Old Herborn University Seminar Monograph 32: Ageing and the Microbiome.
Editors: Peter J. Heidt, John Bienenstock, Thomas C.G. Bosch, Michael Zasloff, and Volker Rusch.
Old Herborn University Foundation, Herborn, Germany: 71-80 (2018).

AGEING AND GUT TO BRAIN SIGNALLING

WOLFGANG KUNZE

The McMaster Brain-Body Institute and Department of Psychiatry,
St Joseph's Healthcare, Hamilton, ON, Canada.

INTRODUCTION

There are several major gut-brain communication pathways. These include signalling via the vagus and spinal nerves, immune factors via cytokines, endocrine signalling via gut hormones, and microbial products that reach the brain via bloodstream (*Holzer* and *Farzi*, 2014). In this brief essay, the focus is on the effects of ageing on gut to brain signalling via the vagus.

AGEING GENERAL

There is a general functional decline in all mammalian physiological systems in advanced old age. For the intestine, this includes a decrease in motility, small intestine permeability and mucosal defence (*Calvani*, 2018). In addition, there are associated changes in lifestyle and dietary habits, reduced somatic mobility and increased probability of hospitalisation and medication use (*Han* et al., 2017). There may also be deficits in memory and increases in anxiety in humans (*Han* et al., 2017) or mice (*Scott* et al., 2017). Other age-related changes include alterations in the HPA axis with decreased negative feedback mechanisms and increased basal glucocorticoid release (*Calvani* et al., 2018). Circadian rhythm disruption and altered sleep/wake cycles have been also been reported to increase in prevalence in old age (*Hood* and *Amir*, 2017; *Calvani* et al., 2018). The microbiome and gut to brain signalling has increasingly been focused on in the ageing research field because of the changing nature of the microbiome during the lifespan and recognition that how the intestine signals to the enteric and central nervous systems alters during ageing. Thus, to understand how the microbiome influences ageing and devise potential remedial interventions it is necessary to understand the gut to brain neural signalling pathway.

MICROBIOME

Eli Metchnikoff proposed that people in Eastern Europe live longer because they consume lactic acid bacteria (*Metchnikoff*, 1908). The link between microbiota and longevity is also supported by the observation that germ-free mice live longer than conventional controls (*Dinan* and *Cryan*, 2017). This association does not mean that the intestinal microbes themselves age. However, here is a decrease in intestinal microbial diversity in the elderly with, for example, an increase in *Bifidobacteria,* but a decrease in their relative proportion (*Claesson* et al., 2011; *Dinan* and *Cryan*, 2017; *Calvani*

et al., 2018). A confounding factor is that age-related alterations in diet can lead to changes in the resident microbiome (*David* et al., 2014). It is at present not established entirely whether changes in microbiota are the cause or result of host ageing.

There is some experimental evidence that for the nematode *Caenorhabditis elegans*, intestinal microbes might manipulate lifespan. The prolongevity effects of metformin in *C. elegans* appear to depend on the suppression of folate metabolism by the worm's intestinal *E. coli* bacteria (*Cabreiro* et al., 2013). In the absence of intestinal *E. coli*, the *C. elegans'* lifespan was extended, but metformin reduced its lifespan. In addition, the effect of supplementary *E. coli* was dependent on the strain used; metformin resistant E. *coli* produce short lifespans in response to metformin, but metformin sensitive *E. coli* strains impaired folate metabolism and slowed ageing (*Cabreiro* et al., 2013). In a separate *C. elegans* model, genetic manipulation of *E. coli* to increase their secretion of the polysaccharide colanic acid increased the worm's lifespan by action on the host's mitochondrial homeostasis (increases fragmentation) and unfolded protein response. Purified colanic acid alone was sufficient to promote longevity (*Han* et al., 2017). Dietary colanic acid also increased lifespan of *Drosophila melanogaster* (*Han* et al., 2017). In other experiments, it was shown that *C. elegans* fed on NO-deficient bacteria have a reduced lifespan whereas worms exposed to NO donors had extended lifespans (*Gusarov* et al., 2013). These remarkable results imply that, for *C. elegans* (and possibly for *Drosophila*); intestinal microbes have a causal effect on the host lifespan.

