Cellular changes in the enteric nervous system during ageing

M. Jill Saffrey*

Department Life, Health & Chemical Sciences, Open University, Walton Hall, Milton Keynes MK7 6AA, United Kingdom

A R T I C L E   I N F O

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- Neurotrophic factors
- Enteric nervous system

A B S T R A C T

The intrinsic neurons of the gut, enteric neurons, have an essential role in gastrointestinal functions. The enteric nervous system is plastic and continues to undergo changes throughout life, as the gut grows and responds to dietary and other environmental changes. Detailed analysis of changes in the ENS during ageing suggests that enteric neurons are more vulnerable to age-related degeneration and cell death than neurons in other parts of the nervous system, although there is considerable variation in the extent and time course of age-related enteric neuronal loss reported in different studies. Specific neuronal subpopulations, particularly cholinergic myenteric neurons, may be more vulnerable than others to age-associated loss or damage. Enteric degeneration and other age-related neuronal changes may contribute to gastrointestinal dysfunction that is common in the elderly population. Evidence suggests that caloric restriction protects against age-associated loss of enteric neurons, but recent advances in the understanding of the effects of the microbiota and the complex interactions between enteric ganglion cells, mucosal immune system and intestinal epithelium indicate that other factors may well influence ageing of enteric neurons. Much remains to be understood about the mechanisms of neuronal loss and damage in the gut, although there is evidence that reactive oxygen species, neurotrophic factor dysregulation and/or activation of a senescence associated phenotype may be involved. To date, there is no evidence for ongoing neurogenesis that might replace dying neurons in the ageing gut, although small local sites of neurogenesis would be difficult to detect. Finally, despite the considerable evidence for enteric neurodegeneration during ageing, and evidence for some physiological changes in animal models, the ageing gut appears to maintain its function remarkably well in animals that exhibit major neuronal loss, indicating that the ENS has considerable functional reserve.

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Introduction

It is established that the mammalian enteric nervous system (ENS) undergoes developmental changes postnatally (e.g., de Vries et al., 2010; Foong et al., 2012). It is also clear that the cells of the adult ENS exhibit plasticity and continue to undergo changes later in life, due both to the highly dynamic nature of the gastrointestinal (GI) system, and to cellular changes that take place as part of the process of ageing.

Throughout life, particularly that of longer-lived animals, the gastrointestinal (GI) system is exposed to dietary variation and also to changes in the microbiota, to disease, inflammation and, in humans, medication. The properties of intestinal cells may be affected as a result of these changing conditions in the gastrointestinal environment. In addition, in long-lived animals, and in animals living in protected laboratory environments, cellular changes that occur simply as a result of ageing will affect the cells of the gut, although different cell types, such as muscle, epithelial cells and neurons, are likely to be affected in different ways. These age-associated cellular changes may impact upon specialised functions, and hence affect GI physiology. There is also evidence from animal models that external factors, including diet, can alter ‘normal’ (i.e. non-pathological) ageing of the ENS.

Understanding the contribution made by normal cellular ageing to the age-associated physiological changes that take place in the gut is important, because gastrointestinal disorders increase in prevalence in older people, and some conditions, such as constipation (see Gallagher and O’Mahony, 2009 Rao and Go, 2010), faecal incontinence (see Chattoor et al., 2007) and gastric reflux (see Franceschi et al., 2009) are very common among the elderly population. These conditions have a serious impact on the quality of life of the elderly, affecting their independence, and also have a significant impact on healthcare costs. Age-associated enteric neurodegeneration (see Bitar et al., 2011; Camilleri et al., 2008; Phillips and Powley, 2007; Saffrey, 2004; Wade and Cowen, 2004; Wiskur and Greenwood-Van Meerveld, 2010) and ageing of the other cells of the gut, such as smooth muscle cells (see Bitar et al., 2011; Bitar and Patil, 2004) are believed to be major contributory factors to the these age-associated GI disorders.

Here, the changes that occur in the cells of the ENS during ageing and the implications of these changes for GI function are reviewed. Ageing of intestinal smooth muscle (see Bitar et al.,
2011; Bitar and Patil, 2004), endocrine cells (see Sandstrom and El-Salhy, 2001) and intestinal epithelial stem cells (Kirkwood, 2004) are reviewed elsewhere. Very few studies have investigated changes in the properties or numbers of Interstitial cells of Cajal and Fibroblast-like cells during ageing.

