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The Role of the Gut-Brain Axis in Neurodegenerative Diseases and Relevance of the Canine Model: A Review

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Abstract

Identifying appropriate animal models is critical in developing translatable *in vitro* and *in vivo* systems for therapeutic development and investigating disease pathophysiology. These animal models should have direct biological and translational relevance to the underlying disease they are supposed to mimic. Aging dogs naturally develop a cognitive decline in many aspects including learning and memory, but also exhibit human-like individual variability in the aging process. Neurodegenerative processes that can be observed in both human and canine brains include the progressive accumulation of β-amyloid (Aβ) found as diffuse plaques in the prefrontal cortex, including the *gyrus proreus*, the hippocampus, and in the cerebral vasculature. A growing body of epidemiological data shows that human patients with neurodegenerative diseases have concurrent intestinal lesions, and histopathological changes in the gastrointestinal (GI) tract occurs decades that evolve before neurodegenerative changes. Gut

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microbiome alterations also have been observed in many neurodegenerative diseases including Alzheimer's and Parkinson's diseases, and inflammatory CNS diseases. Interestingly, only recently has the dog gut microbiome been recognized to more closely resemble in composition and in functional overlap with the human gut microbiome as compared to rodent models. This article aims to review the physiology of the gut-brain axis (GBA), and its involvement with neurodegenerative diseases in dogs and humans. Additionally, we outline the advantages and disadvantages of traditional *in vitro* and *in vivo* models and discuss future research directions investigating major human neurodegenerative diseases such as Alzheimer's and Parkinson's diseases using dogs.

1. Introduction

The gut-brain axis (GBA) is a highly complex bidirectional interactive system, mediated by hormonal, immunological and neural signals between the gut and the brain[1]. A growing body of evidence suggests that the gut microbiota have profound impacts on the neurodevelopmental processes and brain function[2,3]. Specifically, dysregulation of GBA cross-talk is associated with metabolic syndrome[4,5] and psychiatric disorders such as depression, anxiety, autism, Parkinson's disease (PD), and Alzheimer's disease (AD)[6,7]. In turn, these disorders are also frequently associated with alterations in gut microbiota composition and function which may in turn contribute to disruption of molecular interactions between the gut and brain[8,9].

The GBA is formed by the central nervous system (CNS), the enteric innervation that includes extrinsic fibers of the autonomous nervous system (ANS) and intrinsic neurons of the enteric nervous system (ENS), the hypothalamic pituitary adrenal (HPA)-axis and the intestinal microbiota[10]. The extrinsic innervations of the gastrointestinal (GI) tract connect the gut with the brain through vagal and spinal fibers, while the brain sends efferent sympathetic and parasympathetic fibers to the GI tract[10–12]. The HPA-axis is considered the main regulator of the stress response[13]. Furthermore, the HPA-axis regulates different body processes including alimentary function during digestion [Ref]. Corticotrophin-releasing factor (CRF) released by the hypothalamus and different proteins within this family (e.g.,

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CRF, urocortin 1-3) are known to affect GI tract function, i.e. intestinal motility[14], permeability[15], and inflammation[16]. Specifically, changes in the gastroduodenal motility induced by urocortin administration were noted in conscious rats and this study also suggested that the vagal pathway may mediate the central action of urocortin[14]. The rats subjected to stress (i.e., water avoidance stress) and corticosterone injections exhibited region-specific decreases in epithelial tight junction protein levels in the colon and increased colon epithelial permeability as measured by low molecular weight macromolecules[15]. In addition, cortisol and the proinflammatory cytokines interleukin (IL)-6 and IL-8 were founds to be elevated in patients with IBS[16].

