brain are positively correlated with the first dimension and contribute to the construction of this axis. Measures in the caecum and colon are also correlated. The first and second factorial axes bear 67 and 16.4 % of the

information respectively. For M83 transgenic mice, the correlation circle resulting from MFA, shown in Figure 3C, overall gives the same result than in C57Bl/6 mice, but measures from the different regions of the brain appear more correlated. Measures in the intestine are also highly correlated. The first factorial axis bears 63% of the information and the second factorial axis 22% of the information.

Factorial maps, where each point represents a synthesis of the ELISA measures of a-Syn in each sites for each individual mouse, are shown in Figure 2D and 3D for C57Bl/6 and M83 transgenic mice respectively. In C57Bl/6 mice, all unexposed mice have negative abscises in the first factorial axis, whereas all but one exposed mice have positive abscises. The hierarchical clustering achieved on the coordinates of the mice in the first five factorial spaces thus creates one cluster composed of all the 5 unexposed mice and one exposed mouse, whereas the other cluster contains the 7 remaining exposed mice. M83 transgenic mice are also clearly separated along the first dimension but this clustering also shows 2 of the 7 exposed mice are in the cluster containing the 7 unexposed mice. In both mouse lines, exposed mice appear more disseminated than unexposed mice, which might represent heterogeneous individual response to paraquat exposure.

Overall, our analyses show that, despite some individual variations, a 9 weeks continuous exposure to paraquat through drinking water increases the levels of a-Syn, in both wild-type and M83 transgenic mice, throughout the brain, as well as in the caecum and colon.

DISCUSSION

We set up an experimental study on the effects of chronic oral exposure to paraquat in wild type and transgenic mice. Paraquat is one of the pesticides that have been the most extensively studied in relation with the possible role of pesticides as PD risk factors. This was

historically associated with the identification of the molecular similarities between paraquat and MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a chemical that had been associated with acute Parkinsonism following accidental injections through the use of contaminated recreational drugs. The paraquat effects have essentially been studied experimentally after intra-peritoneal or sub-cutaneous injections in mice or in rats, often combined with exposure to maneb, a fungicide, suggested to increase the risk of developing PD when associated to paraquat. As shown by the past studies of Prasad et al., paraquat readily accumulates into the brain after chronic exposure of mice by oral route, even *via* the drinking water route (Prasad *et al.*, 2009).

This allowed the study of the effects of paraquat exposure on a-Syn protein levels, which is a critical criterion since an increase of a-Syn levels represents a well-established risk of PD, as shown by genetic PD cases with duplications or triplications of the SNCA a-Syn encoding gene. Even a modest life-long increase of a-Syn expression may represent an increased PD risk, as recently shown from genome-wide association studies (Soldner *et al.*, 2016). Our data revealed that paraquat exposure induced a widespread increase of a-Syn levels in all the brain regions examined and in the intestine after 9 weeks of oral exposure through drinking water, in both C57Bl/6 wild-type mice and M83 transgenic mice overexpressing A53T mutated human a-Syn. Such an effect was already reported in some experimental studies after intra-peritoneal challenge of wild-type mice and rats, but these studies generally focused on the *substantia nigra* or midbrain (Cristovao *et al.*, 2012; Manning-Bog *et al.*, 2002) or on the striatum (Wills *et al.*, 2012) and this effect could be transitory after paraquat injections (Manning-Bog *et al.*, 2002). A more widespread a-Syn increase was reported in high-salt fractions of the brain stem and cerebellum, but not of the cerebral cortex and hippocampus, in the M83 transgenic mouse model used in our study, but only

after combined intra-peritoneal repeated injections of old mice to both paraquat and maneb (Norris *et al.*, 2007). This last result was shown by Western blot detection with the LB509 antibody that specifically recognizes human a-Syn. In our ELISA conditions, using the C20-R polyclonal antibody for a-Syn detection, only the human a-Syn is detected during examination of M83 tissues. Thus, this indicates that both human A53T a-Syn in M83 mice and murine a-Syn in C57Bl/6 are similarly up-regulated after paraquat oral exposure.

