On α-synuclein In the Human Enteric Nervous System

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Abstract

Parkinson's disease is a neurodegenerative disease resulting primarily from loss of dopaminergic innervation in the striatum subsequent to cell loss in the substantia nigra pars compacta. The abnormal accumulation of the normal pre-synaptic protein α-synuclein (αsyn) forms intraneuronal inclusions known as Lewy neurites and Lewy bodies. The origins of central Lewy pathology have been suggested to lie in the enteric nervous system, ascending through the vagus nerve to the dorsal motor nucleus of the vagus. To ascertain gastrointestinal regions most likely to be the source of central Lewy pathology, αsyn expression was evaluated in the neural elements of gastrointestinal regions receiving the densest vagal innervation. The vermiform appendix was found to have the densest αsyn-immunoreactive innervation in all layers of the gut wall. In addition, macrophages in the appendiceal mucosa were laden with αsyn within lysosomes, consistent with attempts to prevent the spread of disease or to correct synaptic dysfunction.

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Lastly, this thesis is dedicated to my late father, William Alexander Gray. His support was both necessary and sufficient for many for so many of my achievements.

“What is the student but a lover courting a fickle mistress who ever eludes his grasp?”

-Sir William Osler

The Student Life

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List of abbreviations

αsyn	α-synuclein
βsyn	β-synuclein
γsyn	γ-synuclein
µm	micrometre
Ala	alanine
BMDC	bone-marrow-derived dendritic cell
CD68	cluster of differentiation 68
CJD	Creutzfeldt-Jakob disease
CN X	cranial nerve X (vagus nerve)
CNS	central nervous system
DAB	3,3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DLB	dementia with Lewy bodies
dmX	dorsal motor nucleus of the vagus nerve
ENS	enteric nervous system
FDC	follicular dendritic cell
GALT	gut-associated lymphoid tissue
GI	gastrointestinal
Glu	glutamic acid
Gly	glycine
His	histidine
HSP	heat-shock protein
IF	immunofluorescence
IgG	immunoglobulin G
IHC	immunohistochemistry

iLBD	incidental Lewy Body disease
IPAN	intrinsic primary afferent neuron
kDa	kilodalton
KO	knockout
LB	Lewy body
LN	Lewy neurite
LRRK2	leucine-rich-repeat kinase 2
MHC	major histocompatibility class
NAC	non-Aβ component of Alzheimer's disease amyloid
NACP	non-Aβ component of Alzheimer's disease amyloid precursor
OB	olfactory bulb
PD	Parkinson's disease
Pro	proline
PrP^{C}	prion protein (cellular)
PrP^{Sc}	prion protein (scrapie)
SN	substantia nigra
SNpc	substantia nigra, pars compacta
SYP	synaptophysin
tau	microtubule-associated protein tau
Thr	threonine
vCJD	variant Creutzfeldt-Jakob disease
VIP	vasoactive intestinal polypeptide

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Chapter 1: Introduction

1.1 Parkinson's disease

Parkinson's disease (PD) is a movement disorder characterised clinically by resting tremor, rigidity, bradykinesia and postural instability. These motor features are attributed to a loss of the principal dopaminergic input to the caudate-putamen from the substantia nigra pars compacta (SNpc) due to degeneration of the latter.

1.1.1 Historical overview

Defined as a clinical entity in 1817 by James Parkinson (Parkinson, 1817), PD or *paralysis agitans* remains a common neurodegenerative movement disorder. PD was eponymised by Charcot in 1875 (Charcot, 1875). While under the supervision of Charcot at the Salpêtrière, Blocq & Marinesco (also Marinescu) provided the first clues for the localisation of the PD lesion. In 1893, they reported a case of left hemiparkinsonism resulting from a tuberculoma in the right cerebral peduncle – leading to the suggestion that PD symptoms may derive from a midbrain defect, especially in the substantia nigra (SN) (Blocq and Marinesco, 1893).

The histopathological hallmarks of PD, eosinophilic cytoplasmic neuronal inclusions with pale halos (figure 1A), were described by Friedrich Lewy in 1912 (Lewy, 1912). Lewy described these *Kugeln* (balls) he found in the globus pallidus of PD brains as being proteinaceous and iron-containing. The Lewy body (LB) eponym was introduced in 1919 as *corps de Lewy* in the doctoral thesis of Konstantin Tretiakoff (Tretiakoff, 1919). That work was also notable for the definitive association of PD with the degeneration of the SN. The final pre-molecular characterisation of LBs came in 1965 when they were found by electron microscopy to be comprised of filamentous material more tightly packed in the darker core (Duffy and Tennyson, 1965).

Figure 1. Lewy bodies in the CNS

(A) Lewy bodies as demonstrated by Lewy

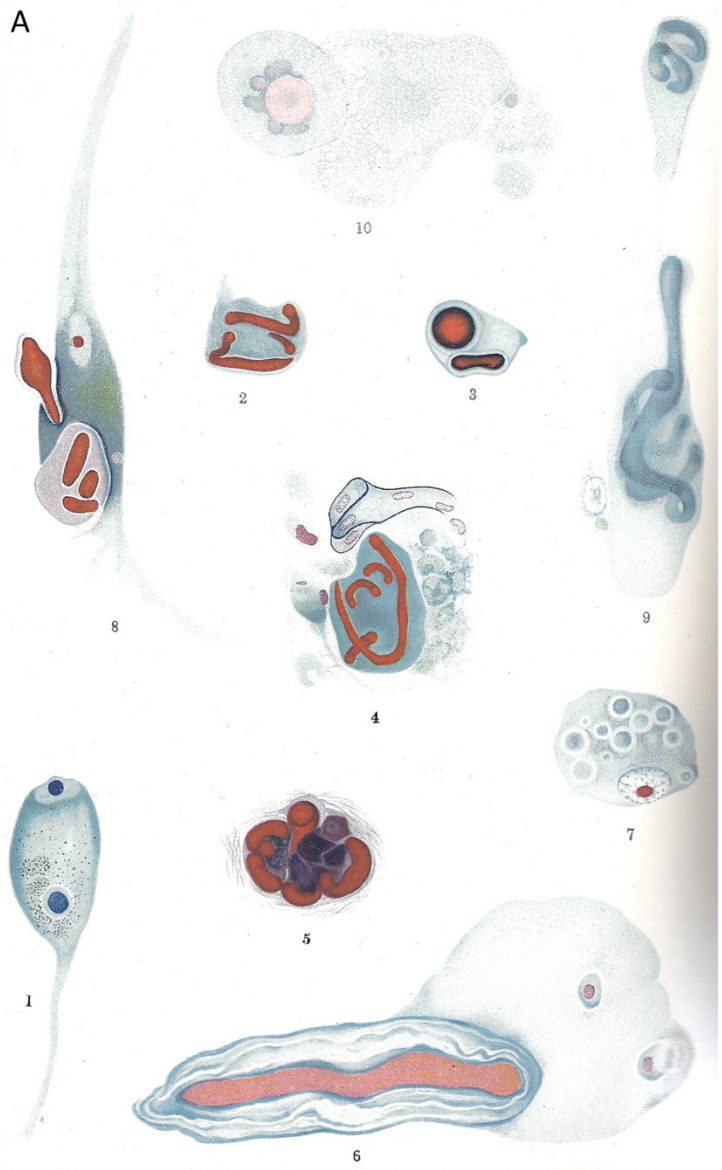
Lewy bodies from the dmX (A1-6) and paraventricular nucleus of the hypothalamus and substantia innominata (A7-10) are demonstrated in formalin-fixed frozen human brain stained using Mann's method (A1-6 & A8) or nitrocellulose-embedded, Mallory-stained sections (A7, A9 & A10).

Adapted from (Lewy, 1912). Copyright permission not required as content is in the public domain

(B&C) αsyn-immunoreactive Lewy bodies and Lewy neurites

αsyn immunostaining (clone MJFR1) in the dmX of a PD patient with (B) or without (C) proteinase-K pre-treatment. From Gray & Woulfe (unpublished).

Scale bars = 100 μm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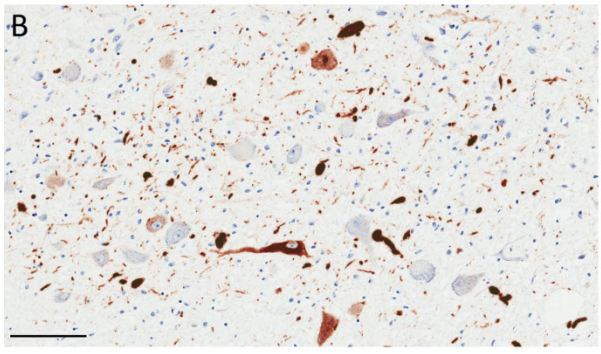
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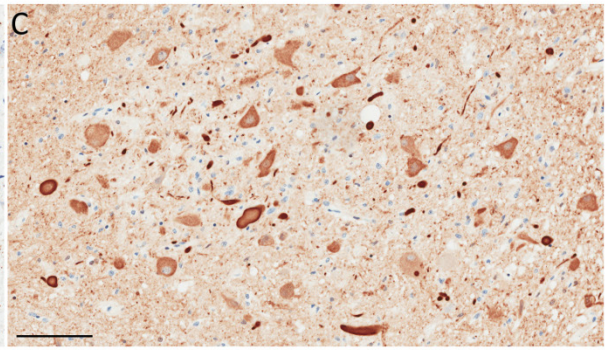

C

In 1988, Lewy bodies were found to display immunoreactivity to ubiquitin using an antibody preferentially recognising conjugated ubiquitin, confirming Lewy's proteinaceous chemical characterisation (Kuzuhara et al., 1988). This provided the first effective immunohistochemical marker for LBs although ubiquitin is a common characteristic of neuronal inclusions. The positivity for conjugated ubiquitin also raised the possibility of other protein components of LBs. Neurofilaments had been suggested to comprise the filamentous structures visible by electron microscopy and originally this was borne out by immunohistochemistry (Goldman et al., 1983) although, upon closer examination, only a small subset of LBs were positive (Forno et al., 1986). Further studies displayed other markers staining subsets of LBs or staining LBs as well as other pathological inclusions (neurofibrillary tangles, Pick's bodies, Mallory bodies). Among these were: UCH-L1 (Lowe et al., 1990), heat-shock protein (HSP) 70 (Namba et al., 1991) and alpha B crystallin (Lowe et al., 1992).

Genetic studies would eventually elucidate further players in LB biology. α-synuclein (αsyn) was first linked to PD when an Italian kindred and three Greek pedigrees with dominantly inherited PD were found to have a G209A substitution causing an Ala53Thr mutation in the αsyn protein (Polymeropoulos et al., 1997). Various other rare mutations in αsyn leading to familial PD have since been described including: Ala30Pro (G88C polymorphism) (Kruger et al., 1998), Glu46Lys (G188A) (Zarranz et al., 2004) and most recently, His50Gly (T150G) (Appel-Cresswell et al., 2013; Proukakis et al., 2013). Certain kindreds with hereditary PD were found to have multiplications of the αsyn locus (Nishioka et al., 2006; Nishioka et al., 2009; Singleton et al., 2003), indicating that αsyn dosage effects could contribute to the development of PD.

αsyn was then quickly discovered to be a key component of LBs (e.g. figure 1B & C) (Baba et al., 1998; Spillantini et al., 1998; Spillantini et al., 1997). This opened new waves of experimentation and many more proteins were found in LBs including: microtubule-associated protein tau (tau), phosphorylated

tau (Arima et al., 1999; Ishizawa et al., 2003), the ubiquitin-binding protein p62 (Kuusisto et al., 2003; Kuusisto et al., 2001; Zatloukal et al., 2002), α-tubulin (Alim et al., 2002), γ-tubulin (McNaught et al., 2002), 14-3-3 (Kawamoto et al., 2002), pericentrin (McNaught et al., 2002), and the ubiquitin-proteasome system components E1 ubiquitin-activating enzyme, PA700 and PA28 (McNaught et al., 2002). Parkin, UbcH7 (Schlossmacher et al., 2002) and synphilin-1 (Wakabayashi et al., 2000) were three proteins found to accumulate exclusively in LBs.

1.1.2 Braak

In 2003, Heiko Braak and others proposed a system for the pathogenesis of PD whereby Lewy pathology begins not in the SN, but rather in the periphery, spreading to the central nervous system (CNS). Subsequent spread within the CNS follows a stereotyped spatiotemporal progression (Braak et al., 2003a; Braak et al., 2003b). This is now commonly referred to as the "Braak hypothesis". Detailed review of the hypothesis and subsequent refinements is beyond the scope of this work, however, brief points merit explanation.

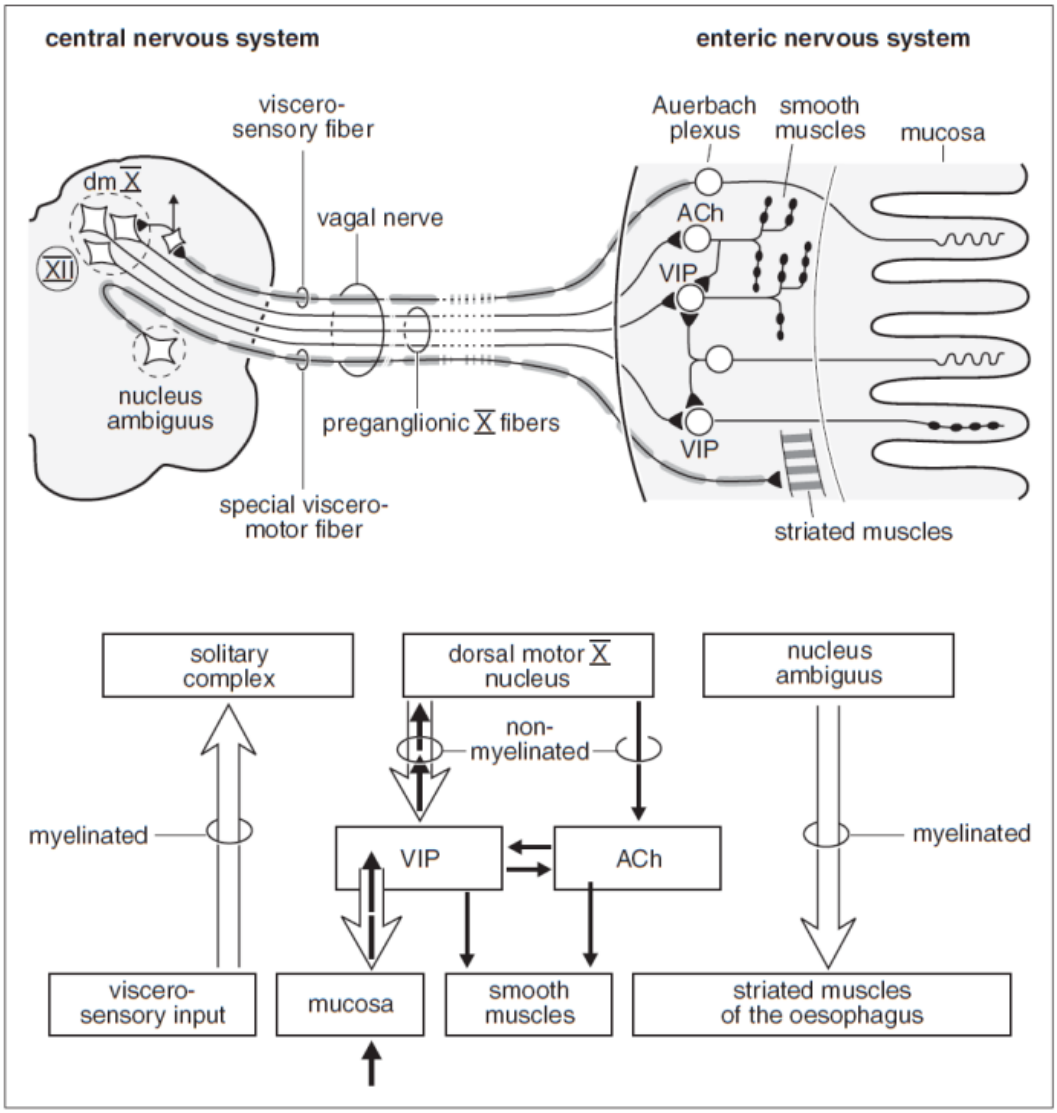
Braak et al. propose that Lewy pathology originates in the olfactory bulb (OB) and the enteric nervous system (ENS) (Braak et al., 2003b). ENS pathology is then suggested to propagate to the medulla, specifically the dorsal motor nucleus of the vagus (dmX) through the vagus nerve (figure 2). Vagal efferents are implicated as they originate in the dmX. Lewy pathology is known to spread retrogradely from axon terminals to cell bodies along microtubules (Freundt et al., 2012; Kramer and Schulz-Schaeffer, 2007; Marui et al., 2002). Afferent vagal fibres originate in the solitary nucleus which remains unaffected in PD. Viscero-motor fibres originating in the nucleus ambiguus end in the striated muscle of the oesophagus and other regions but they do not develop Lewy pathology, possibly due to the thick, myelinated nature of these fibres (Braak et al., 2003a).

