THE AGEING GUT

IMPLICATION OF THE SECOND BRAIN IN NEURODEGENERATION

Literature thesis

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INDEX

GLOSSARY

Autophagosome. Vesicles formed by double-membrane containing cargos ranging from proteins to invading pathogens, involved in clearing the cytoplasm of the cell from potentially toxic structures.

Cholinergic neuron. Neurons distributed in various discrete regions of the brain, such as the striatum or the basal forebrain, projecting to almost all part of the human brain that release acetylcholine (Ach), playing an important role in sensory functions, attention and memory.

Commensal microorganisms. Microorganisms that have demonstrated to act in the host's immune system to induce protective responses preventing the colonization of pathogen and harmful microorganisms, as well as producing antimicrobial products against them.

Intestinal dysbiosis. Phenomenon by which the reciprocal dialogue between the host's immune cells and the enteric microbiome is altered due to a disruption in the microbial homeostasis resulting from shifts in the microbiome composition.

Excitotoxicity. Biological process in which the overactivation of glutamate receptors leads to elevated influx of Na^{+} and Ca^{+2} into the cell resulting in ion homeostasis disruption and finally neuronal dysfunction.

Indels. Type of mutation in which a deletion or insertion of less than 1kb length nucleotides occurs in a region of the genome.

Myenteric plexuses. Layer of the enteric nervous system situated between the circular and longitudinal muscular tissues which provides most of the innervation for intestinal motor function.

Nitrergic neuron. Descending inhibitory neurons controlling the reflex of intestinal peristalsis containing nitric oxide synthase (NOS) enzyme. They release NO acting as an inhibitory neurotransmitter in the gastrointestinal system smooth muscle.

Opportunistic microorganisms. Typically, non-pathogenic microorganisms that takes advantage of certain situations to colonize the host and become pathogenic, for example weakened immune system of the host or permeabilization of the gut barrier. In normal situations, the microorganisms do not cause harm to the host and can act as commensals during long periods of time.

Parasympathetic nervous system. Part of the autonomous nervous system, from the peripheral nervous system, operating independently from voluntary control with the primary function of modulating visceral organs and glands much slower than the sympathetic nervous system.

Proteases. Cellular enzymes responsible for processing, maturation or destruction of specific set of proteins resulting in new protein products and bioactive molecules or modulation of protein-protein interactions.

Senescence. Cellular process in which cells stop dividing and undergo distinctive phenotypic alterations due to, for example, DNA damage.

Single-nucleotide variant. The most common genetic variant in the human genome consisting on the variation of a single nucleotide in a specific genomic position.

Sympathetic nervous system. The other part in which the autonomous nervous system is divided, responsible for producing an immediate and widespread response called the fight-or-flight response when facing stressful conditions.

T cells. Cells representing the major component of the adaptive immune system responsible for killing infected cells from the host, activating complementary immune cells and producing cytokines.

ABSTRACT

Research in the field of ageing has exposed some of the key mechanisms behind this natural event in the human body. In particular in the nervous system, the main cellular and molecular pathways contributing to neuronal ageing were found to be similar in the CNS and the ENS. These findings suggest that, even though the CNS is the major site of integrative neuronal activity, the correct functioning of the GI is essential for the homeostasis of multiple biological systems, including the brain. Even though both systems are located in distant areas of the human body, they maintain a constant crosstalk between nerve fibres that enable direct reciprocal communication. Indeed, a key player in this bidirectional communication cohabiting the human body from its birth is the GM. The co-evolution of humans and their associated microbial composition has led to complex communications between the gut and the brain. This interaction is essential so as to modulate and support neurological processes throughout the lifespan of an organism. In fact, the action of the GM and its secretome is not only limited to the enteric neurons and immune cells along the gut but also extends to the CNS. Likewise, disruptions in biological systems during ageing are directly affecting the GM. During ageing, shifts in the GM composition are known to trigger pathological changes in the immune system, the ENS and the CNS. These natural events are aggravated in cases of neurodegeneration, where specific taxa of pathogens are known to colonise the gut and negatively influence the progression of the disease. In addition, pathological mechanisms of neurodegeneration typically found in the CNS are eventually reported in the ENS, leading to a promising field of research regarding future treatments and diagnosis for neurodegeneration. Nevertheless, further research is needed to better understand how microbiome-gut-brain axis interact in health and disease.

INTRODUCTION

Neurodegenerative diseases are a large group of neurological disorders where multiple pathological features are present among specific subsets of cells from the nervous system. Considering the primary risk factor for developing neurodegeneration is the natural process of ageing, investigation on this event is being made so as to untangle neuronal mechanisms involved in health and disease. Nonetheless, the research on natural ageing is really challenging since neurons from the central nervous system (CNS) do not experience ageing the same way as neurons in the peripheral nervous system do (PNS). Moreover, the correct functioning of the CNS is constantly influenced by inputs coming from other biological systems. In particular, the gut, also known as the second brain, has demonstrated to play a key role modulating the behaviour of the CNS. Therefore, any pathological mechanism during ageing in the GI will directly affect the course of ageing in the CNS. This situation suggests that the progress or even the onset of neurodegeneration in the CNS is linked to the healthiness of the GI.

The aim of this literature review is to unravel the molecular mechanisms behind the pathophysiology of neurodegeneration in both systems, the CNS and the GI, and how the GI can be manipulated so as to modulate or even prevent the progression of the disease. To do so, the multiple mechanisms involved in normal ageing will be explored in both systems. Following, the interaction between the CNS and the GI will be analysed, forming the so-called microbiome-gut-brain axis, to finally discover potential therapeutic and diagnostic strategies to stop or slow the progression of neurodegeneration through GI interventions (Figure 1).

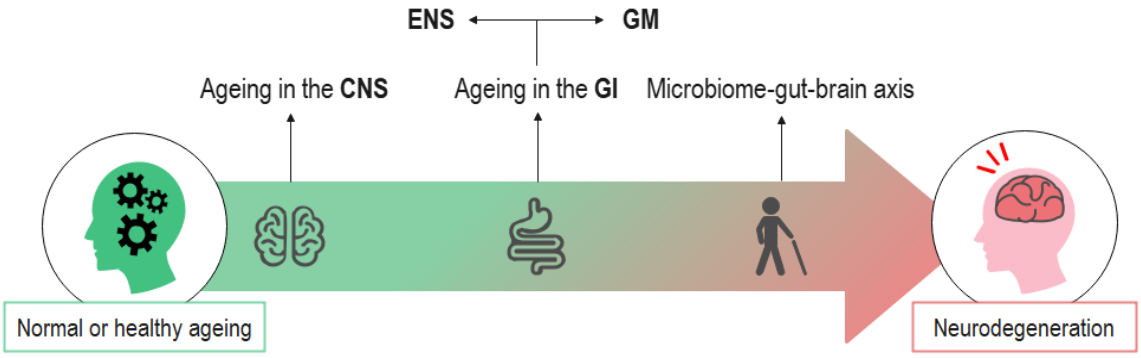

Figure 1 | **Schematic overview of the process of investigation**. Abbreviations ENS and GM refers to the Enteric nervous system and Gut microbiota respectively, being part of the GI. Understanding the normal process of ageing in the CNS and GI, and the interaction between them, is essential for determining the pathological mechanisms involved in neurodegeneration in the CNS.

1. CENTRAL NERVOUS SYSTEM

The mammalian nervous system is formed by two main systems, the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS compromises the brain and the spinal cords, where the information coming from different parts of the human body is processed, analysed and stored. The brain is composed by multiple parts, each of them with specific functions for the survival of the organism.

The process of ageing in the Central Nervous System

The human brain experiences natural changes associated to normal ageing. Given the vulnerability of the cells in the nervous system to external and internal inputs, from dietary energy intake to blood pressure changes, the impact of natural ageing in the CNS is more accentuated than in other biological systems, such as the muscular or the skeletal system(Brown, 2001).