The resident and transient gut microbiota can also influence gut to brain signalling via neuroactive molecules that they generate and release. Intestinal bacteria can release a variety of neurotransmitters including GABA, noradrenaline, dopamine, acetylcholine and 5-HT. These neurotransmitters can cross the mucosal layer to act on neuronal fibres innervating the epithelium (*Dinan* and *Cryan*, 2017; Calvani *et al.*, 2018). Short-chain fatty acids produced by intestinal microbes may affect the enteric nervous system or brain directly or with respect to butyrate via epigenetic modulation through histone deacetylases (Dinan and Cryan, 2017; *Calvani* et al., 2018). Reduced levels of short-chain fatty acids associated with old age have been associated with increased susceptibility to intestinal inflammatory disorders, and it has been speculated that short-chain fatty acid availability may affect human longevity (*Nagpal* et al., 2018).

INFLAMMATION

Ageing is associated with raised background inflammation (inflammageing). Inflammageing is associated with increased risks for morbidity and mortality and age-related diseases (*Franceschi* and *Campisi*, 2014). The increased background inflammation appears to be accompanied by reduced inflammatory response to acute challenges (*Franceschi* and *Campisi*, 2014) perhaps because the acute inflammatory response starts off from a higher background level to a given ceiling. A counterpoint is that chronic inflammation in the very elderly may have an adaptive role possibly underlying tissue remodelling; for example, healthy centenarians can have an increased background inflammation with associated hypercoagulability, but this may be

balanced by other anti-inflammatory mechanisms (*Franceschi* and *Campisi*, 2014).

It has been reported that intestinal commensal bacteria that maintain immune tolerance tend to be reduced in aged people, but opportunistic bacteria that stimulate intestinal inflammation are often increased in number (*Nagpal* et al., 2018). The old age inflammatory phenotype can be manipulated experimentally. For example, when the resident microbiome was transplanted from old to young germ-free mice, there was increased inflammation in the intestines, increased leakage of bacterial components into the circulation and increased T-cell activation systemically (*Fransen* et al., 2017). The importance of inflammageing in gut function is illustrated by the IP injection of the tumour necrosis factor alpha antagonist etanercept in aged mice, which reversed the old age related decrease in colon serotonin transporter and slowing of intestinal motility (*Patel* et al., 2017).

Inflammageing is manifest in the nervous system through the activation of microglia (the tissue macrophages of the brain) and may be associated with neurodegenerative pathologies such as Parkinson's disease (*Jyothi* et al., 2015). The roles of microglia are complex with metabolic and neuroprotective functions, but in old age the microglial sensome can shift from being mainly protective to favouring neuroinflammation (*Hickman* et al., 2013). Microglia activation and/or development are influenced by the gut microbiome (*Erny* et al., 2015), since germ-free mice have defective and immature microglia, and antibiotic treated mice also had immature microglia. Reconstitution of microbiota from donor specific pathogen free mice restored microglia function (*Erny* et al., 2015). The influence that the microbiome appears to have on brain microglia might explain, at least to some extent, the shift in old age to a pro-inflammatory microglial phenotype.

GUT MOTILITY

Chronic constipation, with or without incontinence, tends to increase in prevalence with old age (*Higgins* and *Johanson*, 2004; *De Giorgio* et al., 2015; *Ranson* and *Saffrey*, 2015). In north America, and after age 65 y, 16 or 26% (male vs female) have reported chronic constipation and this rose to 26 or 34% at the 84th year (*De Giorgio* et al., 2015). Beyond psychosocial and economic factors, it is believed that ageing of the gut itself can underlie these numbers.

Changes in gastrointestinal function with ageing occur in both humans and animal models and the upper and lower gut are most at risk. The oesophagus, stomach, colon, and rectum are targeted with difficulties in swallowing and defaecation; there is also an increase in intra-colonic pressure (reviewed in *Hall*, 2002; *Wade*, 2002; *Soenen* et al., 2016). Aged rats have fewer colonic migrating motor complexes as measured by electrodes in fasting conscious animals (*Metugriachuk* et al., 2006). Similarly, aged (2 y old) mice have reduced total faecal output with decreased water content in the pellets compared to their younger (3 mo) counterparts (*Patel* et al., 2012). In particular, there was a decrease in velocity of epoxy coated pellet movement in the colon and an increase in impaction (*Patel* et al., 2012).