Changes in GI physiology in the ageing human population are well-known, but identifying the contribution made by cellular ageing to these changes is difficult, because of the many confounding factors that may also influence GI function in humans. For example, co-morbidity (e.g. vascular disease), medications (e.g. anti-cholinergic medications), change in diet and immobility may all influence GI physiology (e.g. see Rao and Go, 2010). The complications associated with discrimination between changes that are genuinely due to ageing, and those due to extraneous conditions, such as those listed above however, make carefully-maintained laboratory animals a valuable resource for studies of GI ageing; confounding factors such as variations in diet and medication are eliminated or reduced to a minimum.

Most work on ENS ageing has been carried out on the myenteric plexus, and analysis of age-related neuronal changes has focused predominantly on changes in the numbers of neuronal cell bodies, although some studies of the density of the myenteric plexus network and nerve fibre density in smooth muscle have also been performed. Cellular and biochemical properties that have been reported to be changed during ageing of other cell types and markers of cellular senescence have also been investigated in enteric neurons and are discussed here.

The changes that occur in GI neurophysiology of animals during ageing are being increasingly studied, although the GI changes that are common in ageing humans, such as constipation and incontinence, have not been widely looked for in the animals that have been used for the study of ENS ageing. Nevertheless recent evidence indicates that ageing mice do exhibit changes in the size and frequency of stools (Patel et al., 2012), indicating that they undergo changes that are at least similar to some of those exhibited by ageing humans. Physiological and pharmacological studies of the ageing ENS are not discussed here.

Plasticity of the ENS during ageing

The ENS is at all times a highly plastic system in terms of its physical characteristics, because the shape of enteric ganglia changes during and between the distentions that occur during peristalsis and other gut movements (Gabella, 1990). However, in addition to these continual dynamic changes, the size of the GI tract changes during the lifespan. The intestine undergoes considerable growth during adult life. For example, Gabella (1989) reported that the length of the guinea-pig small intestine increases by 27%, and its circumference by 30% during ageing. This growth results in an increase of 70% of serosal area under the conditions studied. Interestingly, the maximum dimensions were measured in middle-aged animals (Gabella, 1989). A number of others have also measured an increase in length and/or circumference with age (e.g. Choi et al., 2008; Gamage et al., in press Phillips and Powley, 2001; Peck et al., 2009). An increase in circumference may be due to changes in the thickness of the mucosa or muscularis externa, or both. Increases in the thickness of the muscle layers during ageing have been reported in several studies (e.g. Peck et al., 2009; Southwell et al., 2010), but not in others (e.g. Marese et al., 2007). With respect to the mucosa, it has been reported that there are changes in villus width and depth (Drozdzowski and Thomson, 2006). Increases in the length and circumference of the intestine and changes in the volume of the different layers of the gut would be expected to have an impact on the arrangement of the enteric plexuses, and potentially on the density of nerve fibres in different layers. It is therefore likely that continued growth, and possibly some rearrangement, of enteric nerve fibres occurs throughout periods of gut growth, which take place during a considerable period of the lifespan.

During such periods of gut growth, for some types of myenteric neuron, such as intrinsic sensory neurons, and those interneurons that project longer distances in the plexus, an increase in the length of their processes would be necessary to maintain contact with their target cells. Such a response may not be necessary for all myenteric neuronal subpopulations however, since an increase in smooth muscle volume for example, could result from an increase in the size or number of muscle cells, and since they are well-coupled (Hoyle and Burnstock, 1989), effective transmission could perhaps continue even if the density of fibres decreased. Nevertheless, it would seem that the ENS, and enteric neurons in particular, must remain plastic during adulthood.