Our ELISA data were limited to measures of the total a-Syn levels, without considering possible translational modifications of the protein. In fact we could not detect any serine 129 phosphorylation of the protein (data not shown), as this can be identified in M83 mice with the characteristic clinical disease that develops during aging in this model (Betemps *et al.*, 2014; Betemps *et al.*, 2015). This is however not unexpected since mice were only 4 months old at the end of the protocol, while clinical symptoms occur spontaneously in older mice (≥ 8 months) during normal aging (Giasson *et al.*, 2002). Further studies will be required to examine other possible a-Syn changes such as intra-cellular aggregation (Manning-Bog *et al.*, 2003), protease resistance (Fernagut *et al.*, 2007) or nitration (Norris *et al.*, 2007), that have already been described in similar studies of paraquat toxicity, but only after intra-peritoneal injections and using immunohistochemistry.

Although the ELISA approach considered here may not allow detailed studies at the tissular and cellular levels compared to immunohistochemistry, it easily allows precise quantifications of changes in a-Syn levels. Prior to the identification of widespread a-Syn up-regulation following oral exposure to paraquat, it first showed, in unexposed mice, that differences in the a-Syn levels between the different brain regions were less important for the human a-Syn in the M83 transgenic mice than for murine a-Syn in C57Bl/6 mice. In these wild-type mice, murine a-Syn was much less abundant in the caudal parts of the brain

(mesencephalon, brain stem and cerebellum). Comparative analysis of the different sections of the intestine by both ELISA and Western blot also showed much higher a-Syn levels in the caecum and colon than in the small intestine, in both mouse lines. Overall the a-Syn levels were however much lower in the intestine than in the brain.

To complete our statistical analysis, we performed an MFA to further represent the overall effect of paraquat, considering the information on the a-Syn level in the different sites as a whole. A hierarchical clustering of the mice was also performed on the coordinates of the MFA without using the status information (exposed *vs* unexposed). It discriminated rather well the two populations of exposed *versus* unexposed mice, even if 1 out of 8 exposed C57Bl/6 mouse and 2 out of 7 exposed M83 mice appeared within the clusters of unexposed mice. The reason of these three misclassifications may be related to the large individual variations of measured a-Syn levels and possibly to some variations of exposure through drinking water. It could also be related to genuine variations in individual response of animals to the pesticide exposure. Such variable responses have already been observed for example when neuronal death was assessed in mice orally exposed to rotenone at moderate doses (10 mg/kg/day) during 28 days whereas this was no longer seen at higher doses (30 mg/kg/day). This clear separation of the two populations illustrates the effect of paraquat on the a-Syn level in all sites.

From a methodological point of view, MFA analysis illustrated here from this first set of data could be used to further include additional variables that could be measured from the same series of samples. As far as the molecular pathogenesis of synucleinopathies is concerned, it is indeed known that regional variations in processes such as autophagy (Malkus and Ischiropoulos, 2012), oxidative stress and neuroinflammation (Cristovao *et al.*, 2012; Mitra *et*

al., 2011; Norris *et al.*, 2007; Tsika *et al.*, 2010) can play crucial roles in a-Syn aggregation and neurodegeneration.

Besides, the PD-related toxicity of rotenone has been experimentally examined after oral exposure, in wild-type (Arnhold *et al.*, 2016; Hu *et al.*, 2016; Inden *et al.*, 2007; Pan-Montojo *et al.*, 2010; Pan-Montojo *et al.*, 2012; Perez-Pardo *et al.*, 2017; Tasselli *et al.*, 2013) or transgenic mice (George *et al.*, 2010). Most of these studies included examination of the contribution of the enteric nervous system (Arnhold *et al.*, 2016; Pan-Montojo *et al.*, 2010; Pan-Montojo *et al.*, 2012; Perez-Pardo *et al.*, 2017; Tasselli *et al.*, 2013), in relation with the Braak's hypothesis regarding the PD pathogenesis in humans. This is in contrast with studies about paraquat, for which, to the best of our knowledge, the enteric nervous system has never been studied. Although a-Syn is much less abundant in the intestine, we similarly identified an increase of total a-Syn levels in the caecum and colon following paraquat exposure of both wild-type and M83 transgenic mice.