MYENTERIC PLEXUS

Since the enteric nervous system (ENS) controls gut motility and secretion, it is reasonable to ask whether the increased prevalence of constipation in the elderly is accompanied by alterations or reductions in number of enteric neurons. Indeed, reductions in the number of myenteric neurons have been reported for the elderly. Along the gut there is an apparent 34% decrease in the number of myenteric neurons in the elderly, with the largest reduction (>38%) reported for the duodenum (*de Souza* et al., 1993). In the human colon myenteric plexus, there was a decrease in the number of choline acetyltransferase positive neurons while neuronal nitric oxide positive neurons did not decline in number (*Bernard* et al., 2009). *Hanani* et al. (2004) reported an increase with age in the proportion of colonic myenteric ganglia with cavities and a decrease in the proportion of normal ganglia.

Similar reductions in myenteric neurons have been reported for animals. Moreover, numerous animal studies suggest that the ENS is more susceptible to age-related degeneration than other nervous systems (*Saffrey*, 2013). Aged Fisher 344 rats showed significant reductions in the number of myenteric neurons (and associated glia) in both small and large intestines, except for the rectum (*Phillips* et al., 2004). The same was true for mice when young (3 mo) were compared to old (24 mo) ones (*El-Salhy* et al., 1999). An analogous reduction in neurons (5 vs 25 mo old) has been reported for the myenteric and submucous plexuses in the small intestine of the guinea pig (*Phillips* and *Powley*, 2007; *Zanesco* and *Souza*, 2011).

The enteric nervous system is a complete nervous system in the sense that it contains primary afferent, inter- and motor neurons. Also, peristalsis and mixing occur *ex vivo* after all nervous connections with the extrinsic nervous systems have been severed, demonstrating that the enteric nervous system can function independently. There are several functional classes of specialised neurons within the ENS subserving different functions (*Kunze* and *Furness*, 1999), but only one class, intrinsic primary afferent neurons (IPANs), is both chemo- and mechanosensitive and serves as an intramural gatekeeper relaying more than two thirds of signals originating from luminal contents to the afferent vagus nerve (*Perez-Burgos* et al., 2014). The remainder (< one third) of afferent chemoceptive vagal signals derive from direct innervation of the intestinal epithelium by the vagus. These considerations make it important to ask, with respect to gut to brain signalling, whether there are enteric neurons particularly sensitive or resistant to the effects of ageing.

Cholinergic (choline acetyltransferase (ChAT) immunopositive) enteric neurons appear to be especially vulnerable to ageing-related decrease when compared to other histochemical phenotypes (*Camilleri* et al., 2008). The number of ChAT positive human myenteric neurons decreased with age while nNOS neurons do not appear to change (*Bernard* et al., 2009). In rodents too, ChAT positive were preferentially lost (after 12 mo of age) with nicotinamideadenine dinucleuotide phosphate diaphorase positive neurons (nNOS containing) being spared (*Phillips* et al., 2003; *Phillips* and *Powley*, 2007). Intriguingly, this loss of enteric cholinergic neurons is paralleled by a preferential decrease in cholinergic innervation in the aged brain (*Casu* et al., 2002). However, enteric

cholinergic neurons belong to several functional classes including IPANs, excitatory motor neurons and interneurons, and the question arises which of these classes are the most vulnerable.

It has been suggested that IPANs are most susceptible to neurodegeneration with age (*Wade*, 2002; *Wade* and *Cowen*, 2004), exhibiting degeneration to a greater extent than other phenotypes, e.g. serotonergic interneurons or nitrergic inhibitory motor neurones. The evidence for this is incomplete, relying mainly on the decrease in old age of vitamin D-dependent 28 kDa calcium binding protein (calbindin) positive neurons or the decrease in substance P positive neurites in the guinea-pig or rodent myenteric plexus (*Wade*, 2002; *Wade* and *Cowen*, 2004). Because IPANs are positive for substance P, choline acetyltransferase (*Phillips* et al., 2003) and calbindin there is at least circumstantial evidence that IPANs may be principally sensitive to the effects of ageing.