Both clinical and experimental evidence suggests that enteric microbiota contribute to regulating the communication and function of the GBA, including the ability to modulate immune mediators (e.g., cytokines and chemokines)[17]. The GBA interacts not only locally with intestinal cells and ENS, but also directly with CNS through neuroendocrine and metabolic pathways[18]. Furthermore, microbiota can influence ENS activity by producing small molecules that can act as local neurotransmitters, such as γ-aminobutyric acid (GABA), amino-acid derivatives (e.g. serotonin, melatonin, and histamine) and fatty-acid derivatives (e.g.acetylcholine)[19] and by generating a biologically active form of catecholamines (i.e., dopamine, norepinephrine) in the lumen of the gut[20]. The ENS is also targeted by bacterial metabolites such as short-chain fatty acids (SCFAs), including butyric acid, propionic acid and acetic acid, which act to stimulate sympathetic nervous system[21], mucosal serotonin release[22] and to influence memory and the learning process[23,24].

2. GBA in Neurodegenerative Diseases

Dysfunction of the gut microbiota-brain axis has been associated with depression and anxiety, as well as neurodevelopmental disorders such as autism, PD, and AD[8,25,26].

Alzheimer's Disease

AD is a neurodegenerative syndrome accompanied by progressive dementia and histologically associated with the accumulation of cerebral amyloid angiopathy (CAA), which is plaques composed of

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misfolded β-amyloid (Aβ) fibrils and oligomers, as well as neurofibrillary tangles consisting of hyperphosphorylated tau protein in the cerebral cortex, locus coeruleus, and hippocampus[27]. Aβ fibrils accumulation leads to demyelination, neuronal cell death, CNS impairment, cognitive dysfunction, and ultimately death[28–30].

One hypothesis for pathogenesis of GBA in neurodegenerative diseases is dysbiosis, which occurs as a result of antibiotic exposure[31], dietary changes[32], probiotics[33], or a variety of other disease conditions[34,35]. Specifically, various studies have shown an association between dysbiosis and aggregation of Aβ peptides in intestinal epithelial cells[36,37] and ENS[38,39]. Different components of the microbiota, such as bacteria, can excrete immunogenic mixture of functional lipopolysaccharides (LPSs), amyloids, and exudates from their outer membranes into the local intestinal environment[40,41]. Amyloids and LPSs are usually soluble, although they can polymerize and form insoluble fibrous protein aggregates, leading to stimulation of oxidative stress and cross-seeding of further protein aggregation[42,43]. For example, *E.coli* endotoxin was shown to enhance the formation of Aβ fibrils in an *in vitro* model[44]. Also, another study showed that co-incubation of Aβ peptide with LPS potentiates amyloids fibrillogenesis[44], and systemic injection of LPS in wile-type and transgenic AD mice result in greater amyloids deposition and tau pathology[46–49]. Moreover, studies suggest that the structural overlaps in the bacterial amyloid proteins to human Aβ may induce molecular mimicry, an immune response against the self-antigens stimulated by a foreign antigen sharing structural similarities with self-antigens, causing greater inflammatory responses to cerebral Aβ due to altered gut microbiota[32–34].

Another hypothesis is “prion concept” given the fact that many neurodegenerative diseases exhibit accumulation of fibrillary, misfolded protein and its propagation similar of what has been seen in prionopaties[45]. Prionopathy also involves GBA and the local immune system when prions accumulate in follicular dendritic cells within Peyer’s patches and other lymphoid follicles once entering into the intestinal epithelium[46]. Interestingly, a study with a senescence-accelerated mouse model, systemic senile amyloid proteins were identified in Peyer’s patches[47] Combining these findings, then, by