Neurons are continuously facing metabolic and ionic stress due to their high electrochemical activity. With age, increased amounts of oxidative stress, altered metabolic and ionic homeostasis and the accumulation of toxic proteins within the brain, lead to functional and morphological alterations in neuronal cells (Satoh et al., 2017). Even though specific parts of the human brain have the ability to generate new cells in the CNS, a process called neurogenesis, it is dramatically reduced during ageing (Seib & Martin-Villalba, 2015). Therefore, the continuous loss of cells due to progressive neuronal dysfunction is ultimately manifested in brain atrophy, memory impairment and cognitive decline.

Nevertheless, the process of ageing does not affect everybody in the same rate. The individual variability can be explained since some genetic or environmental factors are known to influence the progress of brain ageing. For example, overweighted or obesity patients experience accelerated hippocampal atrophy as compared to non-obesity subjects(Cherbuin et al., 2015). In this regard, different efforts to untangle the principal mechanisms underlying this natural process in the brain are being made. A summary of the molecular mechanisms during CNS ageing can be found in **Figure 2A**.

DNA damage

The human genome constantly faces endogenous DNA damage due to spontaneous errors during cell replication and metabolism end products derived from normal cell functioning. However, cells from the nervous system are more susceptible to DNA damage than other cell types due to their longevity, energy demands and high production of reactive oxygen species (ROS) during cellular respiration (Coon & Benarroch, 2018). Likewise, specific regions of the brain accumulate higher rates of DNA defects than others, such as the hippocampus or the cerebellum (Rutten et al., 2007). Interestingly, when focusing on the type of DNA damage, a recent study using a new sequencing protocol called NanoSeq reported that post-mitotic neurons showed a higher proportion of indels than other cell types (Abascal et al., 2021). In addition to these results, a mutational signature consisting on single-nucleotide variant (SNV) C>T and T>C

was found to increase with age in neurons independent from their region. Indeed, the presence of SNVs seems to slowly increase throughout the lifespan of the human brain and is aggravated in cases of neurodegeneration (Lodato et al., 2018). Moreover, the proportion of copy number variants mutations (CNVs) seemed to increase in brains from elderly patients and enhances the vulnerability towards cognitive decline and loss of synaptic function(Chronister et al., 2019). These findings suggest the presence of a clock for mutational processes in ageing cells, independently of their division rates (Alexandrov et al., 2015).

In response to this deleterious event, cells are provided with a wide variety of DNA repair mechanisms active throughout different phases of the cell cycle. However, every type of DNA damage does not activate the same pathways for DNA repair. When in presence of single strand damage such as SNVs or indels, the mismatch repair (MMR), base excision repair (BER), nucleotide excision repair (NER) and single strand break repair (SSBR) act removing or replacing the error by the original base. On the contrary, when the damage consists of double strand breaks, non-homologous end joining (NHEJ) and homologous recombination (HR) become activated(Chatterjee & Walker, 2017). In particular in neurons, the BER mechanism play a key role repairing the DNA damage derived from oxidative stress. In the CNS, the generation of ROS due to the activation of critical pathways for correct neuronal functioning, for example synaptic plasticity, is counterbalanced by the presence of antioxidant defences. However, during ageing, ROS tends to accumulate due to a reduction of these defences as well as impaired ability to remove oxidatively damaged molecules (Paul et al., 2007.). Since ROS are highly unstable and reactive molecules, they can interfere with nucleic acids and damage DNA bases. In line with these observations, the aforementioned study using the protocol NanoSeq found that the presence of C>A variants associated with oxidative DNA damage significantly increased with age in healthy neurons(Lodato et al., 2018).

Despite the powerful machinery to maintain DNA integrity, the accumulation of nuclear and mitochondrial DNA damage together with a reduced ability to repair these defects exacerbates while ageing in the CNS. Therefore, the identification of specific mutational signatures associated to brain ageing can act as an indicator of the healthiness of the process and may shine a light for potential therapeutic interventions. The final outcome of high loads of non-repaired mutations accrued over a lifetime finally lead to neuronal disruption and consequent neuronal death.

Neuroinflammation

As what happens with other biological systems, the immune system maintains a close communication with the nervous system. The innate immune system plays a key role regulating neuronal plasticity and stress resistance mechanisms in the brain. However, aberrant immune activation contributes to synaptic degeneration and functional impairment. With age, chronic inflammation in the CNS, also called inflamm-ageing, takes place due to a cascade of events (Franceschi et al., 2000).

The CNS is formed by multiple cell types besides neurons: microglia, astrocytes and macrophages. The microglia are the only cells in the CNS originated from the yolk sac which migrates into the brain during

embryogenesis. They constitute the major cellular component in the innate immune system in the brain responsible for protecting neurons from pathogens by scanning the CNS from external threats (Bachiller et al., 2018). During ageing, the inflammatory response driven by the microglia becomes constantly activated (Scheiblich et al., 2020). Consequently, the permanent production of pro-inflammatory cytokines, among them interleukins (IL-1β, IL-6) and tumor necrosis factor α (TNF- α), exacerbates a feedforward loop while activating more microglia, leading to chronic inflammation. In addition, the activated microglia produce large amounts of nitric oxide (NO) leading to oxidative stress in neurons (Cross, Sarah J. Linker, Kay E. Leslie, 2018). The inflammatory boost consecutively activates another cell type also implicated in the immune response, the astrocytes. As a result, the reactive astrocytes start secreting pro-inflammatory factors, such as NF-κB or IL-1β, and lose their ability to remove glutamate from synapses, avoiding excitotoxicity**,** and stop producing neurotrophic factors necessary for synaptogenesis and neuronal survival (Liddelow et al., 2017). This huge amplification of the innate immune response results in progressive neuronal damage, synaptic degeneration, functional impairment and, ultimately, neuronal death.

Neuronal death

During early life development, large numbers of immature neurons undergo apoptosis through proteases, the so known programmed cell death. However, the rate of cell death stabilizes and slows down during adult life until it accelerates again in late life due to natural processes of ageing (Yuan & Yankner, 2000).

As previously mentioned, oxidative stress tends to aggravate during ageing due to progressive mitochondrial dysfunction and accumulation of oxidatively damaged molecules. When the DNA damage persists, programmed cell death is activated so as to delete genome instability among the affected cells (Cross, Sarah J. Linker, Kay E. Leslie, 2018). On the other hand, persistent activation of the brain immune system also creates neuronal stress ending up in neuronal death. However, other factors can be involved in the activation of cell death in the CNS, as an example dysregulation in calcium homeostasis. It is known that Ca^{+2} dynamics within the neuron becomes impaired during ageing, partly due to age-related reductions in the neuroprotective Ca^{+2}-binding protein calbindin, resulting in elevated concentrations of intracellular Ca^{+2} levels (Geula et al., 2003). The continuous Ca^{+2} intracellular influx is known to enhance ROS production and continuously activates glutamate receptors, leading to impaired glucose uptake and consequent neuronal damage due to excitotoxicity (Mattson, 2003).

In line with this neurotoxic event, the accumulation of damaged proteins in form of insoluble plaques and tangles within or outside the cell is strongly linked to ageing. In fact, natural enzymatic degradation carried out by cytosolic proteases, lysosomes and proteasome, is progressively reduced in neurons while ageing (Saez & Vilchez, 2014). Increased production of neurotoxic factors or the accumulation of autophagosomes with undegraded cargos is a common event among neurodegenerative disorders, resulting in synaptic dysfunction, inflammatory boost and final neuronal death (Mattson, 2003).