Figure 2. Descending vagal pathways to the ENS

(Top) Cartoon of the neural connections between various brainstem nuclei and the ENS

(Bottom) Schema of the normal neural connections (empty arrows & descending black arrows) with the putative pathway of ascending αsyn pathology in short black arrows

Adapted from (Braak et al., 2003b), permission to reproduce found in appendix A


central nervous system
enteric nervous system
viscero-
sensory fiber
Auerbach
plexus
smooth
muscles
mucosa
dm X
vagal nerve
ACh
XII
VIP
nucleus
ambiguus
preganglionic X fibers
VIP
special viscero-
motor fiber
striated muscles
solitary
complex
dorsal motor X
nucleus
nucleus
ambiguus
non-
myelinated
myelinated
VIP
ACh
myelinated
viscero-
sensory input
mucosa
smooth
muscles
striated muscles
of the oesophagus

The dual origin of Lewy pathology in the OB and the ENS has been termed the “dual-hit hypothesis” (Hawkes et al., 2007; Hawkes et al., 2009). The authors of this theory suggest that PD begins concurrently in the OB, with subsequent anterograde transport to and within the CNS, and the ENS with spread as detailed above. The agent initiating the aggregation of αsyn is proposed to be a neurotropic pathogen, likely a virus. The main issue with this is clearly that αsyn pathology has yet to be shown to spread in an anterograde fashion, rendering the spread from the OB questionable. Further to this, any pathogen could only reach the ENS by penetrating the mucosa; this fault is acknowledged by Braak which leads his group to suggest that Lewy pathology may begin in the stomach where the mucosa is often lesioned and has been shown to be susceptible to infections with organisms such as *Helicobacter pylori*. Suggestions of a viral cause for PD may date to 1932 when Lewy noted similarities between LBs and the Negri bodies of rabies (Lewy, 1932).

The presence of lipofuscin or neuromelanin in a neuron is widely considered as requisite for the development of Lewy pathology (Braak and Del Tredici, 2009; Hirsch et al., 1988; Zecca et al., 2006). Light microscopy reports of ENS lipofuscin in the normal gut date from the earliest systematic studies (Dogiel, 1895) and have been complemented by demonstration of accumulation in storage diseases (Itoyama et al., 1978). More recent work using autofluorescence as a lipofuscin marker has attempted to situate the ageing pigment within certain neuronal subtypes in the rat (Corns et al., 2002) and in man (Brehmer et al., 2004). The utility of these studies at mapping susceptibility to lipofuscin development has been fairly limited although it appears that neurocalcin-expressing neurons are resistant to this phenomenon (Itoyama et al., 1978) and nitrergic neurons may present a different pigmentation pattern than non-nitrergic neurons (Brehmer et al., 2004).

1.2 Synucleins

α-synuclein (αsyn) is a 140 amino-acid protein encoded, in humans, by the *SNCA* gene found on chromosome 4 at position 4q21 (Chen et al., 1995; Shibasaki et al., 1995; Spillantini et al., 1995). αsyn was initially described as NACP, the precursor to NAC (non-Aβ component of Alzheimer's disease amyloid) (Ueda et al., 1993). It was later determined that NACP was normally localised to pre-synaptic terminals and ultrastructurally, to synaptic vesicles (Iwai et al., 1995). Based on these findings and sequence homology to rat (*Rattus norvegicus*) and electric ray (genus *Torpedo*) synuclein, NACP was determined to be a human synuclein and was named α-synuclein (Jakes et al., 1994). The synuclein family in humans is composed of three proteins: α-, β- and γ-synuclein; encoded by the *SNCA*, *SNCB* and *SNCG* genes respectively (Jakes et al., 1994; Lavedan et al., 1998). The prototypical synuclein was initially isolated by Maroteaux *et al.* from the electric organ of the Pacific electric ray (*Torpedo californica*) and was thought to be expressed mainly in presynaptic terminals (*syn*) and the nucleus (*nuclein*) (Maroteaux et al., 1988).

β-synuclein (βsyn) is also enriched at presynaptic terminals (Nakajo et al., 1994), however it is not found in LBs (Galvin et al., 1999). In fact, increases in βsyn have been shown to decrease the rate of αsyn aggregation (Hashimoto et al., 2001; Shaltiel-Karyo et al., 2010). In contrast, mutations in βsyn (e.g. P123H) are associated with familial DLB (Fujita et al., 2010).

γ-synuclein (γsyn) was discovered independently in several laboratories in the late 1990s (Buchman et al., 1998; Ji et al., 1997; Lavedan et al., 1998; Surguchov et al., 1999). As with all synucleins, γsyn is abundantly expressed in the brain. γsyn is unique among the family members in its association with neoplasms, specifically of the breast and ovaries (Jia et al., 1999). Overexpression of γsyn in mice does cause neurodegeneration, possibly through downregulation of HSPs or disruption of neurofilaments (Ninkina et al., 2009), however, this has no cognate human condition.

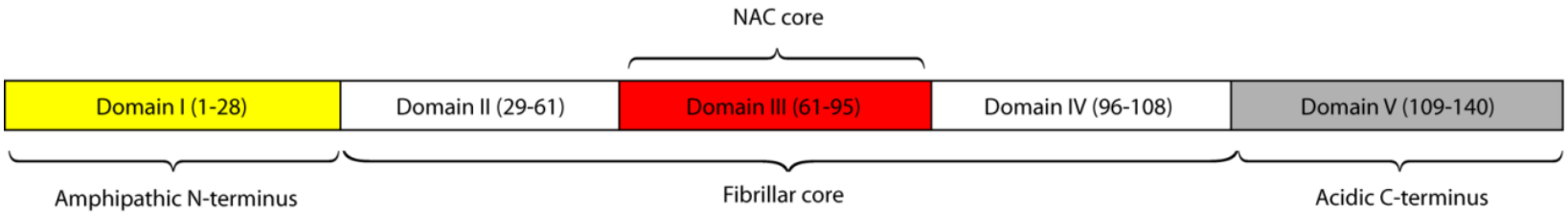
1.2.1 αsyn structure & aggregation

αsyn is generally considered to exist naturally as an unstructured monomer (Weinreb et al., 1996). Recently, some authors have claimed that its true native state is a helical tetramer (Bartels et al., 2011; Bartels and Selkoe, 2013; Dettmer et al., 2013; Wang et al., 2011) although this claim has not been widely held and the experiments generally are not reproducible (Burré et al., 2013; Fauvet et al., 2012). It is further suggested that the tetrameric structure is resistant to aggregation and maintenance of that form would therefore be beneficial.

Despite the orthodox view of monomeric αsyn as an intrinsically disordered protein, there exist intramolecular long-range interactions which are purported to impose a tertiary structure important for auto-inhibition of oligomerisation. Five main functional domains can be discerned (figure 3), grouped in three regions: the N-terminal domain I, central domains II-IV and the C-terminal domain V. Domain I (aa 1-28) has a weak tendency to form α-helices (Jao et al., 2004), domains II-IV form the ordered core of αsyn fibrils with domain III (aa 61-95) constituting the NAC core also found in AD amyloid plaques (Giasson et al., 2001). The acidic domain V (aa 109-140) binds endogenous polyamines (Fernandez et al., 2004).

The carboxy-terminus of αsyn is particularly interesting as regards the auto-inhibition of oligomerisation. Hydrophobic interactions exist between: the area surrounding residue 120 and the amino-terminus at residue 20, residues 110-130 in the C-terminal tail and 85-95 in the NAC core, and between residues 105-115 and 120-130 within the C-terminus (Bertoncini et al., 2005; Dedmon et al., 2005; Zhou et al., 2010). The maintenance of these interactions as well as the acidic charge of the C-terminus are necessary for the prevention of αsyn oligomerisation (Bertoncini et al., 2005; Hoyer et al., 2004; Levitan et al., 2011; Murray et al., 2003). This may relate to the abundance of post-translational modifications described in the C-terminus in diseased states. Tyrosine nitration (Tyr^{125}) and truncation of αsyn at the C-terminus are frequently found in synucleinopathies and have

Figure 3. Functional structure of the αsyn protein


NAC core
Domain I (1-28)
Domain II (29-61)
Domain III (61-95)
Domain IV (96-108)
Domain V (109-140)
Amphipathic N-terminus
Fibrillar core
Acidic C-terminus

been shown to induce fibrillation *in vitro* (Chavarria and Souza, 2013; Oueslati et al., 2010). A polar C-terminal tail is characteristic of chaperone proteins of which αsyn is one (Guha et al., 1998). With other chaperone proteins, the tail interacts with the hydrophobic region of a separate denatured protein, however, in αsyn it acts as an intramolecular chaperone. αsyn truncated at the C-terminus lacks this auto-chaperone property (Souza et al., 2000) and aggregates at an increased rate compared with the full-length counterpart (Crowther et al., 1998; Hoyer et al., 2004; Murray et al., 2003). When the C-terminus is intact, protonation with metal ions (Lowe et al., 2004; Nielsen et al., 2001) or charge neutralisation by polyamine binding (Antony et al., 2003; Goers et al., 2003; Grabenauer et al., 2008) induce αsyn aggregation.

Monomeric αsyn aggregates along a pathway from dimer to oligomer to protofibril and finally fully formed fibrils (Marques and Outeiro, 2012; Stefanis, 2012). Various oligomeric species exist and off-pathway oligomers can also form. These oligomers are the true toxic species of PD and oligomer levels are directly linked to neurotoxicity (Gosavi et al., 2002; Lo Bianco et al., 2002; Winner et al., 2011). In fact, mutants with defects specifically in fibrillation display increased toxicity (Karpinar et al., 2009). The PD-linked αsyn mutants A30P and A53T act by accelerating oligomerisation, not fibrillation (Conway et al., 2000).

Normal αsyn is found predominantly in presynaptic terminals and mitochondria of neurons where it can be cytoplasmic or membrane-bound (Lee et al., 2002; Liu et al., 2009; Zhang et al., 2008). Membrane-bound synuclein may influence the aggregation of the cytoplasmic species. Pathological, aggregated αsyn can be differentiated biochemically from normal αsyn by molecular weight or insolubility to various detergents. Histologically, in brain immunostaining for αsyn normally reveals a punctate pattern consistent with synapses. In Lewy body disease, this normal staining is joined by the concentrated intraneuronal or intraneuritic immunoreactivity associated with LBs and LNs

respectively (e.g. figure 1C). Pathological αsyn accumulates initially at the presynapse in a manner which cannot be differentiated visually from normal αsyn using conventional techniques (Kramer and Schulz-Schaeffer, 2007; Schulz-Schaeffer, 2010; Tanji et al., 2011; Tanji et al., 2010). Pathological αsyn, however, is resistant to degradation by proteinase K, similar to pathological prion protein. Therefore, treating tissue sections with proteinase K prion prior to αsyn immunostaining allows the revelation specifically of abnormally accumulated αsyn without the background neuropil staining of normal αsyn (e.g. figure 1B). The conformational basis for proteinase K resistance is unclear but given that proteinase K cleaves after hydrophobic amino acids (Ebeling et al., 1974) and the wholesale sequestration of the hydrophobic central domain in αsyn fibrils, it is conceivable that proteinase K simply cannot access as many hydrophobic cleavage sites in aggregated αsyn.

1.2.2 "Prion-like" transmission

The spread of misfolded protein to the CNS via the ENS as suggested by Braak is not an idea born in the world of Lewy body disease. Indeed the hypothesis takes root in the field of prion disease. Prion disease in humans results from the aberrant folding of the normal cellular protein PrP^{C}. Innocuous in its native form, the misfolded variant (PrP^{Sc}) aggregates forming amyloid plaques and prompting an aninflammatory astrogliosis (Mabbott and MacPherson, 2006). The unique aspect of PrP^{Sc}, however, is its autocatalytic ability. That is, PrP^{Sc} alone is sufficient to induce the transformation of PrP^{C} to another molecule of PrP^{Sc} (Cohen et al., 1994; Come et al., 1993; Pan et al., 1993).

The prion diseases which include Creutzfeldt-Jakob disease (CJD), kuru, scrapie and chronic wasting disease often take hold following the ingestion of diseased, PrP^{Sc}-containing tissue such as beef in the case of variant CJD (vCJD). Following ingestion, the PrP^{Sc} found in the offending meal takes the obvious route to the intestines (Mabbott and MacPherson, 2006; Natale et al., 2011). In animal models, once PrP^{Sc} reaches the ileum it can be taken up and cause PrP^{Sc} to be found in lymphoid structures known

as Peyer's patches. In human cases of vCJD, lymphoid follicles in both the tonsils and the vermiform appendix show accumulation of PrP^{Sc} (Hilton et al., 1998; Hilton et al., 2002; Hilton et al., 2004; Joiner et al., 2002). This appendiceal prion protein, along with accumulations in ileal Peyer's patches, has been implicated in the oral to CNS spread of prion disease.

In prion disease, PrP^{SC} is taken up from the lumen by M-cells and/or bone-marrow-derived dendritic cells (BMDC) and then spreads to the gut-associated lymphoid tissue. Proteins taken up by M cells are transported to the intra-epithelial pocket on the abluminal side (Heppner et al., 2001). From there, prion protein can be taken up by macrophages or dendritic cells. BMDCs play an essential role in the gut-to-CNS spread of PrP^{Sc}. BMDCs can take up PrP^{Sc} from the intra-epithelial pocket or directly from the gut lumen by extending dendrites between the tight junctions of epithelial cells (Liu and MacPherson, 1993; MacPherson and Liu, 1993). PrP^{Sc} within BMDCs is directed to one of two fates: (1) Entry into lysosomes with rapid degradation into short peptides and antigenic presentation in concert with MHC class II or (2) survival in a native state (Huang et al., 2000; Luhr et al., 2004; Luhr et al., 2002; Mohan et al., 2005; Wykes et al., 1998). PrP^{Sc} which survives in its infectious state can spread from BMDCs to other cells, where it catalyses the formation of more PrP^{Sc}. The prevailing theory currently is that BMDCs migrating from the epithelium transfer PrP^{Sc} to follicular dendritic cells (FDC) in lymphoid follicles (Huang et al., 2002). The absence of Peyer's patches impairs or at least delays spread of prion disease to the CNS indicating that GALT is necessary or eminent in this propagation (Prinz et al., 2003b).

The exact route of neuroinvasion remains unclear in prion disease (and PD). Direct invasion is possible in nerve fibres that closely circumscribe intestinal epithelium (Jeffrey et al., 2006). Also, PrP^{Sc}-containing follicular dendritic cells in appendiceal lymphoid follicles and ileal Peyer's patches have

been shown by electron microscopy to directly contact ENS nerve fibres (Chiocchetti et al., 2008; Defaweux et al., 2005; Prinz et al., 2003a).

ENS Lewy pathology has been confirmed experimentally to follow the vagal route in mice (Pan-Montojo et al., 2010; Pan-Montojo et al., 2012), however, there has been little progress in man since the idea was proposed over a decade ago.

What has developed recently is our understanding of the auto-catalytic role of fibrillar αsyn in the propagation of αsyn pathology. The initial evidence for cell-to-cell spread of αsyn pathology was derived from autopsy studies demonstrating that that fetal mesencephalic stem cells transplanted into PD patients went on to develop LBs despite having a true age of 16 years at most (Braak and Del Tredici, 2008; Kordower et al., 2008; Li et al., 2008; Li et al., 2010). In 2009, it was shown by Virginia Lee's lab that exogenous fibrils of recombinant αsyn can induce αsyn aggregation, phosphorylation and ubiquitination in cultured cells that over-express αsyn (Luk et al., 2009). In 2011 they expanded this to primary neurons (Volpicelli-Daley et al., 2011). Rapidly the Lee lab demonstrated that intracerebral injections of recombinant fibrillar αsyn can cause spreading Lewy pathology in transgenic (Luk et al., 2012b) and wild-type mice (Luk et al., 2012a). Others have since shown that brain homogenate from mice carrying mutant human αsyn is sufficient to induce disease (Mougenot et al., 2012) and that injections of soluble αsyn (cf. PrP^{C}) are non-pathological whereas fibrillar αsyn (cf. PrP^{Sc}) induces Lewy pathology (Masuda-Suzukake et al., 2013). The latter also demonstrated that sarkosyl-insoluble brain extract from human DLB was sufficient to induce LB/LN formation. This despite the fact that the exogenous αsyn was undetectable after one week - three months before disease onset (Masuda-Suzukake et al., 2013).

1.3 Organisation of the enteric nervous system

The enteric nervous system is a network of neural plexus[1] innervating the gastrointestinal tract from as far rostral as the upper oesophagus to the internal anal sphincter as well as the pancreaticobiliary system (figure 4). These plexus contain both sensory and motor fibres and are responsible for most enteric functions including motility, secretion and absorption (Furness, 2006).