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interacting with dendritic cells, the misfolded protein might be transported to ENS then ultimately spreading to the CNS[46] and this could explain the pathogenesis in AD with Aβ accumulation. A significant amount of functional amyloid was shown to be generated by certain bacterial strains, including *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhimurium*, *S. enterica*, and *Staphylococcus aureus*, and may contribute to the pathology of AD through the accumulation of misfolded Aβ oligomers and fibrils[40,48]. Some bacterial species, such as *Lactobacillus* spp. and *Bifidobacterium* spp. (both gram-positive facultative anaerobic or microaerophilic bacteria) are able to metabolize glutamate to produce GABA, the major inhibitory neurotransmitter[28]. These observations suggest that alteration of the gut microbiota can compromise the endogenous production of GABA[28]. Indeed, alteration of GABA signaling is linked to cognitive impairment, AD, anxiety and depression[45,49–51]. Alternatively, gut bacteria can affect the peripheral nerve function including ENS, is by its metabolites such as short-chain fatty acid (SCFAs)[21]. The SCFAs, such as butyric acid, propionic acid, and acetic acid, are produced by a bacterial fermentation of dietary fiber in the colon. They not only are a part of the critical energy source for colonic epithelial cells, but also can stimulate sympathetic nervous system and release serotonin, then ultimately influence the CNS processes including memory and learning[22]. Importantly, lower levels of SCFAs are shown to negatively affect immune responses, epithelial cell growth, and possibly affect the function of both the central and peripheral nervous systems[52,53].

Parkinson's Disease

Patients with PD show classic motor symptoms such as asymmetric resting tremor that are caused primarily by the loss of dopamine resulting from degeneration and death of dopaminergic neurons in the midbrain[2]. The pathophysiology in PD neurodegeneration have not been definitely established; however, abundant evidence suggests that there are neuroinflammation and glial cell activation in PD patients. Proinflammatory signaling molecules including cytokines (i.e. IL-1β, IL-6, and TNF-α) or enzymes (i.e. nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2)), and oxidative stress are considered key mechanisms that contribute to neurodegeneration and cell death in PD[54].

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Another highly relevant factor in PD pathogenesis is α-synuclein (αSYN), which is the protein present in numerous cell types throughout the body with increased expression at presynaptic terminals of neurons[55]. This protein is highly soluble and regulate the release of synaptic vesicles which contains important neurotransmitters[55]. The αSYN is also expressed as a normal component of the ENS, and it can be detected in submucosal neuronal structures in the intestinal tissues in a large percentage of neurologically intact humans[56–58]. However, under certain circumstances[59], αSYN adopts a β-sheet structure, loses its membrane-binding capacity, therefore leads to aggregation of such misfolded proteins, which ultimately leads to the histological hallmark of PD-Lewy neurites and Lewy bodies in especially dopaminergic neurons in the substantia nigra and noradrenergic neurons in the locus coeruleus[59]. Aggregates of misfolded αSYN impair mitochondrial complex I activity, reduce mitochondrial function and lead to oxidative stress in the neuron[54–56]. Individuals with mutations in the αSYN gene *SNCA* or multiplication of wild-type *SNCA* gene allele are known to develop early-onset, rapidly-progressive PD[60]. PD pathology that involves αSYN spreads from the ENS to the CNS by trans-synaptic cell-to-cell transmission in intact sympathetic and parasympathetic nervous systems[55,56], which is the foundation of “prion concept” in PD pathophysilogy[61]. Interestingly, studies have reported distinct αSYN immunoreactivity in intestinal biopsies taken from clinically normal individuals who would later develop PD[57,62,63], indicating that abnormal enteric αSYN is present before CNS neurodegeneration has advanced sufficiently to produce motor symptoms[2]. Various clinical gastrointestinal signs or the characteristic PD ENS pathology often occur before function of the brain is affected, with constipation being the most common GI complaint in PD[57]. This is likely due to prolonged intestinal transit time, which has been reported to affect both the small intestine[64] and the colon in PD patients[65]. It has been clearly shown that constipation can manifest as a pre-motor symptom years before CNS degeneration[66,67]. In addition, a growing body of data indicates that PD patients have increased intestinal permeability compared to healthy controls[68]. Interestingly, studies also suggest that there are increased risk of developing dementia[69] or Parkinson’s disease[70] in patients with irritable bowel syndrome.

