Preface

During my PhD I had the possibility to work in two different laboratories and be involved in two different topics.

I spent the first period of the PhD at the department of Medical, Surgical and Neurological Sciences in Siena. Here, I investigated genetics variants found in patients with clinical suspicion of leukoencephalopathies and leukodystrophies. In particular, in the context of molecular diagnosis of CADASIL, CARASIL and Alexander's disease, patients DNA was screened for mutations in the genes of interest- NOTCH3, HTRA1 and GFAP respectively.

On the other hand, in Cambridge I had the chance to approach basic research; I gained new practical skills, becoming confident with immunohistochemistry and biochemistry methods, other than acquiring knowledge on Parkinson's disease.

Working in two different fields allowed me to widen my spectrum of knowledge, hence acquiring a more aware working method,

and to carry on basic and clinical applied researches, which was my goals as a PhD student.

Part 1: Genetic analysis of leukoencephalopathies

Background

In the first part of my PhD, I worked in the Neurogenetics laboratory of the Department of Medical, Surgical and Neurological Sciences, in the University of Siena. During this period I studied the genetics basis of leukoencephalopathies and leukodytrophies, particularly, CADASIL, CARASIL and Alexander's disease. Here I report a brief description of CADASIL, CARASIL and Alexander's disease and the genetic studies I carried out.

CADASIL (*MIM* 125310) and CARASIL (*MIM* 600142) belong to the group of familial small vessel diseases (SVDs), a useful model to better understand the pathomechanisms involved in the common sporadic ischemic small vessel disease of the brain (Tikka *et al*, 2014; Di Donato *et al*, 2017). SVDs are known as the main cause of vascular cognitive impairment and stroke in the human adult. They include all pathologies affecting mainly small arteries and arterioles, but also capillaries and veins of the brain. They result from extensive lesions occurring in the cerebral white matter (leukoaraiosis) and multiple lacunar infarcts in both white and deep gray matter. There is no specific treatment for SVDs, mainly due to the poor understanding of their pathogenesis (Di Donato *et al*, 2014; Choi, 2015; Haffner *et al*, 2016).

CADASIL is caused by mutations in the *NOTCH3* gene, identified as the defective gene in 1997 (Joutel *et al*., 1997). CADASIL has been reported worldwide in all ethnic groups, with a prevalence still unknown. In Italy, the minimum prevalence is estimated in 4.1 per 100.000 adult inhabitants (Bianchi *et al,* 2015).

The clinical phenotype is characterized by migraine with aura, recurrent subcortical ischemic events, mood disturbances, apathy and cognitive impairment leading to dementia. The disease has a progressive course and the onset usually ranging from 30 to 60 years, therefore sensibly younger with respect the common sporadic form. Life expectancy is reduced, with death occurring at about 65 years in men and 71 years in women (Chabriat *et al*., 2009). Neuropathological features of the disease include diffuse lesions of the hemispheric white matter, brain stem, and deep brain nuclei, and brain atrophy (Chabriat *et al*., 2009).

Arteriopathy affects the small penetrating cerebral and leptomeningeal arteries. It is characterized by thickening of the arterial wall, leading to lumen stenosis, and by morphological alterations of smooth muscle cells (Chabriat *et al*., 2009).

Pathognomonic of CADASIL is the accumulation of GOM (Granular Osmiophilic Material), within microvessels, vascular smooth muscle cell membranes, and perivascular cells; GOM deposits are probably due to multimerization of the extracellular domain of the protein. However, the mechanisms of NOTCH3 multimerization and GOM deposition are not fully understood (Yamamoto *et al,* 2013). GOM can be detected all along the vasculature throughout the body, although clinical manifestations of CADASIL are almost restricted to the central nervous system.

NOTCH3 gene is predominantly expressed in smooth muscle cells of small arteries, and also in the pericytes of brain capillaries (Chabriat *et al*., 2009). It belongs to the Notch family, which includes evolutionarily conserved transmembrane receptors involved in cell fate specification during the embryonic development. *NOTCH3* encodes a single pass trans-membrane protein composed by a large extracellular domain containing 34 epidermal growth factor-like repeats (EGFRs), a single transmembrane domain and an intracellular domain (Chabriat *et al.,* 2009).
NOTCH3 has 33 exons, mutations in exons 2-24, encoding the EGFRSs, are responsible for CADASIL (Joutel *et al*., 1997; Dotti et al, J Neurol Neurosurg Psychiatry 2005; Chabriat *et al*., 2009). More than 95% of the mutations occurring in *NOTCH3* are missense mutations, the others are splice site mutations, small deletions or insertions. The certain pathogenic missense mutations involve cysteine residues and the pathogenicity of mutations not involving a cysteine residue is still debated (Rutten *et al,* 2014).

The likely pathogenic mechanism of CADASIL is toxic gain of function, related to unpaired cysteine in Notch3-extracellular domain. The aggregation of Notch3 extracellular domain within blood vessel walls leads to degeneration of vascular smooth muscle cells (VSMCs) and a small vessel disease (SVD) type dementia (Yamamoto *et al,* 2011; Tikka *et al,* 2014).

The aggregation/accumulation of Notch3ECD in the brain vessels is likely to promote the abnormal recruitment of functionally important extracellular matrix (ECM) proteins, as TIMP3 and vitronectin. The dysregulation of these ECM proteins could contribute to

mutant Notch3ECD toxicity by impairing extracellular matrix homeostasis in small vessels (Monet-Lepretre et al, Brain 2013). Recently, a dysregulation of the TGF-β pathway, a major regulatory system of vascular function and homeostasis, has been hypothesized in CADASIL development (Kast et al, Acta Neuropathol Commun. 2014).

CARASIL (Cerebral Autosomal Recessive Arteriopathy with Subcortical Infarcts and Leukoencephalopathy) is rare form of inherited SVD, earlier almost exclusively reported in the Asian population, characterized by leukoencephalopathy, lumbago, alopecia and progressive motor dysfunction and dementia. In most cases, and when it is present, alopecia predates the neurological signs (Fukutake *et al,* 1985; Hara *et al*., 2009; Nozaki *et al,* Stroke 2014). Lumbago and alopecia, together with a typical onset between the second and third decade of life, are the main clinical signs differentiating CARASIL from CADASIL. Neuropathological signs include diffuse and focal demyelination and severe arteriosclerotic changes of meningeal and small arteries in the cerebral white matter (Maeda *et al.,* 1976).

The pathology is linked to mutations in *HTRA1* gene, and transmitted as autosomal recessive disease. *HTRA1* encodes a serine protease expressed in the blood vessels, skin, and bone that represses the inhibition of TGF-β family signaling. Dysregulated TGF-β signaling promotes CARASIL pathogenesis, but the underlying molecular mechanisms are incompletely understood (Hara et al 2009; Nozaki et al, 2014; Beaufort et al, PNAS 2014).

Recently, heterozygous HTRA1 mutations have been reported as an important cause of familial small vessel disease (Verdura *et al*, 2015) . The underlying pathogenic mechanism is still unclear, but probably related to an impaired Htra1 activation cascade or to a lower tendency to form trimers (Nozaki *et al*, 2016).

Alexander's disease (MIM 203450) is a genetic leukodystrophy, first described by Dr Stewart Alexander in 1949. Based on the age of onset it is defined as infantile, (<2years), juvenile (2-12 years) and adult (≥ 13 years) disease. The infantile form is the most severe, leading to death by 10 years of age. It is characterized by seizures, megalencephaly and progressive psychomotor retardation (Li *et al*., 2005,1-2). Myelin is markedly rarefied in the frontal lobes. The juvenile and adult forms have slower

course and less severe symptoms. The age-related Alexander's disease phenotypes have been extensively reviewed by Prust M. *et al*. (Neurology 2011,77:1287-94)

The hallmark of the disease are the Rosenthal fibers, cytoplasmic inclusions accumulating in astrocytes. They are composed of protein glial fibrillary acidic protein (GFAP), heat shock protein 27 and αB-crystallin (Johnson & Bettica, 1989; Tomokane *et al*., 1991; Iwaki *et al*., 1989).

Detection of Rosenthal fibers during brain biopsy or autopsy has been the only way to make a diagnosis for long time. The molecular basis of Alexander's disease remained indeed obscure until it was observed that transgenic mice overespressing the *GFAP* developed Rosenthal fibres in great amount (Messing *et al.*, 1998). In 2001, Brenner *et al*. identified missense point mutations in *GFAP* as a genetic base for the disease.

In infantile cases, *GFAP* mutations frequently occur *de novo*, while in the adult form, both sporadic cases with *de novo* mutations and familial cases with autosomal dominant transmission have been described (Pareyson *et al.,* 2008). Furthermore, a study carried out on 28 unrelated cases of Alexander's disease determined that the paternal chromosome carried the *GFAP* mutation in 24 of them, suggesting that most mutations occur during spermatogenesis rather than in the embryo (Li *et al*., 2006).

To date, 111 pathogenic mutations have been reported, most of which are missense mutations in the coding region of *GFAP* gene (most in exons 1,4,6). The left part includes deletions, insertions, frameshift mutations and one intronic mutation.

Some *GFAP* mutations have been associated with all the 3 forms of AxD, hence the issue of genotype-phenotype correlation is a matter of debate (Pareyson *et al.*, 2008).

A genotype-phenotype correlation was described for the 2 most frequently mutated arginine residues, R79 (8 patients) and R239 (10 patients), with the phenotype of the R79 mutations appearing much less severe than that of the R239 mutations (Rodriguez *et al*., 2001). Other genetic or unknown environmental factors might influence the phenotype; this would explain the variability in clinical manifestation and the reduced penetrance of some variants, even detected in asymptomatic subjects (Pareyson *et al.*, 2008), as Asp78Glu and Leu331Pro.

Synthesis of my research experience in the genetic analysis of leukoencephalopathies

Patients with clinical and neuroimaging features suggesting a genetic white matter disorder with still undefined origin have been assigned to two different groups, of either adult vascular leukoencephalopathies or adult onset leukodystrophies. The clinical screening let us select 124 patients with probable vascular leukoencephalopathy and 32 patients with late-onset leukodystrophy.

Following informed consent, total genomic DNA was isolated from peripheral blood leucocytes from the probands using standard procedures. PCR was performed on genomic DNA for exons 1-9 and *GFAP* gene, 1-24 of *NOTCH3* and 1-9 of *HTRA1* and flanking intron regions. Sanger bioinformatics analyses of the identified variant were carried out with five software tools: Polyphen-2 (http://genetics.bwh.harvard.edu.pph), SIFT (http://sift.jcvi.org/www/SIFT_enst_submit.html), Mutation Taster (http://www.mutationtaster.org/), Panther (http://www.pantherdb.org/) and PROVEAN (http://provean.jcvi.org/index.php). Before starting the analysis, a validation step was carried out by checking programs predictions for pathogenicity of known mutations and polymorphisms collected from both literature and databases.

In the *Notch3* negative patients of the first group, we screened for *HTRA1* mutations. We found the first compound heterozygous case of CARASIL. The patient, recently reported in the 2014 issue of Neurology (see below the PDF file, PDF-a) was a 29-year-old Romanian woman with a previous diagnosis of multiple sclerosis. Her neurologically normal parents, showed mild white matter signal changes at brain imaging. This interesting and still unexplored finding let us to suggest that the carrier condition in CARASIL may be associated with subclinical abnormalities.