Ageing also affects the neurites (processes) of myenteric neurons. Age-related development of dystrophic nerve fibers alters the shape of human (*Hanani* et al., 2004) and guinea pig enteric ganglia (*Abalo* et al., 2007), presumably due to the presence of swollen fibres. In aged mice (>18 mo old) calbindin containing and nNOS immunoreactive neurons developed multiple swollen processes when compared to young mice (3 mo) (*Gamage* et al., 2013). Other signs of degeneration and pathology include the accumulation in guinea pig or rat of lipofuscin (pigment granules composed of lipid-containing residues of lysosomal digestion) (*Phillips* et al., 2004; *Abalo* et al., 2007) and the presence of α-synuclein immunoreactive aggregates and hyperphosphorylated microtubule-associated Tau protein (*Phillips* et al., 2009). It should be noted that glial cells and interstitial cells of Cajal also decrease with age, although neuronal reduction seems to occur first (*Phillips* and *Powley*, 2007; *Camilleri* et al., 2008; *Saffrey*, 2013, 2014).

Despite neural loss and degeneration, the ageing gut and the enteric nervous system have a significant functional reserve since intestinal motility, although reduced, appears largely intact until the animal is very old (*Saffrey*, 2013).

EXTRINSIC AFFERENTS

The intestine is innervated by nerve fibres whose cell bodies lie outside the gut wall. These fibres run within mesenteric neurovascular bundles that branch off arterial arcades supplying intestinal segments. The nerve bundles contain vagal, and spinal and sympathetic nerve fibres.

Single unit (extracellular action potentials from single nerve fibres) mesenteric nerve fibre recordings have been made using *ex vivo* segments of human ileum or sigmoid colon (*Yu* et al., 2016). For these recordings, background firing rates correlated negatively with age (from 24 to 77 y old), and number of single units showing burst firing patterns also decline with age. There was also a decrease in the number of substance P containing nerve fibres (presumably either intrinsic or extrinsic) innervating the luminal mucosa (*Yu* et al., 2016). Paradoxically, there was also an increase in the density of mucosal mast cells and ileal enterochromaffin cell numbers with age, which the authors attributed to a compensatory mechanism for the

sensory neurodegeneration (*Yu* et al., 2016). These results parallel others that report decreased intestinal visceral pain perception in old age (*Lasch* et al., 1997; *Lagier* et al., 1999).

The diminished mesenteric nerve fibre discharge seen in elderly humans has been replicated in ageing mice. Single and multiunit spike recordings have been made from the mesenteric nerve of young and aged (3 mo vs 12 and 24 mo old) C57bl/6 mice (*Keating* et al., 2016). Baseline mesenteric multiunit spiking activity was significantly decreased for both jejunal and colonic mesenteric afferent fibres in old (24 mo) compared to young (3 mo) mice. Mesenteric nerve fibre responses to intraluminal fluid distension were also reduced in old mice. The TRPV1 receptor is also a mechanoreceptor in the intestine mediating the response of mesenteric afferents to fluid distension (*Rong* et al., 2004). It is therefore of interest that intestinal TRPV1 receptor expression was diminished in the older mice (*Keating* et al., 2016).

There appears to be little functional data on the effects of ageing on the afferent vagus compared to the mixed mesenteric nerve. There are, however, reports in the literature that the vagus nerve of old animals displays degenerative anatomical changes. In aged rats, vagal afferents appear to have swollen varicosities in fibres innervating the myenteric plexus, smooth muscle and mucosa (*Phillips* and *Powley*, 2007). It is not established that there is an actual decrease in the number of vagal fibre endings supplying the myenteric plexus but there are dystopic changes including dilations and swellings (*Phillips* et al., 2010) in the NIH Fisher 344 rat model of ageing. The extent of the terminal arbors is also reduced compared to young rats. Similar degenerative changes are seen in vagal afferents to mucosal villi and the musculature (*Phillips* et al., 2010). It is thought that this reduction or the pathological changes in vagal innervation results from a decrease in trophic factors released from the targets of vagal innervation (*Phillips* et al., 2010) in particular NT-4 (*Phillips* et al., 2010).

Sympathetic nerve fibres originate from prevertebral sympathetic ganglia to innervate the ENS, intestinal arteries and smooth muscle (*Phillips* and *Powley*, 2007). Tyrosine hydroxylase staining can be used to identify the sympathetic innervation of the intestine, and positive fibres that supply the myenteric and submucous plexuses of aged (24 mo old) rats display axonopathies such as swollen axons and small sparse terminals (*Phillips* and *Powley*, 2007). Functionally, old age has been associated with decreased ability of the sympathetic nervous system to adapt to environmental or interoception of stimuli (*Hotta* and *Uchida*, 2010).