Neurogenesis disruption

The presence of two neuronal stem cells populations in the brain, located in the dentate gyrus and the olfactory bulb, are well known to generate new neurons during adulthood(Ming & Song, 2011). However, recent studies demonstrated that age-related oxidative stress, impaired DNA repair or inflammation in the progenitor cells population contributed to a reduction in their neurogenesis ability (Regnell et al., 2012).

Altogether, the accumulation of errors in multiple cellular systems together with a reduced ability to generate new neurons, results in a progressive neuronal dysfunction and loss of cells during ageing in the CNS.

Normal ageing vs neurodegeneration

Ageing is considered the greatest risk factor for developing neurodegenerative disorders. However, normal brain ageing and neurodegeneration are not strictly the same. Indeed, discrepancies on how neurodegeneration should be considered with regard to normal ageing exists among the scientific community. On the one hand, brain ageing can be considered as a natural event during the lifecycle of an organism, whereas neurodegeneration is a brain disorder where the aforementioned critical hallmarks of ageing are accelerated and aggravated (Hou, 2019). In some cases, research on the etiology behind neurodegeneration place neurodegenerative diseases as secondary outcomes from primary cardiovascular pathology(Castillo et al., 2019). Finally, some studies state that apart from the normal mechanisms of brain ageing, other processes are additionally triggered in neurodegeneration, for example the fragmentation of neuronal Golgi apparatus due to misfolded proteins accumulation(Jellinger, 2010).

As what happens with ageing, the progression and onset of neurodegeneration differs among individuals depending on their genetic background and individual lifestyle habits. One example is the presence of certain polymorphisms located in genes involved in the inflammatory response that have demonstrated to increase the risk for developing Alzheimer disease (AD) (Eikelenboom et al., 2006). Moreover, studies in animal models demonstrated that diet-induced obesity, diabetes and insulin resistance increased the vulnerability to suffer from AD by accelerating normal mechanisms of brain ageing, such as the disruption of glucose metabolism (Luchsinger & Gustafson, 2009).

Nonetheless, neurodegeneration not only strictly affects the CNS but also other biological systems maintaining a reciprocal interaction with the brain. This is the case of the gastrointestinal system (GI) which is known to experience, through pathological features, the progression of neurodegenerative diseases with onset in the CNS. Likewise, some mechanisms found in the GI are known to influence the pathophysiology of neurodegeneration.

2. GASTROINTESTINAL SYSTEM

The GI consists of multiple organs distributed from the mouth to the anus responsible for digestion, absorption, excretion and protection of ingested food. Among the different tissues forming the digestive track, part of PNS has evolved into an almost independent nervous system, the so called enteric nervous system (ENS), considered by the scientific community as the second brain (Cheng et al., 2010).

Given its vital role for defence against potentially pathogenic organisms acquired from the environment, the GI hosts the largest population of immune system cells in the body, located in the mucosa. A continuous cross-talk between the ENS and the immune system is stablished so as to tolerate or activate an inflammatory response regarding the gut microbiome colonization (Mason et al., 2008). The gut microbiota (GM) has cohabited the human gut to the point of establishing its own niche within the GI and becoming a key player in the development of the intestinal immune system and the correct functioning of the ENS (Cerf-Bensussan & Gaboriau-Routhiau, 2010). Indeed, the GM is responsible for training the immature T cells that recognized them in the colon to develop into regulatory T cells and, thus, inducing tolerance to these microorganisms in early life (Katsnelson, 2011).

The GI experiences a functional decline during ageing. Nevertheless, cellular and molecular changes exhibited in the ageing gut vary depending on the tissue observed and can be influenced by genetic background or lifestyle habits.

Enteric nervous system

The ENS constitutes an essential part of the GI responsible for the regulation of secretion, digestion, absorption, excretion, mucosal maintenance and immunological defence along the gut. The human ENS contains more than 100 million neurons, at least as many as the spinal cord, organized in small ganglia mainly distributed in two plexuses connected with each other forming microcircuits (Furness et al., 2014). Indeed, not only neuronal bodies but glial cells resembling those found in the CNS are also contained in the enteric ganglia. Even though the ENS can act independently from the CNS innervated by its own neurons, both systems maintain a constant communication through extrinsic parasympathetic, sympathetic and sensory nerves modifying the behaviour of the ENS (Rao & Gershon, 2016). This bidirectional communication is also known as the brain-gut axis. A summary of the principal mechanisms during ageing in the ENS can be found in **Figure 2B**.

Hallmarks of ENS ageing: similarities with the CNS

As what happened with the CNS, the GI experiences natural ageing changes. Indeed, it is thought that neurons in the ENS might be more vulnerable to this process than neurons in the CNS due to its constant exposure to external factors (Saffrey, 2013). Some of the confounding factors influencing the progression of this natural event might be the effect of the medication prescribed for complaints in other biological

systems or changes in the microbiome composition (Saffrey, 2013). Due to the wide variability of factors influencing the mechanisms of ageing in the ENS, the investigation on this topic is really challenging. However, recent advances in new model systems and genetic tools are facilitating the research in this field.

Neuronal loss

Some key physiological changes have been reported during ageing in the human ENS. Interestingly, the vulnerability to age-related damage is not equally distributed along the 16 different subpopulations of neurons found in the ENS, being cholinergic and nitrergic neurons the most affected ones (Robert et al.,2003). Moreover, there is conflicting evidence regarding neuronal loss along the gut. While some authors state significant loss of neurons and glial cells in the enteric ganglia of older subjects, others do not completely agree with this conclusion (Saffrey, 2013). Some explanations behind the variability between the results might be related to the anatomy of the gut and how it is analysed in each study. For example, the size of the intestinal track experiences changes over the lifetime and the distribution and size of the enteric ganglia are arbitrary and individual-dependent (Saffrey, 2004). Other methodological aspects, such as the neuronal markers selected for neuronal quantification or the effect of the diet adopted by each analysed participant, may also have masked some results and made the studies non-comparable (Saffrey, 2013). Despite the differing opinions to determine whether neuronal loss is dramatically accelerated while ageing in the gut, cell death is a key feature of this natural process in both the CNS and the ENS.

Oxidative stress and DNA damage

The principal mechanisms behind the enteric neuronal dysfunction and loss during ageing are the same as found in the CNS, however the factors triggering them may vary between the two systems.

Oxidative damage has a detrimental effect on neurons and increases while ageing, being the ENS particularly affected by this event. As stated above, elevated ROS production interacts with cell components such as the genome, triggering DNA damage. Indeed, DNA damage was found to induce cellular senescence phenotype in a population of myenteric neurons from the small intestine in aged mice (Jurk et al., 2012). Regarding the mutation rate, it was found to be tissue-specific in middle and old age mice, being the small and the large intestines the most affected ones along the GI (Ono et al., 2006).

Some sources of oxidative stress in the gut are changes in the intestinal microbiome or dietary habits. On the one hand, the gut microbiome regulates the correct functioning of the GI and stablishes a balance between beneficial and pathogenic species in the GI. When gut homeostasis is altered due to harmful colonizing species, these microorganisms can induce oxidative stress by triggering molecular pathways related to neuronal apoptosis, epithelial barrier disruption or inflammation. Two examples of pathogenic bacteria triggering oxidative stress by disrupting intestinal homeostasis are the family of *Helicobacter* and *Salmonella typhimurium* (Y. Wang et al., 2020). On the other hand, the consumption of high levels of saturated fat have demonstrated to induce chronic states of metabolic inflammation through the generation

of white adipose tissue that continuously secrets proinflammatory factors, exacerbating oxidative stress(B. L. Tan & Norhaizan, 2019). Meanwhile, calorically restricted diets have demonstrated to be potentially beneficial against ROS generation and reducing myenteric neuronal loss in adult animals(da Silva Porto et al., 2012; Saffrey, 2013).