In the group of undefined leukodystrophies, patients were screened for *GFAP* mutations causing Alexander disease. In particular, we found a 46 year-old female patient with progressive walking imbalance and episodic falls with onset 3 years before and unremarkable family history carrying a novel missense variant in exon 8, very likely to be pathogenic. The case is discussed in detail in the paper enclosed in PDF file (PDF-b).

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Part2: Characterization of a new transgenic mouse model of spreading of α-synucleinopathy from intestine to brain

Abstract

Parkinson's disease (PD) is the second most common neurodegenerative disorder, characterized by motor symptoms which include rigidity, bradykinesia, postural instability, gait dysfunction and tremor.

The pathological hallmark of the disease is represented by Lewy bodies (LBs) and Lewy neurites (LNs), cytoplasmic inclusions found in many areas of the central, peripheral and enteric nervous system of PD patients, and mainly composed of α-synuclein.

According to the neuropathological studies, PD may begin in the nerve cells of enteric nervous system many years before the onset of motor symptoms and would then spread to the brain by cell to cell propagation of misfolded α-synuclein, following a stereotypic anatomic pathway.

The characteristic motor symptoms of PD only appear when the pathology reaches the substatia nigra pars compacta (SNPc).

The present study shows the analysis of a newly-generated mouse model of intestinal α-synucleinopathy, where truncated α-synuclein (α-Syn (1-120)) is expressed under the control of the murine villin promoter. By biochemistry and immunohistochemistry experiments, it was shown that in αSyn (1-120) mice, the transgenic protein is expressed in intestine only and is transferred to the myenteric plexus. αSyn (1-120) mice were crossed with two full-length α-synuclein expressing lines. The transgenic protein induced aggregation of full-length protein, at least in one of these line, which makes these mice a promising model of spreading.

Introduction

Pathogenesis and clinical features

Parkinson's disease (PD) is the second most common neurodegenerative disorder. It is characterized by rigidity, bradykinesia, postural instability, gait dysfunction and tremor. In addition, a wide spectrum of non-motor symptoms are present including psychiatric problems, dementia, cognitive and sensory dysfunctions, autonomic symptoms, and rapid eye movement sleep behaviour disorder (RBD). Dysosmia, RBD and constipation, frequently appear many years before the onset of motor symptoms.

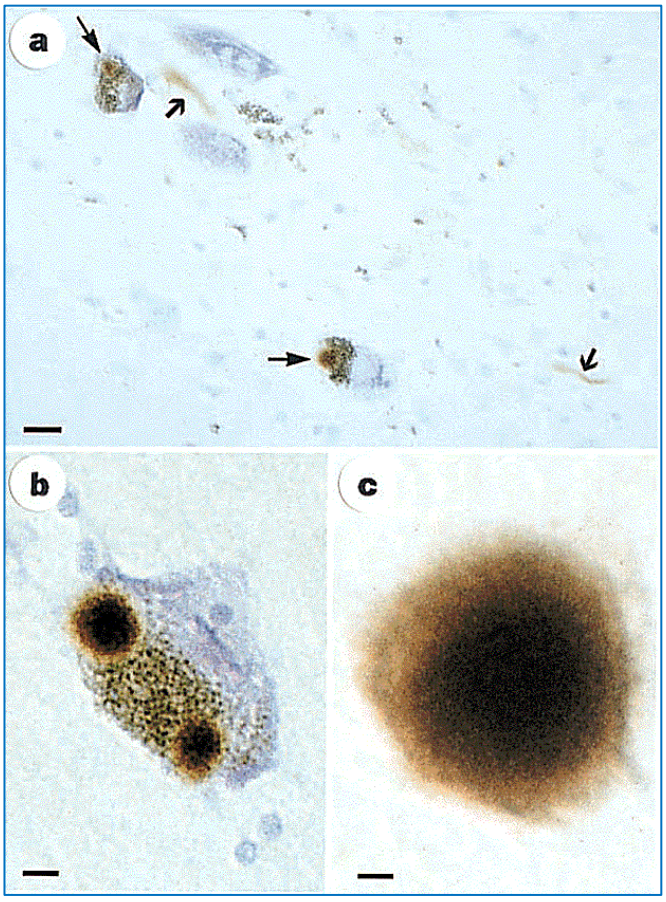
The motor clinical signs appear later as a consequence of a progressive dysfunction of the nigrostriatal dopaminergic pathway and loss of dopaminergic neurons in the SNPc (Crossman *et al*., 1989; Albin *et al*., 1989; Litvan *et al*., 2003). Though, non-dopamine neurons in the upper and lower brainstem are also affected in PD, as well as olfactory system, cerebral hemisphere, spinal cord, and autonomic nervous system.

The pathological hallmark of PD is represented by cytoplasmic inclusions known as Lewy bodies (LBs) and Lewy neurites (LNs). LBs usually present as spherical or reniform, acidophilic inclusion bodies with smooth surfaces, varying in shape and size. At times, ill-defined and weakly immunopositive "pale bodies" (Dale *et al*., 1993; Gibb *et al*., 1991; Irizarry *et al*., 1998) appear between the pigment deposit and the cell nucleus or adjacent to a LB. Thick LNs are club- or corkscrew-shaped. Others are short and stubby or long and thread-like. Both LBs and LNs can be found in many areas of the central nervous system (CNS), and peripheral nervous system e.g. in celiac ganglia and in enteric nervous system of PD patients (Zarow *et al*., 2003; Wakabayashi *et al*., 1990; Forno, 1996; Braak *et al*., 2006). The severity of PD-related pathology varies among cases and ranges from a single LN in the dorsal motor nucleus to extremely high densities of inclusion bodies at multiple sites, including the cerebral cortex, and peripheral nervous system (Zarow et al., 2003; Wakabayashi et al., 1990; Forno, 1996; Braak et al., 2006)

The most mildly affected cases usually display lesions in the dorsal motor nucleus or intermediate reticular zone and frequently, in the anterior olfactory

nucleus (Pearce *et al*., 1995). The brain stem lesions in mildly involved cases consistently remain confined to the medulla oblongata and pontine tegmentum. Cases with moderate involvement exhibit additional lesions in mesencephalic and prosencephalic nuclei, and all cases with severe pathology, most of them with clinically diagnosed PD, show additional deterioration of the neocortex. Dorsal motor nucleus of the vagus (DMV), intermediolateral nucleus in the spinal cord, locus coeruleus, olfactory bulb, and peripheral nervous system are the mainly affected regions (Zarow *et al*., 2003; Forno, 1996; Braak *et al*., 2006).

Different neuronal populations show different susceptibility to develop LNs/LBs (Braak *et al*, 2003).



PD was first described in 1817 by James Parkinson, whose name was later adopted by Charcot for the pathology. In 1912, Friedrich Lewy described the inclusion bodies, and the loss of dopamine as a core pathological event in PD was first described with successful treatment of patients with L-dopa in the ’60s.

α-synuclein and its major role in PD

Two decades ago, in 1997 it was revealed that a small 140 amino-acid protein, α-synuclein, constitutes the main structural component of LBs and LNs (Spillantini *et al*., 1997; Duda *et al*., 2000), (Fig. 1). A crucial role for α-synuclein in PD etiology was confirmed by the observation that inherited PD can arise following missense mutations (Polymeropoulos *et al*., 1997; Kruger *et al*., 1998; Zarranz *et al*., 2004) as well as duplication and triplication in the α-synuclein gene (Fuchs *et al*., 2007, Singleton *et al*., 2003). α-synuclein is encoded by the *SNCA* gene, located on the long arm of chromosome 4 at position 22.1 (4q22.1). It is a 140-aa protein that is natively unfolded, however it may adopt an α-helix-rich conformation when bound to negatively charged lipids, such as phospholipids present in cellular membranes (Ferreon *et al*., 2009). The protein is composed of three distinct regions: an amino terminus (residues 1–60), containing lipid-binding motifs, which are predicted to form amphiphilic helices conferring the propensity to form α-helical structures on membrane binding; a central hydrophobic region (61–95), called NAC (non-amyloid β component), which confers the β-sheet potential, and a carboxyl terminus, highly negatively charged and prone to be unstructured (Maroteaux&Scheller, 1991; Ueda *et al*., 1993; Maroteaux *et al.*, 1988). α-synuclein is a member of the synuclein family of proteins, which also include β- and γ-synuclein.

In the physiological conditions α-synuclein is located predominantely in presynaptic terminals (Takahashi & Wakabayashi, 2001), though sometimes it is contained at high levels in cell bodies, which is the case of DMV neurons. Small amounts of α-synuclein are contained in cellular cytoplasmic vesicles and can be released from neuronal cells (Lee *et al*., 2005). α-synuclein can be found in body fluids like plasma, cerebrospinal fluid (CSF) and brain interstitial fluid. In particular, α-synuclein in CSF and brain interstitial fluid seems to derive from CNS neurons, while sources of plasma α-synuclein are unclear (Mollenhauer *et al.*, 2012).

During normal ageing, α-synuclein starts accumulating as a nonpathological phenomenon in the substantia nigra, but not in other dopamine neuronal nuclei such as the ventral tegmental area (Baba *et al*., 1998).

Modifications of α-synuclein associated with its pathology

Mutations

Five α-synuclein missense mutations (A30P, E46K, H50Q, G51D and A53T) have been described as causes of familial PD and certain single-nucleotide polymorphisms in the gene coding for α-synuclein are associated with increased PD risk (Siddiqui *et al.,* 2016; Maraganore *et al*., 2005). Additionally, duplications and triplications of the wild-type protein were reported to be associated with familial forms of PD (Chartier-Harlin *et al.,* 2004; Singleton *et al.,* 2003). This finding suggests that increased production of wild-type α-synuclein is sufficient for the development of PD. Mutations in other genes, including *Parkin*, *LRRK2, PINK1* also contribute to the development of familial forms of PD (Paisan-Ruiz *et al*., 2004; Kitada *et al*., 1998; Polymeropoulos *et al*., 1997), however most of the cases are sporadic and due to unclear aetiologies.

Phosphorylation

Phosphorylation at serine 129 (pS129) is also associated with α-synuclein pathology. It has been reported to alter α-synuclein propensity for aggregation (Fujiwara *et al.,* 2002; Chen&Feany, 2005; Gorbatyuk *et al*., 2008) and toxicity (Kragh *et al.,* 2009). However, considerable controversy exists over the effects of pS129 on neurodegeneration in PD, and studies on different animal models showed discordant results, suggesting that the effects of pS129 may be highly model-dependent (Stewart *et al*., 2015).

Truncation

It has been reported that LB extracts are enriched in C-terminally truncated α-synuclein (Baba *et al*., 1998; Tofaris *et al*., 2003) and that human α-synuclein lacking the last C-terminal 20 amino acids (α-Syn120) assembles into filaments *in vitro* faster than either wild-type or mutant protein (Crowther *et al.,* 1998; Serpell *et al*., 2000; Murray *et al*., 2003). By generating transgenic mice expressing human α-Syn120 under the rat tyrosine hydroxylase (TH) promoter, Tofaris *et al*., (2006) showed that such animal models reproduced many typical features of human α-synucleinopathy. It is currently unclear how truncated α-synuclein can be generated in cells under pathological conditions, however it has been suggested that it may be a proteasome mediated mechanism (Tofaris *et al*., 2001; Liu *et al*., 2003) and occur after assembly,

since isolated α-synuclein filaments from human brain are made of the full-length protein (Spillantini *et al*., 1998; Takao *et al*., 2004). Tofaris *et al*. (2006) suggested that C-terminal region of α-synuclein is a negative regulator of self-assembly and that different post translational modifications such as oxidation, nitration, and phosphorylation may act on the propensity of α-synuclein to aggregate *in vivo* in a way similar to truncation.