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In recent years, the relationships between intestinal microbiota and PD pathology and their link to deranged GI motility have been studied, and some of the reported differences include decrease in *Prevotella* spp. and *Clostridium* spp. in PD patients[71,72]. These intestinal bacteria are prominent producers of SCFAs, such as butyrate as well as folate (vitamin B9) and thiamine (vitamin B1) which are important for maintenance of epithelial barrier function[71,72]. Interestingly, all of these SCFAs are associated with the amelioration of PD pathology[72–74]. For molecular mimicry in the pathophysiology of PD, Tobacco Mosaic virus (TMV)[43] has been implicated but needs more investigation to make absolute conclusion.

3. Experimental Approaches to Investigating the GBA

Both static and dynamic *in vitro* models have been utilized to advance the understanding of pathogy of the GBA in neurodegenerative diseases. The schematic of the major benefits and disadvantages are summarized in Figure 2. It is important to note that cognitive dysfunction is a highly prevalent not only in AD but also in the non-motor symptoms of PD[75]. In *in vivo* model section, main focus will be on *in vivo* AD models; however, findings from these *in vivo* models for cognitive impairment would be relevant to both AD and PD. The summaries of similarity and differences between clinical and histological differences are stated in Figure 3.

3.1. *In vitro* Models

Static Systems

Development of useful *in vitro* model is critical for elucidating pathophysiology and developing effective therapies especially in the neurodegenerative diseases. Only about 7% of investigational agents tested in phase III trials progress into the market in neurology. This is worse than the average of 11% of drugs marketed for all disease categories[76,77].

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The blood-brain barrier (BBB), a unique compartment that constitutes the interface between the peripheral circulation and the CNS, is the key compartment to understand the GBA[78]. The BBB not only supplies nutrients to the CNS, but also removes waste products (such as urea or potassium) and, prevents blood-borne pathogens and toxic products from harming the brain[78]. The most unique characteristic of the BBB is the network of tight junctions between individual capillary endothelial cells that lack fenestration with reduced capacity for pinocytosis, which ultimately maintains the molecular integrity of BBB[79].

Attempts to craft an *in vitro* model to recapitulate the complexity of the BBB has been attempted and the most traditional BBB *in vitro* culture models, include brain microvascular endothelial cells and astrocytes in a static Transwell culture[66]. Leveraging its similarity with conventional 2-dimensional (2D) culture system and relative simplicity, the Transwell BBB system has been widely used in a research setting[66]; however, it does not provide the shear forces that are critical for maintenance of endothelial polarization and tight junction (TJ) formation[66]. These critical shortcomings result in endothelial permeability that is higher in this model than physiologically seen, which leads to overestimation of compounds that poorly penetrate across the BBB *in vivo* (e.g., sucrose) can now readily diffuse across the endothelial monolayer in the static model[80].

Additionally, current in vitro models include brain microvascular endothelial cell (BMVEC) and astrocyte elements as the BBB models do not replicate the close physiological cross-talk between pericytes and the capillary endothelium[81]. Significant improvements were seen in these BBB models with addition of intraluminal flow in a hollow fiber *in vitro* model and the presence of astrocytes on the abluminal surface, which accomplished more physiologically realistic polarization of the endothelial cells and strengthens the integrity of TJs[82].

Attempts were made to study the GBA using a transwell culture system as also[83]. This system includes only a few components of the GBA and it is important to note that Caco-2 cells, immortal cells from human epithelial colorectal adenocarcinoma, are used to model the enteric epithelial cells in this

system.[83] Given these collective limitations as well as the lack of integration of microbiome/ENS in the *in vitro* system, the results derived from these studies are of questionable translational relevance.

Dynamic Model Systems Using Microfluidics

It is only recent that a novel technology called an organ-on-a-chip (organ-OAC) has emerged[84,85]. The microfluidic device contains microtubing that allow continued flow of media and comprises of multiple cell culture channels allowing co-culture of different cell types[86,87] The multiple small channels compartmentalized by a flexible or a rigid porous membrane allow this innovative model system to recapitulate the tissue-tissue interface[88].