Oxidative stress occurs when there is an imbalance between the production of ROS and its elimination through antioxidant defences. In order to maintain this balance, antioxidant defence mechanisms involving neurotrophic factors, GDNF and NT-3, and endogenous enzymatic antioxidants are found in myenteric neurons (Thrasivoulou et al., 2006). Moreover, dietary nutrients, such as vitamins, proteins or fats, obtained from the environment also play a key role to restore normal levels of ROS (Y. Wang et al., 2020). Unfortunately, disruptions in the production of neurotrophic factors or nutrient-poor diets occurs during ageing, exacerbating the susceptibility to oxidative damage and leading to neuronal degeneration and death (camilleri et al., 2008) . Additional mechanisms such as calcium dysregulation or protein aggregation have also been found to contribute to the ageing ENS (Saffrey, 2013).

Neuroinflammation

There is a continuous immune crosstalk between the ENS and intestinal macrophages, the responsible for initiating the immune response. In particular in the gut, a specific population of macrophages named muscularis macrophages (MM) are located along nerve fibres and within myenteric ganglia(Veiga-Fernandes & Mucida, 2016). An imbalance in ROS production is one of the factors triggering an inflammatory response in the gut. Similar to the CNS, normal immune response involves the production of anti-inflammatory and neurotrophic growth factors promoting ENS regeneration after mucosal inflammation, which can be localized in specific areas of the GI (Margolis et al., 2016). However, when the MMs response is exacerbated due to persisted inflammation, altered neuronal hyperexcitability and neuronal loss leads to disruption in the ENS(Brierley & Linden, 2014).

Other processes have also been described to trigger an inflammatory response, for example significant alterations in the GM. In order to maintain the correct homeostasis of GM, intestinal epithelial cells secrete antimicrobial substances protecting against external pathogens (Miron & Cristea, 2012). During ageing, there is an increase in mucosal permeability due to a disruption in the production of antimicrobial factors(Tran & Greenwood-Van Meerveld, 2013). This event facilitates pathogenic and non-pathogenic microorganisms to get access into the gut disturbing the stablished microbiome homeostasis. As a consequence, a reduction in commensal colonizing bacteria and the rise for opportunistic species are observed in the aged gut, stimulating a proinflammatory state which contributes to inflammageing (Claesson et al., 2012).

During ageing, scientific evidence confirms that a low-graded chronic inflammatory state is not only characteristic from the aged CNS but also the ENS. This exacerbated immune response is also contributing

to a decrease in enteric neuronal density, by activating neuronal apoptosis, and the reduction in enteric neurogenesis ability for neuronal replacement (Becker et al., 2018).

Neurogenesis

In order to maintain ENS homeostasis facing continuous neuronal loss, adult neurogenesis was discovered in mice carried out by the enteric neural crest-derived cells (ENNCC). Similar to the CNS, enteric neural stem cells showed the ability to differentiate into neurons *in vitro* (Nancy M Joseph 1, Shenghui He, Elsa Quintana, Yun-Gi Kim, Gabriel Núñez, 2011). However, no evidence on the presence of neuronal formation has been found *in vivo* under different conditions including ageing (Joseph et al., 2011). In this regard, it was though that no on-going neurogenesis was done in the healthy and mature intestine but was restricted to repair after significant injury. This paradigm shifted when a recent study using novel tools in *in vivo* microscopy showed proliferating enteric neural progenitor cells (ENPCs) replacing almost 90% of adult myenteric neurons in the mice gut. This neurogenesis event allowed to maintain the original number of neurons located in the myenteric ganglia after significant neuronal loss in adult individuals (Kulkarni et al., 2017). Moreover, some factors influencing the correct functioning of the GI are also known to influence the ENPC behaviour, for example diet or the microbiome composition (Saha et al., 2018; Yarandi et al., 2020).

Further investigation on cellular and molecular pathways regulating neurogenesis in the ENS needs to be done so as to determine the etiology of gut pathologies where neuronal loss plays a key role in the progression of the disease.

In conclusion, the study of cellular ageing in the ENS is very challenging since multiple factors can influence its progression. Even though some molecular pathways have been discovered in the gastrointestinal track from animal models, some physiological changes remain poorly understood and need further investigation on human subjects

The gut microbiome

Considering microbes have been colonizing the human body from its origin, it is not surprising to think they play a crucial role for the species to survive. Indeed, the human has co-evolved together with its microbiota, to the point that the microbiome is involved in the correct functioning and regulation of different systems (Cryan et al., 2019). Among the different niches of the human body where the microbiome resides, the highest proportion is found in the gut. In fact, not only bacteria but other microorganisms such as viruses or parasites co-exists in the gut environment (Eckburg et al., 2005).

The composition of the GM is highly variable and depends on both the genetic background and the environment of the host. Not only intra- individual differences exist across time but the diversity of the GM also differs between individuals (Caporaso et al., 2011). Indeed, specific combinations of prokaryotic

species, also known as compositional signatures, have been identified among individuals (Lin & Zhang, 2017). Interestingly, family members are often showing more similar GM taxa than non-related individuals; either because they share common environments or genetic composition(Goodrich et al., 2014).

Constant changes in the host lifestyle, such as diet, medication or physical activity are constantly shaping the composition of the GM community within the host (Amato et al., 2021). This dynamic response in the GM composition is tightly regulated by the host-immune system, responsible for eliminating harmful microbes and allowing commensal species to recolonize the gut. Given that the immune response declines while ageing and the intestinal integrity is subject to age-related changes, disruptions in the microbiome homeostasis start appearing while ageing (Jeffery et al., 2016).

Microb-ageing

While human ages, its GM composition undergoes significant changes at three remarkable time points: the initial colonization after birth and subsequent development, when the infant reaches 2 years of age (which has started to transform into adult form) and when entering the old age. These periods are known to be the time windows where other bodily systems, such as the immune system or the CNS, undergo development or decline (Palmer et al., 2007). During the latest stage of the human lifespan, slow processes of deterioration start appearing in the gut. From degenerative changes in the ENS to gastric motility disorders, the composition and correct functioning of the GM is dramatically affected (Konturek et al., 2015). In addition, environmental factors such as the dietary pattern, physical activity or immune strength, which normally deteriorates during ageing, together with recurrent infections can have a greater impact later in life (Vauzour et al., 2017).

The characterization of the microbiome ageing, microb-ageing, has been challenging since the GM composition not only differs between individuals but is also subjected to cultural and geographical variances. When ageing, the correct functioning of the immune system and the intestinal epithelium barrier declines, resulting in the onset of a phenomenon called dysbiosis. The main characteristic of this event is a decline in the GM diversity; which has been observed from the 8th decade of life (Kim & Benayoun, 2020). In this regard, a decrease in the beneficial anti-inflammatory taxa *Lactobacillus* and *Bifidobacterium* was observed in elderly patients (Claesson et al., 2012). However, the presence of *Enterobacteriaceae, Clostridium, Streptococcus spp., Staphylo-coccus spp.,Enterococcus spp.,*and *Enterobacter spp*. was found to increase with age promoting infection and intestinal inflammation (Toward et al., 2012).

Interestingly, these changes in the microbiota play a key role determining whether the subject will experience a healthy or unhealthy process of ageing, referring to unhealthy ageing as the natural process where pathological consequences of ageing are aggravated. When referring to healthy gut ageing, specific microbial taxa including *Bacteroidetes, Clostridium cluster XIVa* and *Faecalibacterium prausnitzii* are associated with improving health due to its immune-regulatory effects and anti-inflammatory properties

(Shintouo et al., 2020). Supplementation on animal models of *Bifidobacterium* and *Caenorhabditis elegans* resulted in improvement of locomotor functions and longevity(Komura et al., 2013). On the contrary, the bacteria family of *Porphyromonadaceae* is linked to cognitive decline and increasing the risk for affective disorders(Collins et al., 2012). The parasite nematode *Trichinella spiralis* also demonstrated the induction of IL-1 cytokines and decrease in food intake(McDermott, 2006). In addition, the phylum Proteobacteria was found to promote pro-inflammatory cytokines IL-6 and IL-8 while Ruminococcus lactaris suppressed them(Kim & Benayoun, 2020). Finally, transplantation of *Christensenella* to animal models showed reduction in weight gain(Goodrich et al., 2014).