The example of paraquat represents a quite different situation to that of the rotenone since it readily accumulates into the brain after oral exposure (Prasad *et al.*, 2009). Regarding implications of these observations to the knowledge of the etiology of synucleinopathies, it should be emphasized that PD is only a subset of these diseases. The pathogenesis of other synucleinopathies with earlier cortical involvement such as dementia with Lewy bodies is still less clear, as well as their etiology (Galasko, 2017). Moreover, besides synucleinopathies, the contribution of a-Syn changes to human neurodegenerative diseases has also been recently emphasized with mounting evidence that cerebral accumulation of soluble oligomeric a-Syn forms was tightly associated with cognitive impairment in Alzheimer's disease (Larson *et al.*, 2017; Larson *et al.*, 2012). This reinforces the need of further experimental studies about the possible contribution of environmental factors to a-Syn changes, keeping in mind that,

beside PD, the relationship between pesticides exposures and cognitive decline has also been repeatedly reported by epidemiological studies (Baldi *et al.*, 2011; Blanc-Lapierre *et al.*, 2013; Ismail *et al.*, 2012).

Our study gives new insights in the effect of low dose oral exposure to paraquat on a-Syn levels in two mouse lines. Also, we provide here the basis of a useful and easily manageable experimental model for further studies of pesticides effects in relation with the a-Syn protein.

ACKNOWLEDGEMENT

Nicolas Naudet was supported by Région Auvergne Rhône Alpes – ARC 3. Authors would like to thank Emilie Antier, Damien Gaillard and Coralie Pulido for their involvement in the mouse care, and Cyril Curtis for his help with the manuscript.

FIGURE CAPTIONS

Figure 1:

A-Syn distribution in the brain and intestine of C57Bl/6 wild-type and M83 unexposed transgenic mice. a-Syn was detected in 7 regions of the brain and in 5 regions of the intestine, representing the whole organs, with 2 antibodies, C20-R, a C-terminal antibody and clone 42 (C42), recognizing the 91-96 sequence of a-Syn protein. Each frame is positioned at ~18 kDa, where a-Syn appears under a monomeric form. C57Bl/6 brains: 200 µg tissue equivalent per lane and exposed 20 seconds, intestines: 3 mg of tissue equivalent per lane and exposed 1 hour. M83 brains: 40 µg tissue equivalent per lane and exposed 30 seconds, intestines: 200 µg tissue equivalent per lane and exposed 60 seconds. Stain-free gels, used to control that equivalent quantities of proteins were loaded in each lane, are

shown in supplemental figure 1. OB: olfactory bulbs, CTX: cerebral cortex, HIP: hippocampus, STR: striatum, MES: mesencephalon, CBM: cerebellum, BS: brainstem, DUO: duodenum, JEJ: jejunum, ILE: Ileum, CAE: caecum, COL: colon, KO: samples from the whole brain, caecum or colon derived from mice which do not express murine a-Syn.

Figure 2:

Oral exposure to paraquat increases murine a-Syn levels throughout the brain and in the caecum and colon of C57Bl/6 mice. A-B ELISA was performed on brain and intestine homogenates (C57Bl/6 – brain: 1/500 – intestine: 1:10), using clone 42 antibody for capture (1/2000) and C20-R antibody for detection (1/10000), measuring levels of murine a-Syn. Each point represents the mean OD measured from three repetitions for each exposed (red plots) or unexposed (green plots) mouse in each of the brain (A) or intestine (B) regions examined. Purple dots represent the samples that were selected for the Western blot analysis shown in Figure 1. C - Correlation circle of MFA. D – Representation of the hierarchical clustering on the factorial map.