The hypothesis of prion-like spreading of α-synuclein pathology in PD.

Staging of PD

Investigating the connection between α-synuclein aggregates and the development of PD, Braak *et al*. suggested that in post mortem brain tissue from idiopathic PD patients, Lewy pathology appears in a stereotyped pattern based on how advanced the disease is. They suggested that α-synuclein pathology first appears in the peripheral nervous system and olfactory bulb, ascends towards the brainstem and into the midbrain and then eventually spreads to the forebrain. The disease assumes an essentially upward course and eventually extends into the cerebral cortex. They described the progressive development of PD in six stages.

At stage 1, PD-related inclusion bodies are only found within the neurons of the dorsal motor nucleus and, in some instances, in neurons of the intermediate reticular zone. Typically, the first observable changes are LNs. In advanced cases, LNs initially outnumber the small and inconspicuous LBs.

At stage 2, a more accentuated affection of both the dorsal motor nucleus and the intermediate reticular zone is observed. The key feature at this stage, is the first appearance of LNs and LBs in neurons of caudal raphe nuclei and reticular formation; the gigantocellular reticular nucleus is the most exposed to the pathology developing within the reticular formation (Braak *et al*., 2000). LNs precede again the appearance of LBs.

The principal characteristics of stage 3 are the affection of a subset of melano-neurons in the substantia nigra and the involvement of lipofuscin-laden neurons in the nuclei of the basal forebrain. At this stage, there is no indication of macroscopically

detectable depigmentation of the substantia nigra (Braak&Braak, 1999). The high number of LNs within nigral subnuclei precede the development of LBs in melano-neurons, whereas non-melanized nerve cells within the area of destruction don't develop LNs/LBs.

Extranigral melano-neurons still display no signs of the inclusion body pathology. Other mesencephalic sites start to develop LNs, and so also do the magnocellular nuclei of the basal forebrain. The hypothalamic tuberomammillary nucleus accumulates large numbers of globular inclusion bodies. At this stage, mesocortex and neocortex areas are not involved in the pathology, while the cortical and subcortical regions joined to the anterior olfactory nucleus can be mildly affected. Additionally, a plexus of long LNs extending throughout the second sector of the Ammon's horn (Dickson *et al*., 1991; Dickson *et al.,* 1994) generally begins to develop. At stage 4, a significant loss of neurons in the substantia nigra is observed, especially in the posterior regions of the pars compacta, compared to control cases. Nigral LNs appear to be slightly reduced in thickness and the accumulation of extraneuronal pigment granules increases. LBs and LNs appear in the neurons of the oral raphe nuclei. The neurons of the compact portion of the pedunculopontine tegmental nucleus are severely affected, and a thick web of long LNs indicates the position of the nucleus.

Stage 4 cases show severe involvement of the nuclei of the basal forebrain, and lesions of similar densities are encountered in the hypothalamic tuberomammillary nucleus. Inclusion bodies develop in the interstitial nucleus of the stria terminalis, the accessory cortical and central nuclei of the amygdala and the ventral claustrum (Braak *et al*., 1994). Lesions are also seen in specific subnuclei of the thalamus (Rüb *et al*., 2002). The anterior olfactory nucleus is usually severely damaged, while the damage seen in cortical areas associated with this nucleus is variable. The recurrent feature of this stage is the development of LNs and LBs in mesocortex.

During stages 5 and 6, the degree of damage seen in all of the previously mentioned cortical structures increases. Neurons in the vulnerable regions of the substantia nigra almost vanish completely. The number of LNs and LBs gradually decreases, whereas that of the extraneuronal neuromelanin aggregations increases. There is a visible loss of melanized neurons of the DMV, intermediate reticular zone, reticular formation, and coeruleus–subcoeruleus complex and the affection of olfactory areas is severe. Degeneration of neocortex begins with presence of LNs at the level of the temporal mesocortex, with subsequent involvement of nearly the entire neocortex.

Transfer of LBs from PD brain to grafted cells

The spatio-temporally defined progression of pathology in PD suggests the existence of a specific factor that mediates transmission of the pathology from cell to cell. In 2008 it was reported that the neural cells grafted to the brains of PD patients may develop Lewy pathology after long-term survival in the host brain. Two cases described by Li *et al*. in 2008 exhibited the presence of LBs in the grafted cells 11-16 years following the transplantation. The LBs in grafted cells were α-synuclein- and ubiquitin-immunoreactive and were positive to Ser129-phosphorylated α-synuclein (Li *et al*., 2008). Also, Kordower *et al*. (2008), reported a female patient affected with PD who had been transplanted with solid pieces of mesencephalon from human embryos. An improvement of the disease was achieved along the first four years after transplantation, but later the disease started to worsen and the patient died from cardiac arrest, 14 years after transplantation. Post mortem examination of her brain showed, in many neurons, the presence of aggregated Lewy body-like structures that stained for α-synuclein and ubiquitine. These results show that abnormal Lewy body-like structures may develop in implanted neurons, suggesting host-to-graft transfer of pathological α-synuclein that may happen independently of the developmental age of the neurons.

Prion-like spreading of α-synuclein

Cell-to-cell transfer of misfolded fibrillar forms has been described for various neurodegenerative disease-related proteins. This mechanism is based on uptake of the aggregates by the cell, where it can serve as a template to convert natively unfolded protein present endogenously in the recipient cell into misfolded form that then tends to aggregate and is released to be taken up by another cell (Meyer-Luehmann *et al*, 2006; Clavaguera *et al*., 2009; de Calignon *et al*., 2012; Hansen *et al*., 2011; Desplats *et al*., 2009; Aguzzi&Rajendran, 2009). Based on the above mentioned clinical data it has been suggested that such a mechanism involving α-synuclein may be responsible also for progression of PD pathology (Angot&Brundin, 2009; Angot *et al*., 2010; Goedert *et al*., 2010; Brundin *et al*., 2010; Aguzzi&Rajendran, 2009; Ferreon *et al*., 2009; Prusiner, 2007; Jao *et al*., 2008). A series of experimental findings using *in vitro* and *in vivo* models strongly supported this hypothesis.

It was shown that in cultured cells, α-synuclein monomers and aggregates can be released by exocytosis (El-Agnaf *et al*., 2003). However, its level is increased under many stress conditions as mitochondrial, proteasomal and lysosomal dysfunction, as well as oxidative stress. Under such conditions, translocation of α-synuclein into vesicles is increased as well (Jang *et al.,* 2010*;* Bae *et al.*,2013*;* Lee *et al*., 2011). In this way, it is likely that α-synuclein monomers, oligomers, and fibrils can be then internalized by cultured neuronal cells, either by direct diffusion across the plasma membrane or through an endocytic pathway (Lee *et al*., 2008). Furthermore, Volpicelli *et al*. (2011) demonstrated that seeds derived from α-synuclein amyloid fibrils generated with recombinant full length and truncated human wild-type α-synuclein, when directly added to mouse primary hippocampal neurons, are internalized and induce the recruitment of endogenous soluble α-synuclein into insoluble pathologic LB-like and LN-like α-synuclein aggregates, resembling those found in human α-synucleinopathies.

Role of enteric nervous system in spreading of α-synucleinopathy

Aggregated α-synuclein is frequently detected in autonomic plexi of the gastrointestinal tract of neurologically intact individuals suspected of having preclinical PD (Minguez-Castellanos *et al*., 2007).

Many studies have been carried out supporting the hypothesis according to which α-synuclein aggregation starts in the enteric nervous system (ENS) and reaches the brainstem via vagus nerve and then spread to the SNPc.

To make a PD diagnosis, the presence of motor symptoms as bradykinesia, tremor, rigidity, postural instability is needed. However, studies showed that non motor symptoms can precede the motor signs up to 10 years, though some patients don't mention them. Among these symptoms, constipation is reported by 28-80% of PD where it is six times more common than in age-/sex-matched controls (Edwards *et al.*, 1991; Chaudhuri *et al*., 2006; Martinez-Martin *et al.*, 2007; Pfeiffer, 2011). The whole gastro-intestinal tract can be affected at the pre-motor stage, and later, the gastro-intestinal symptoms can increase.

The most likely cause of constipation in PD patients during the early stages of the disease is the neurodegeneration of the ENS, while degeneration of the CNS arises at a more advanced stage (Cersosimo & Benarroch, 2008). The pathophysiological basis of it is a reduced colon motility (Jost & Schrank, 1998; Edwards *et al.*, 1992).

The Braak staging first identified the olfactory bulb and the DMV as the sites where the PD neurodegenerative process starts, a process known as dual-hit mechanism (Reichmann, 2011; Hawkes, 2007). The fact that both ENS and olfactory bulb are exposed to the environmental factors, combined with the possibility that PD starts in the olfactory and gastrointestinal system, suggested the possibility that environmental factors can trigger PD. Studies have shown that pesticides, herbicides and some metals are linked to the presence of high levels of α-synuclein in brain (Lai *et al*., 2002; Gorell *et al.,* 1998; Manning-Bog *et al*., 2002). In particular, pesticides have been shown to inhibit the mitochondrial complex I and increase the oxidative stress (Tanner *et al*., 2011).

On the other hand, environmental factors such as caffeine and cigarette smoking are associated with a lower risk to develop PD (Baron, 1986; Scheperjans *et al*., 2015). The hypothesis at the basis of this is that they are able to modify the gut microbiota making it less prone to develop inflammation, which results in reduced α-synuclein aggregation (Derkinderen *et al.,* 2014). This is consistent with studies showing that PD patients have an altered gut microbiota. In particular, some bacteria populations are less represented in PD patients gut, while one population is much increased compared to the controls (Scheperjans *et al*., 2015, *(b)*). Nonetheless, further studies are needed to confirm the involvement of intestinal microbiota in PD risk.

To date, PD animal models have been produced by administration of toxins such as rotenone and MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) through different ways, but no model can reproduce the whole PD pathology process, nor the Braak staging. Studies also showed that α-synuclein spreads from intestinal neurons to DMV via vagus nerve after the injection of monomeric, oligomeric and fibrillar α-synuclein in the intestinal wall and that hemivagotomy before gastric rotenone treatment delays the development of motor but not gastrointestinal symptoms (Pan Montojo *et al*., 2012; Holmqvist *et al*., 2014). This is supported from results of a wide epidemiologic study where patients who underwent truncal vagotomy had a lower risk to develop PD compared to general population (Svensson *et al.,* 2015).

These results are in line with the suspected long pre-motor period of PD and suggest that the vagus nerve is the caudo-rostral way of transport of PD to the CNS.

Aim of the thesis

The aim of the thesis was to characterize the expression and spreading of α-synuclein in a new transgenic mouse model of Parkinson's disease. In particular, mice expressing α-Syn (1-120) under the villin promoter were crossed with a mouse model expressing endogenous α-synuclein and with transgenic mice expressing human α-synuclein to confirm whether spreading of α-synuclein pathology occurs from the intestine to the brain.