Representing an exception towards this natural trend of diversity reduction during ageing, one study where GM from centenarians and supercentenarians subjects was analysed, found out that this population reported a high diversity of GM species(Santoro et al., 2018). In these subjects, subdominants species of beneficial microbiota taxa *Akkermansia, Bifidobacterium* and *Christensenellaceae* were finally stablished as the dominant ones due to long-term adaptation; promoting longevity and healthy effects in this elderly population (Biagi et al., 2016).

In summary, the GM composition are not only subjected to physiological changes while ageing but it contributes to the progress of this natural event. Significant shifts in the GM composition can increase the susceptibility to aged-related disorders, such as the onset and progression of neurodegenerative diseases; whereas beneficial taxa can ameliorate key pathological features improving the quality of life. A summary of microb-ageing characteristics can be found in **Figure 2C**.

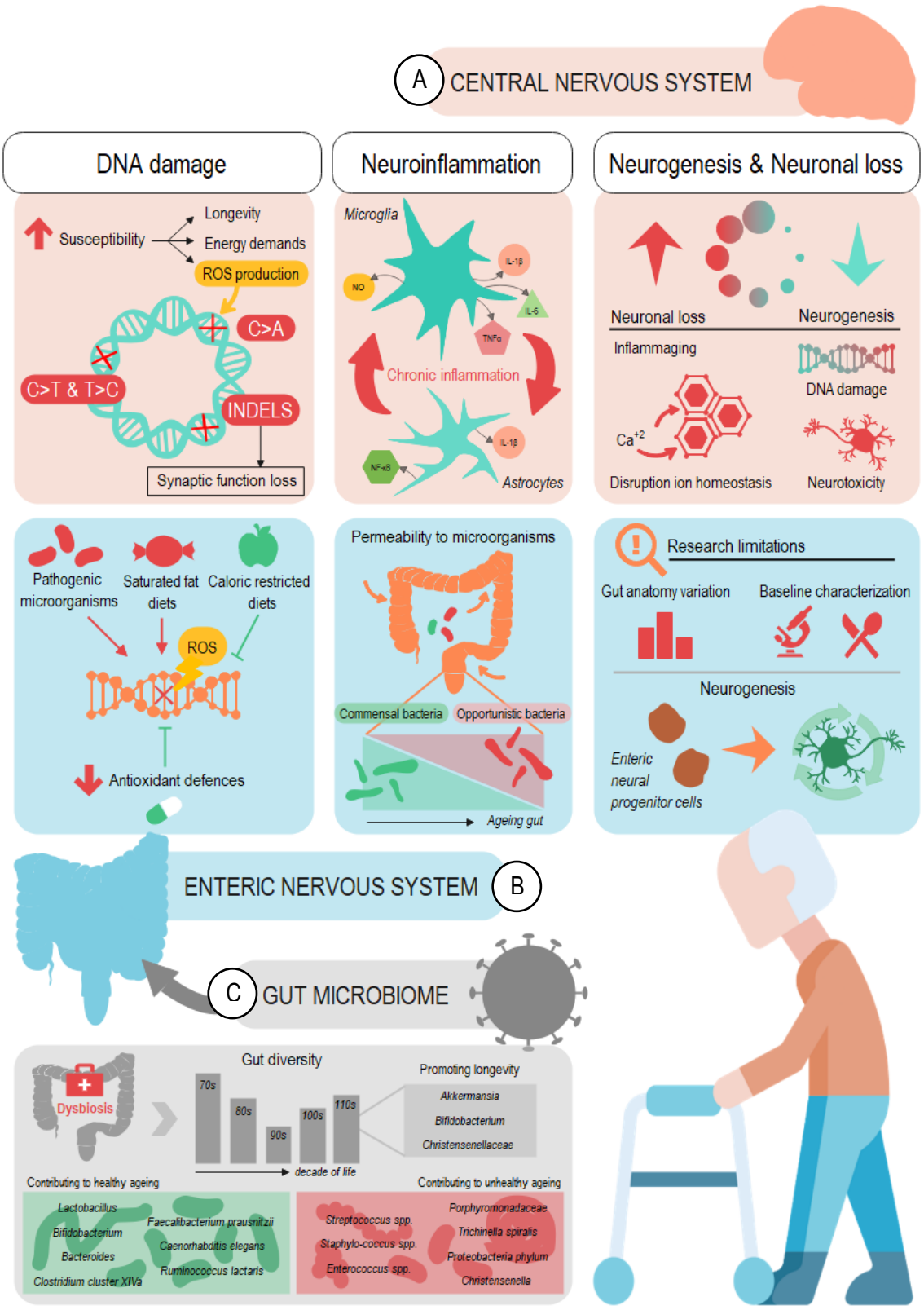

Figure 2 | **The process of ageing in the different systems: CNS, ENS and GM**. (**A-C**) Natural processes occurs during ageing in the (**A**) CNS (**B**) ENS and (**C**) GM. While CNS and ENS share common molecular mechanisms regarding DNA damage, neuroinflammation, neuronal loss and neurogenesis, the ageing GM consists on compositional shifts in microorganisms taxa influencing the progression of pathological pathways in the CNS and ENS.

3. MICROBIOME-GUT-BRAIN AXIS

The microbiome-gut-brain axis refers to the network of neuronal connections linking the GM, the ENS and the CNS allowing bidirectional communication between them. As previously reported, multiple neuronal pathways connect the gut with the brain in order to maintain a constant crosstalk influencing the functioning of the ENS and the behaviour of the CNS. In addition, the GM has also stablished a direct communication with the ENS through the secretion of certain neurotransmitters, amino acids and microbial metabolites into the gut lumen (Obata et al., 2020). These chemical signals can either interacts with the host immune system, transferring the signals to the ENS (**Figure 3A**), or contacting terminal ends of enteric neurons (**Figure 3B**) (Morais et al., 2021). However, the molecular mechanisms by which the microbe-mediated immune response interacts with the ENS are not fully understood. As an example, it remains unclear whether the ENS is the responsible for initiating an immune response after sensing the luminal signals produced by the GM or whether the ENS acts after the activation of immune response propagating this signal (Yoo & Mazmanian, 2017).

The action of this GM chemical signalling is not only restricted to local neurons in the gut but it can also travel through afferent pathways connecting the gut with the brain, such as the vagus nerve. Indeed, it has been reported that peripheral ends of the vagus nerve can get activated through mechanoreceptors or chemoreceptors sensing luminal signals produced by the enteroendocrine cells (EECs) located in the gut epithelium (**Figure 3C**) (H.-E. Tan et al., 2020). The EECs modulates the production of endocrine products under the presence of GM chemical signals (Aresti Sanz & el Aidy, 2019). Therefore, the GM can also have an impact in the CNS behaviour, for example regarding host's appetite, through indirect chemical signalling. Another mechanism by which the GM modulates the CNS is through the secretion of cytokines that activates the immune system. In this case, the secretion of cytokines can be done either from the GM and travel through the blood brain barrier (BBB) to the CNS (**Figure 3D**) or released by activated immune cells in the brain, such as the microglia(Morais et al., 2021). Interestingly, the permeability of the BBB is also subjected to the GM composition and any possible pathogenic infection in the mice gut (Braniste et al., 2014). Finally, the GM is also known for modulating neuroplasticity and gene expression in the brain through the release of lipids, in particular short-chain fatty acids (SCFAs), after the fermentation of fibre from the ingested food (Dalile et al., 2019).