Figure 3:

Oral exposure to paraquat increases human a-Syn levels throughout the brain and in the caecum and colon of M83 transgenic mice. A-B ELISA was performed on brain and intestine homogenates (M83 – brain: 1:5000 – intestines: 1:500), using clone 42 antibody for capture (1/2000) and C20-R antibody for detection (1/10000), measuring levels of human a-Syn. Each point represents the mean OD measured from three repetitions for each exposed (red plots) or unexposed (green plots) mouse in each of the brain (A) or intestine (B) regions examined. Purple dots represent M83 mouse selected for the Western blot analysis shown in Figure 1.

C - Correlation circle of MFA. D – Representation of the hierarchical clustering on the factorial map.

Supplemental figure 1:

Visualization of protein loads in each lane of Western blot shown in figure 1. Proteins were visualized using the BioRad Stain Free technique using 12% gels, a method replacing Ponceau Red staining. Gels were activated by UV for 1 minute and protein auto-fluorescence was detected by Chemidoc apparatus.

Supplemental figure 2:

Oral exposure to paraquat increases murine a-Syn levels throughout the cerebellum, mesencephalon and brain stem of C57Bl/6 mice. ELISA was performed on brain homogenates at 1/50 dilution, using clone 42 antibody for capture (1/2000) and C20-R antibody for detection (1/10000), measuring levels of murine a-Syn. Each point represents the mean OD measured from three repetitions for each exposed (red plots) or unexposed (green plots) mouse in each part of the brain examined.

Supplemental figure 3:

Statistical analyses of a-Syn level between PQ-exposed and control mice using Mann-Whitney test.