The Gut-OAC (GOAC), which contains multiple-compartment microenvironment, allows researchers to investigate intercellular interactions between intestinal epithelium, immune components, and living gut bacteria or probiotics[86,89]. This technology can be used to investigate the contributions of the gut microbiome, probiotics, or compounds on intestinal pathophysiology and to elucidate pathophysiologies of the *in vitro* environment that are not possible using conventional/static *in vitro* systems[86].

Recently, a BBB-OAC was established and showed physiological barrier functions[90], using ENS and enteroendocrine cells (EEC)-OAC combined together to assess the GBA microenvironment[91]. Advancement in bioengineering techniques will allow incorporating multiple compartments in one *in vitro* system such as a GBA-OAC[92,93]. Despite the great promise of the Organ Chip technology, the transfer of cells from a macroscopic environment (e.g., well-plates) to a microfluidic system requires a significant revision and optimization of cell culture protocols. In fact, multiple factors distinguish microfluidic from macroscopic cell cultures, such as different culture channel surfaces (hydrophobic vs hydrophilic) and the need of reduced media volumes which can magnify the air bobbles blocking the cell-medium contact within the culture channel[72]. Despite these limiting factors including the technology being labor intensive, GOACs are a fast-growing model system which holds greater potential to investigate primary GI diseases and the GBA microenvironment. environment. It is important to note that our group recently established canine primary enteroid and colonoid culture system[94,95]. This is

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canine intestinal stem cell (ISC) culture system which faithfully mimic physiologic structure and function of in vivo intestines[96]. We can establish such in vitro system from both healthy and diseased individuals, which allow investigation of the pathophysiology and treatment effect using this model. Integration of canine primary enteroid/colonoid to the GOAC system is a primary area of research for the further drug development currently being investigated by our group. The GOAC technology can provide alternative and translatable methods for drug absorption, toxicity, and efficacy screenings, and holds a promise to explore avenues of personalized therapy for GI and neurologic diseases in the near future[94].

3.2. *In vivo* animal Models

One of the main obstacles in studying the GBA is the lack of an animal model system that successfully replicates a healthy or diseased individual's gut microbiome. Another obstacle is that the current rodent models for neurodegenerative diseases only allow investigation of short-term exposure to suspected triggers. Investigation on the GBA effect with certain diets or probiotics requires studies in natural models to have translational significance. Although the traditional rodent models for neurodegenerative diseases have been and will be allowing investigators to assess a targeted question (i.e. transgenic mice with deleted gene and how such gene deletion affects the pathology), it is critical to realize the current flaws in utilizing such *in vitro* models in pharmaceutical development, especially after looking at the poor success rate in drug discovery[76,77]. Since rodent diets differ substantially from that of humans, making comparisons between human and mouse gut microbiota studies is inherently difficult[97,98]. Mice preferentially consume grains and cereals, which are low in ascorbic acid, and they hold the ability to synthesize this essential cofactor while humans have lost this ability[99]. Also, presumably because of their ancestors' ingestion of different xenobiotics, mice and humans have different complements of cytochrome P450 enzymes and different patterns of xenobiotic metabolism[100,101]. At least in part for this reason, toxicology testing in mice has been a poor predictor of human toxicity[102]. While studies have been performed using conventional mouse models to investigate diseases involving the GBA, the

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alterations seen in the intestinal bacterial populations in mice are usually not reciprocated by human data[103].

Another factor as to why rodent models do not mirror human pathophysiology is due to the contrived nature of these induced disease models. As discussed before, AD is histologically characterized by progressive dementia and the presence of CAA due to Aβ aggregates in the walls of cerebral vessels[29,104]. However, rodent models do not produce human sequence Aβ naturally[105] which limits their investigative utility. Transgenic mouse models with over expressing the mutant human amyloid precursor protein (APP) alone or combined with transgenic presenilin 1 (PS1) and presenilin 2 (PS2) gene have secondary Aβ plaque formation in the brain histologically mimicking AD[106]. However, these transgenic mouse models naturally have cellular and behavioral resistance to Aβ pathology and therefore do not develop the extensive neuronal loss seen in the AD patients[107]. Also, there is a fundamental difference in the anatomic folding of the cerebral cortex; with humans having a gyrencephalic brain and rodents having a lissencephalic brain[108].