Nonetheless, this microbiome-brain communication is not unidirectional since the GM can also receive information from the CNS. Different signals with origin in the brain, from the secretion of stress hormones to neurotransmitters, can act in the ENS via the efferent pathway of the vagus nerve, leading to changes in the gut environment and GM composition (**Figure 3E**). As exposed in this review, the vagus nerve is a key player in the bidirectional communication of the microbiome-gut-brain axis(Bonaz et al., 2013).

In conclusion, multiple pathways are involved in the continuous communication between the three biological systems throughout the lifecycle of an organism. Therefore, any disruption compromising the integrity of any

of these parts will lead to pathological consequences in the other two. During ageing, it is not a mere coincidence that progressive cognitive decline is normally accompanied by changes in the GM composition. As mentioned above, dysbiosis is a recurrent phenomenon during ageing leading to significant shifts in GM composition. This situation is indirectly recognized by the CNS and the ENS through the secretion of gut microbiome-derived molecules, influencing the immune system and promoting neuroinflammation(Ma et al., 2019).

Given the critical role that GM plays towards neuronal homeostasis and that the CNS and ENS actively shapes the GM environment, multiple translational research on animal models is being made so as to untangle the pathological mechanisms triggered during neurodegeneration. First, enteric consequences will be investigated as a result of neurodegenerative disorders in the CNS. Then, GM will be analysed as key modulators on the progression of neurodegeneration. Finally, future perspectives regarding therapeutic strategies or markers for neurodegeneration will be discussed.

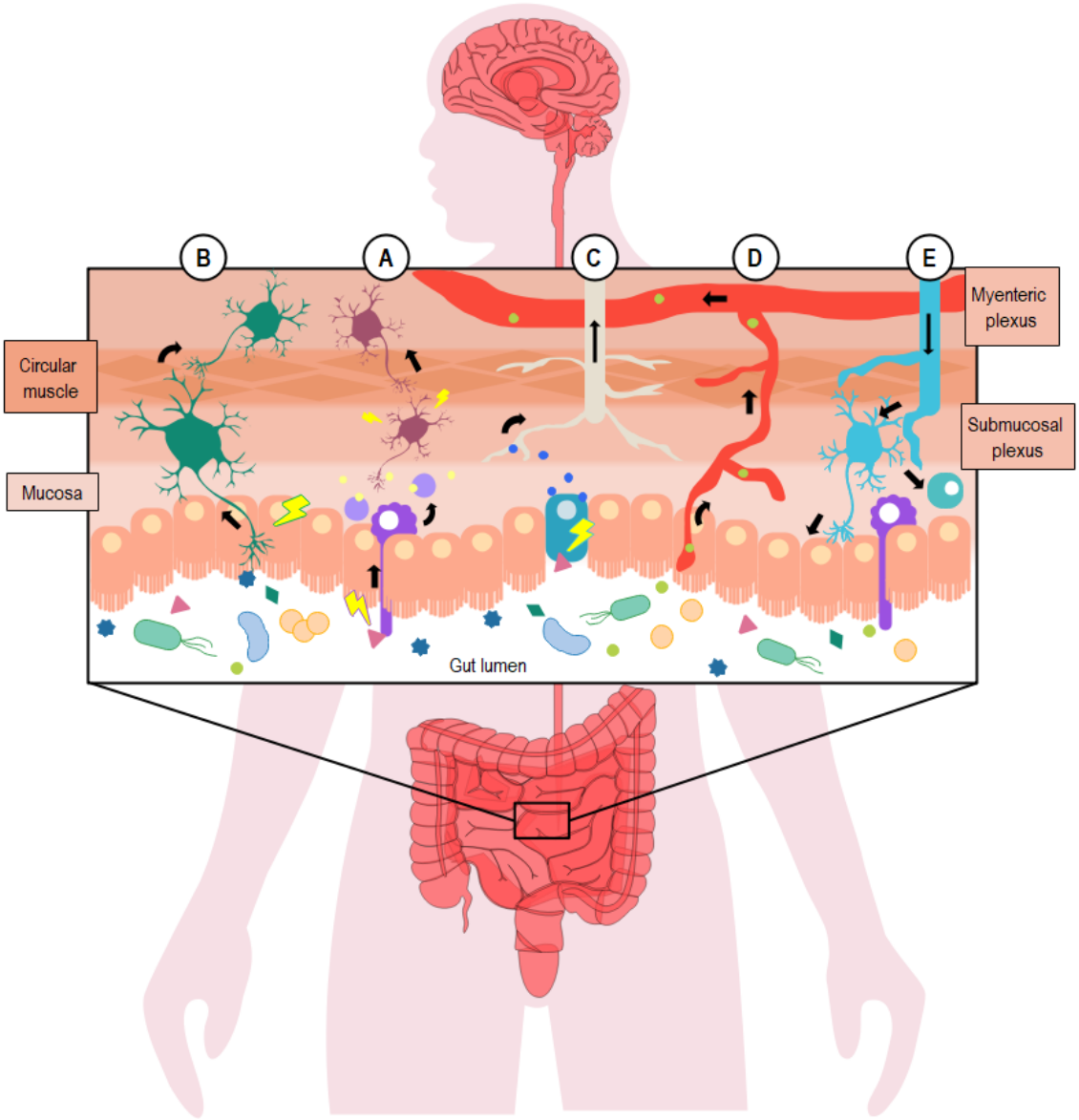

Figure 3 | **The microbiome-gut-brain axis**. Bidirectional communication between the gut microbiome, the ENS and the CNS. (**A-B**) The GM secretes certain molecules to the gut lumen that can interact with either (**A**) the host immune system, in this case first dendritic cells and T cells (purple) transferring the signal to the enteric neurons (magenta), or (**B**) directly activating terminal ends of the ENS (green). (**C-D**) However the GM chemical signalling can also affect the CNS by (**C**) activating EECs (blue) that transfer the signal to peripheral ends of the afferent vagus nerve (white) or (**D**) GM cytokines (green) traveling through the portal blood (red) to the BBB, relaying these signals to the brain. (**E**) Finally, the CNS is also able to transmit signals to the gut environment through the efferent pathway of the vagus nerve (blue).

Enteric manifestations of neurodegenerative disorders

Mounting evidence suggests that neurodegeneration is not limited to the CNS but its effects can also be observed in other nervous systems. Given the considerable degree of cellular and molecular similarities during ageing between the CNS and the ENS, some neurodegenerative disorders have been found to show enteric consequences. In some cases, key pathological conditions behind degeneration in the CNS can even originate in the ENS much earlier than in the brain. In this Review, the focus of investigation will be Parkinson disease (PD) and AD.

Parkinson disease

PD is the second most prevalent neurodegenerative disorder affecting 0.3% population worldwide. It is characterized by the inability to control unvoluntary movements due to alterations in the substantia nigra of the brain. Different pathological mechanisms results disrupted in PD: aggregation of insoluble amyloid α-Syn fibrils and degeneration of midbrain dopaminergic neurons, mitochondrial dysfunction, neuroinflammation due to microglia activation and excessive production of ROS (Blandini et al., 2000). Among the characteristic symptoms of this disease, pathological manifestations in the GI such as dysphagia, delayed gastric emptying or faecal incontinence are found in PD patients.

The intracellular aggregation of insoluble amyloid α-Syn fibrils in neurons is the histological hallmark of PD and is tightly related to motor dysfunction(Hawkes et al., 2007). In this regard, multiple studies in transgenic mice models have investigated the presence of these phosphorylated inclusions in the ENS, and are summarized in **Table 1**.