The earliest reports of neural elements in the gut come from the work of Robert Remak who, in 1847, described the innervation of the gastrointestinal tract of the rooster and proposed that this demonstrated the existence of a "*selbständiges Darmnervensystem*" or independent intestinal nervous system (Remak, 1847). This was expanded upon when Remak later reported the presence of ganglia in the stomach wall of man and other mammals (Remak, 1852b). By analogy with other regions of vagal innervation in the heart and lungs, Remak was also able to demonstrate vagal efferents leading to ganglia in the muscular and "glandular" areas of the stomach in a series of animals, supposing therefore both motor and secretory functions for this innervation (Remak, 1852a).

Most of the early developments that followed in the field also came from Germanic anatomists. Despite Remak's considerable body of work, it was left to Georg Meissner to first describe the plexiform arrangement of nerve fibres in the *tela submucosa* now known as Meissner's plexus or *plexus submucosus internus* (Meissner, 1857). This work was expanded by Billroth (Billroth, 1858).

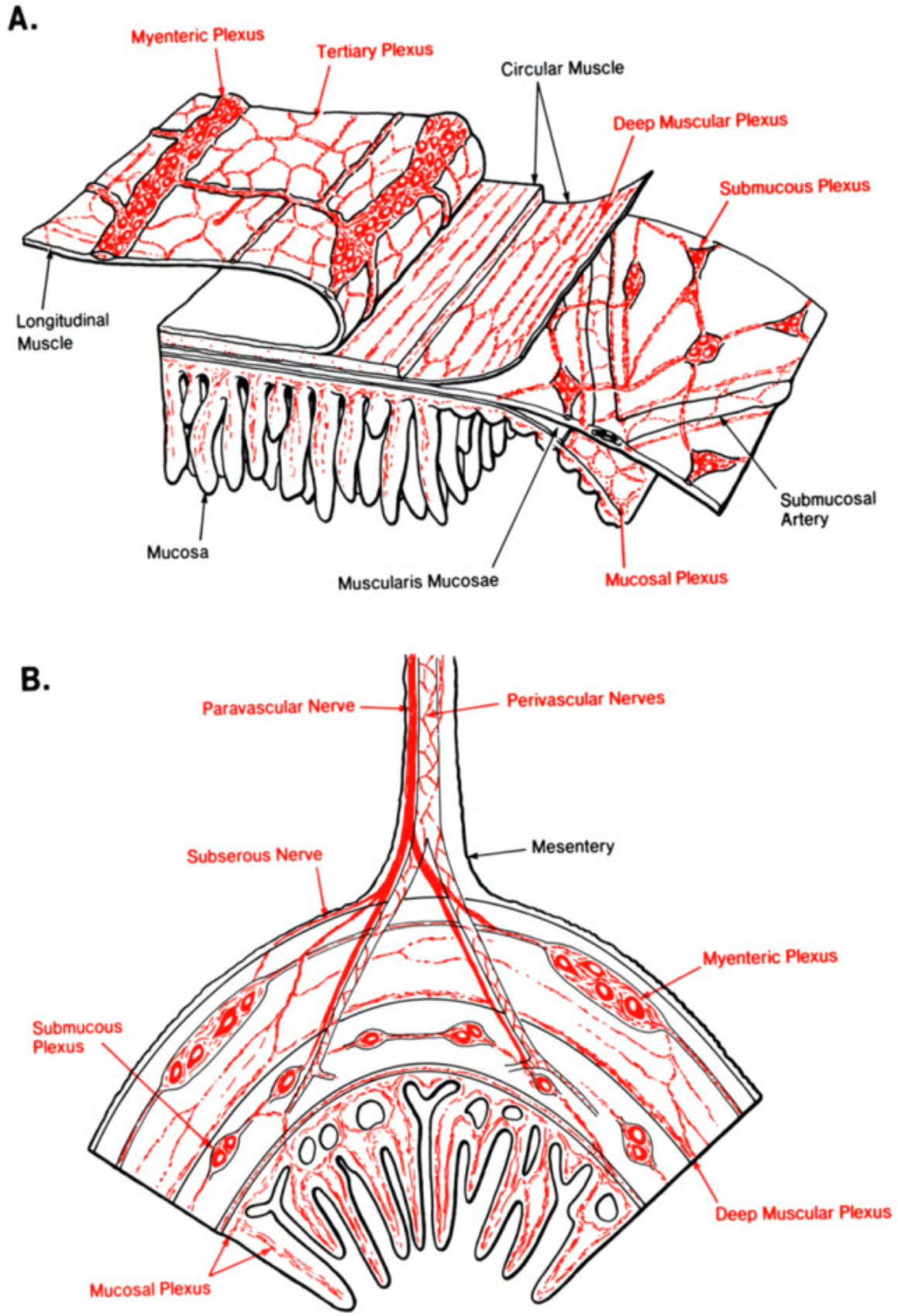
Leopold Auerbach described his eponymous plexus, also known as the *plexus myentericus*, between the circular muscle and longitudinal muscle layers in vertebrates in 1862 (Auerbach, 1862a; Auerbach, 1862b). The Ukrainian histologist Arnold Schabadasch later described a third nerve plexus, this also

[1] Note that the proper plural for "plexus" is also "plexus" and not "plexuses" or "plexi" (Wedel et al., 1999; Young, 1810)

Figure 4. Organisation of the ENS

Plexus of the ENS and other GI tissues dissected (A) and *in situ* (B).

Adapted from (Furness and Costa, 1980). Copyright permission in appendix B


A.
Myenteric Plexus
Tertiary Plexus
Circular Muscle
Deep Muscular Plexus
Submucous Plexus
Longitudinal Muscle
Submucosal Artery
Mucosa
Muscularis Mucosae
Mucosal Plexus
B.
Paravascular Nerve
Perivascular Nerves
Mesentery
Subserous Nerve
Myenteric Plexus
Submucous Plexus
Deep Muscular Plexus
Mucosal Plexus

in the submucosa but distinct from Meissner's plexus (Schabadasch, 1930). This plexus is found in the outer submucosal, proximal to the circular muscle layer, whereas Meissner's plexus is adjacent to the muscularis mucosa. Unfortunately, Schabadasch's work was largely unrecognised as, misreading an early work by Jakob Henle, he mistakenly associated this plexus with the latter scientist – naming his own discovery "Henle's plexus", also known now as the *plexus submucosus externa*.

The functional properties of the ENS were described by Bayliss & Starling who defined peristalsis – calling it the "law of the intestine" – and demonstrating that it could occur in the absence of CNS innervation (Bayliss and Starling, 1899; Bayliss and Starling, 1901).

1.3.1 Mucosal innervation

The intestinal mucosa (comprising the *lamina epithelialis* (epithelium), the *lamina propria mucosæ* and the *lamina muscularis mucosæ)* is the adluminal layer of the gastrointestinal tract. It is responsible mainly for absorption and secretion. In contrast to the detailed studies on the innervation of the outer layers of the intestinal wall, the innervation of the mucosa has always been less precisely defined. Reports of mucosal innervation are contemporary to other plexus (Berkley and Baltimore, 1893; Billroth, 1858; Drasch, 1881) but the origins of the fibres were never clear likely because they are so heterogeneous. Mucosal nerve fibres can originate in the myenteric plexus (Reiche and Schemann, 1999) and both submucosal plexus (Hens et al., 2000; Hens et al., 2001; Porter et al., 1999; Timmermans et al., 2001) as well as the mucosal plexus.

The mucosal plexus itself is a ganglionated plexus known since 1881 (Drasch, 1881) while more work a century later described various subplexus within the mucosa (Balemba et al., 1998; Balemba et al., 2002; Furness and Costa, 1980). The most recent of these describes seven subplexus: lamina muscularis mucosae, outer proprial, interglandular proprial, inner proprial, villous, subepithelial and

perivascular (Balemba et al., 2002). The villous subplexus is obviously absent in the large intestine which lacks villi.

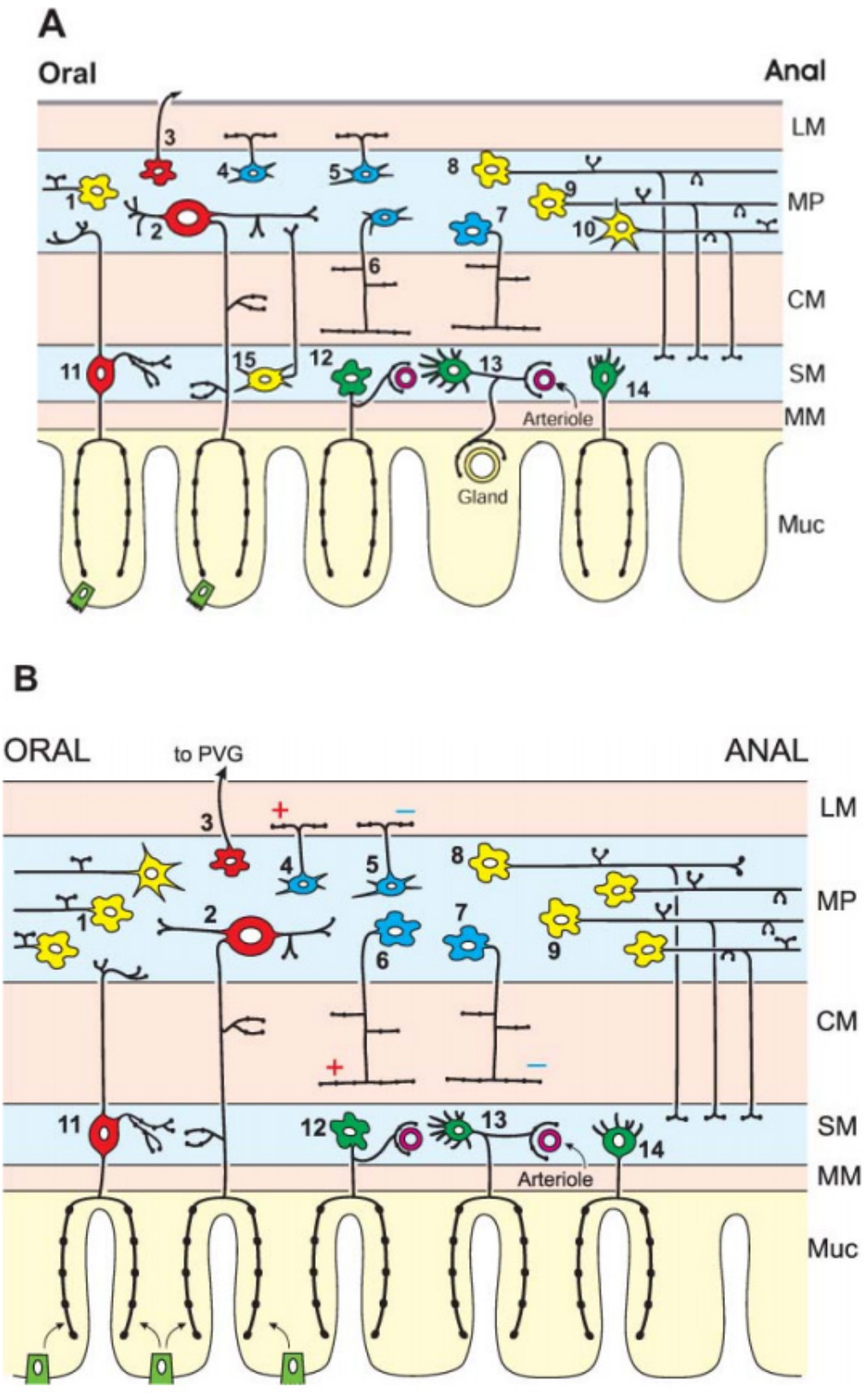
In the guinea pig, four separate neuronal types are known to innervate the mucosa (figure 5): submucosal intrinsic primary afferent neurons (IPANs), cholinergic and non-cholinergic (VIPergic) secretomotor vasodilator neurons as well as cholinergic secretomotor (non-vasodilator) neurons (Furness, 2006). The secretomotor subtypes have largely similar roles. The combination of vasodilation and secretomotor signalling in neurons allows the coupling of these functions. Physiologically this means that at the instigation of fluid secretion, the relevant tissue regions are immediately backfilled with fluid supplied from the recently dilated vessels.

IPANs are Dogiel type II neurons innervating the mucosa which relay information on mechanical distension of the gut and chemicals reaching the mucosa. These neurons can have their cell bodies in the gut wall (intestinofugal neurons), in dorsal root ganglia (spinal afferents) or in vagal nuclei (vagal afferents). Chemically, IPANs recognise and respond to signals such as pH changes, short-chain fatty acids, serotonin and glucose (Bertrand et al., 1997; Kirchgessner et al., 1996; Kunze et al., 1995). Distortion of the mucosa with nitrogen gas or balloon results in neuronal activity detectable by c-Fos staining (action potentials) and styryl dye uptake for synaptic vesicle cycling (Kirchgessner et al., 1996; Kirchgessner et al., 1992). Binding of isolectin B_4 (IB4) is a property reserved - in the ENS - to IPANs (Furness, 2006; Hind et al., 2005; Thacker et al., 2006). Given that IB4 stains only nociceptive afferents in dorsal root and trigeminal ganglia, a similar role in pain sensation has been attributed to IPANs in the ENS. Functionally, this ascribes a role to IPANs in the forceful expulsion (i.e. diarrhoea) of noxious infectious agents or toxins (e.g. cholera toxin or rotavirus) both to protect the host from injury or as a propagatory mechanism for infectious agents (Lundgren, 2002; Lundgren and Jodal, 1997; Lundgren et al., 2000).

Figure 5. Subtypes of neurons in the ENS and their distribution in the guinea pig
The distribution of ENS neuron subtypes as mapped in the guinea pig small intestine (A) and colon (B). Four neuronal subtypes innervate the mucosa: submucosal intrinsic primary afferent neurons (11), non-cholinergic secretomotor/vasodilator neurons (12), cholinergic secretomotor/vasodilator neurons (13) and cholinergic secretomotor (non-vasodilator) neurons (14).

Adapted from (Furness, 2006). Copyright reproduction permission found in appendix C

LM – longitudinal muscle, MP – myenteric plexus, CM – circular muscle, SM – submucosa, MM – muscularis mucosae, Muc - mucosa


A
Oral
Anal
LM
MP
CM
SM
MM
Muc
Arteriole
Gland
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
B
ORAL
to PVG
ANAL
LM
MP
CM
SM
MM
Muc
Arteriole
+
−

Mucosal nerve fibres are closely apposed to both entero-endocrine cells and immune cells in the stomach and intestine (Furness, 2006; Schemann et al., 2001). Innervation is difficult to demonstrate visually but isolation of the mucosa or submucosa allows for functional studies of their differential effects on endocrine cells (Saffouri et al., 1984; Schubert et al., 1992).

Such studies have also allowed for analysis of the flow of sodium and chloride – therefore water – across the mucosal membrane (Hubel, 1978; Hubel and Shirazi, 1982). This is mediated mainly by VIP-immunoreactive fibres which are abundant in the mucosa (Balemba et al., 2002) and intrinsic to the submucosal/mucosal plexus, not degenerating after vagotomy and intestinal myomectomy (Costa and Furness, 1983; Keast et al., 1984). Retrograde labeling shows that this innervation of the mucosa is fairly localised with cell bodies lying not more than 3-4 mm along the oral-anal axis from their terminals (Porter et al., 1999).