Accumulated data shows that the dog provides a complementary model system to the transgenic mouse model to investigate the physiology of aging associated with neurodegenerative diseases, and ultimately to develop therapeutics[109]. The dog is a particularly relevant species since it shares similar environmental, genomic, and intestinal physiologic features with humans[110]. Canine natural models also offer additional predictive validity before transitioning to human clinical trials in many different diseases including neurodegenerative diseases[111]. A recent study suggests that in the process of domestication in dogs, genes associated with digestion have been selected to thrive on a starch-rich diet unlike wolves and more similar to humans[112]. Interestingly, a study with polynomial regression analysis showed that middle aged beagles between 5 and 9 years show similar aging process to humans between 40 and 60 years regarding cognitive function, while beagles over 9 years are similar to humans over 66 years[113].

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3.3. Canine Models as Natural Models for Neurodegenerative Diseases: similarities and differences

Aged dogs with canine cognitive dysfunction (CCD) spontaneously develop varying degrees of progressive cognitive decline and particular neuropathological features, similar to changes seen in AD[114]. CCD dogs show similar abnormal MRI or gross histological findings as AD patients including cortical atrophy[115,116] and ventricular enlargement[117]. Neurodegenerative changes which have been identified in the aged dog brain are similar to those seen in AD, including diffuse Aβ plaque deposition[110,118] and accompanied CAA[119], together with neuronal loss[120] and dysfunction of neurotransmitter systems[121]. Moreover, another major neuropathological hallmark of AD besides Aβ plaques is hyperphosphorylated tau proteins[62] and they are rarely found in aged dogs compared to that in human AD[122]. Interestingly, one of the biomarkers of AD, plasma $A\beta_{42}$ level, is also increased in CCD dogs[123]. This biomarker will allow early identification of those patients that are most likely to develop AD in human (or CCD in dogs) and possibly amenable to early intervention to slow down disease progression.

Canine multiple system degeneration (CMSD) is a fatal, familial movement disorder first described in Kerry Blue Terriers[124], then in Chinese Crested dogs[125], and these breeds could be considered as natural models for PD. Affected dogs are normal until 3–6 months of age, when they develop cerebellar ataxia[125]. This progresses to akinesia (i.e., impairment in voluntary movement) and severe postural instability ultimately necessitating euthanasia by 1–2 years of age[125]. Histologically, CMSD is characterized by loss of cerebellar Purkinje cells followed by degeneration of the olivary nucleus, substantia nigra, putamen, and caudate nucleus[124,126]. Interestingly, the CMSD locus includes a segment that contains *PARK2*, the gene for parkin, and mutations in human *PARK2* is known to cause familial PD, which has clinical and pathological similarities to CMSD[125].

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In addition to the similarity in clinicopathological changes in human and canine neurodegenerative diseases, a recent study showed the similarity in their microbiome and the diet response between dogs and humans compared to traditional rodent models[127].

No animal models are perfect and it is recognized that the canine model has limitations as well. For example, it has been recently shown that dogs lack aldehyde oxidases (AOXs) which catalyze the oxidation of aldehydes or N-heterocycles metabolism[128]. This fact has physiological, pharmacological, and toxicological relevance because AOXs are believed to represent an important metabolic system capable of oxidizing a large array of endogenous and exogenous substrates[129]. Also, human and canine have different CYP3A isoforms (i.e., canine CYP3A12 is equivalent to human CYP3A4) and it is important to recognize the species differences when interpreting permeability, toxicity, and metabolism analysis using both *in vitro* and *in vivo* system[130]. A parallel assessment between *in vivo* expressions of such transporters and receptors and those found in *in vitro* system is required to demonstrate translatability. Also, it possible that differences in activity and substrate specificity/inhibitors and inducers are observed in the dog ; therefore, utilizing *in vitro* systems from multiple different species would allow us to supplement other *in vitro* systems that might not completely mimic human physiology[130].