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Figure

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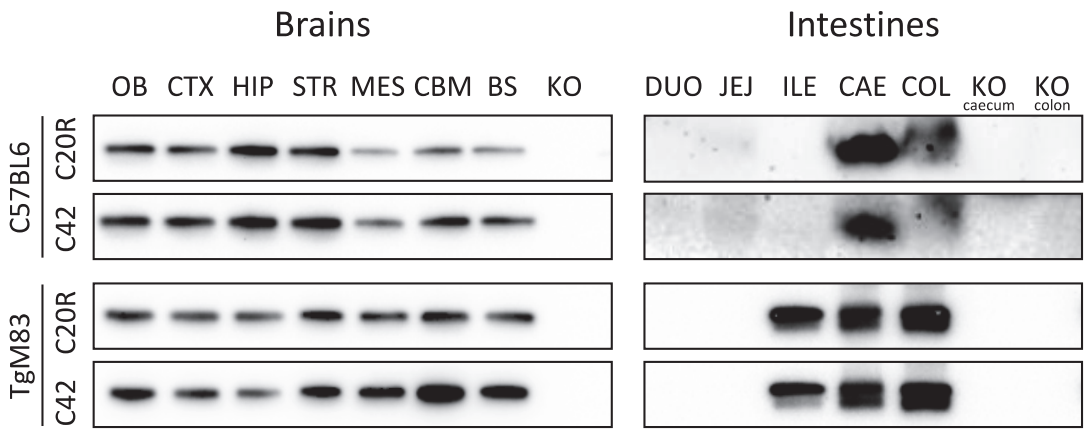
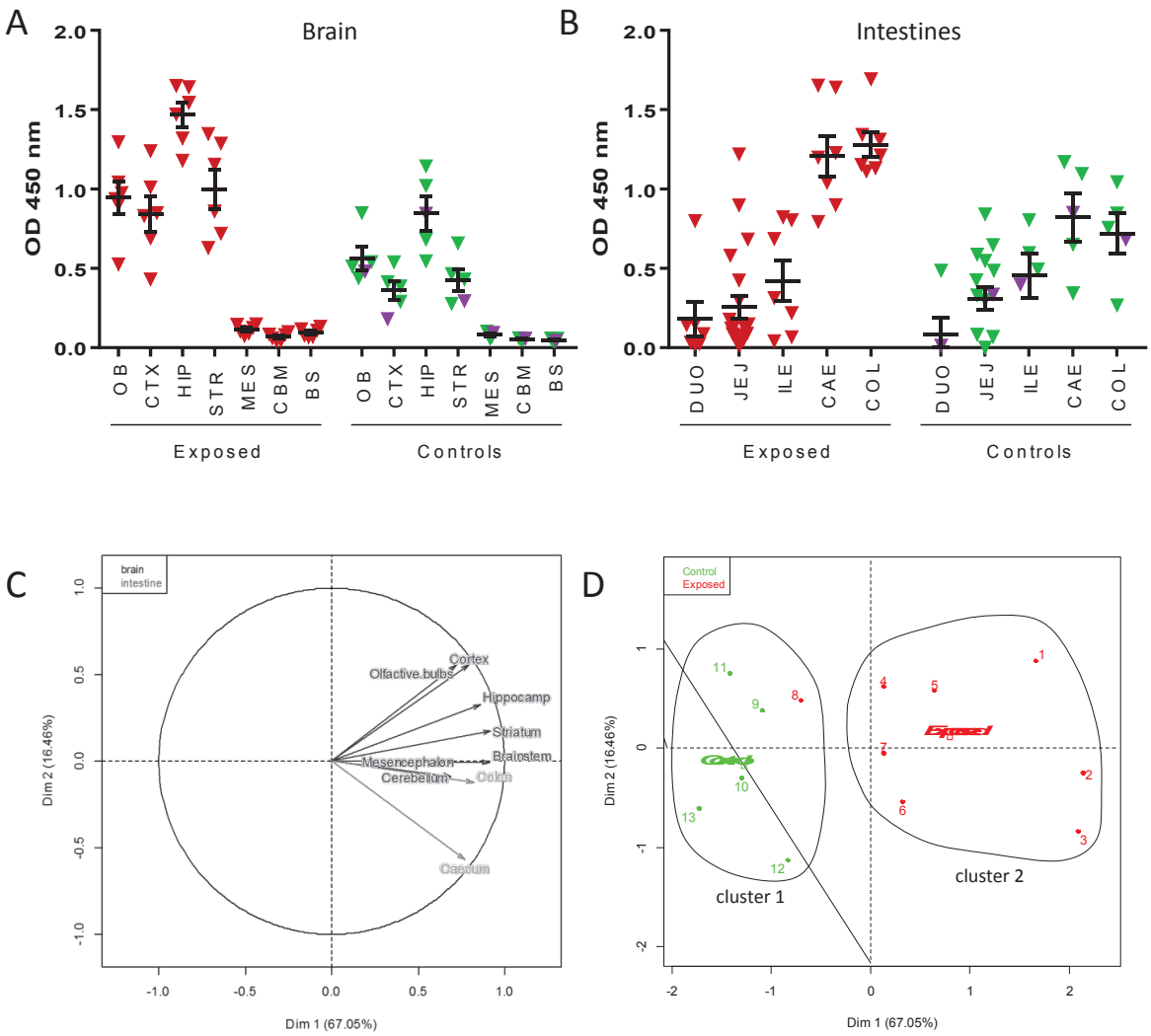