Disrupted mechanism	Pathological manifestation	Authors
Non endogenous α-synuclein proteinase	Inclusion bodies within enteric neurons expressing α-Synuclein-immunoreactivity	Kuo et al., 2010
Overexpression of human wildtype α-synuclein	Aggregation of α-Synuclein in axonal fibres and cell bodies in enteric neurons, accumulation of proteinase-K insoluble α-Synuclein, progressive functional changes in the GI and other peripheral organs.	Hallett et al., 2012
Overproduction of human pre-synaptic α-synuclein	Aggregation of -Synuclein in myenteric ganglia and impairment in defecation to stimuli.	L. Wang et al., 2012

Overexpression of human α-synuclein	Expression of α-synuclein limited to efferent fibres projecting from the dorsal motor nucleus of the vagus nerve (DMV) to the ENS. Older transgenic mice had lower density of α-synuclein in the GI than younger mice, suggesting disruption in efferent vagal fibres during ageing.	Noorian et al., 2012
Injection of large doses of misfolded α-synuclein into the pyloric and duodenal muscle	Vague nerve involved in the centripetal spread of misfolded α-synuclein from the gut to the brainstem. Loss of dopaminergic neurons and motor and non-motor symptoms.	S. Kim et al., 2019
Truncal vagotomy and α-Synuclein deficiency	Prevention of α-Synuclein spread from the gut to the brain due to elimination of the vagal nerve and associated terminal ends in the GI.	S. Kim et al., 2019
Duodenal inoculation of α-synuclein preformed fibrils	Gastrointestinal deficits and progression of α-synuclein pathology in aged mice, but not younger ones, to the midbrain and consequent motor deficits.	Challis et al., 2020

Table 1 | Modelling PD in mice models and consequent pathological manifestations.

In summary, studies in animal models evidence that aggregation of α-synuclein in enteric neurons is not only linked to GI disfunction but also motor deficits related to the CNS. Moreover, these findings suggests that pathogenic deposition of α-synuclein can spread from the ENS to the CNS through afferent autonomic fibres, for example via the vagus nerve, therefore the pathology of PD may start in the gut. Although these promising results, studying pathological characteristics of a human disease in animal models arise translational problems. Some experimental limitations are that the cellular mechanisms observed in animal models do not necessarily occur in the same way in human subjects, or that the model does not accurately mimic the pathogenesis of the disease

To solve these inter-species boundaries, recent investigation on gut samples from PD patients is being made and are summarized in **Table 2**.

Sampling methodology	Pathological manifestation	Authors
Autopsy of enteric plexuses	PD patients exhibited aggregation of Lewy bodies in both plexuses while only 8 out of 24 controls showed it.	Wakabayashi et al., 1988
Autopsy	Identification of Lewy bodies inclusions in specific subpopulations of neurons in brainstem nuclei from PD	Braak et al., 2001

	subjects, in particular projections of efferent fibres from the vague nerve.	
Autopsy	Presence of Lewy pathology in the peripheral vague nerve and the enteric plexuses in the stomach and oesophagus from PD patients	del Tredici & Duda, 2011
Biopsies of the distal colon	Immunostaining revealed α-synuclein staining in nerve fibres of the colonic submucosa in early subjects with PD disease, but not in control subjects.	Shannon et al., 2012
Autopsy	Expression of α-synuclein was universally detectable in PD patients but not in AD or control subjects.	Gold et al., 2013
Colonoscopy biopsies	Accumulation of α-Syn aggregation was found in the gut mucosa from early stages of non-treated PD patients.	Hilton et al., 2014
Vagotomy	Subjects who underwent full vagotomy, eliminating vagal projections to the GI, had lower risk of developing PD than patients without this treatment.	Svensson et al., 2015

Table 2 | Studies of PD pathophysiology in human patients.

Interestingly, α-synuclein inclusions were not only seen in animal models but also in enteric tissue from patients suffering from PD. These findings, together with previously reported results from mice models, made the scientific community restate the hypothesis that the vagal nerve can serve as a connection for the retrograde propagation of the α-synucleinopathy from the ENS to the brainstem finally reaching the cerebral cortex. Indeed, one study where subjects underwent removal of vagus nerve fibres connecting the GI with the CNS, showed a protective effect of this treatment against developing PD later in life (Svensson et al., 2015). Latest insights in this disease indicate that ingested environmental pathogens can be the trigger behind the formation of α-synuclein inclusions in the ENS (Travagli et al., 2020).

In conclusion, the main hallmark of PD is not restricted to the CNS but can also be found in the ENS. However, some relevant information is still lacking; for example, whether the presence of Lewy bodies in the ENS is necessarily pathological specific for PD and relates to the severity of the disease, since they've also been reported (in smaller proportions) in the aged gut from healthy subjects, or which neurons in the ENS are more affected by this mechanism (Böttner et al., 2012).

Alzheimer disease

AD is a degenerative disorder and the most common form of dementia in the world. It is well known by its devastating effects in disability and dependency among patients suffering from it. Since no effective prevention and treatment is available, multiple efforts to untangle the pathophysiology of AD are being made.

AD is a complex disorder where numerous molecular pathways are disrupted. One of the main hallmarks is the progressive accumulation of extracellular amyloid beta (Aβ) plaques triggering neuroinflammation and neuronal disruption (Schliebs, 2005). As what happened with PD, AD patients experience symptoms of GI disruption such as diarrhea or bowel incontinence (Li et al., 2018). When focused on the amyloid pathology, cells from the ENS including neurons and glia express the precursor of Aβ plaques, the so-called Amyloid precursor protein (APP). Indeed, the presence of this protein is necessary for the normal functioning of gastrointestinal motility, immunity and secretion (Puig et al., 2012). This finding suggested that AD pathological manifestations can also be found in the ENS.

Contrary to what happens with PD, very few studies concerning the ENS in AD human subjects are available and can be found in **Table 3**.

Sampling methodology	Pathological manifestation	Authors
Autopsy	No difference in neuronal density or tau pathology in small and large intestine between AD patients and elderly healthy controls.	Shankle et al., 1993
Biopsies of intestinal submucosa	Immunoreactive Aβ plaques in the submucosa of AD patients.	Joachim et al., 1989
Autopsy	Elevated Aβ immunoreactivity in the colon from AD patients. This study did not include controls.	Puig et al., 2015

Table 3 | **Studies of AD pathophysiology in human patients.**

In general, amyloid pathology seems to be present in the ENS from patients of AD. However, a lot of information is still lacking regarding the implication of the GI in the pathophysiology of AD. For example, whether amyloid deposition in the ENS occur later in the disease as a consequence of CNS disruptions or whether this event contributes to the progression of the disease.

Since data from human subjects is very sparse and the results do not seem consistent at all, research in animal models is being made. The available data is summarized in **Table 4**.

Animal model	Pathological manifestation	Authors
Ageing rats	Detection of hyperphosphorylated tau aggregates forming neurofibrillary tangles in the myenteric plexus from ageing rats.	Phillips et al., 2009
APP transgenic mice	Amyloid deposition was present in transgenic and wild-type mice. Alterations in the ENS and deposition of Aβ plaques in the enteric plexuses do not seem to be primarily involved in the pathogenesis of AD but are secondary events during the progression of the disease.	van Ginneken et al., 2011
APP transgenic mice	Amyloid deposition in myenteric neurons of the bowel and reduction of neuronal density was observed in AD models but not in wild-type.	Semar et al., 2013
Transgenic mice expressing mutant forms of APP involved in familial AD	Progressive accumulation of Aβ between enteric neurons leading to increased neuronal loss, dysmotility and enteric inflammation.	Puig et al., 2015
APP transgenic mice	Accumulation of Aβ and overexpression of phosphorylated Tau were present in myenteric neurons. Expression of pro-inflammatory factors was increased and the density of cholinergic and nitrergic neurons was decreased compared to control mice.	Han et al., 2017

Table 4 | **Modelling AD in animal models and pathological manifestations.**

In this regard, studies in animal models may confirm the results seen in AD patients. On the one hand, Aβ plaques are present in the myenteric plexus from transgenic mice for APP protein, as normally occurs in the brain. In addition, phosphorylated tau protein forming neurofibrillary tangles, which are another recurrent pathological feature in AD, are also present in myenteric neurons.