In the model it was investigated α-synuclein transfer from enterocytes to myenteric neurons.

Experimental procedures

Animals

All procedures were performed according to the United Kingdom Animals Scientific Procedures Act 1986.

Generation of transgenic Vitras line

A construct containing human (1-120) α-synuclein was prepared and cloned into a *pBS-KS-Villin-MES-SV40-polyA* plasmid, and provided to the Biological Sciences facility of the University of Cambridge. The construct was microinjected into the egg cells derived from a female wild-type C57BL/6J mice (Charles River, UK). The presence of transgene in the offspring was detected with Polymerase Chain Reaction (PCR) using the DNA isolated from ear punch biopsies (see below). The animals with confirmed expression of transgenic DNA were used for crossing to establish homozygous transgenic Vitras line on endogenous mouse α-synuclein-positive background. Additionally, transgenic mice were back-crossed with α-synuclein-null C57Bl/6OlaHsd line (Harlan, UK) to establish homozygous transgenic Vitras line on endogenous mouse α-synuclein-positive background to be used as a control for *Vitras x wild-type* line. The presence or absence of transgenic or endogenous α-synuclein was detected in both lines with PCR using DNA isolated from ear punch biopsies.

DNA isolation from ear punch biopsies

Animals were ear notched, the tissue was collected and DNA was isolated by incubation in 0.05 M KCl, 0.01 M Tris, pH 9.0, 0.1% Triton X100 , 0.4 mg/ml Proteinase K overnight at 55°C, followed by 40-min incubation at 95°C. The samples were then chilled in ice.

Genotyping

PCR reaction was performed with a reaction mix of 20 µl containing 100 ng of total DNA as template, Green GoTaq Flexi buffer (GoTaq® G2 Polymerase, Promega), 20

pmol of each primer, dNTPs mix at concentration of 200 μM for each dNTP, 2 mM MgCl2, and 0.5U of GoTaq G2 DNA polymerase (GoTaq® G2 Polymerase, Promega),

Primers used:

Vitras
VF: 5'CGCGACGCGTTCAAGGATCCACAGGCATATCTTCCAGAATTC
VR: 5'GTAGGGAGGTCGAGGCTAAAGAAGAGC

Mopas
MF: 5'ATACTGGGGATTGCTGGGATCGAACCCAGGGATAGGTTTTTAGTTTCT
MR: 5'TCGACTCGAGTTAGGCTTCAGGTTCGTAGTCTTGATACCCTTCC

Endogenous α-synuclein (wild-type)
EF: 5' GTTCCAAACACTGCTCTGAGG
ER: 5' GGCTTCAGGCTCATAGTCTTG

The PCRs were performed in a BioRad T100Thermal Cycler under the following conditions:

Vitras and Mopas

Initial denaturation step	4-min at 96°C	
Denaturation	45-s at 96°C	←
Annealing	45-s at 66°C	× 35
Elongation	45-s at 72°C	—
Final elongation step:	10-min at 72°C	

Endogenous α-synuclein

Initial denaturation step	4-min at 96°C	
Denaturation	45-s at 96°C	←
Annealing	45-s at 61°C	× 35
Elongation	45-s at 72°C	—
Final elongation step:	10-min at 72°C	

Antibodies

Primary antibodies used in the experiments were the following:

- Mouse anti-α-synuclein Syn1 (BD Bioscience), a purified monoclonal antibody with reactivity against rat, human and mouse α-synuclein. Working concentration used was 1:500 dilution from stock solution.

- Mouse anti-α-synuclein syn204 (abcam), a monoclonal antibody against human recombinant α-synuclein. Working concentration used was 1:200 dilution from stock solution.

- Rabbit anti-α-synuclein filament antibody [MJFR-14-6-4-2] (abcam), a monoclonal conformation-specific antibody against mouse, rat, human α-synuclein filament. Working concentration used was 1:5000 dilution from stock solution.

- Sheep anti-α-synuclein antibody (ab21976) (abcam), a polyclonal antibody against human α-Synuclein (aa 108-120). Working concentration used was 1:800 dilution from stock solution.

Secondary antibodies used for western blotting and dot blotting were the following:
- Peroxidase-conjugated polyclonal swine anti-rabbit IgG antibody (DAKO)
- Peroxidase-conjugated polyclonal rabbit anti-mouse IgG antibody (DAKO)

Secondary antibodies used for the immunohistochemistry were the following:

- biotinylated Horse Anti-Mouse IgG antibody (VECTOR laboratories)

- biotinylated Goat Anti-Rabbit IgG antibody (VECTOR laboratories)

- biotinylated Rabbit Anti-Sheep IgG antibody (VECTOR laboratories)

Proteinase K digestion

Samples were digested in 50 µg/ml Proteinase K solution. The digestion was performed for 45 minutes at 37°C. To stop the digestion, the samples were moved to a 5mM PMSF in 0.2Mm isopropanol solution. Subsequently, staining was carried out with Syn1 antibody.

Immunohistochemistry

The animals were anesthetized and transcardially perfused with PBS followed by 4% paraformaldehyde (PFA), and the intestine and brain were isolated and postfixed overnight in 4% PFA at 4°C. Brain and part of small intestine were paraffinized, cross-sectioned at 5 µm using slide microtome (Leica, UK) and collected on Superfrost glass slides (Thermo Scientific, UK). The other parts were used for isolation of muscle/myenteric plexus whole mounts for free floating staining. Prior to the immunohistochemical staining, the paraffinized tissue was re-hydrated by incubation in xylene and 100%, 95%, 70% and 50% EtOH and in H_2O. The parameters of the immunohistochemistry procedure were then the same for both mounted sections and free-floating wholemounts. An antigen retrieval step was carried out by a 30 minutes incubation of the tissue in 10 mM sodium citrate buffer, pH 8.5 at 80°C. After 3 washes in PBS + 0.3% Triton-X100 (PBST), the samples were incubated for 1 h in 3% H_2O_2, 20% MetOH in PBST. After 3 washes in PBST, a blocking was performed by incubating the tissue in 5% normal horse serum (VECTOR laboratories) in PBST for 1 h. After one wash with PBST , the samples were incubated overnight at 4°C with the primary antibody diluted to the specific working dilution with PBST. Then, after five washes with PBST, the samples were incubated with the secondary antibody for 3 h and washed six times with PBST. Next, the tissue was incubated in avidin/biotin solution

(VECTASTAIN Elite ABC HRP Kit Peroxidase, Standard, VECTOR laboratories) for 1 h, washed 6 times with PBST, and the reaction was developed using DAB substrate kit for peroxidase (VECTOR Laboratories) and counterstained using Nissl procedure (incubation in 0.1% cresyl violet (Sigma) in 0.3% acetic acid). Prior to this step the whole mount samples were mounted onto glass slides. Following the Nissl staining the tissue was de-hydrated using descending concentrations of EtOH, de-fatted with Xylene and coverslipped using DPX mountant for histology (Sigma, UK).

Western blotting

In order to analyze the expression pattern of transgenic protein, different tissues (eyes, olfactory bulbs, cerebrum, brain stem, cerebellum, spinal cord, heart, lungs, liver, spleen, adrenal glands, kidney cortex, kidney medulla, stomach, small and large intestine, testicles, muscles, skin) were collected from Vitras and α-synuclein-null mouse (negative control). All the samples were homogenized with RIPA buffer supplemented with Protease Inhibitor Cocktail (Roche) on ice. The homogenates were then centrifuged at 13000 x g at 4 °C in a Microfuge 22R Centrifuge (Beckman Coulter) and the supernatants were collected. Total protein in lysates was determined by the BCA Protein Assay kit (Merck Millipore) and the protein concentration in individual samples were equalized. Samples were then mixed (2:1) with 3 x sample buffer (120 mM Tris, 30% glycerol, 6% SDS, 15% β-mercaptoethanol, 0.015% bromophenol blue, pH 6.8), and the proteins were denatured by 5-min incubation at 95°C. Proteins were then separated on 15% polyacrylamide gel using Tris-Glycine running buffer (20 mM Tris, 192 mM Glycine, 0.1% SDS) and then subjected to 3 hours transfer to 0.2 μm nitrocellulose membrane (Bio-Rad, UK), at 300 mA, at 4°C using Tris-Glycine transfer buffer (20 mM Tris, 192 mM Glycine, 10% methanol). After the transfer, the membrane was incubated in 0.01% glutaraldehyde and 4% PFA in PBS for 30 minutes in order to crosslink the proteins with the membrane. A blocking step was later carried out by incubating the membrane in 5% milk in Tris-buffered saline with 0.05% Triton-X100 (TBST) Then the membrane was incubated overnight at 4°C with syn1 primary antibody diluted in 5% milk in TBST. Next day, after 5 washes, the membrane was incubated with the secondary antibody diluted in 5% milk in TBST for 3 hours and washed 6 times with TBST. The blot was visualized using the

chemiluminescent SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) and exposed in a ChemiDoc MP System (Bio-Rad).

Protein aggregate filtration assay

Tissue was homogenized in ice using in PBST with Protease Inhibitor Cocktail (Roche). The homogenates were centrifuged at 13000 x g at 4°C for 10 minutes and the supernatants were collected. Protein was measured with a BCA assay kit and the protein concentrations were equalized in the individual samples. DNA was then digested by incubation of the samples with 50 µg/ml DNAse 1 in 50mM Tris pH 8.0 with 10 mM $MgCl_2$ for 1 hour at 37°C. A 2 µm pore size cellulose acetate membrane was mounted in a dot blot system (SCIE-PLAS), samples were loaded into the wells and vacuum was applied. After the whole volume of samples was completely passed through the membrane, 200 µl of 5% SDS was added to and vacuum was applied. The membrane was subsequently washed in TBST for 1 min, blocked with 5% milk in TBST and subjected to immunodetection using the same procedures as described for the western blot.

Dot blotting

The tissue was homogenized and centrifuged using the same procedure as for protein aggregate filtration assay, and protein concentration in the samples were equalized following BCA assay protein measurements. A 2 µm pore size nitrocellulose membrane was mounted in a dot blot system (SCIE-PLAS), samples were loaded into the wells and incubated for overnight to allow proteins to bind to the membrane. Next, the liquid was removed from the wells, membrane was dried and then soaked in TBST followed by blocking with 5% milk in TBST and subjected to immunodetection using the same procedures as described for the western blot.

Generation of α-synuclein preformed fibrils (pff) using protein misfolding cycling amplification (PMCA)

Aliquotes of 100ul reaction mixtures containing 70 uM recombinant α-synuclein monomers in conversion buffer (1% Triton X-100, 13 mM NaCl, Complete Protease

Inhibitor Mixture (Roche); in 1×PBS) were subjected to cycles of 20 seconds sonication and 29:40 minutes incubation at 37°C, for 18 hours using a Misonix 4000 sonicator at 200 watts power setting.

Thioflavin T (ThT) fluorescence assay

In order to estimate pffs generated with PMCA, Thioflavin T (ThT) fluorescence assay was performed. 5 µl of sample was added to 95 µl of ThT solution (20 µM ThT, 50 mM glycine, pH 8.5, with KOH). Fluorescence was measured with an Infinite 200 PRO. Multimode microplate reader with 450 nm excitation and 480 nm emission.