4. Therapeutic approaches for modulating the GBA and value of the canine model in AD

In this section, we focus on therapeutic approaches for modulation of the GBA with a special emphasis on AD. However, similar approaches could be of benefit for the management of non-motor symptoms of PD, such as cognitive dysfunction.

4.1. Dietary interventions

Many human epidemiological studies have shown that nutrition and other lifestyle factors affect cognitive function and some of those factors show ameliorating effect in developing AD[131]. Decreased microbial diversity in the GI tract induced by high-fat diets has been associated with development of

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368 various neurological diseases including AD and PD[132]. The multi-hit hypothesis in neurodegenerative
369 diseases is that certain diets lead to dysbiosis[133], then bacterial amyloids (e.g., molecular mimicry)
370 activate AD pathogenesis by providing immunostimulatory misfolded amyloids, while the gut
371 microbiome enhances inflammatory responses to cerebral accumulation of Aβ[43]. This suggests that
372 modulating the gut microbiome through specific dietary interventions with prebiotics and/or probiotics
373 can be an effective strategy to correct dysbiosis, reduce chronic gut inflammation and Aβ aggregation
374 to slow down the progression of AD.
375
376 The ketogenic diet, which has anticonvulsant properties, was developed in the 1920s to mimic
377 physiological state seen in prolonged fasting[134]. The traditional ketogenic diet is very high in fat and low
378 in carbohydrates, which shifts the energy balance to lipolysis (i.e., to metabolize body fat), which leads
379 to ketogenesis, which is β-oxidation of fatty acids, and ultimately to the production of acetoacetate, β-
380 hydroxybutyrate, and acetone[135]. These substances can easily cross the BBB and be used as
381 precursors for the generation of adenosine triphosphate (ATP)[135]. Several mechanisms exist for
382 explaining how ketone bodies exert anti-convulsant actions,[136] including increased ATP production,
383 altered brain pH affecting neuronal excitability, and/or their direct inhibitory effects on ion channels[137].
384 Since some glucose is required for the synthesis and homeostasis of glutamate, which is the most
385 abundant excitatory neurotransmitter, a ketogenic diet that is very low in carbohydrates may prevent
386 seizures by minimizing the formation of the excitatory neurotransmitter that could lead to seizure
387 activities[138]. Ketones are also structurally similar to GABA, which is an inhibitory neurotransmitter, and
388 may have direct anticonvulsant or even antiepileptogenic effects[139]
389
390 Recent findings further suggest that caloric restriction also prevents age-related neuronal damage and
391 may be useful in the prevention and treatment of AD[140]. Several mechanisms for its beneficial effects of
392 caloric restriction include anti-inflammatory properties, reduction of oxidative stress, promotion of
393 synaptic strength as well as induction of various neuroprotective factors[140]. Caloric restriction also