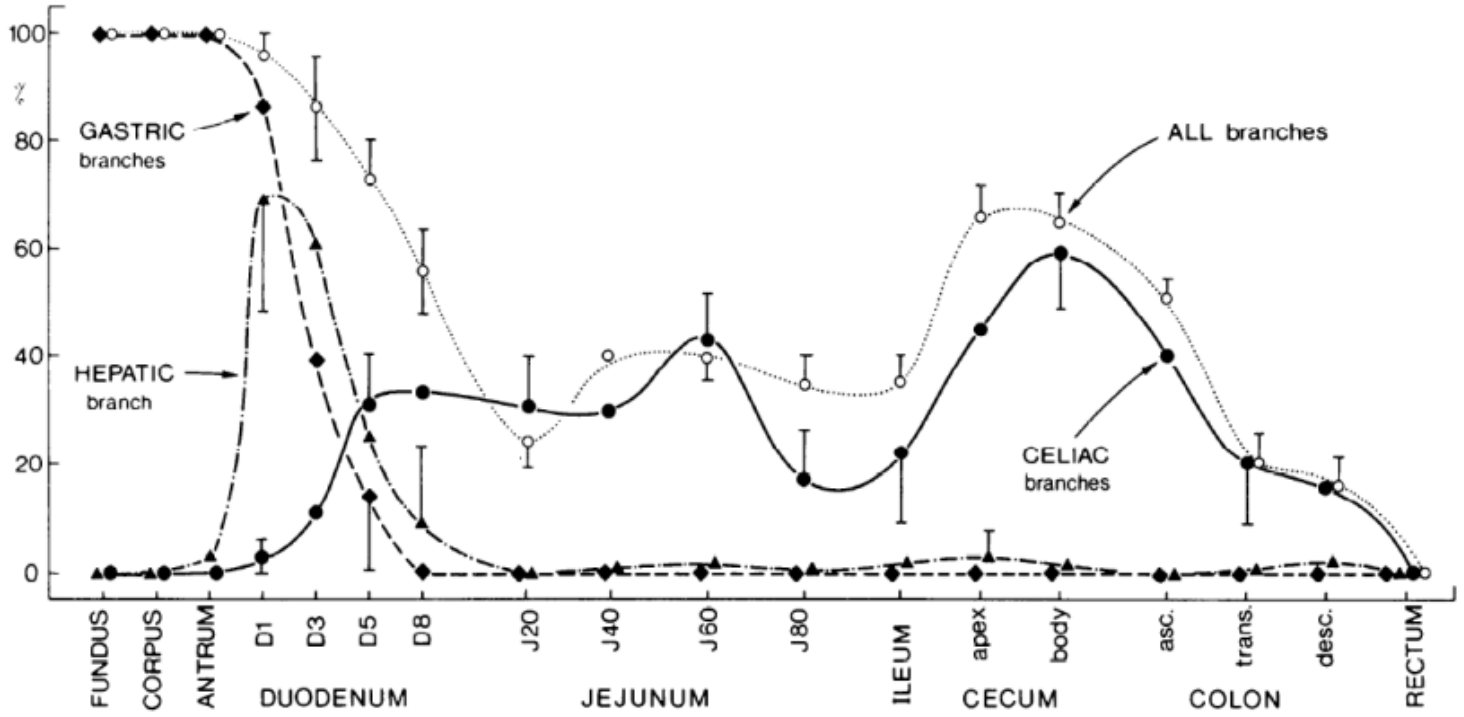
1.4 Aims of this work

ENS Lewy pathology spreads to the dmX via the vagus nerve (Pan-Montojo et al., 2010; Pan-Montojo et al., 2012), therefore regions receiving especially dense vagal innervation are more likely to be the origin of dmX pathology. Studies in rodents have determined that the stomach and caecum, cf. mammalian caecum & appendix (Smith et al., 2009), receive the densest levels of vagal innervation (Altschuler et al., 1991; Berthoud et al., 1991; Berthoud et al., 1990; Cao et al., 2007; Holst et al., 1997) (figure 6). In light of the concept that αsyn aggregation begins in axon terminals in the ENS, and that this is initiated by an environmental pathogen, it stands to reason that different regions of the gastrointestinal tract should be differentially vulnerable to αsyn aggregation depending on their density of αsyn -containing axon terminals. Thus, we sought to determine the normal distribution of αsyn in and surrounding vagal-innervation-rich regions of the GI tract. To achieve this goal, archived surgically-excised tissue was obtained from neurologically intact individuals: stomach from gastrectomies and terminal ileum, appendix and ascending colon from right hemicolectomies.

Figure 6. Percentage of myenteric ganglia receiving vagal innervation

Percentage (means ± SE) of myenteric ganglia receiving anterogradely DiI-labelled vagal terminals at indicated locations of GI tract in control rats with all abdominal vagal branches intact (n=8), and in experimental rats with either only the 2 gastric branches intact (n=5), the hepatic branch intact (n=6) or the 2 coeliac branches intact (n=6). # of enteric neurons contacted by vagal terminals in a given ganglion is not taken into account in this analysis but is generally decreasing at more anal locations.

Adapted from (Berthoud et al., 1991), no permission required for reproduction in thesis


100
%
80
60
40
20
0
GASTRIC
branches
HEPATIC
branch
ALL branches
CELIAC
branches
FUNDUS
CORPUS
ANTRUM
D1
D3
D5
D8
J20
J40
J60
J80
ILEUM
apex
body
asc.
trans.
desc.
RECTUM
DUODENUM
JEJUNUM
CECUM
COLON

Chapter 2: Materials & methods

2.1 Specimen acquisition

Formalin-fixed, paraffin-embedded blocks were retrieved from archived surgical specimens in the Department of Pathology and Laboratory Medicine of The Ottawa Hospital. These specimens were accrued from patients with no diagnosed synucleinopathy who underwent right hemicolectomy (n=10, average age=73) or subtotal gastrectomy (n=10, average age=63). Pertinent clinical and demographic information is presented in Table 1. Only non-lesional tissue was used. This study was conducted with the approval of The Ottawa Hospital Research Ethics Board.

2.2 Immunohistochemical staining

Sections of 5 µm thickness were cut from formalin-fixed, paraffin-embedded tissue blocks. Staining was performed using the automated Leica Bond Max stainer (Leica Microsystems; Concord, Ontario, Canada). Sections were deparaffinised and heat-induced epitope retrieval (HIER) was achieved by incubating the sections at 98°C for 20 minutes in Bond Epitope Retrieval Solution 2 (Leica), a pH 9.0 EDTA-based solution. Endogenous peroxidase activity was blocked using the Bond Polymer Refine Detection kit (Leica) following manufacturer's instructions. Immunohistochemical staining was performed with the antibodies listed in Table 2 using the indicated dilutions and conditions. Staining was concluded using the Bond Polymer Refine Detection kit. The chromogen was 3,3'-diaminobenzidine (DAB) and haemotoxylin was used as a counterstain. CNS tissue was used as a same-slide positive control. Negative control sections were processed in the same manner but with the primary antibody omitted.

Table 1. Patient information

Demographic information and pathological diagnosis of patients for gastrointestinal tissues.

Patient number	Procedure	Sex	Age (years)	Neurological disease	Diagnosis
Terminal ileum, appendix & ascending colon					
1	Right hemicolectomy	F	67	-	Adenocarcinoma
2	Right hemicolectomy	M	90	-	Adenocarcinoma
3	Right hemicolectomy	F	68	-	Adenocarcinoma
4	Right hemicolectomy	M	81	-	Adenocarcinoma
5	Right hemicolectomy	M	76	-	Adenocarcinoma
6	Right hemicolectomy	M	63	-	Adenocarcinoma
7	Right hemicolectomy	F	83	-	Adenocarcinoma
8	Right hemicolectomy	M	82	-	Adenocarcinoma
9	Right hemicolectomy	F	30	-	Adenocarcinoma
10	Right hemicolectomy	M	91	-	Adenocarcinoma
Stomach					
11	Subtotal gastrectomy	F	48	-	Carcinoma
12	Subtotal gastrectomy	M	62	-	Gastrointestinal stromal tumour
13	Partial gastrectomy	F	56	-	No pathology
14	Subtotal gastrectomy	M	70	-	Carcinoma
15	Distal gastrectomy	F	70	-	Lymphoma
16	Oesophagogastrectomy	F	74	-	Oesophageal cancer
17	Total gastrectomy	M	80	-	Gastric carcinoma
18	Proximal gastrectomy	M	67	-	Oesophageal cancer
19	Sleeve gastrectomy	F	45	-	Chronic gastritis
20	Wedge gastrectomy	F	64	-	Hiatal hernia

Table 2. Antibodies used

Antigen [Clone]	Source	Dilution
α-synuclein [LB509]	Inter Medico Mouse monoclonal #18-0215	1:50 (IHC)
α-synuclein [MJFR1, previously 12.1]	Abcam (Epitomics) Rabbit monoclonal #ab138501	1:100 (IF)
CD68 [KP1]	Dako Mouse monoclonal #M0814	1:25
Peripherin	Biosensis Chicken polyclonal #C-1339-50	1:400
Synaptophysin [SY38]	Dako Mouse monoclonal #M0776	1 :20
UCH-L1 [13C4/I3C4]	Abcam Mouse monoclonal #ab8189	1 :10
VIP [H-6]	Santa Cruz Biotech Mouse monoclonal #sc-25347	1 :100
Goat anti-Mouse IgG Alexa Fluor 594	Invitrogen #A-11032	1:500
Goat anti-Rabbit IgG Alexa Fluor 488	Invitrogen #A-11034	1:500
Goat anti-Rabbit IgG Alexa Fluor 594	Invitrogen #A-11012	1:500
Goat anti-Chicken IgY Alexa Fluor 488	Invitrogen #A-11039	1:500

Some peroxidase staining was performed manually with all reagents other than primary antibodies from the VECTASTAIN ABC Mouse IgG kit (Vector Laboratories). In this case, 5 µm sections were deparaffinised and rehydrated through xylenes and an ethanol series. Following rehydration, HIER was effected by heating sections in solution of sodium citrate pH 6.0 for 10 minutes in a 700W microwave oven and allowing heated sections to cool for 30 minutes at room temperature. After antigen retrieval, blocking was performed by incubating for 30 minutes with 1.5% normal horse serum diluted in phosphate-buffered saline pH 7.4 (PBS). Primary antibodies were diluted in 1.5% normal horse serum as listed in Table 2 and incubated overnight at 4^{o}C. After washing in PBS, biotinylated horse anti-mouse IgG was applied at a dilution of 1:200 for 45 minutes at room temperature. Sections were then developed using the avidin-biotin complex method with DAB as the chromogen.

The immunohistochemical procedure of Beach et al. for the detection of pathological αsyn was not used as this study focused on the detection of normal αsyn (Beach et al., 2008).

Immunofluorescence was performed manually. Deparaffinisation and antigen retrieval were carried out as in manual peroxidase staining. After antigen retrieval, blocking was performed by incubating for 30 minutes with 5% normal goat serum (Santa Cruz Biotechnologies; Santa Cruz, California, USA) diluted in phosphate-buffered saline pH 7.4 (PBS). Primary antibodies were diluted in 5% normal goat serum as listed in Table 2 and incubated overnight at 4^{o}C. For double labeling, antibodies to different antigens were applied simultaneously. After washing in three changes of PBS, secondary antibodies as listed in Table 2 were applied for one hour. Slides were again washed in three changes of PBS and to diminish autofluorescence, slides were incubated for 10 minutes in 5 mM $CuSO_4$ in 50 mM ammonium acetate pH 5.0 (Schnell et al., 1999). Following a brief PBS wash, slides were mounted using VECTASHIELD HardSet Mounting Medium with DAPI (#H-1500, Vector Laboratories; Burlington, Ontario, Canada). No labeling was observed when primary antibodies were omitted from this

protocol. In co-labeling experiments, no cross-species reactivity was observed when secondary antibodies to the incorrect species were used.

2.3 Analysis

Sections processed using the immunoperoxidase technique were scanned using a Scanscope CS system (Aperio; Vista, California, United States of America) and analysed using Imagescope (Aperio). Immunofluorescent sections were imaged on an Axioskop II microscope (Carl Zeiss Canada; Toronto, Ontario). Images were acquired through a Zeiss Axiocam and processed using Zeiss Axiovision.

2.4 Animal work

Mice with murine αsyn expression ablated by introduction of a neomycin-resistance gene in place of exons 4 & 5 (αsyn-null) were obtained from Dr Robert Nussbaum (Cabin et al., 2002). Mice with expression of a human A53T αsyn transgene driven by the mouse PrP prion promoter on an αsyn-null background were also obtained from Dr Nussbaum (Cabin et al., 2005; Kuo et al., 2010). Caeca from five-month old mice were excised and processed similarly to human tissue with immersion fixation in 10% neutral buffered formalin following by embedding in paraffin. All animal work was approved by the University of Ottawa Animal Care Committee.

2.5 Antibody characterisation

Two anti-αsyn monoclonal antibodies were used: LB509 for peroxidase-based immunohistochemistry and MJFR1 for immunofluorescent double-labelling. LB509 is a mouse monoclonal antibody raised against Lewy bodies purified from the brains of patients with dementia with LBs (Baba et al., 1998). The epitope recognised by LB509 is amino acids 115-122 of human αsyn (Jakes et al., 1999). Its specificity for αsyn has been characterised previously in the CNS by immunoblotting (Baba et al., 1998) and it has been used for immunohistochemistry in the human ENS (Annerino et al., 2012). MJFR1 (previously 12.1) is a rabbit monoclonal antibody raised against full-length human αsyn and

recognises amino acids 118-123 (Mollenhauer et al., 2012a; Mollenhauer et al., 2012b). Its specificity for αsyn by immunohistochemistry is demonstrated here.

The anti-synaptophysin clone SY38 is a mouse monoclonal antibody raised against purified synaptic and coated vesicles of bovine brain (Wiedenmann and Franke, 1985) and binds synaptophysin between amino acids 269-299 (Knaus and Betz, 1990). It has been shown in knockout mice to be specific to SYP by immunoblotting (Eshkind and Leube, 1995) and has been used to mark axonal varicosities by immunohistochemistry in the human (Harrington et al., 2010) and rodent (Liu et al., 2008) ENS.

The mouse monoclonal antibody 13C4 (also I3C4) recognises the neuronal protein UCH-L1 (also PGP9.5) (Day and Thompson, 1986). It displays immunoreactivity within all nervous tissues including enteroendocrine (argentaffin) cells not recognised by some UCH-L1 antibodies (Wilson et al., 1988).

The mouse anti-CD68 clone KP1 was raised against a lysosome-enriched fraction of human lung and it is able to immunoprecipitate a 110 kDa protein band from radio-labeled human spleen homogenate (Pulford et al., 1989). However, KP1 is unable to immunoprecipitate CD68 from transiently-transfected COS cells indicating that the epitope is likely dependent on a specific glycosylation pattern (Holness and Simmons, 1993).

Vasoactive intestinal peptide (VIP) was detected using the mouse monoclonal IgG_{2b} clone H-6 raised against amino acids 1-95 of the human protein (manufacturer's datasheet). It has been used previously for IHC in the human ENS (Jaafari et al., 2008).

The chicken polyclonal anti-peripherin antibody was raised by Dr Gerry Shaw (University of Florida) against recombinant full-length rat peripherin isolated from *Escherichia coli* (Sekerkova et al., 2008).

This antibody specifically recognises a 57 kDa band by western blotting (manufacturer's information) and labels filamentous structures in peripheral nerves of rats (Sekerkova et al., 2008)

Chapter 3: Results

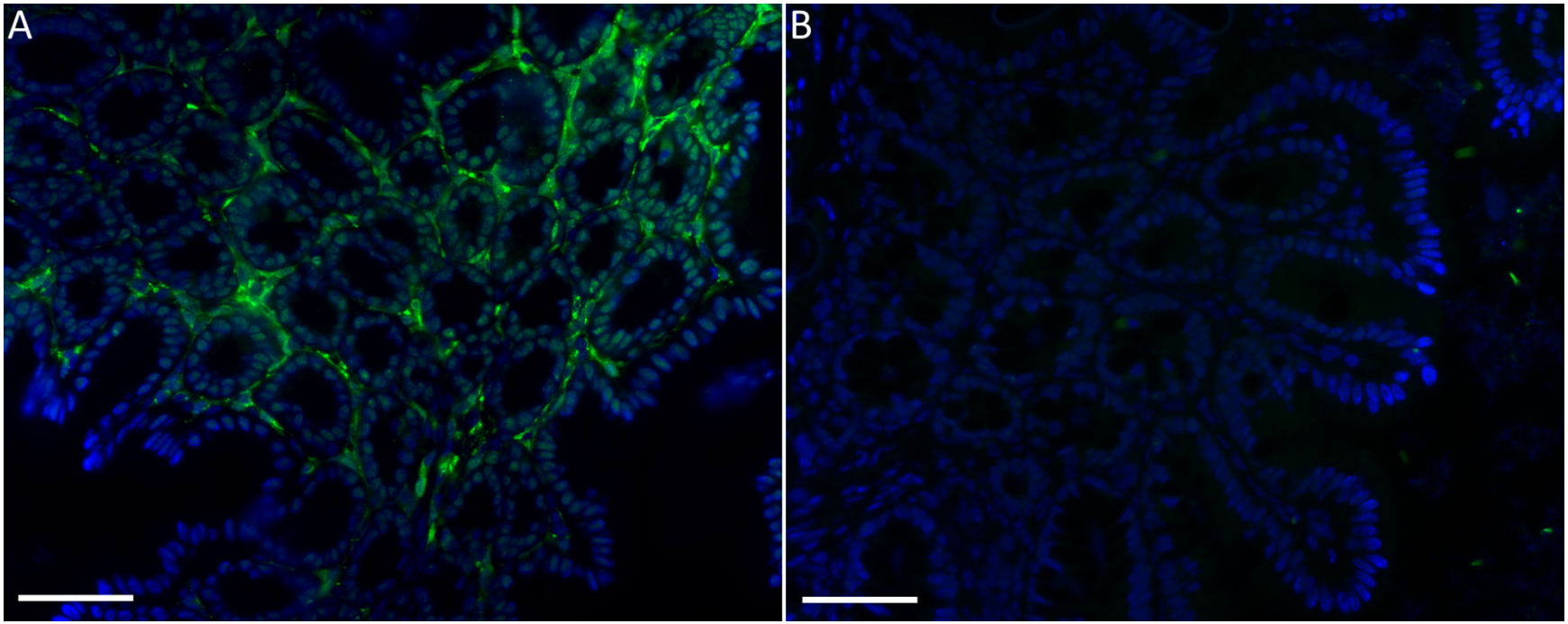
3.1 Characterisation of the anti-αsyn clone MJFR1 in immunohistochemistry

It is essential to confirm the specificity of antibodies to their target prior to their use. This is especially important in IHC where cross-reactivity or background staining cannot be differentiated from true staining (unlike, for example, western blotting where molecular weight can aid). Before pursuing the use of MJFR1 for IHC, I determined its reactivity on tissue from αsyn transgenic and knockout mice. The anti-αsyn rabbit monoclonal clone MJFR1 was developed in the laboratory of Dr Michael Schlossmacher. Under the previous appellation 12.1, its utility has been demonstrated in ELISA (Mollenhauer et al., 2012a; Mollenhauer et al., 2013). αsyn-null mice were developed by Dr Robert Nussbaum (Cabin et al., 2002) and αsyn-A53T mice expressing human αsyn with the pathogenic A53T mutation on the αsyn-null background were also created in the Nussbaum lab (Cabin et al., 2005; Kuo et al., 2010). The epitope of MJFR1 excludes the A53T mutation site therefore the αsyn-A53T mice were taken as representative of the reactivity of MJFR1 towards normal human αsyn. The use of tissue from knockout animals is a particularly effective method for antibody validation in IHC (Saper, 2005; Saper and Sawchenko, 2003).