Figure 1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brain
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Cortex
Olfactive.bulbs
Hippocamp
Striatum
Mesencephalon
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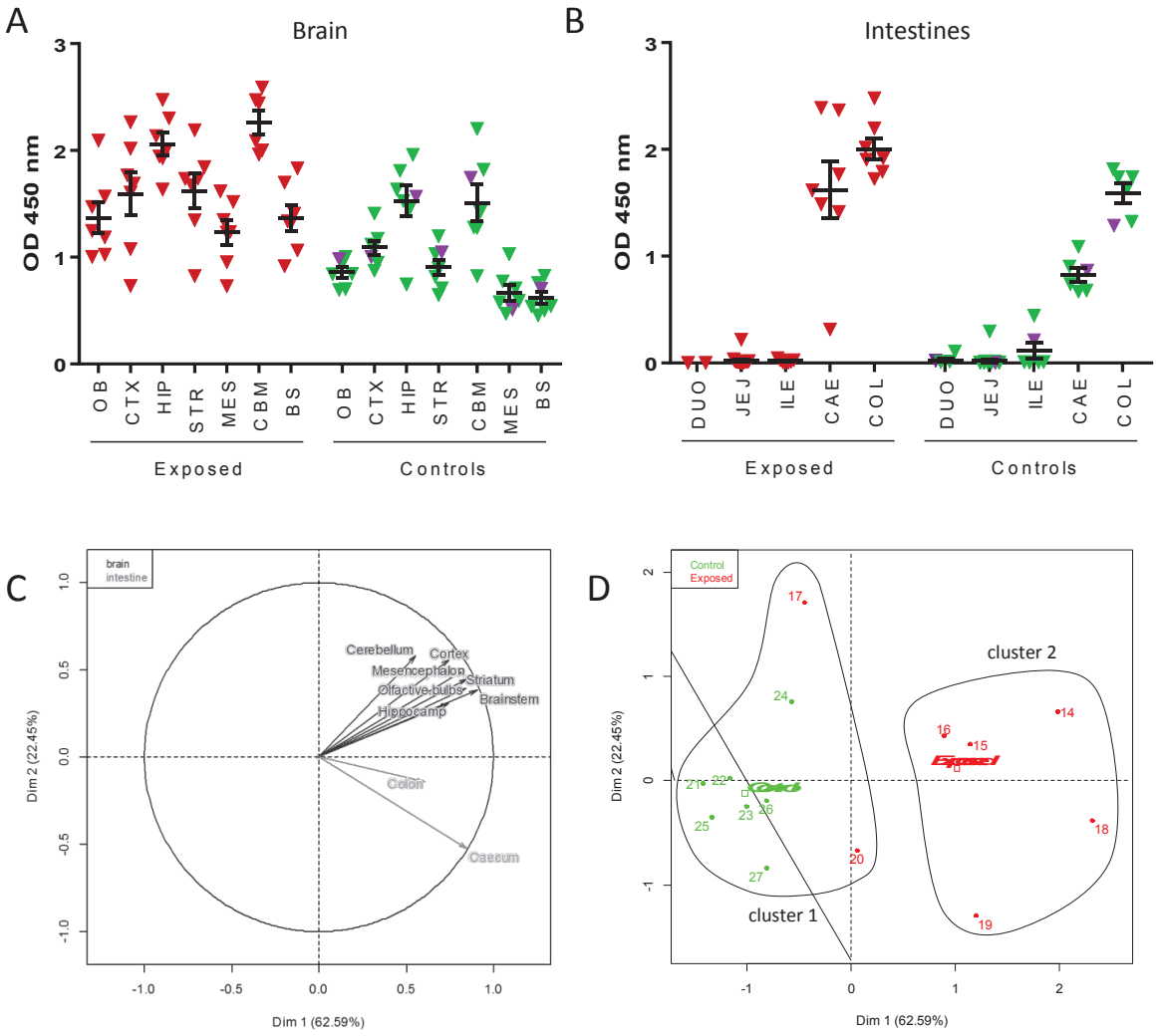
Dim 2 (16.46%)
Dim 1 (67.05%)
D
Control
Exposed
cluster 1
cluster 2

Figure 2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intestine
Cerebellum
Cortex
Mesencephalon
Striatum
Olfactive bulbs
Brainstem
Hippocamp
Colon
Caecum
Dim 1 (62.59%)
Dim 2 (22.45%)
D
Control
Exposed
cluster 1
cluster 2

Figure 3