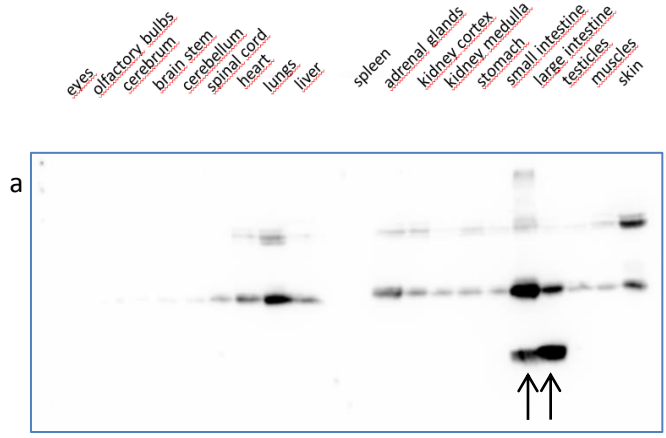
Results

Characterization of Vitras mice

Tissue distribution of transgenic α-Syn(1-120) protein

The Vitras mice were tested for the expression and tissue distribution of the transgenic protein. The western blot was performed on tissue homogenates from 19 different organs in order to prove that the transgene was only expressed in the intestine, using Syn204 antibody, which recognizes human α-synuclein. A~15 kDa band, corresponding to monomeric truncated α-Syn(1-120) was found exclusively in the small and large intestine from the transgenic mouse, but was absent in the other organs from the same animal (Fig. 1a). In the control, non-transgenic animal, α-Syn band was absent in all the organs including intestine (Fig. 1b).

These data prove that transgenic α-Syn is exclusively expressed in the intestine of in Vitras mice, hence these animals represent a proper model to study spreading of synucleinopathy from intestine.



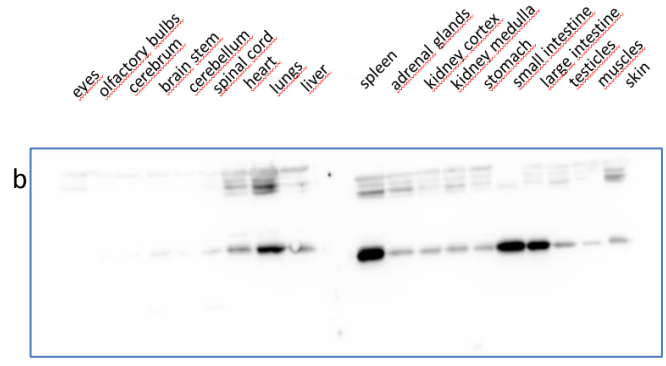


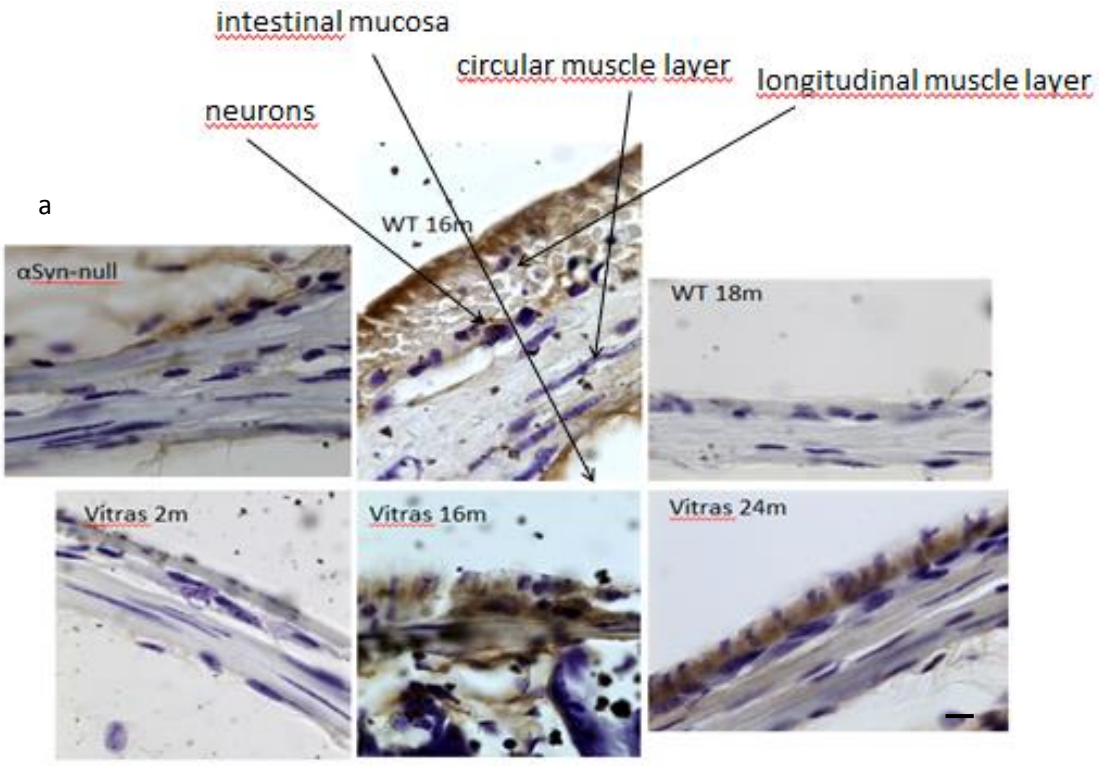
Fig. 1, Intestine-specific expression of α-Syn(1-120) protein in Vitras mice (**a**), compared to αSyn-null mouse (**b**). Lysates from different organs from control (αSyn-null) and Vitras mice were analyzed by western blot for the presence of α-Syn using human-specific Syn204 antibody. Only small and large intestine contained transgenic, human, truncated α-Syn (arrows).

Localization of α-Syn(1-120) protein in the intestinal tissue

Intestine from 2-24 months old Vitras mice was analyzed using immunohistochemistry to identify the pattern of transgenic α-synuclein protein distribution and its age-dependent changes. Both cross sections from paraffin-embedded tissue and mechanically isolated myenteric plexus were used in this study.

Cross section immunostaing

The cross section immunostaining was performed using three different antibodies: Syn1, recognizing both murine and human α-synuclein, ab21976, against human α-synuclein, and MJFR-14-6-4-2, specific for α-synuclein filaments.



b

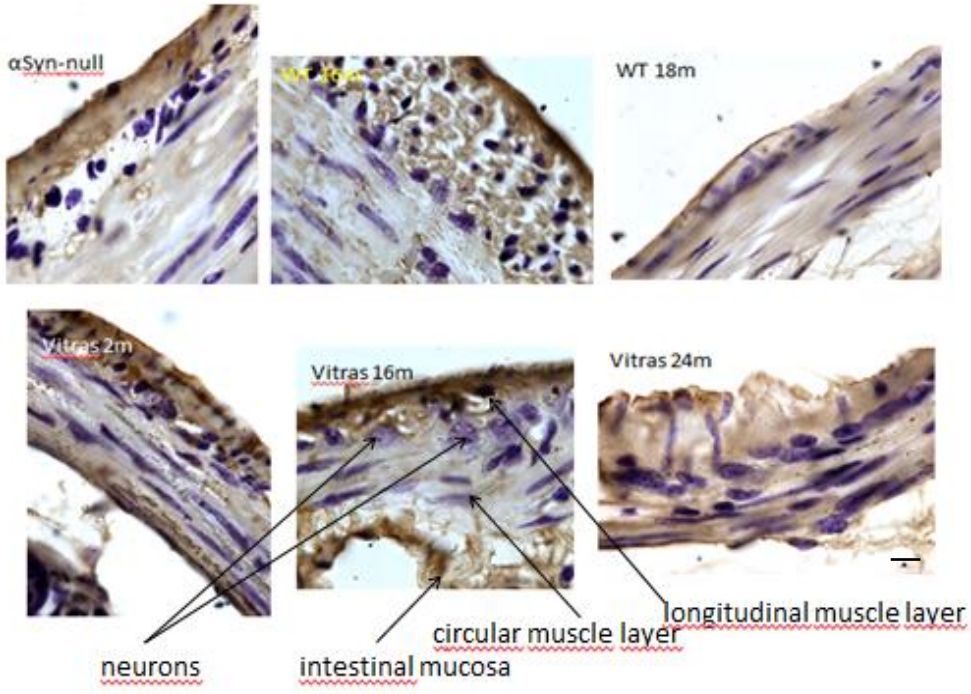


Fig. 2. Cross sections staining with syn1 (a) and ab21976 (b) antibodies. The strong background doesn't allow to distinguish between specific and non-specific staining. *Scale bar* indicates 100 nm.

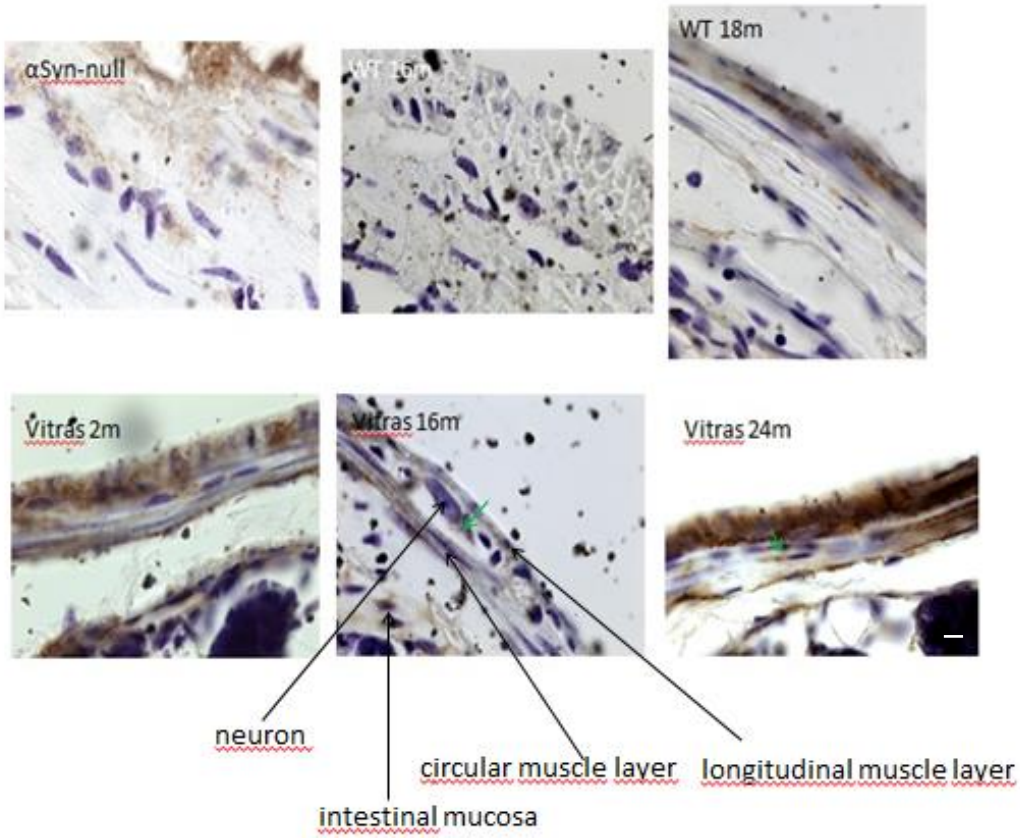


Fig. 3. Immunohistochemistry with MJFR-14-6-4-2**.** A brown area is visible around neurons nuclei (green arrows) only in 16 and 24 years old Vitras mice, compared to the α-Syn-null and the wild-type samples. *Scale bar* indicates 100 nm.