MJFR1 staining of formalin-fixed, paraffin-embedded caecum from αsyn-A53T mice revealed a fine reticular network of αsyn-immunoreactive nerve fibres in the mucosal lamina propria (figure 7A). Staining of identical tissue from αsyn-null mice showed a complete lack of signal (figure 7B). Nuclear staining of enterocytes was also lost in the KO animal. Despite some controversy, it has been definitively shown that αsyn has a nuclear localisation in addition to its well-known axon terminal location (Andringa et al., 2003; Yu et al., 2007) and this is confirmed again here in the mouse caecum.

Figure 7. Characterisation of the anti-αsyn clone MJFR1.
Immunofluorescent staining of caecal mucosa from humanised A53T αsyn (A) and αsyn-null mice (B). MJFR1 clearly marks nerve fibres in the lamina propria of the humanised animal as well as reacting with enterocyte nuclei (A). No specific reactivity was noted for MJFR1 in αsyn-null mice although some fluorescence, likely endogenous, is noted in the intestinal lumen (B).

Scale bars = 50 μm


A
B

3.2 αsyn expression in enteric muscularis propria and submucosa

Previous studies on αsyn expression in the ENS have focused on the classic duo of Auerbach's & Meissner's plexus. To validate our approach and to compare previous reports (all excluding appendix) to our new data, I examined αsyn expression within Auerbach's & Meissner's plexus in normal human stomach, terminal ileum, vermiform appendix and ascending colon.

Almost no αsyn-immunoreactive perikarya were visible in the muscularis propria of stomach, similar to reports in rat of <3% positivity in myenteric ganglia (Phillips et al., 2008). αsyn positivity in ganglia was almost exclusively found in axons terminals synapsing on neurons and possibly glial cells (figure 8). Roughly half of submucosal (Meissner's) neurons in the stomach expressed αsyn (figure 8A).Within the smooth muscle, various axons could be found containing αsyn-positive varicosities *en passant*, possibly originating from those rare αsyn-positive neurons.

Consistent with Phillips et al., proceeding anally resulted in an increase in αsyn-positive ganglionic cells (Phillips et al., 2008). The terminal ileum had two thirds of submucosal neurons and one third of myenteric neurons expressing αsyn (figure 8C&D). Even further along, the large intestine evidences a large shift with nearly 100% of submucosal perikarya and 75% of those in Auerbach's plexus containing αsyn. In both regions, many intensely stained αsyn punctae were found on ganglionic cell bodies, likely representing axon terminals. (figure 8E & F)

Between the ileum & ascending colon, the vermiform appendix was unexpectedly rich in ganglionic αsyn positivity. All ganglionic neurons in mucosal ganglia (figure 9A) - discussed below - in all ten patients examined, as well as all submucosal neurons (figure 9B) were αsyn-positive. Approximately 90% of perikarya in myenteric ganglia expressed αsyn (figure 9C & D). Large-calibre axons with distinct αsyn varicosities were readily visible exiting ganglia to innervate muscle and coursing

Figure 8. αsyn expression in ENS ganglia

Immunohistochemical staining of submucosal (A, C, E) and myenteric (B, D, F) ganglia in the gastric fundus (A, B), terminal ileum (C, D) and ascending colon (E, F).

Scale bars = 50 μm

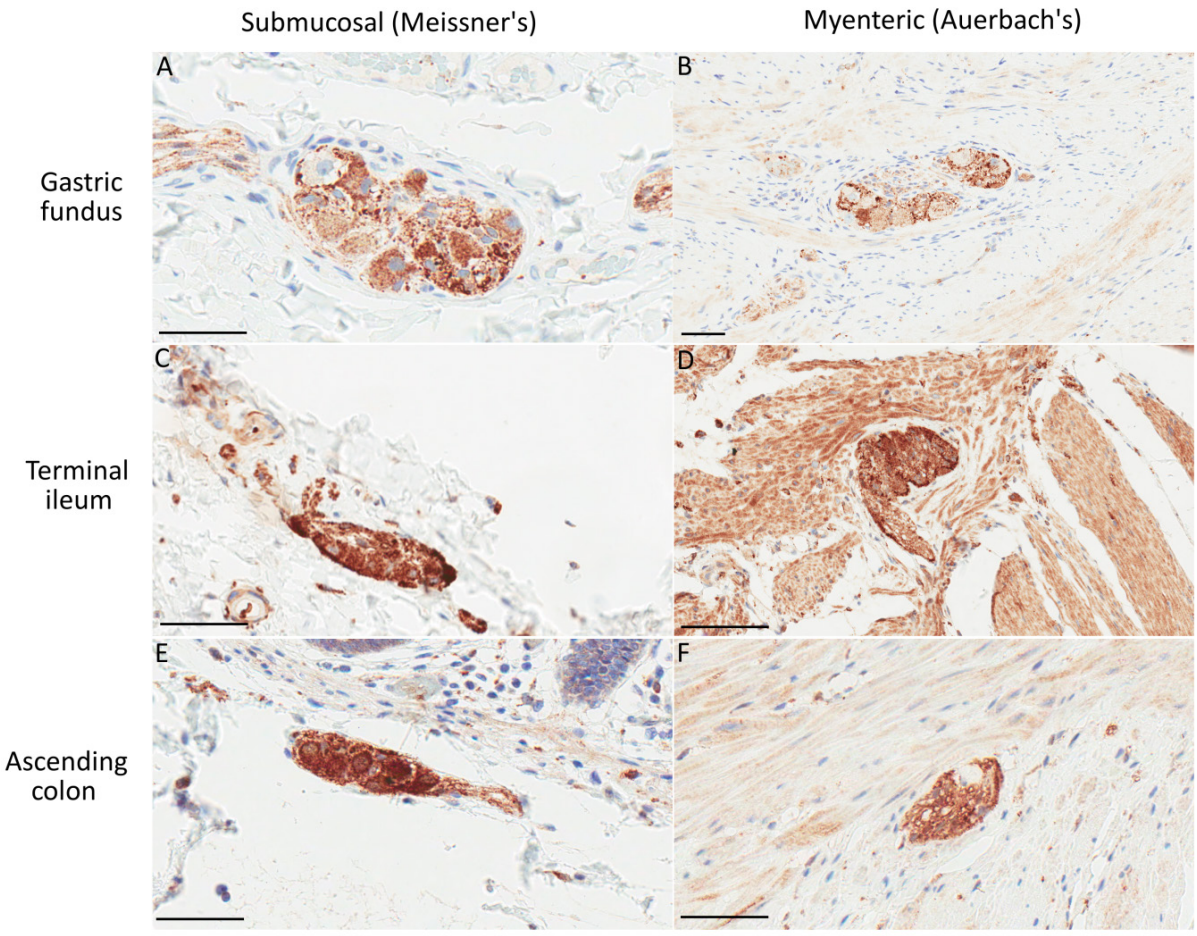

Submucosal (Meissner's)
Myenteric (Auerbach's)
Gastric fundus
Terminal ileum
Ascending colon
A
B
C
D
E
F

Figure 9. αsyn in ganglia of the vermiform appendix

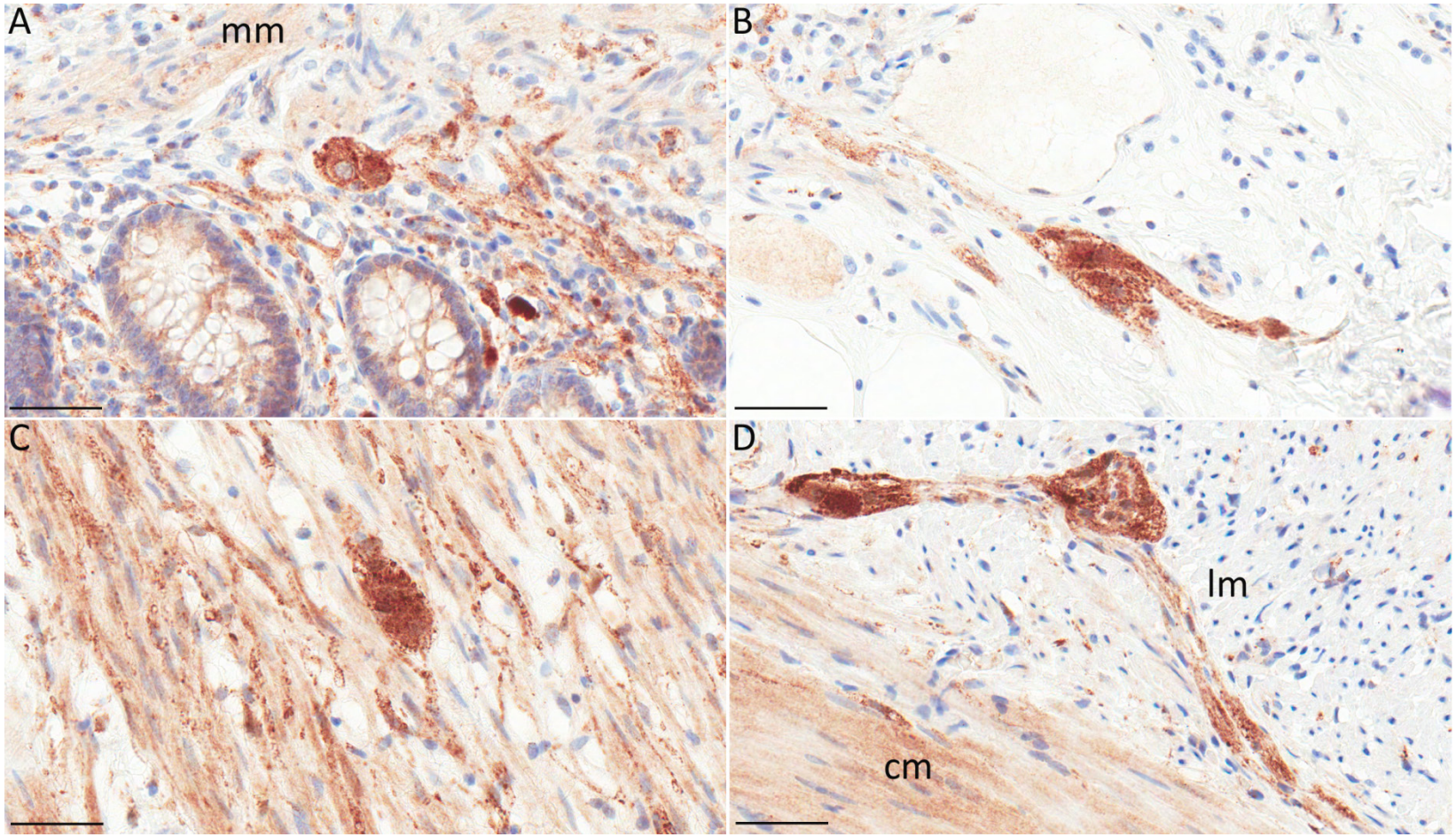
A) Mucosal ganglion in the basal lamina propria of the vermiform appendix near the muscularis mucosae (mm). Nerve fibres of the mucosal plexus can be seen exiting the right side of the ganglion.

B) Submucosal ganglion two αsyn+ neurons and processes directed towards the mucosa.

C) αsyn-immunoreactive ganglion with three αsyn+ neurons in the circular muscle layer on a bed of αsyn-positive nerve fibres

D) Two interconnected myenteric ganglia between the circular muscle (cm) and longitudinal muscle (lm) layers. Large nerve fibres containing αsyn-immunoreactive varicosities are directed towards smooth muscle.

Scale bars=50 µm


A
mm
B
C
D
lm
cm

between ganglia. Long axons with αsyn varicosities filled the muscularis propria. The normal pattern of αsyn-positive axon terminals was seen in all ganglia.

3.3 αsyn expression in intestinal laminae propriae

Within the sections of stomach analysed, αsyn staining was most prominent in the gastric corpus. The majority of αsyn was found in light punctate fields decorating gastric glands (figure 10A & B) – possibly representing innervation related to control of gastric secretion. At higher power, scarce αsyn-immunoreactive nerve fibres could be discerned (figure 10B, arrow). Mucosal ganglia were practically non-existent throughout the stomach wall.

In the lamina propria of the terminal ileum, any αsyn immunoreactivity could at best be described as disorganised (figure 10C & D). Recognisable neural elements, including αsyn+ ganglia, were rare. αsyn-immunoreactive nerve fibres were especially noticeable circumscribing cryptal epithelium (figure 10D, arrow)

The ascending colon displayed a marked transition from more oral GI regions. The lamina propria contained almost no αsyn+ nerve fibres (figure 10E & F). In contrast, mucosal αsyn immunoreactivity was largely confined to small, round cells close to the intestinal lumen (figure 10F, arrowhead). These were later determined to be colonic macrophages, known to express αsyn (*infra* at 3.5). Mucosal ganglia were relatively abundant; however, in opposition to outer ganglia in the colon, these perikarya generally did not stain for αsyn.

αsyn immunostaining in the mucosa of the appendix revealed a dense, reticular network of filamentous structures (figure 11A) consistent with descriptions of nerve fibres in the mucosal plexus (Balemba et al., 1998; Balemba et al., 2002; Furness and Costa, 1980; Kramer et al., 2011).In the apical lamina propria, these fibres were fine and discretely separated. They were often arranged in a reticular or “chicken-wire” pattern (figure 11B). These fibres also visibly ran perpendicular to the

Figure 10. αsyn in GI lamina propria

αsyn immunoreactivity in the mucosa of the gastric corpus (a, b), terminal ileum (c, d) and ascending colon (e, f). In the stomach, a low-power view demonstrates intense αsyn staining on the epithelial cells of some glands (A). Higher magnification shows rare αsyn-positive nerve fibres (b, arrow). In the terminal ileum, spare αsyn immunoreactivity is visible at low power (c), submucosal ganglia are also visible (*). αsyn+ nerve fibres can be seen, particularly circumscribing epithelium (d). Little immunoreactivity is seen in the colon (e). Higher power views illustrate αsyn+ cells in the apical lamina propria (f).