Although these results may look promising, translational problems exist when modelling human neurodegeneration in animal models. In addition, limitations on the methodology followed in each study and the presence of contradictory results challenges the comparison between the results.

The impact of the GM in neurodegeneration

Recent studies have demonstrated the GM to be a crucial factor modulating the correct functioning and developing of both the ENS and the CNS regarding neuroimmune modulation; for example, by regulating the correct development, maturation and activation of microglia (Luck et al., 2020). Since the host immune system is always compromised in neurodegenerative diseases, alterations in GM composition can either worsening molecular mechanisms behind the pathology or improving the prognosis of the disease.

In this case, even thought a lot of research is being done towards the GM in the progress of neurodegenerative diseases, in particular AD and PD, some questions remain unknown. As an example, whether the presence of pathogenic strains appear early in the disease, contributing to the onset and progression of neurodegeneration, or they colonize the body later in the course of the disease is still under debate.

Disease	Model	Disrupted mechanism	Authors
PD	Human patients	Increased abundance of *Enterobacteriaceae* in GI from PD patients correlates to the severity of pathological symptoms and motor phenotype.	Scheperjans et al., 2015
		Enterococcus faecalis has an enzyme that reduces the efficacy of PD treatments by decreasing the rates of drug activation.	Maini Rekdal et al., 2019
	Mice model	Transplant of GM from PD patients into germ-free mice over-expressing human α-synuclein worsened motor symptoms when compared with GM from healthy individuals.	Sampson et al., 2016
		Proteus mirabilis promotes motor déficits driving PD-like pathology.	Choi et al., 2018
		Intestinal infection of *Citrobacter rodentium* can aggravate motor symptoms in PD.	Matheoud et al., 2019
		Pathogenic strains of *Escherichia coli* produce a protein capable of promoting α-synuclein aggregation in the gut and the brain.	Sampson et al., 2020
		Motor dysfunction or dopaminergic neurodegeneration can be reduced through probiotic administration of *Bifidobacterium*, *Lactobacillus* and *Lactococcus* strains	Hsieh et al., 2020
		Increasing abundance of *Escherichia coli* and *Shigella* associated to pro-inflammatory phenotype in the gut and	

AD	Human patients	reduction in the abundance of anti-inflammatory *Eubacterium rectale.*	Cattaneo et al., 2017
		Decreasing abundance of *Firmicutes* and *Bifidobacterium* and an increase in *Bacteroidetes* associated with inflammation and amyloid deposition.	Vogt et al., 2017
	Mice models	Systemic injection of LPS, principal component of *Bacteroidetes*, resulted in amyloid deposition and tau-related pathology.	Asti & Gioglio, 2014
		Akkermansia is correlated to elevated levels of pathogenic Aβ42 in the brain.	Harach et al., 2017

Table 5 | Scientific evidence on GM composition towards pathological mechanisms.

Shifts in the microbiota have been described in individuals with neurodegeneration, mostly due to dysbiosis. Moreover, disruptions in the gut barrier leading to increasing permeability and alterations in the correct functioning of the host immune system due to neurodegeneration, provide the best scenario for pathogenic taxa to colonize the damaged gut. In this context, pathogenic microorganisms not only disrupt the GM homeostasis and prevent commensal microorganisms to recolonize the gut but aggravate molecular mechanisms that naturally occurs during ageing and are involved in neurodegeneration, for example enhancing gut inflammation or deposition of pathogenic proteins in the ENS and CNS (**Figure 5**). However, some beneficial strains have also demonstrated to act as protectors against other pathogens and prevent from inflammation promoting gut integrity.

In this regard, future investigation on how beneficial taxa of GM can be used in the treatment of pathological conditions and to improve the quality of life is required.

CONCLUSION AND FUTURE PERSPECTIVES

The process of ageing in the nervous system compromises complex mechanisms in which multiple factors are involved. The characterization and quantification of changes in the ENS and CNS, for example the presence of specific mutations associated to normal ageing and oxidative damage, offers the possibility to track them so as to determine whether the subject is experiencing a healthy process of ageing. Likewise, understanding how the GM is involved in normal ageing, can allow the scientific community to stablish therapeutic strategies focused on these microorganisms. Indeed, all three separated systems maintain a constant bidirectional communication in which any disturbances in one of them will affect the rest of the systems. In the field of neurodegeneration, the CNS has been the main focus of investigation. However, recent findings in the pathological implication of neurodegeneration in other peripheral systems, such as the ENS, offers a new opportunity for research regarding therapeutic strategies and diagnosis interventions.

The exciting results regarding neurodegeneration in the ENS and the modulation of its severity through GM composition, should encourage researchers to further explore the microbiome-gut-brain axis in health and disease.

When investigating the ENS and neurodegeneration, two main problems arise. On the one hand, animal models do not exactly exhibit the same molecular mechanisms as those affected in the human body, and the progression of the disease cannot mimic the human lifespan scale. On the other hand, the methodology and the baseline characterization carried out by each research group differ among them, making the comparability of the results a challenging process. Thus, stablishing protocols and key markers for the correct identification of cellular and molecular components of interest, for example enteric cholinergic neurons or enteric ganglions, needs to be conducted. As a result, the experimental design would be the more reproducible and transparent as possible, leading to valuable conclusions and scientific progress.

Analysis of specific types of DNA damage in neurons from the nervous system, during ageing and in specific diseases, can have a potential application for clinical approaches. For example, the presence of mutational signatures characteristic from pathological mechanisms in neurodegeneration could be used in the future as a tool for early diagnose of possible candidates for the disease. More importantly, the presence of mutational indicators in the ENS identical to those found in the CNS would allow for less invasive techniques to identify or even prevent pathological disruptions related to neurodegeneration. Thus, further development of genetic approaches and methods attributing each mutational signature to a pathogenic event in the field of neurodegeneration needs to be done in the future.

In line with this approach, neurodegeneration in the GI needs further exploration. Considering that some hallmarks of neurodegenerative diseases have been reported in the ENS along the gut, such as amyloid pathology or Lewy bodies, new methodological approaches should emerge. For example, in PD the presence of α-Syn fibrils aggregations in the enteric tissue, particularly the mucosa and submucosa, could

be used for diagnostic of the disease. In this case, routinary endoscopic biopsies, that are easier to obtain that samples from the brain, could reveal the presence of Lewy bodies in the gut and therefore indicate the risk for developing PD. Nevertheless, the molecular marker for the diagnosis, in this case α-Syn fibrils aggregates, should be exclusive and unique from this disease; whereas pathological features of neurodegeneration are normally seen in natural ageing. Finally, the gut sample should take as many layers of the ENS as possible, to have a proportional view of the GI of the patient. Otherwise, some regions of enteric neurons expressing higher amounts of pathological markers could be hidden.

Even though research in animal models has a lot of drawbacks, it contributes to unravel the link between the GI and the CNS. Indeed, exciting insights in the complex relationship between the GM and neurodegeneration inspire the research community to develop therapeutic strategies using specific beneficial GM compositions. In the same way GM can influence the progression of the disease by worsening disrupted mechanisms, probiotic supplementation and fecal microbiota transplant are being developed as future treatments for neurodegeneration. However, evidence for these treatments in neurological disorders is still limited or even non-existent.

In conclusion, there is an urgent need for interdisciplinary approach so as to explore the brain in health and disease in multiple levels, including the GI and the GM inhabiting it.

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