A strong background staining is evident using syn1 and ab21976 antibodies. In particular, in syn1 stained slices the 18 months old wild-type and 2 months old Vitras appear very weakly stained (Fig. 2). It is likely that during the staining on the slide, the antibody does not equally reach all the regions of the tissue. The sections appear strongly stained along the edges, nonetheless brown staining is visible in the whole thickness of the slices.

The staining with MJFR-14-6-4-2 antibody shows a brown area surrounding neurons nuclei in 16 and 24 years old Vitras mice, compared to the α-Syn-null and the wild-type samples (Fig.3).

Whole mounts immunohistochemistry

The first step to obtain α-syn transfer from intestine to the brain is to see protein spreading from enterocytes to myenteric neurons, which is the starting point for spreading to the brain through vagus nerve. Hence, whole mount preparations made of myenteric plexus and muscle layer were isolated from small intestine, and stained with syn1 and MJFR-14-6-4-2 antibodies. Ab21976 did not show any specific structure. A third staining was carried out with syn1. In order to reduce the strong background which this antibody shows when used in intestine, a step of 45-min PK digestion was carried out before the staining to prevent its binding to monomeric α-synuclein. Syn1 staining showed the presence of not digested material around the myenteric neurons in 16, 20 and 24 months old Vitras mice. Additionally, staining with MJFR-14-6-4-2 revealed the presence of intracellular structures, surrounding the nuclei, that were more pronounced in 20 and 24 months old mice compared to younger animals.

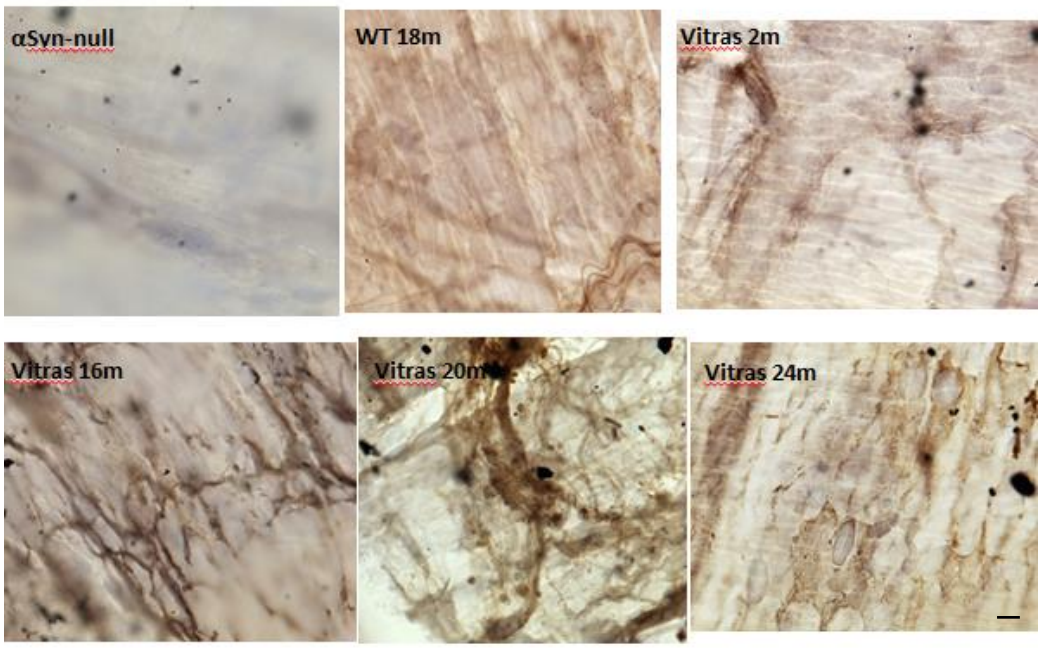


Fig. 5. Staining with syn1 after PK digestion. Before the staining with syn1 antibody, a 45-min PK digestion step was carried out at 37°C in order to reduce the background which was always evident with this antibody. *Scale bar* indicates 100 nm.

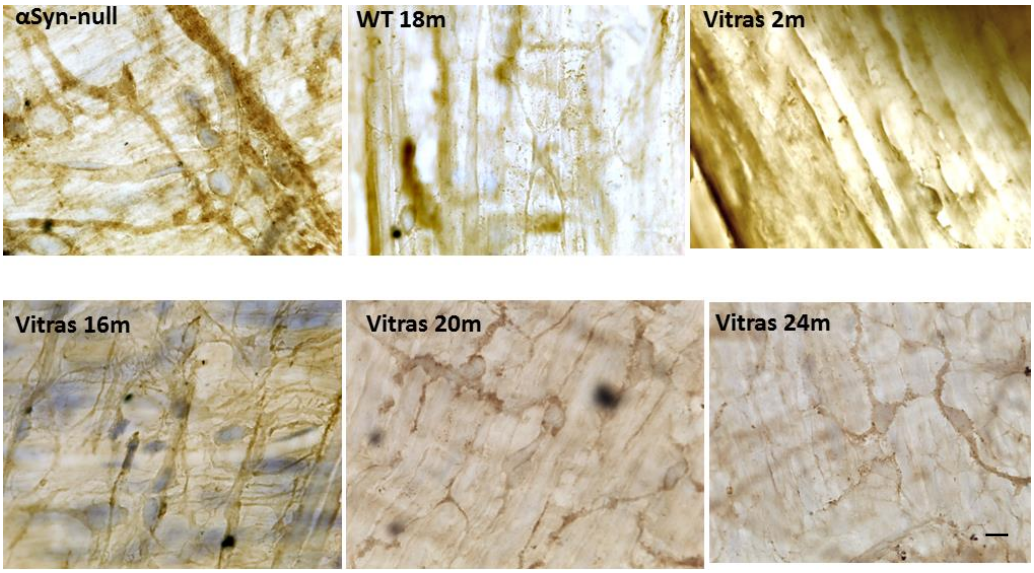


Fig. 6. Immunohistochemistry using human-specific ab21976 . The myenteric plexus is evident in all the samples and there is no evidence of specific staining. *Scale bar* indicates 100 nm.

Immunohistochemistry with MJFR-14-6-4-2 showed cell-shaped dark brown structures, surrounding the nuclei. Such structures were more pronounced in 20 and 24 months old Vitras mice, while the wild-type mouse showed a less compact pattern. These data suggest that there is a transfer of transgenic αSyn to myenteric neurons, and accumulation of this α-synuclein with the age.

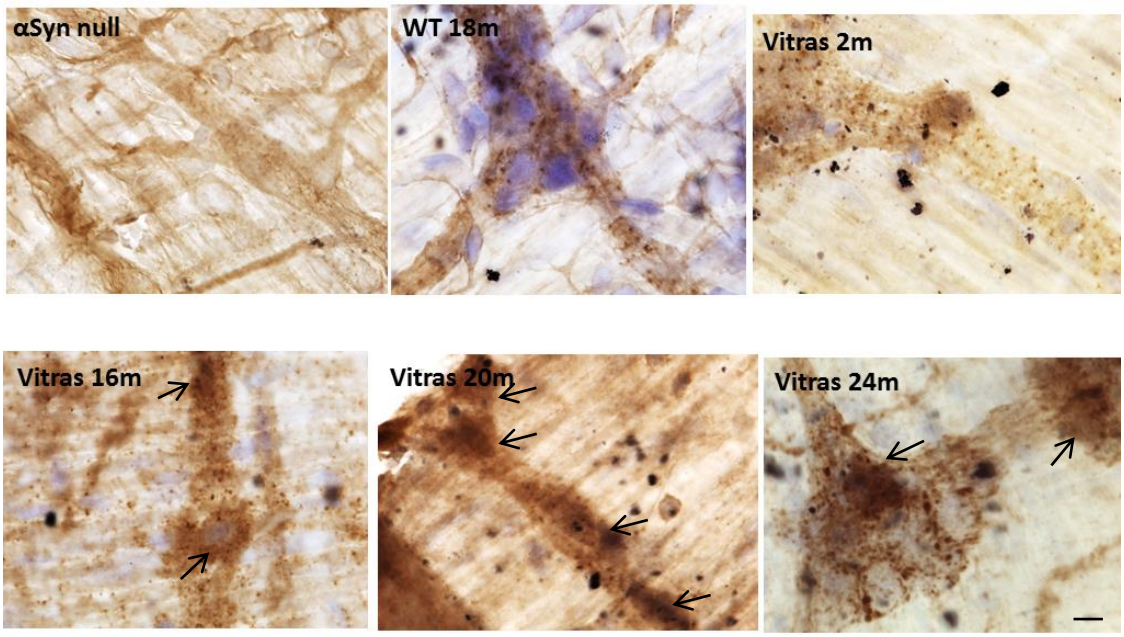


Fig. 7. Immunohistochemistry with MJFR-14-6-4-2 antibody. Cell-shaped dark brown structures were evident around the nuclei, especially in the oldest transgenic mice (arrows), compared to a less compact pattern in the wild-type. *Scale bar* indicates 100 nm.

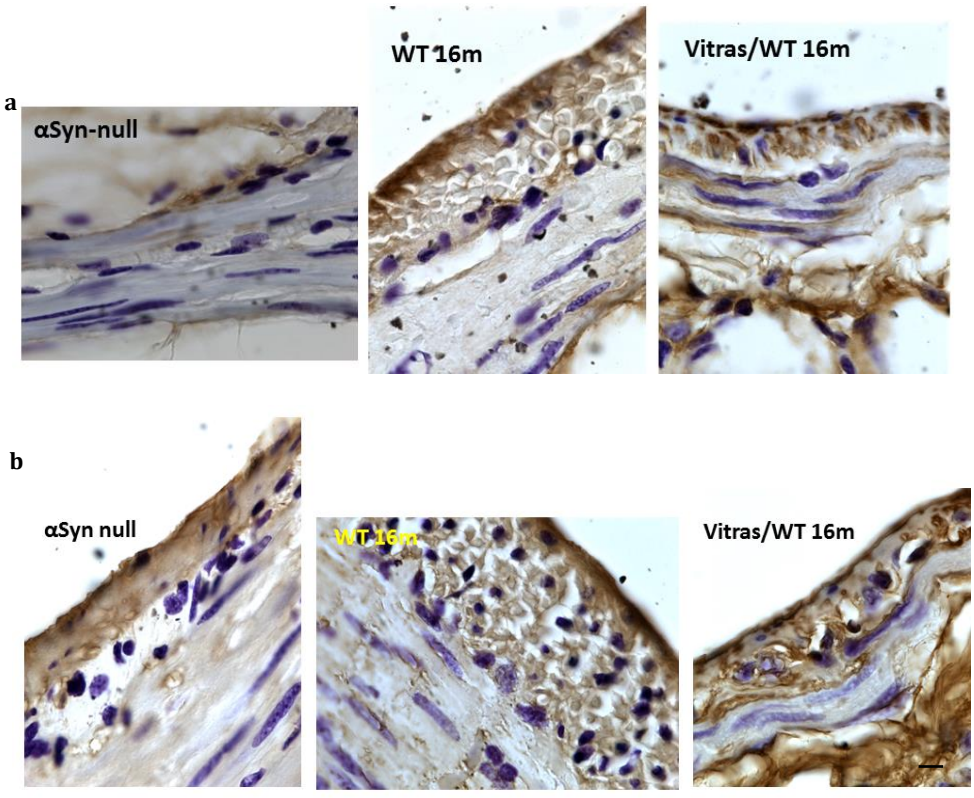
Generation of mouse models of α-Syn spreading

Vitras x wild-type line

Double homozygous (aSYN(1-120)+/+, aSYN endo +/+) animals were analyzed using immuohistochemistry as mentioned before.