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394 induces fatty acids oxidation (FAO) in intestinal stem cells, which are known to be reduced with
395 aging[141].
396
397 Interestingly, some of these dietary interventions have been investigated in dogs. Similar positive
398 effects were observed with ketogenic diets incorporating medium chain triglyceride (MCT) in epileptic
399 dogs with up to <50 % reduction in seizure activity[142], and now commercially available. Aged dogs
400 receiving MCT diets showed significantly improved mitochondrial function, decreased APP levels, and a
401 trend towards a decrease in total Aβ levels most prominent in the parietal lobe[143].
402 Lifestyle and nutrition are suspected to play a role in the development of CCD in dogs and intensive
403 training on cognitive tasks during their lifetime as well as supplementation of food with antioxidants can
404 delay the onset or mitigate cognitive decline[144]. Similarly, aged dogs fed an antioxidant-enriched diet
405 had significantly less age-dependent cognitive impairment than aged dogs fed the control diet[144].
406
407 **4.2. Probiotics**
408 Probiotics are living microorganisms with potential health benefits to the host[38]. As discussed before,
409 GABA is the major inhibitory neurotransmitter in the CNS, and it is produced by *L. brevis* and *B.*
410 *dentium* via glutamate metabolism[145]. Postmortem studies of the cortical areas of AD patients have
411 shown reduced frontal, temporal, and parietal GABA concentrations[146]. There are numerous studies in
412 rodent models assessing the impact of probiotics on cognitive behavior[147]. Stress-induced memory
413 impairment in mice can be restored by administering a daily treatment of probiotics (*L. rhamnosus*
414 R0011 + *L. helveticus* R0052)[147]. Treatment with *L. fermentum* NS9 mitigated an ampicillin-induced
415 spatial memory impairment and inhibited the ampicillin-induced reductions in N-methyl-D-aspartate
416 (NMDA) receptor, which is a glutamate receptor as well as an ion channel, expression in rats[148]. The
417 probiotic *L. helveticus* NS8 was also shown to significantly mitigate cognitive impairment in the
418 hippocampus of rats[149]. Treatment with VSL#3 was shown to induce significant increase in intestinal
419 *Actinobacteria* and *Bacterioidete)*, which correlated with ameliorated age-related deficit in VSL#3-
420 treated aged rats[150]. More importantly, a recent randomized, double-blind, and controlled clinical trial

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demonstrated that a mixture of probiotics (*L. acidophilus* + *L. casei* + *B. bifidum* + *L. fermentum*) consumption for 12 weeks had a positive effect on cognitive function and some metabolic statuses in AD patients[151]. Dysbiosis assessment in CCD dogs and potential therapeutic benefit of probiotics in CCD dogs are needed to further investigate parallel therapeutic options in cognitive dysfunction in both humans and dogs.

5. Conclusions and perspectives

The collective scientific evidence supports the hypothesis that the GBA plays a critical role in the pathophysiology of various neurodegenerative diseases, such as AD and PD. Murine models are informative tools to investigate specific hypotheses in many research settings; however, given the induced or genetically modified nature of their disease state, there has been very little translatability in testing of new therapeutic interventions to humans. Aged dogs with CCD syndrome naturally recapitulate the key features of human aging, making them particularly useful for investigating preventative or therapeutic interventions particularly for AD. Also, the dog gut microbiome has been shown to overlap more with the human microbiome compared to that of conventional murine model.

The most recent analyses suggest that one of the most expensive therapeutic areas in terms of drug research and discovery (R&D) costs is neurology[152]. This is because drugs in this category experience particularly lower success rates and that approximately 7% of investigational agents tested in phase III trials make it onto the market in neurology[76]. A barrier to achieving better attrition rate in neurology drug R&D is the lack of utilization of good natural models of true patient benefit. As we discussed earlier, the dog is a particularly relevant species since it shares multiple features with humans. Also, CCD dogs can be utilized as a natural model for AD as well as PD, and novel therapeutic trials can be done prior to entering human trials to assess its effect (i.e. reverse extrapolation). It is important to note that because organoids are derived from individuals with different genotypes and environmental risk factors, they are a highly relevant model system for personalized therapy. Integration of such organoid culture system with GOAC technology could hold natural or patient-specific disease characteristics and could

448 be utilized to screen for potential therapeutic discovery during early exploratory R&D phase. In the near
449 future, combination of data from GOAC models and clinical trials using dogs as natural disease models,
450 as well as bioinformatics, such collaborate studies can be used not only to screen novel therapeutics
451 but also to predict the outcome of novel therapeutics prior to entering human trials to assess its effect
452 (i.e., reverse extrapolation).
453
454 CONFLICT OF INTEREST:
455 JM, AJ, KA, and HJK are founders of a company, 3D Health Solutions, which is offer canine intestinal
456 oganoid culture as an assay system to improve the selection of the most promising candidate in
457 pharmaceutical research and development. YMA is a recent addition to the company.
458
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