Scale bars = 50 µm

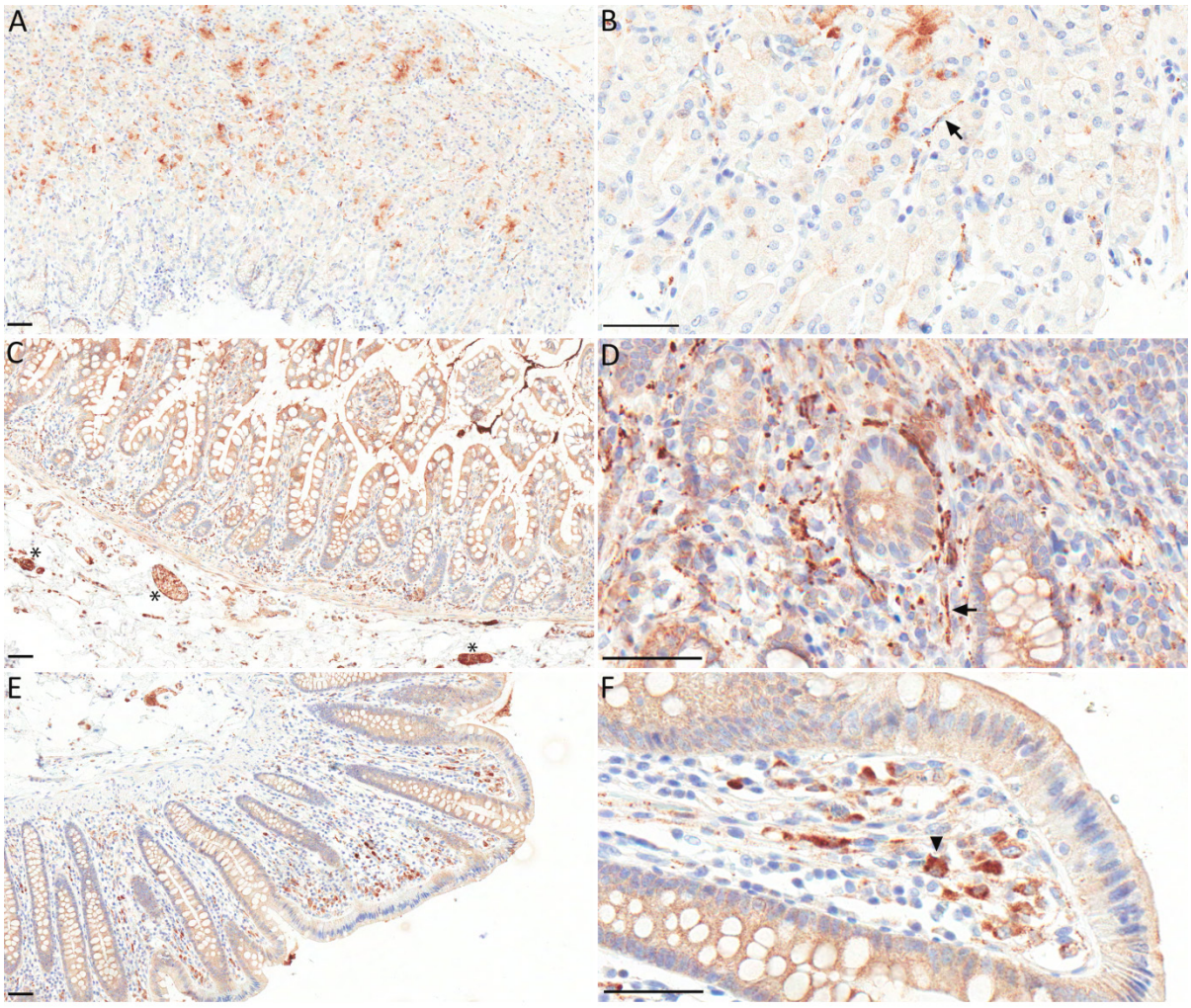
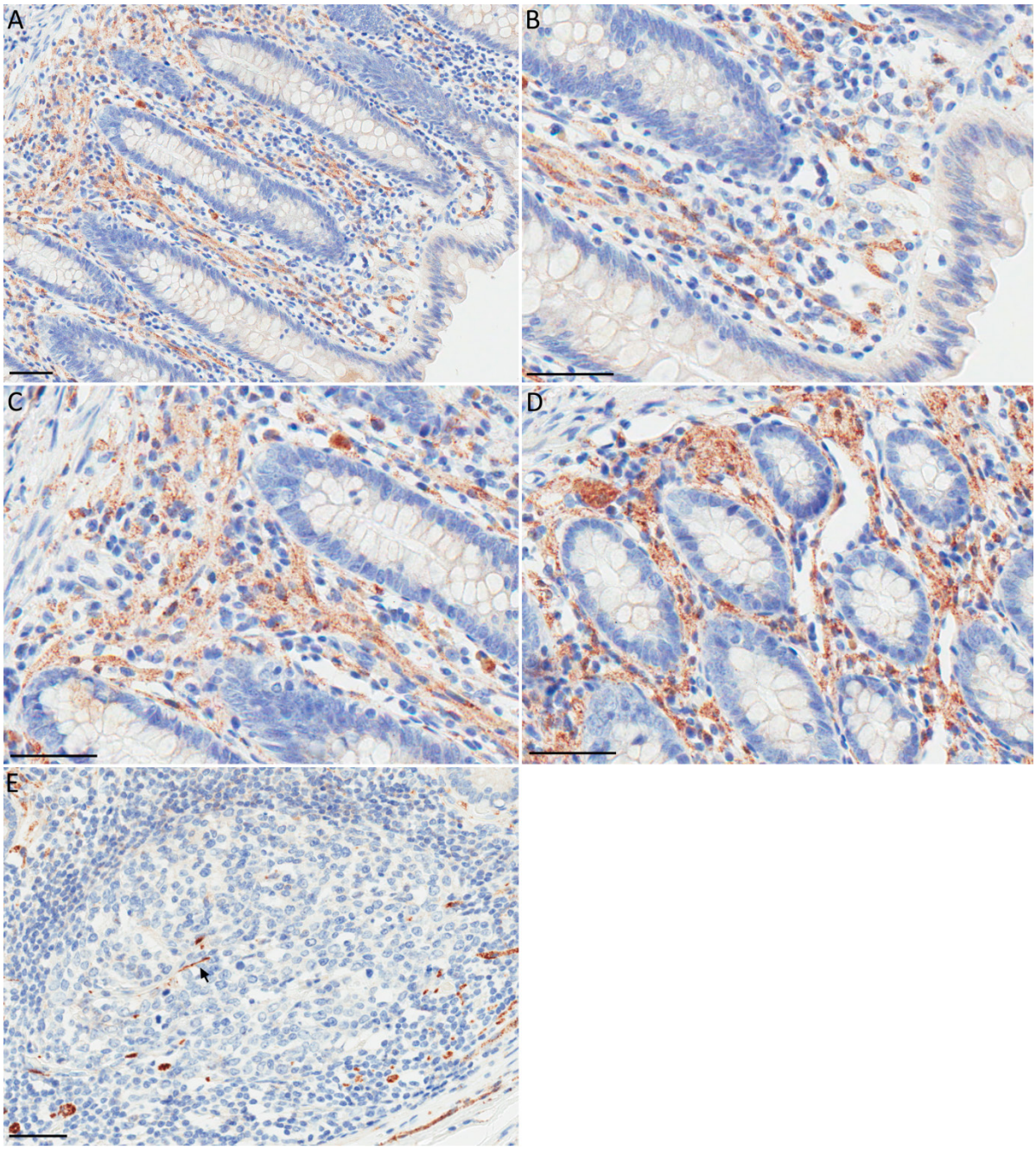

A
B
C
D
E
F

Figure 11. αsyn immunoreactivity in the lamina propria of the vermiform appendix
A) Low-power view of αsyn staining **B-D**) Higher magnification images showing the reticular pattern of αsyn staining in the inner proprial subplexus (**B**), with denser staining in the external propria (**C**) and pericryptal (D) mucosal subplexus. Nerve fibres with αsyn immunoreactivity are noted with the lymphoid follicles (E, arrow).

Scale bars = 50 μm


A
B
C
D
E

muscularis mucosae. At the apex of the lamina propria, fibres appeared to contact the base of cells in the epithelium or course along the epithelium for a brief distance. Approaching the muscularis mucosae, in the basal lamina propria, individual fibres become quite indistinct (figure 11C). Rather than perpendicular, nerves begin to run parallel to the muscularis mucosae. Rare fibres also appear to cross the smooth muscle layer. What mucosal ganglia exist are also found in this region (e.g. figure 9A or figure 11D). Surrounding appendiceal crypts was the most intense αsyn+ innervation seen in the whole GI tract (figure 11D). These fibres circumscribed the crypts and, adjacent to the muscularis mucosae, seemed to fill the entirety of the lamina propria. Finally, αsyn+ nerve fibres could also be noted variably within lymphoid follicles at the base of the appendiceal lamina propria (gigure 11E). Rarer bulbous or botryoid αsyn-immunoreactive structures could also be noted in the appendix and not elsewhere. The nature of these structures is dicussed below (section 3.5).

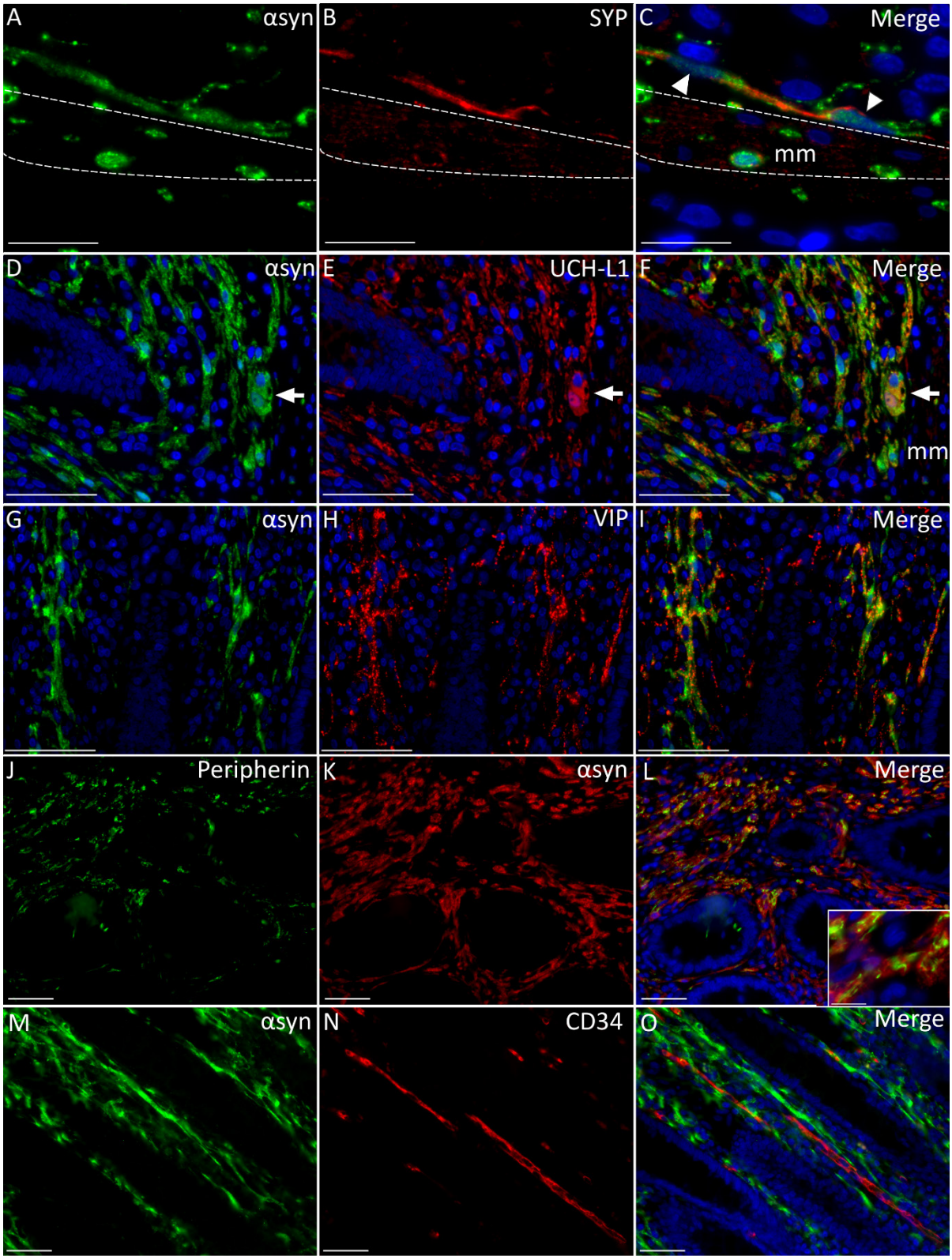
3.4 αsyn staining in the appendiceal lamina propria co-localises with neural markers

αsyn immunoreactivity in the appendiceal lamina propria appeared to be distributed along a ramified, filamentous network. Given the preferentially neuronal localisation of αsyn it was likely that this represented nerve fibres in plexus, however, the synaptic character of αsyn made it possible that this instead was indicative of extensive innervation of other mucosal networks such as vasculature. To ascertain the nature of mucosal αsyn staining, I performed double-label immunofluorescence with MJFR1 and neural (synaptophysin, UCH-L1, VIP & peripherin) or endothelial cell (CD34) markers. αsyn co-localised with all neural markers in the appendiceal lamina propria but did not co-localise with CD34.

The pre-synaptic protein synaptophysin (SYP) co-localised with fellow pre-synaptic protein αsyn throughout the course of αsyn-positive mucosal networks (figure 12A-C). However, given that both

Figure 12. αsyn fibres in the appendiceal mucosa co-localise with neural markers
Double-labelling immunofluorescence demonstrating: **a-c**) colocalisation of αsyn (**a**) and SYP (**b**) , **d-f**) of αsyn (**d**) and UCH-L1 (**e**) and **g-i)** of αsyn (**g**) and VIP (**h**) in mucosal nerve fibres. **j-l)** j lack of co-localisation of αsyn (**j**) and CD34 (**k**) in vascular channels of the appendiceal lamina propria. Merged images in **c, f, i** and **l**.Note Schwann cell nuclei apposing nerve fibres (arrowheads) and a fine punctate pattern of SYP staining in the muscularis mucosae (mm in b&c). An αsyn+/UCH-L1+ mucosal ganglion is also readily visible in d-f (arrow).

Scale bars = 20 μm (a-c), 60 μm (d-f), 67 μm (g-i), 50 μm (j-o). Inset scale bar = 12.5 μm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markers are pre-synaptic proteins; this does not differentiate varicosities *en passant* within nerve fibres from other structures receiving innervation.

UCH-L1 (also PGP 9.5) is a pan-neuronal marker both in the CNS and the peripheral nervous system including the ENS (Beiler et al., 2004; Sams et al., 1992; Tomita, 2012). UCH-L1 immunoreactivity was found in ganglionic perikarya and nerves fibres in all layers of the appendix. In the lamina propria, the entirety of αsyn staining co-localised with UCH-L1-immunoreactive elements (figure 12D-F) including mucosal ganglia (figure 12F, arrow). Rare UCH-L1-positive, αsyn-negative neural elements could be identified.

Vasoactive intestinal polypeptide (VIP) functions to stimulate water secretion and enteric smooth muscle contraction. In the outer layers a subset of neurons/fibres express VIP. However, VIP immunoreactivity is widespread and possibly omnipresent in mucosal nerve fibres (Anlauf et al., 2003; Sams et al., 1992), providing a solid double-labeling partner to determine the nature of mucosal αsyn. Indeed, in the appendiceal lamina propria VIP-immunoreactive structures were found throughout (figure 12H). The VIP staining within these structures was uniformly bead-like, consistent with previous reports (Larsson et al., 1976). The finest fibres resembled pearls on a string and thicker fibres appeared simply as less organised clusters of beads. All αsyn immunoreactivity co-localised with VIP (figure 12G-I). There was a significant VIP-positive, αsyn-negative nerve fibre population – larger than the UCH-L1 equivalent – indicating that αsyn may only be present in a subset of mucosal nerve fibres.

Peripherin, a type III intermediate filament protein, is an established marker of intestinal plexus (Ganns et al., 2006; Kramer et al., 2011; Yuan et al., 2012). In the lamina propria, peripherin stained only the larger nerve fibres near the muscularis mucosa (figure 12J-L), a pattern found with many neurofilament markers (Balemba et al., 2002). Here αsyn did co-localise with peripherin but it was somewhat difficult to distinguish individual nerve fibres.

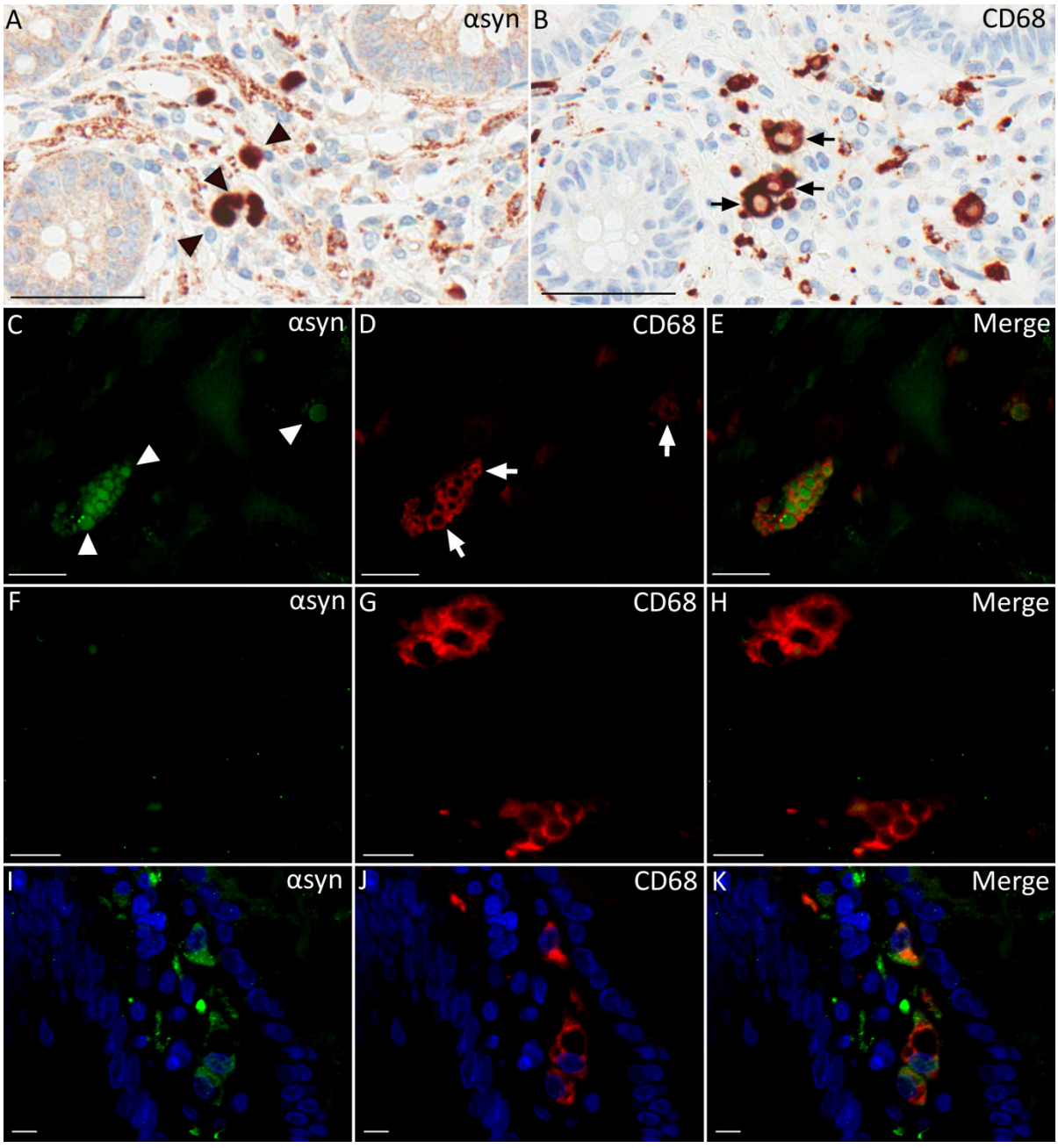
Although filamentous αsyn staining co-localised reliably with neural markers, for completeness it was confirmed that αsyn immunoreactivity did not represent synapsing along vascular walls. CD34 is a glycoprotein present on blood vessel endothelial cells (Pusztaszeri et al., 2006). In the appendix, CD34 staining labeled apparent artery-vein pairs in muscle and serosa (not shown) and numerous small vessels in the lamina propria (figure 12N). Mucosal, filamentous αsyn staining did not run along CD34+ vascular beds (figure 12O). Any areas of co-localisation appeared to be incidental where αsyn-staining nerve fibres crossed over CD34+ vessels.