Cross-sections immunohistochemistry

As for the Vitras line, immunohistochemistry on cross sections did not produce any reliable results; in particular, the background staining was too strong when the Syn1 antibody was used; neither specific structures could be detected with this antibody nor with the human specific ab21976 and with the α-synuclein filament-specific MJFR-14-6-4-2 (Fig. 8, a;b;c).



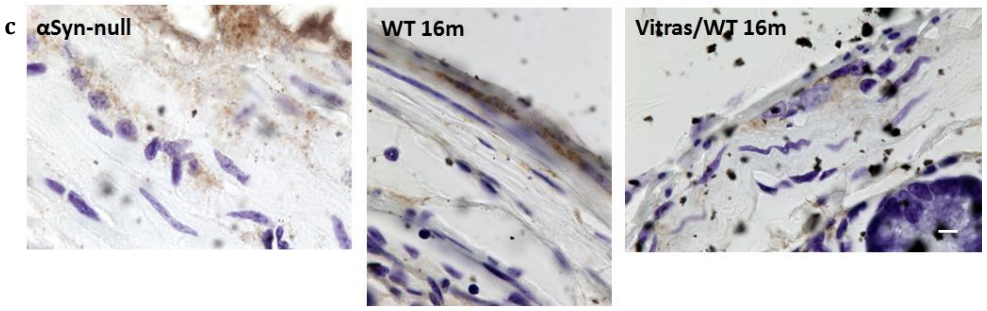
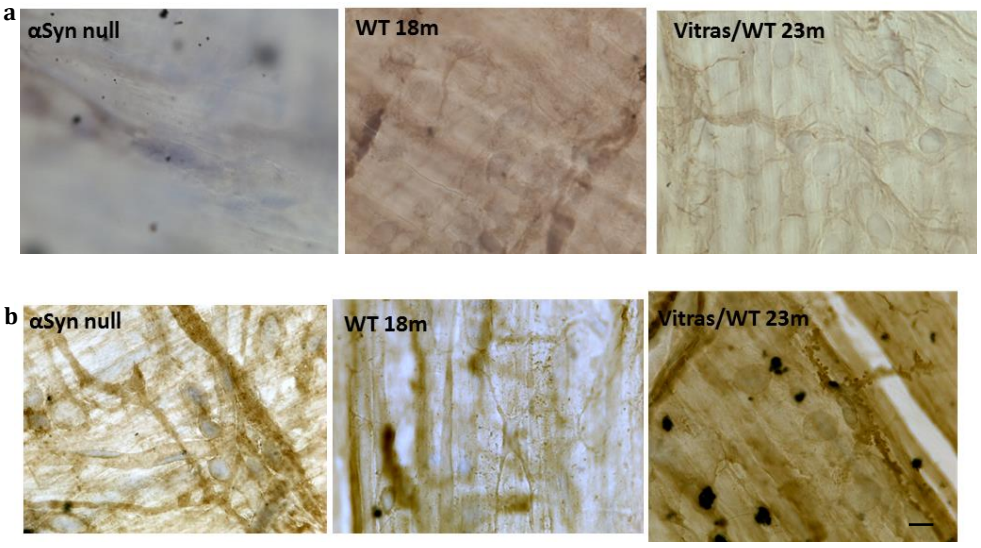


Fig. 8. Cross-sections immunohistochemistry with syn1 (**a**), with ab21976 (**b**) and with MJFR-14-6-4-2. Background staining, particularly strong when syn1 was used, and method related issues (see *Discussion and Conclusions*) did not allow detection of any specific structures. *Scale bar* indicates 100 nm.

Whole mounts immunohistochemistry

Similarly to the Vitras line, immuohistochemistry with ab21976 antibody showed no specific staining in the myenteric plexus whole mount samples from the Vitras x α-Syn endo mice (Fig. 11-b). Syn1 antibody however, allowed for detection of a more defined myenteric plexus net after a 45-min digestion with PK at 37°C (Fig. 11-a), compared to the smoother pattern in the wild-type, suggesting presence of PK-resistent structures in the transgenic mouse.

Immunostaining with MJFR-14-6-4-2 produced evidence of a compact structures surrounding nuclei. Such structures, apparently really similar to the ones observed in oldest Vitras, are absent from the wild type mouse (Fig. 11-c).



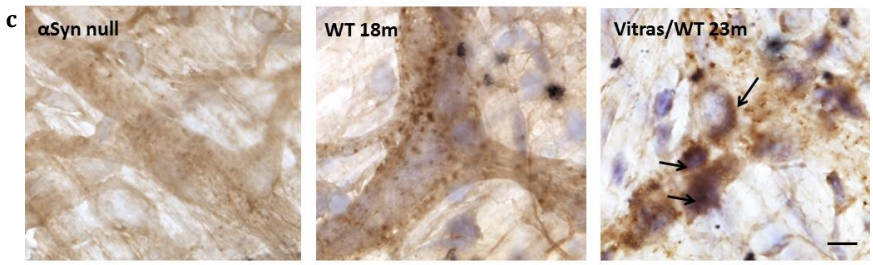


Fig 11. (**a**): myenteric plexus immuostaining with syn1 after 45-min PK digestion at 37°C. The Vitras/WT mouse shows a more defined brown net compared to the smooth structure in the control; (**b**) myenteric plexus immuostaining with ab21976, showing not specific structures; **(c**): myenteric plexus immuostaining with MJFR-14-6-4-2; compact brown structures surround neurons nuclei in both Vitras andVitras/wild type mice, while they are absent from the wild type mouse. *Scale bar* indicates 100 nm.

Vitras x Mopas line

Vitras mice were crossed with Mopas mice, a mouse line expressing human full length α-synuclein under the control of the mouse prion protein. This line, expressing truncated human α-synuclein in the gut and full-length human α-synuclein in most types of neurons, was generated to overcome the problem of species-specific barrier between human and mouse α-synuclein that could potentially obstruct the progression of α-synuclein pathology spreading in Vitras x wild type line. Vitras x Mopas animals were crossed to obtain a line that was Vitras gene-homozygous to induce the pathology in the gut easily, and Mopas gene-heterozygous in order to limit potential spontanous aggregation in other regions.

Characterization of α-synuclein expression in Mopas mice

In order to successfully transmit pathology, full-length α-synuclein needs to be widely expressed in the gut and brain, therefore Mopas mice were first characterized for the expression of transgenic protein in enteric and CNS.

Expression of α-synuclein was analyzed in small intestine of Mopas mice, using western blot with syn1 antibody. A 16 kDa band was recognized corresponding to full-length α-synuclein (Fig. 12).

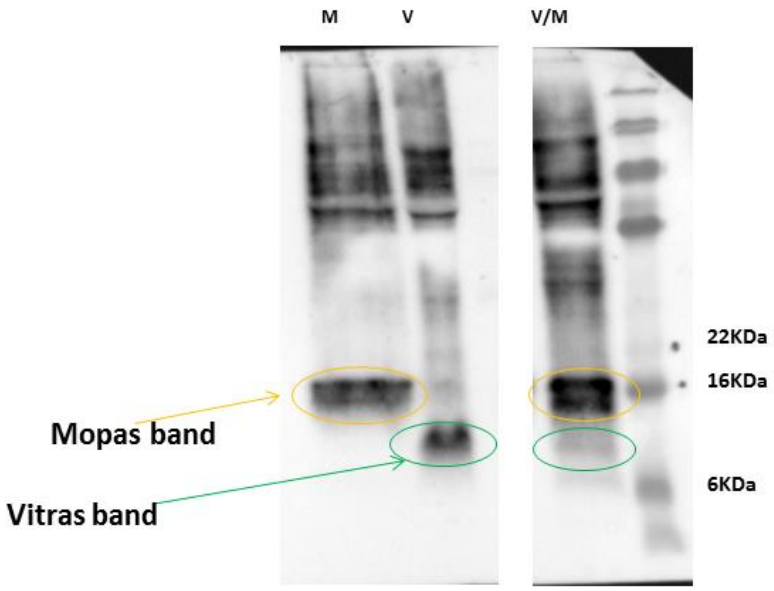


Fig. 12. Western blot with syn1 antibody in intestine of Vitras, Mopas and Vitras x Mopas mice.

Abbreviations: M:Mopas; V:Vitras; V/M: Vitras x Mopas

Distribution of α-synuclein in the enteric and CNS of Mopas mice was analyzed using immunohistochemistry. Transgenic protein was found in myenteic plexus in both fibers and cell bodies (Fig. 12). Intense staining was also revealed in brain (Fig. 13).

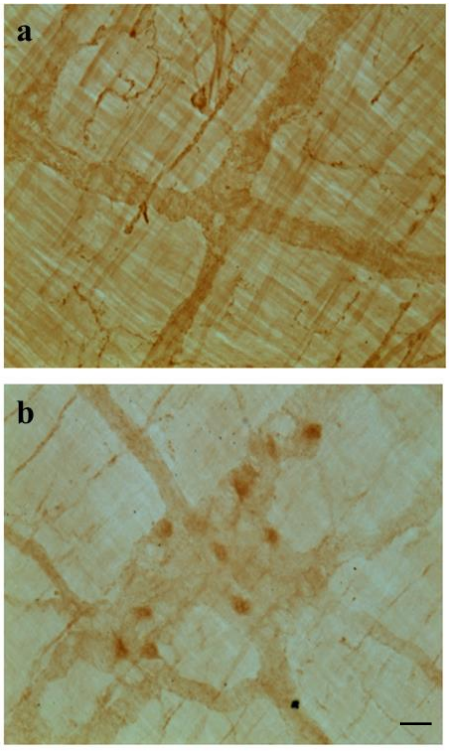


Fig. 12. α-synuclein immunohistochemistry performed on myenteric plexus with human specific antibodies LB509 (**a**) and PER7 (**b**). *Scale bar* indicates 100 nm.

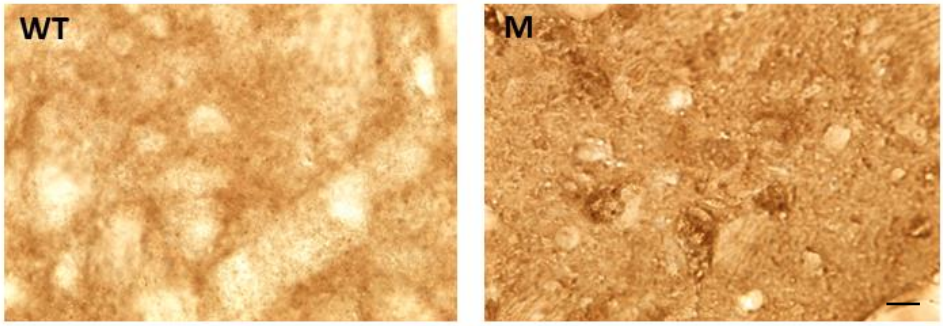


Fig. 13. α-synuclein immuohistochemistry in striatum of Mopas mice performed using syn1 antibody. Mopas mice overexpression of α-synuclein compared to the wild-type animal. *Scale bar* indicates 100 nm.