INTRAMURAL GATEKEEPER

90 to 95% of sensory neuron processes innervating the intestinal epithelium arise from the ENS, with the rest originating from neurons whose somata are located outside the intestine (*Keast* et al., 1984; *Ekblad* et al., 1987). In agreement with this anatomical data on epithelial innervation density, is the recent discovery that more than two thirds of vagal afferent signals evoked by a luminal probiotic is relayed to the vagus via the enteric neurons (*Perez-Burgos* et al., 2014). Neuroactive luminal molecules first excite juxtaepithelial neurites belonging to IPANs whose cell bodies are located within

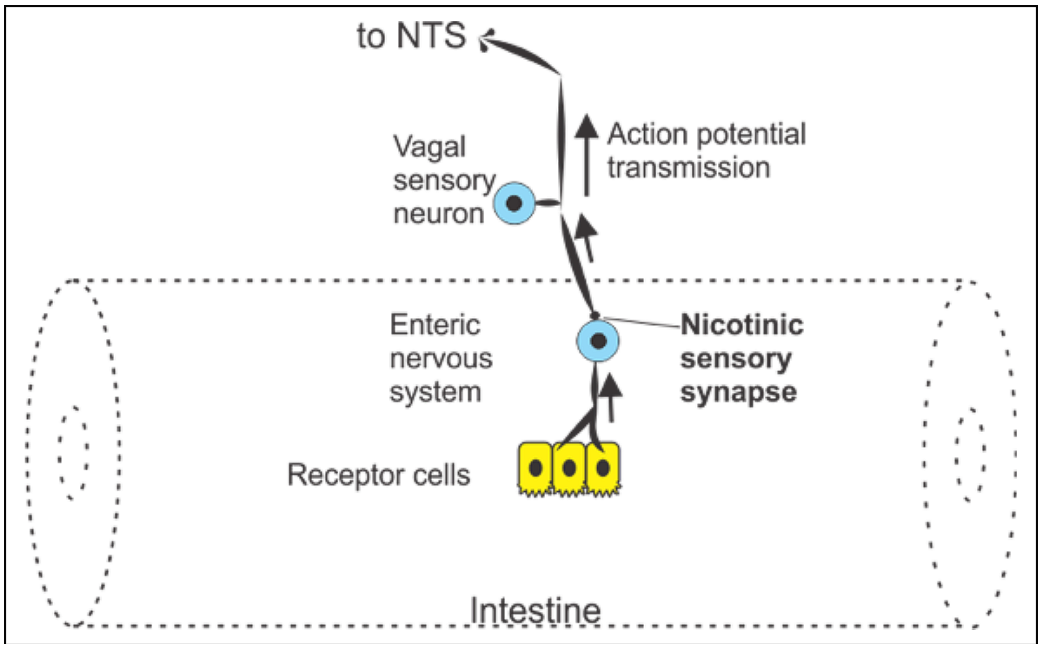

Figure 1: Gut to brain afferent signalling pathway. Sensory neurons within the enteric nervous system respond to luminal stimuli and relay information to the brain via a nicotinic sensory synapse that activates the afferent vagus.

the enteric nervous system. The excited IPANs release acetylcholine and perhaps other neurotransmitters to activate vagal intraganglionic laminar endings (IGLEs) which closely surround and abut the IPANs (*Berthoud* et al., 1997; *Perez-Burgos* et al., 2014). The IPAN to IGLE nicotinic sensory synapse is perfectly positioned to act as a gatekeeper to regulate microbial to brain signalling (Figure 1). Accordingly, the amount of information transmitted to the brain via the vagus would be markedly influenced by whether IPANs are refractory or readily responsive to luminal stimuli, and by the density of IPAN sensory innervation of the epithelium.

Given the important role of enteric IPANs in gut to brain signalling, the vulnerability of the enteric nervous system to old age in terms of numbers and degeneration would have a significant impact on the amount and quality of information reaching the brain from the gut. Thus, even if the number of vagal afferent fibres are not appreciably reduced it can be predicted that there would be decreased constitutive and stimulus evoked vagal afferent responses to luminal microbial stimuli in old age. Exacerbating these potential impediments in gut to brain signalling there is the added problem of decreased microbial diversity (*Nagpal* et al., 2018) demonstrated in old age. In summary, there are several reasons why gut microbes to brain signalling via the vagus is compromised in old age, with a degenerating or dysfunctional enteric nervous system being a major contributor to the reduced signalling.

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