3.5 αsyn accumulates in CD68+ lysosomes in the appendiceal lamina propria

IHC staining of appendices for αsyn frequently revealed large, irregular shaped clusters which appeared to be contained within a single cell (figure 13A). This did not resemble any classical neural element and was of unknown nature. Staining for the macrophage/lysosomal membrane glycoprotein CD68 on adjacent sections outlined a complementary pattern suggesting αsyn may be contained within CD68+ lysosomes (figure 13B). This was confirmed by double-label IF in the appendix showing botryoid lysosome clusters enclosed αsyn accumulations (figure 13C-E). Autofluorescence, present inside clustered lysosomes, confirmed their identity as such. This signal likely represents lipofuscin as it was detectable even in unstained sections and exhibited saffron-coloured fluorescence under a filter suited for detection of DAPI (Carl Zeiss filter set 02, excitation maximum=365 nm, detection with a long-pass filter >420 nm) (Gray and Woulfe, 2005) (not shown). The signal was slightly attenuated by copper (II) sulfate (Schnell et al., 1999) but not glycine, osmium tetroxide or crystal violet (not shown). Although macrophages with clustered CD68+ lysosomes were also detected in Peyer's patches of the terminal ileum, these were never found to contain αsyn (figure 13F-H). The cellular αsyn of the colonic lamina propria (*supra*) was found within CD68+ macrophages although both proteins were diffusely distributed and the cells were located in the adluminal lamina propria (figure 13J-K).

Figure 13. αsyn is found in lysosomes of appendiceal mucosal macrophages
Peroxidase staining demonstrates globular αsyn staining (A) and a complementary CD68 pattern (B) in appendiceal lamina propria. Immunofluorescence confirms that, in the appendix, αsyn localises to the interior of botryoid CD68+ lysosomes in macrophages (C-E). In ileal Peyer's patches, clustered CD68+ lysosomes (G) were found; however, these did not contain αsyn (H). αsyn-immunoreactive cells in the apical lamina propria of the ascending colon (I) also expressed CD68 (J &K).

Scale bars = 50 μm (A, B), 10 μm (C-K)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Chapter 4: Discussion

4.1 αsyn distribution

This work expands the body of knowledge regarding the normal distribution of αsyn within the enteric nervous system, specifically in man. In rat, ganglionic αsyn positivity has been described as an increasing rostrocaudal gradient from the stomach through the small intestine (Phillips et al., 2008) Here, that increasing gradient is confirmed and extended through to the ascending colon, although the appendix represents an unexpected peak with more positive neurons than the colon (figures 4 & 5).

In the appendix, αsyn-positive ganglia were noted within the circular muscle, distinct from the myenteric (Auerbach's) plexus (figure 9C). Ganglia have been noted there previously, as the appendix has a unique organisation of ganglia in the muscularis propria (Emery and Underwood, 1970). Rather than existing solely between the two muscle layers, they are found in a concentric pattern with 3 layers: within the longitudinal muscle, between the muscle layers and within the circular muscle (Hanani, 2004; Reiser, 1932).

αsyn-positive axon terminals were seen in apposition to neurons in all ganglia regardless of their regional or intramural location. This is not unexpected conceptually as αsyn is present at most, though not all, presynaptic terminals (Braak et al., 2000) and the plexus that make up the ENS would not be true plexus if they did not interconnect via synapses. Indeed, axon terminals can be distinguished on all neurons of the myenteric (Cook and Burnstock, 1976; Komuro et al., 1982; Pompolo and Furness, 1988) and submucosal plexus (Wang et al., 1995; Wilson et al., 1981).

Among the GI regions examined, the appendix is endowed with the greatest abundance of αsyn. The apicobasal density of the fibres (figure 11A) can likely be attributed to differential organisation of mucosal subplexus. In swine, Balemba et al. have described six subplexus within the mucosal plexus

of the large intestine (figure 14) (Balemba et al., 2002). Following this classification, the finer, sparser fibres found in the apical lamina propria clearly represent the inner proprial subplexus (figure 11B). The denser basal staining corresponds to the outer proprial and interglandular subplexus (figure 11C). The singular fibres surrounding crypts define the subepithelial network (figure 11D).

In all gut regions, αsyn+ nerves fibres were most evident in the mucosal subplexus that circumscribe intestinal epithelium (figures 10 & 11). Similar to prion disease (Jeffrey et al., 2006), this raises the possibility that exogenous misfolded αsyn in the intestinal lumen could directly infect the ENS.

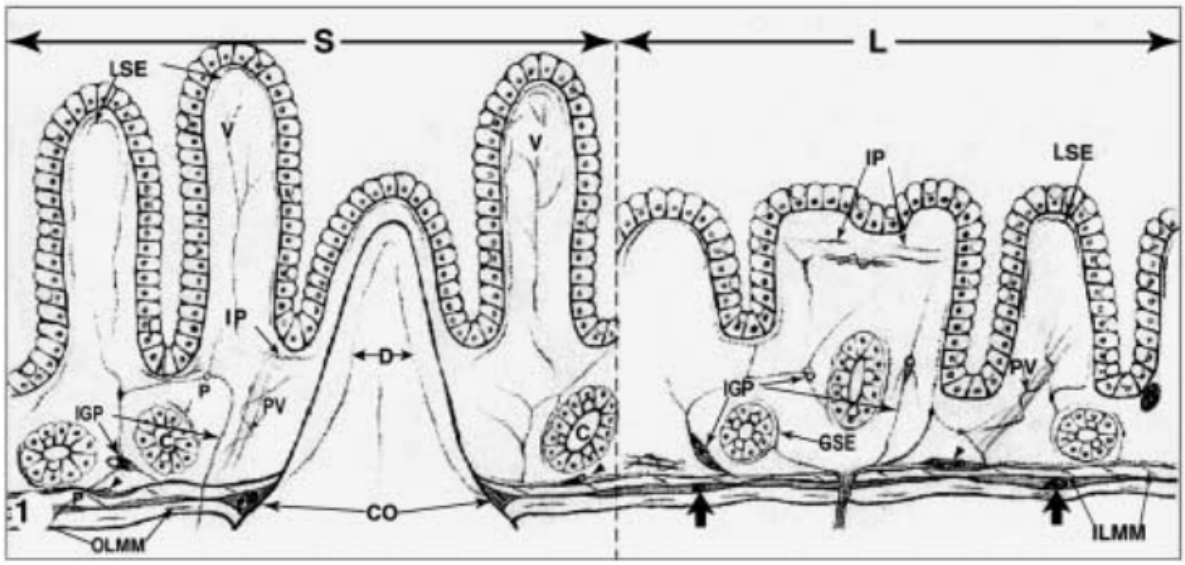
Since the proposal of the peripheral to central and specifically ENS to dmX spread of Lewy pathology by Braak and others starting in 2003 (Braak et al., 2003a; Braak et al., 2003b), the ENS has garnered increased attention from researchers in the PD community. This had led others to examine the expression pattern of αsyn in normal and diseased states. In humans, previous work seems to suggest that αsyn is frequently absent in the ENS of healthy patients and present in PD. In rat, Terry Powley at Purdue University has demonstrated in well-controlled studies that αsyn is normally widespread in the myenteric plexus and in the colonic submucosa, is found in a subset of neurons (Phillips et al., 2013b; Phillips et al., 2009). Given the scientific nature of those studies and the similarity in most senses between the human and rodent ENS (Furness, 2006), any evidence to the contrary should have to stand up to strict scrutiny.

In man, Shannon et al. have reported αsyn immunoreactivity in pre-diagnosis colonic biopsies from three PD patients with none in 23 healthy controls (Shannon et al., 2012a). These findings are clearly in opposition to ours which show obvious, neural αsyn staining in all ten healthy patients. That said, Shannon et al. do not specify where in the colon their staining was found and the figures they present lack any discernible neural elements. It is unclear how these findings can be

Figure 14. Subplexus of the mucosal plexus
Arrangement of the subplexus of the mucosal plexus in the small intestine (left). The large intestine (right) is similarly arranged save for the lack of a villous subplexus.

Adapted from (Balemba et al., 2002), permission to reprint contained in appendix B.

GSE & LSE – subepithelial subplexus, IGP – interglandular subplexus, ILMM & OLMM - lamina muscularis mucosae subplexus, IP – inner proprial subplexus, OP – Outer proprial subplexus, PV – Perivascular subplexus, V – villous subplexus


S
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CO
OLMM
IP
LSE
IGP
GSE
PV
ILMM

interpreted. Although they use the LB509 anti-αsyn clone as we have done for peroxidase staining, they pre-treat with an uncharacterised formic acid and citric acid antigen-retrieval protocol.

In a concurrently published article, Shannon et al. purport to analyse αsyn expression in the colonic submucosa in early PD using the LB509 clone (Shannon et al., 2012b). They then show that αsyn staining in the "lamina propria of the colonic submucosa" is present in biopsies of 70% of PD patients and 8% of healthy control colon, again contrary to our staining. Of course, intestinal submucosa does not possess a lamina propria which is reserved to mucosal surfaces. In reality, Shannon et al. present photomicrographs of colonic mucosa. The αsyn immunostaining therein does not contain any discernible neural elements and no evidence is presented to counter that.

Böttner et al. evaluated αsyn expression in the normal human rectosigmoid colon where they discovered αsyn expression in all myenteric and submucosal ganglia (Böttner et al., 2012). Their choice of rectosigmoid colon was not explained but may have been simply due to ease of access to samples. Phosphorylated-Ser129 αsyn was also observed variably. This is in contrast to to findings in rodents (Phillips et al., 2009; Phillips et al., 2008) and was not correlated to CNS disease.

A Toronto group found αsyn expression only in 52% of colons from the normal autopsy population using the uncharacterised anti-αsyn clone KM51 (Gold et al., 2013). In contrast 100% of PD colon expressed αsyn.

The factors underlying the discordance between our findings and these previous reports are uncertain. However, they may be related to differences in immunohistochemical technique, antibodies used or patient populations. Gold et al. used tissue from autopsies, introducing the possibility of autolytic degradation of colonic αsyn, and also use the less well known anti-αsyn clone KM51, which likely has a reactivity different from LB509. Post-mortem autolysis is most rapid in the GI tract, beginning in under 24 minutes in swine (Cross and Kohler, 1969).

Shannon et al., however, used both surgical specimens and LB509 in both of their studies. The deparaffinisation technique and antigen retrieval method are different than used here and the lack of histological detail provided sheds serious doubt on the validity of the results in their controls. The smaller sample size of this work is unlikely to be a factor in these differences as previous reports put the αsyn immunoreactivity in colon at 0, 8 or 52% in normal patients. We would therefore expect at least half of our cases to show no staining where in fact all cases were positive. This should not be surprising in light of the fact that αsyn is normally present in axon terminals throughout the brain of all human subjects. On the basis of our results, we conclude that αsyn is present in axon terminals and axonal varicosities of the ENS in all humans.

4.2 The appendix in PD

Much like the mucosal plexus, the vermiform (worm-like) appendix is the oft-forgotten member of the GI tract. The appendix is a blind outpouching of the caecum in the human large intestine and is rich in lymphoid tissue (Gorgollon, 1978). Embryologically, the appendix and caecum develop together from the caecal diverticulum which swells from the caudal limb of the midgut loop during the sixth week of development.

In terms of GI motility, the appendix is relatively inert with little movement of its content. This relative stasis may lead to the development of fecaliths (Silen, 2012) and, consequently, the vermiform appendix is known for the right lower quadrant pain of appendicitis (Cope, 1928) and little else. Here I have shown that the appendix is notable for an innervation particularly rich in αsyn, both in ganglia and resultant nerve fibres. Of special note is the significant mucosal αsyn-positive innervation – distinct from other GI regions.

αsyn is ubiquitous at the preganglionic axon terminals of vagal efferents (Phillips et al., 2008) which synapse on ENS ganglia. Therefore for an uninterrupted chain of αsyn+ terminals (figure 2) the

postganglionic presynapse must also be positive. Postganglionic nerve fibres are obviously present in the muscularis propria and the submucosa, however, those most accessible to the outside, by virtue of their proximity to the GI lumen and lack of blood-brain barrier protection (*infra* at 4.3) lie in the mucosal lamina propria. The route of mucosal axon terminal → αsyn+ ganglion → dmX efferent fibre is best developed in the appendix, making it an attractive site for the initiation of dmX-petal Lewy pathology.

At least three factors make the vermiform appendix the most attractive locus for the initiation of ENS, and therefore dmX, Lewy pathology: (1) rich vagal innervation, (2) significant αsyn content, and (3) substantial immune component.

4.2.1 Vagal innervation

αsyn aggregation and Lewy pathology begins not in the soma but at the presynaptic axon terminal (Chung et al., 2009; Kramer and Schulz-Schaeffer, 2007; Nakata et al., 2012; Schulz-Schaeffer, 2010; Tanji et al., 2011). Therefore, it is likely that the Lewy pathology observed in the dmX must originate at the terminals of those neurons' axons. In mice, it has been shown that rotenone treatment or proteasome inhibition localised to the GI lumen or wall can cause dmX Lewy pathology (Miwa et al., 2006; Pan-Montojo et al., 2010; Pan-Montojo et al., 2012). This clearly implicates dysfunction at the axon terminal as key to αsyn aggregation; the cell body of dmX neurons are not only at least 50 cm away, but safely ensconced behind the blood-brain barrier to which the ENS is not privy (discussed below).

The vagal innervation of the GI tract is most pronounced in the stomach/duodenum and the caecum/appendix (figure 6). It is notable that the vagal innervation of these regions is differentiated by the branches of CN X they receive. In rat, as expected, practically the entirety of the vagal input to the stomach comes from the gastric branches, in duodenum the hepatic branch dominates and in the

caecum/appendix it is the coeliac branch (figure 6). Vagotomy, or interruption of the course of CN X, is a common laboratory investigative procedure but is also a human surgical treatment. Vagotomy severing particular branches, if done prior to the peripheral to central spread of Lewy pathology, would allow deduction of the sites from which the pathology arose. Before the discovery of the pathogenic role of *Helicobacter pylori*, severing branches serving the stomach allowed for treatment of refractory peptic ulcers (Martin, 2005). Two procedures would be informative in the context of our goals: truncal vagotomy (severing the abdominal vagus completely) and selective vagotomy (which transects both gastric branches). Although the first would not provide specific data, it would be of particular interest to examine the dmX of patients who received selective vagotomies to determine if this prevented dmX Lewy pathology or frank Lewy body disease – which would confirm the stomach as the source of dmX disease. In more recent years, although vagotomy for ulcers has all but ceased, the utility of abdominal vagotomy has spread to the treatment of obesity, thereby expanding the potential population for such studies.

Further along in the gut, appendectomy, an even more common surgical procedure, provides a potential epidemiological study population. Here it would be necessary to compare patients who underwent appendectomy or not and dmX Lewy pathology as well as rates of PD or DLB. The fact that appendectomy is often performed at a young age would lessen the risk that dmX disease was established prior to surgery.