Protein Filtration Assay

Protein filtration assay was performed to detect the presence of large aggregates in small intestine of Vitras, Mopas and Vitras x Mopas mice. When using syn1 antibody, a light signal was visible for Mopas genotype, while a strong concentration of insoluble material results from filtration of intestine homogenate from Vitras and Vitras x Mopas intestine lysate (Fig. 14-a).

The analysis performed with the LB509 antibody, that recognizes only human full-length α-synuclein showed a strong signal for Vitras x Mopas sample and a weack signal for Mopas, while the Vitras homogenate showed no signal, as expected (Fig. 14-b).

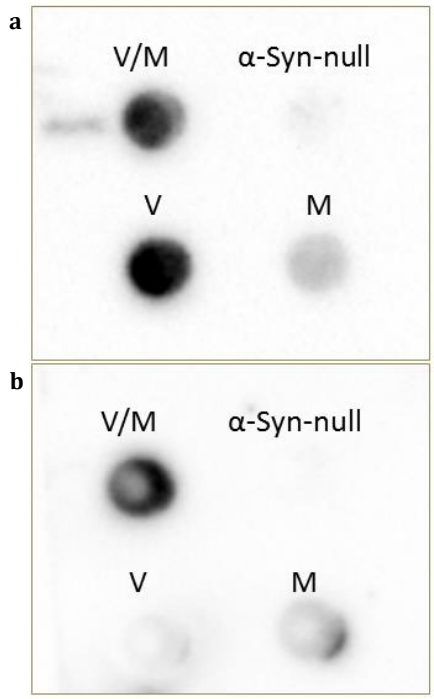


Fig. 14. (**a**) Protein filtration assay with syn1 antibody. Vitras and Vitras x Mopas intestine lysate appears rich in insoluble materials. Compared to them, Mopas shows a much weaker signal. (**b**) Protein filtration assay with human full length α-synuclein specific LB509 antibody. Vitras x Mopas sample sample shows the highest density in insoluble compounds, while a weak signal is detected in Mopas. The Vitras homogenate results negative, as expected.

Discussion and conclusions

According to the neuropathological studies, PD may begin in the nerve cells of ENS many years before the onset of motor symptoms. The pathology would then spread to the brain by cell to cell propagation of misfolded α-synuclein, following a stereotypic anatomic pathway. When the pathology reaches the SNPc, the characteristic motor symptoms appear.

In the present study I analyzed a newly-generated mouse model of intestinal α-synucleinopathy, Vitras mice, where truncated α-synuclein (α-Syn (1-120)) is expressed under the control of the murine villin promoter. This truncated form of α-synuclein has been reported to have higher propensity to aggregation than other forms of protein (Crowther *et al*., 1998; Serpell *et al*., 2000; Murray *et al*., 2003). Additionally, mice expressing α-Syn (1-120) under Tyrosine Hydroxylase promoter in catecholaminergic cells were reported to display a series of nigrostriatal phenotypes resembling those observed in Parkinson's patients, showing that this form of protein is toxic to cells in vivo (Garcia-Reitbock *et al.*, 2013). Villin promoter in Vitras mice was used to direct the expression of the transgene to the intestine.

Western blot confirmed that in Vitras mice the expression of human truncated α-synuclein is limited to the small and large intestine. Hence, a transfer from enterocytes to the neurons in myenteric plexus is necessary to initiate the spreading to the brain. This transfer could occur through the diffusion or cell to cell transfer, from enterocytes to the neuronal cell body through neuronal processes.

The distribution of the transgenic protein was initially analyzed with immuohistochemistry using paraffinized cross sections from small intestine. This method was supposed to allow the analysis of myenteric plexus and enterocytes. Unfortunately, the non-specific staining in the intestine is common with most methods; for this reason, searching for good protocol and antibody to use was a challenging and time consuming task since the beginning of this study. Further, intestinal mucosa revealed to be one of gut most sensitive regions to non-specific staining. An additional issue was tissue damaging, either due to the tiny thickness of the slices, cut at 5μn to reduce the background staining as much as possible.

Next, mechanically isolated myenteric plexus was analyzed in order to reveal if the transgenic protein can be transferred to the neurons from the enterocytes. Different ways to isolate it were tried, e.g. collagenase digestion, before reaching the goal and obtain myenteric plexus bound to the longitudinal muscle layer. Though no specific staining was evident using ab21976 antibody, the immuostaining with Syn1 after Proteinase K (PK) digestion showed the presence of PK-resistant α-synuclein in the neurons in 16, 20 and 24 months old Vitras samples, that was not present in the control animals or younger Vitras mice. Additionally, staining with MJFR-14-6-4-2 antibody revealed the presence of cell-shaped dark brown structures in the tissue from Vitras animals, surrounding the nuclei, that were more prominent in 20 and 24 months old mice. Since MJFR-14-6-4-2 recognizes α-synuclein filaments, this result suggests the presence of aggregated α-synuclein in the myenteric plexus of Vitras mice.

Vitras mice were crossed with the wild-type mice expressing endogenous mouse α-synuclein and with Mopas mice, expressing human full length α-synuclein under the murine prion protein promoter (Mopas line) in order to generate the model where α-synucleinopathy initiated in the intestine by the aggregation of truncated α-Syn (1-120) would spread to the brain based on the cell to cell propagation of aggregated α-Syn, using either mouse or human α-Syn for generation of new cellular aggregates.

The following step was to check if the presence of the endogenous protein could interfere with aggregates formation in Vitras x wild type line. A previous study reported indeed that accumulation of prefibrillar oligomers and fibril formation from human α-synuclein is inhibited in the presence of the mouse protein, *in vitro* (Rochet *et al*., 2000).

The results obtained from immunohistochemistry with antibodies syn1 and MJFR-14-6-4-2 in Vitras x wild type line showed α-synuclein deposits in the cell bodies, very similar to the Vitras line. Hence, an experiment to perform in the future should be staining of myenteric plexus with mouse-specific antibody, e.g. D37A6, to verify if the observed aggregates are only made from human truncated α-synuclein or if the endogenous protein was recruited into them.

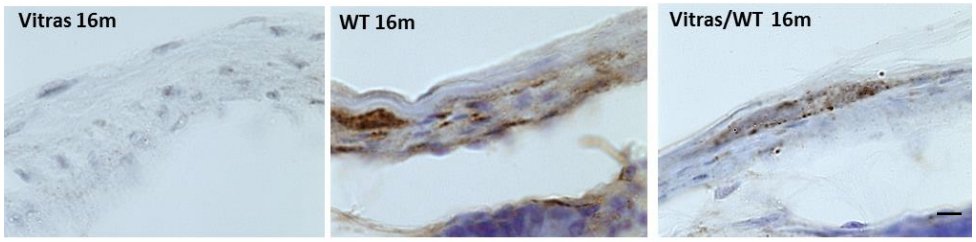


Fig.1 D37A6 mouse specific antybody which recognizes endogenous α-synuclein proved to be really specific when used on 12µn cross sections from 16 months old mice paraffinized intestine. *Scale bar* indicates 100 nm

The results obtained from staining of the myenteric plexus whole mounts isolated from Vitras x wild type line are the same obtained for Vitras mice: immunohistochemistry with ab21976 shows no specific staining. However, both syn1 after PK digestion and MJFR-14-6-4-2 immunostainings show smoother α-synuclein pattern in the wild-type compared to the Vitras x wild typemouse. In particular, the myenteric plexus appears as a more prominent structure after the digestion with PK and syn1 staining suggesting the presence of insoluble material in the transgenic mouse compared to the smoother pattern in the controls. Staining with the MJFR-14-6-4-2 antibody shows a dark structure surrounding the neurons nuclei, which is absent from wild-type sample. Though, both MJ-14-6-4-2 and PK-syn1 antibody show some staining in control mouse. This suggest the presence of insoluble endogenous material in control mice, or non-specific staining with these two methods that recognize also normal physiological α-synuclein. In the normal conditions there is accumulation of α-synuclein in myenteric neurons (in rats), and presence of PK-resistant aSYN in intestinal biopsies from control, non-PD patients.

In Vitras x wild type model, endogenous α-synuclein is expressed under its own promoter; hence this model should reproduce the human pathology with more reliability compared to Vitras x Mopas line, where α-Syn(1-120) is expressed in the enterocytes and the human full length α-synuclein is expressed under the murine prion protein promoter. In these mice, full length human α-synuclein may be overespressed. This may facilitate the propagation of α-synuclein pathology, by eliminating α-synuclein species-specific barrier that could potentially prevent the seeding of aggregation in Vitras x wild type line, and by widespread availability of high levels of full-length α-synuclein for the synthesis of new aggregates. On the other hand, however, the potential drawbacks of this model should be also considered, like spontanous aggregation of full-length α-synuclein due to its overexpression and abnormal anatomical pathways of α-

synucleinopathy propagation due to different pattern of expression of prion promoter used in Mopas mice compared to α-synuclein promoter expression pattern in patients or wild-type mice.

Immunohistochemistry using the myenteric plexus whole mounts and striatum sections from 13 years old mice, confirmed α-synuclein overexpression in these tissues. However, no evidence of aggregation was seen. According to immunohistochemistry results, in the case of α-synuclein spreading and presence of aggregates in the brain of Vitras x Mopas line, using Mopas mice as a control would allow to normalize the results, based on the background.

Protein filtration assay showed presence of syn1-positive material retained on the membrane in Vitras and Vitras x Mopas small intestine, absent in α-Syn-null control mice, suggesting the presence of large aggregates in the transgenic mice. On the other hand, the presence of a weak signal in Mopas sample suggests self-aggregation of full-length α-synuclein even in the absence of α-Syn(1-120).

Vitras x Mopas line was checked at the beginning of the study, to compare the two different models. However, the spreading in these mice was not further investigated in this study, which was focused on Vitras and Vitras x wild type analysis.

In conclusion, based on this study:

1. αSyn (1-120) is expressed in intestine only in Vitras mice, what makes them a suitable model of intestinal synucleinopathy;

2) αSyn (1-120) is transferred to the myenteric plexus. This is a required step in this model to initiate the potential spreading to the brain;

3) αSyn(1-120) mice were crossed with two full-length α-synuclein expressing lines, and αSyn (1-120) protein induced aggregation of full-length protein , at least in Mopas. This makes Vitras mice a promising model of spreading;

4) further analysis is necessary to characterize the spreading of pathology to the brain.

Future perspectives

In the case of a positive result for the implication of the endogenous protein in fibrils formation in myenteric plexus, the immunohistochemical and biochemical analysis of the other organs according to the Braak staging will be performed.

Once the most appropriate model will be chosen, it will allow to study the earliest stages of the pathology, in order to search for markers of the disease when its motor symptoms have not yet arise. Additionally, a good model of spreading is a necessary tool to investigate new molecules to be used as drugs.

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