The need to select specific surgical populations, however, may not be necessary. Caecum- (and likely appendix) projecting neurons in the dmX are found only along the lateral aspect of the nucleus whereas gastric-projecting neurons are found mostly medially (Altschuler et al., 1991; Cao et al., 2007). Thus it may be possible to determine the origin of dmX Lewy pathology simply by determining the topography of LBs in the dmX.

4.2.2 αsyn content

I have demonstrated that the vermiform appendix has the most abundant αsyn content, both in cell bodies and nerve fibres, of the cadidate regions of origin for Lewy pathology in the dmX. This led to the conclusion that the appendix was the most likely source of that disease. There is an established link between αsyn dosage and predilection for Lewy body disease. Families with duplications or triplications of the αsyn locus have a markedly increased rate of PD (Nishioka et al., 2006; Nishioka et al., 2009; Singleton et al., 2003). On a cellular level, rat brain regions with perikaryal αsyn expression correspond to areas of human brain more apt to develop Lewy body disease (Andringa et al., 2003).

Although αsyn is a major (likely *the* major) component of LBs and LNs and exogenous fibrillar αsyn is sufficient for their formation (Luk et al., 2012a; Luk et al., 2009; Volpicelli-Daley et al., 2011), it has not been definitively proven that αsyn is necessary for their formation. Ubiquitin-immunoreactive structures formed after proteasome inhibition in cells lacking αsyn are structurally distinct from the bona fide LBs formed in the presence of αsyn (Rideout et al., 2004). Therefore, even if αsyn is not required for the development of neuronal inclusions, it is almost certainly required for the formation of true Lewy pathology and higher levels of αsyn will aid in that process.

Using typical prion disease as a model, PrP^{C} in the ENS is required for prion spread after intraluminal inoculation with extract from PrP^{Sc} contaminated tissue (Glatzel and Aguzzi, 2000; Peralta and Eyestone, 2009). Given that both ENS PrP^{C} (previous) and Peyer's patches (Prinz et al., 2003b) are required for disease spread, the PrP^{C}-expressing nerve fibres found in animal Peyer's patches and the neuroimmune connections thereof have been claimed as important for pathogenesis (Defaweux et al., 2007; Defaweux et al., 2005). If PD truly does follow a prion-like sequence, the αsyn-positive innervation found in lymphoid follicles only in the appendix (figure 11E) may be essential.

Of particular relevance with respect to the prion hypothesis of αsyn spread, it has been demonstrated that injection of human DLB brain extract into the gastric wall of A53T-αsyn-overexpressing transgenic mice was sufficient to induce punctate aggregates in myenteric neurons (Lee et al., 2011). That said, these same effects were not observed when the extract was given to wild-type animals, indicating that αsyn dosage may play a role.

4.2.3 Immune content

The third pillar of the possible appendiceal origin of dmX Lewy pathology is the rich immune component of the vermiform appendix. The appendix has the largest relative lymphoid content of any GI section and may play a role as a "safe-house" for commensal bacteria, re-establishing their population after loss elsewhere in the gut (Laurin et al., 2011; Randal Bollinger et al., 2007; Zahid, 2004). In rabbit, which serves as the laboratory animal model of appendiceal function, the appendix has been shown to be the mammalian equivalent of the avian bursa (Archer et al., 1963; Weinstein et al., 1994). That is, B cells can be selected against foreign and self-antigens with certain B-cell populations being expanded after interaction with CD5 (Lanning et al., 2000; Pospisil et al., 2006; Pospisil and Mage, 1998). The rabbit appendix is also a site for expansion of the antibody population including V(D)J recombination (Zhai and Lanning, 2013). The human appendix is equivalent to the rabbit in that it is rich in plasma cells which can release their products (i.e. antibodies) (Gorgollon, 1978).

The immune system is linked to PD through two main pathways: LRRK2 and human leukocyte antigen (HLA) loci linked to neuroinflammation. Leucine-rich repeat kinase 2 (LRRK2) and its G2019S variant are associated with PD, especially in Ashkenazi Jews. The reasons for this correlation were initially unclear, but very recently research has linked this to alterations in immune function and autophagy. The connection to the appendix lies in the role of LRRK2 in modulating Crohn's disease. Loss of LRRK2

results in an enhanced susceptibility to Crohn's disease and inflammation in general (Liu et al., 2011; Liu and Lenardo, 2012). LRRK2 is found in circulating and tissue-based immune cells. Therefore it has been suggested that the circulating LRRK2-containing cells link peripheral inflammation with the CNS (Hakimi et al., 2011). Specific HLA variants have been linked to PD in genome-wide association studies (GWAS) namely: HLA-DR and –DQ of major histocompatibility complex (MHC) class II (Ahmed et al., 2012; Hamza et al., 2010; Hill-Burns et al., 2011; Sun et al., 2012). HLA expression has been linked to neuroinflammation in PD and generally in neurodegeneration (McGeer et al., 1988a; McGeer et al., 1988b). Although neuroinflammation remains to be linked definitively to the pathogenesis of PD, it has been shown to be part of the disease process at both a cellular (Banati et al., 1998; Brochard et al., 2009; Mirza et al., 2000) and molecular level (Hirsch and Hunot, 2009; Hunot and Hirsch, 2003; Mann et al., 1994).

4.2.4 Appendix versus stomach

Braak et al. suggest that the stomach would be especially susceptible to a luminal pathogen due to the friability of its epithelium, ease of infection (as evidenced by *H. pylori)* and the long incubation time of the food bolus there (Braak et al., 2003a; Braak et al., 2003b). Gastric LBs are somewhat common although they have not been linked to dmX disease (Braak et al., 2006). Phillips and colleagues aptly combatted the gastric hypothesis by pointing out that with age, the intestinal epithelium becomes quite permeable to large molecules (Ma et al., 1992; Mullin et al., 2002; Phillips et al., 2008). This is consistent with the extra-gastric ENS Lewy pathology reported by many groups (Beach et al., 2010; Kupsky et al., 1987; Pouclet et al., 2012a; Pouclet et al., 2012b; Wakabayashi et al., 1990; Wakabayashi et al., 1993; Wakabayashi et al., 1988; Wakabayashi et al., 1989). The mere observation of LBs or LNs in a specific GI segment will likely not be sufficient to claim the origin of dmX Lewy pathology.

4.3 The blood-ENS barrier

The plenitude of αsyn+ fibres in the mucosal plexus of the appendix make it an enticing candidate as a target for any luminal mediator of αsyn aggregation. This could include ingested/inhaled misfolded αsyn similar to true prion disease, a neurotropic pathogen as proposed by Braak or another ingested toxin (Braak et al., 2003a; Braak et al., 2003b). The presence of αsyn-positive axon terminals in close proximity to mucosal blood vessels (figure 12M-O) brings to mind a novel route. It is possible that a blood-borne agent could reach αsyn-positive terminals via the leaky blood vessels of the intestinal lamina propria. This blood-borne substance could be, as in the GI lumen: a pathogen, misfolded αsyn or another chemical toxin. The haematogenous route, however, allows one to consider that the *agent agrégateur* could originate in plasma or blood cells. These would include: auto-antibodies, immune effector cells, serum iron or systemic infectious agents not found in the GI lumen.

There has been some controversy over the degree to which ENS is accessible by external agents. Most peripheral ganglia have fenestrated capillaries and are exposed to blood-borne substances (Arvidsson et al., 1973; Azzi et al., 1990; Jacobs et al., 1976). Enteric ganglia do not possess intrinsic vasculature (Gabella, 1972; Gershon and Bursztajn, 1978) leading some to assume it would be protected. It has been pointed out that CNS-excluded drugs such as hexamethonium acts on the ENS, therefore any possible blood-ENS barrier would have to be less selective than the blood-CNS barrier (Brehmer, 2006; Furness, 2006).

Myenteric ganglia in particular are resistant to the direct entry of any blood-borne substance (Gershon and Bursztajn, 1978). This, despite the fact that many enteric neurons lie naked on the surface of the ganglion (Gabella, 1972). In contrast, the extracellular spaces of all enteric ganglia readily label with tracers administered intravenously (Allen and Kiernan, 1994). This would seem to indicate that cells in ganglia are impervious to outside molecules but many enteric neurons contain

albumin, indicating that chronic exposure likely allows serum proteins access to the ENS (Allen and Kiernan, 1994). Enteric axons rapidly label after intravenous injection of tracers, however, cell bodies take much longer demonstrating that any perikaryal labelling may be due to retrograde axonal transport (Allen and Kiernan, 1994; Jacobs, 1977). Intravenous HRP or fluoro-gold also label dmX neurons, however the path through which this happens remains unelucidated (Broadwell and Brightman, 1976; Merchenthaler, 1991).

4.4 αsyn in appendiceal macrophages

Although CD68 is not restricted to macrophages in much of the human body, it does specifically mark macrophages in the intestinal mucosa (Rogler et al., 1998), and therein specifically labels lysosomes (Saito et al., 1991; Tsang and Chan, 1992). I have shown here, for the first time, that CD68+ macrophages in the intestinal mucosa have engorged lysosomes containing αsyn (figure 13). This lends itself to two somewhat divergent interpretations: macrophages have taken up αsyn from neural tissue to prevent disease spread or they are processing exogenous αsyn for antigen presentation and/or neuron infection.

Our findings in macrophages are readily analogous to those of Phillips & Powley who have shown that in the aged rat, macrophages of the muscular wall are closely apposed to myenteric neurons (Phillips and Powley, 2012). These macrophages frequently infiltrated ganglia. Of particular interest, muscularis macrophages then contacted, surrounded, and phagocytosed αsyn aggregates in myenteric neurons and their processes. The authors suggest that this uptake of αsyn represents a coming together of two roles of muscularis macrophages: maintaining synaptic homeostasis and eliminating damaged cellular material or products of normal ageing. This suggestion extends to the possibility that these macrophages could be performing synaptic stripping, removal of damaged synapses often done by microglia in the CNS. Although true destruction of the presynaptic terminal is

not a requisite process in synaptic stripping (Perry and O'Connor, 2010), the opportunity arises for intestinal macrophages to attempt to remove axon terminals containing aggregated αsyn before it can spread retrogradely to the cell body.

Phillips et al. have gone on to demonstrate in a later article that, despite engulfing a significant portion of it, intestinal macrophages in rats were unable to completely clear aggregated αsyn from the GI tract (Phillips et al., 2013a). Aggregated αsyn therefore joins PrP^{Sc} and β-amyloid in the repertoire of proteins incompletely cleared by microglia/macrophages. Hughes et al. have shown that phagocytes in the brains of prion-infected animals are highly activated and readily take up latex beads or apoptotic cells (Hughes et al., 2010). Despite this, PrP^{Sc} is not effectively cleared. Intracerebral treatment with lipopolysaccharide increased the production of IL-1β, cellular infiltration and apoptosis but still failed to result in any additional clearance of PrP^{Sc}.

Taken as a whole, these findings suggest that mucosal macrophages in the appendix may attempt to extract misfolded αsyn from neural components in the lamina propria to prevent spread of aggregation to the CNS.

In prion disease, it is traditionally considered that PrP^{Sc} taken up by macrophages in the intestinal mucosa is destined for degradation. In recent years, this idea has been expanded and it now seems that macrophages may play a dual role with some degrading PrP^{Sc} and others being responsible for the transport of exogenous prion protein (Wathne and Mabbott, 2012). Macrophages can carry M-cell-absorbed PrP^{Sc} from the M-cell pocket to deeper lymphoid tissues (Takakura et al., 2011). They are also able to transfer PrP^{Sc} from Peyer's patches to other regions including peripheral nerve fibres. αsyn-positive nerve fibres in the lymphoid follicles of the appendix (figure 11E) allow for this possibility in Lewy pathology. New research suggests that CD68-positive cells in the intestinal mucosa are capable of transporting PrP^{Sc} directly across the intestinal wall to the target organs (Piercey

Akesson et al., 2012). This includes transfer to lymphoid follicles which receive prion-susceptible ENS innervation. In a cell-culture study using spleen cells from mice infected with the Chandler prion, CD68+ macrophages were found to be the predominant species able to transfer PrP^{Sc} to neuronal cells (Tanaka et al., 2012). In this system, cell-to-cell contact was found to be the most efficient manner of prion transmission.

These recent changes in our understanding of macrophage handling of PrP^{Sc} could be extended to αsyn and Lewy body disease if we consider the analogy between the spreading of prion disease and synucleinopathy. If exogenous misfolded αsyn is the origin of Lewy body disease, the presence of αsyn in appendiceal macrophages suggests that the appendix would be the site of macrophage transfer of abnormal αsyn from lumen to nerve or to further lymphoid tissue and therefrom to the ENS.

Macrophages are also classically known as antigen-presenting cells. In the synuclein context, this would allow a coming together of both previously suggested roles of macrophages. αsyn taken up by appendiceal macrophages will at least be partially degraded. Macrophages then migrate to lymphoid follicles where they can present that αsyn to B cells. The appendix is unique in the intestine as it is a site for development and selection of B cells, including those recognising foreign & self-antigens (Pospisil and Mage, 1998). Therefore, the appendix represents a potential site for the development of anti-αsyn antibodies frequently found in humans (Gruden et al., 2011; Gruden et al., 2012; Papachroni et al., 2007; Smith et al., 2012; Woulfe et al., 2000; Yanamandra et al., 2011).

4.5 Summary

Given the proposed role for the ENS in the development of αsyn-based Lewy pathology, there have been many recent attempts to determine the normal enteric distribution of αsyn. In contrast to previous reports, the present study has clarified that αsyn is expressed in the ENS of all healthy human subjects. Regional variations in ganglion neuron αsyn positivity follow an increasing oral-anal gradient from the stomach to the ascending colon in the myenteric and submucosal plexus. However, the vermiform appendix does not follow this gradient and represents the apex of ganglionic αsyn positivity. In addition, αsyn-immunoreactive nerve fibres are present throughout the mucosa of the appendix but not other GI regions. We have also detected lysosome-bound αsyn in macrophages of the appendiceal mucosa. Taken along with the intense vagal innervation of the appendix, these results suggest that the appendix may be at the origin of dmX Lewy pathology. This hypothesis can be tested both epidemiologically and histologically by methods suggested herein.

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Contributions of collaborators

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Appendices

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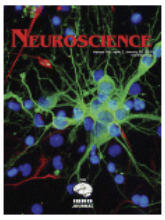

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Curriculum vitae

Madison Tanner Gray

Education

2012-2013	**M.Sc. in Biochemistry – Pathology & Experimental Medicine** University of Ottawa Thesis: On α-synuclein in the Human Enteric Nervous System Supervisors: Drs John Woulfe & Doug Gray
2010-Present	**Doctor of Medicine (M.D.)** University of Ottawa
2007-2010	**General B.Sc. with minor in Biochemistry (*summa cum laude*)** University of Ottawa University gold medal for highest average

Scholarships

2013-Present	Bourse Fondation Baxter & Alma Ricard - $42,000
2012-13	Toth Family Fellowship (Parkinson's Research Consortium) - $10,000
2012-13	Frederick Banting & Charles Best CGS Master's Award (CIHR) - $17,500
2012-13	Ontario Graduate Scholarship - $15,000 (declined)

Publications

Coulombe J, Gamage P, **Gray MT**, Zhang M, Tang MY, Woulfe JM, Saffrey J, Gray DA. Loss of UCHL1 promotes age-related degenerative changes in the enteric nervous system. (submitted)

Gray MT, Middeldorp J, Gray DA, Munoz DG, Woulfe JM. Hypothesis: A Role for EBV-induced Molecular Mimicry in Parkinson's Disease (submitted)

Gray MT, Munoz DG, Gray DA, Schlossmacher MG, Woulfe JM. α-synuclein in the appendiceal mucosa of neurologically intact subjects. *Mov Disord* (in press)

Gray MT, Rutherford MN, Bonin DM, Patterson B & Lopez PG. Hairy-cell Leukemia Presenting as Lytic Bone Lesions. *J Clin Onc* (2013) 31(25):e410-2

Conference abstracts (presenting author underlined)

Gray MT, Tsang JJ, Munoz DG, Woulfe JM. 2013. Exploring the biological significance of molecular mimicry between EBV and α-synuclein. Canadian Association of Neuropathologists.

Gray MT, Woulfe JM. 2013. Striatal blood-brain barrier permeability in PD: re-evaluating the spread of Lewy pathology, Canadian Association of Neuropathologists.

Gray MT, Munoz DG, Gray DA, Woulfe JM. 2013. α-synuclein in the human vermiform appendix . American Academy of Neurology, San Diego. (Poster)

Gray MT, Munoz DG, Gray DA, Woulfe JM. 2013. α-synuclein in the human vermiform appendix. United States & Canada Academy of Pathology, Baltimore. (Poster)

Academic work

Summer 2012	Summer Student Supervisor: John Woulfe Focus: Epstein-Barr Virus and the aggregation of α-synuclein
2009-11	Summer Student Supervisor: Doug Gray Focus: UCH-L1 and the oxidative stress response
Fall 2009	Teaching assistant Principles of Chemistry (English & French) University of Ottawa Supervisor: Alain St-Amant