Changes in the gross morphology of the myenteric plexus during ageing

Changes in the general morphology of the enteric ganglia during ageing have been described in some studies. Gabella (1989) has reported that there is greater separation between myenteric ganglia in old guinea-pigs, and that myenteric neurons appear to be less densely packed within the ganglia. Abalo et al. (2005) described a reduction in size of myenteric ganglia of guinea-pig ileum with age, and that myenteric ganglia occupied a reduced area. No change in the number of ganglia per unit area during ageing was measured in the same study. A change in the size and the appearance of myenteric ganglia with age has also been described in humans (Hanani et al., 2004); in samples from older individuals the overall ganglionic area was found to be larger, and gaps or spaces were observed within the ganglia. Ganglia from older individuals had increased numbers of spaces, and also an increased proportion of ganglia with spaces, in both the ileum and colon. The extent of these changes correlated with advancing age, and was greater in the colon than the ileum. No difference between males and females was measured. The authors speculated that the spaces could occur due to a stretch effect resulting from gut growth, but pointed out that this was unlikely, because normal ganglia, without spaces, were seen adjacent to abnormal ganglia.

Changes in the general morphology of enteric neurons during ageing

The size, general shape and distribution of myenteric neurons have also been reported to change during ageing. Gabella (1989) reported that myenteric neurons were smaller in the ageing guinea-pig small intestine and Gomes et al. (1997) also measured a reduction in myenteric neuronal perikaryon area in ageing human colon. Santer and Baker (1988); however, found no change in the size of myenteric neurons in the rat small or large intestine, while others have measured an increase in neuronal cell body size in older rats (Phillips et al., 2003). Some of the discrepancies between these results may be due to the methods used (see section on challenges of analysis of changes of neuronal numbers in the ageing gut).

The shape of neurons in the ageing guinea-pig gut has been reported to be different from that in younger animals; neurons in ageing animals were found to have a ‘horny’ profile (Gabella, 1989). Some single neurons, which were large and with a smooth profile, were found to be present at the edges of myenteric ganglia in old animals in the same study. Such peripherally-located
Table 1
Changes in myenteric neuronal numbers during ageing.

<table>
<thead>
<tr>
<th>Species &amp; strain</th>
<th>Regions studied</th>
<th>Ages studied</th>
<th>Populations studied</th>
<th>Diet</th>
<th>Loss (notes)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAT Wistar ♂</td>
<td>Jej, il, prox col, rect</td>
<td>6, 24m</td>
<td>Total (NADHd)/area</td>
<td>N/S</td>
<td>~40% loss in SI &amp; rectum, ~60% loss in colon [Changes in intestinal dimensions not corrected for]</td>
<td>Santer and Baker (1988)</td>
</tr>
<tr>
<td>Wistar ♂</td>
<td>SI ileum</td>
<td>4, 24, 30m</td>
<td>Nitrigeric (NADPHd)</td>
<td>AL</td>
<td>15% loss at 24m, also at 30m Increased proportion of nitrigeric neurons in colon, but not in ileum. [analysis independent of intestinal dimensions]</td>
<td>Santer (1994) Belai et al. (1995a)</td>
</tr>
<tr>
<td></td>
<td>Colon</td>
<td>4, 6, 24m</td>
<td>Nitrigeric (NADPHd), as proportion of total (PGP 9.5) /ganglion</td>
<td>CR</td>
<td>15% loss NADHd neurons but no change in PGP 9.5-IR neurons, or NADPHd positive or neurons expressing any other markers</td>
<td>Johnson et al. (1998)</td>
</tr>
<tr>
<td>Sprague-Dawley</td>
<td>Ileum</td>
<td>4, 24m</td>
<td>Total (NADHd and PGP 9.5), Sub pop's: NADPHd, ChAT-, SP-, ENK-, VIP-, NPY-, SOM-IR /area</td>
<td>AL, CR</td>
<td>~50% loss of total, 64% loss cholinergic neurons in AL-fed only. Nitrigeric neurons spared.</td>
<td>Cowen et al. (2000)</td>
</tr>
<tr>
<td>Fischer (F344XBN) F1</td>
<td>Mid-colon</td>
<td>4–8, 22–28m</td>
<td>nNOS/ganglion, NOS protein &amp; mRNA, PGP 9.5 protein levels</td>
<td>DCR</td>
<td>Significant reduction in number of nNOS-IR neurons per ganglion. Reduction in NOS protein &amp; mRNA. No change in PGP 9.5 levels. [analysis independent of intestinal dimensions]</td>
<td>Takahashi et al. (2000)</td>
</tr>
<tr>
<td>Fischer 344 ♂</td>
<td>Stomach SI regions</td>
<td>3,12, 21, 24, 27m</td>
<td>Total (Cuprolinic Blue)/area</td>
<td>AL</td>
<td>Differential loss in regions. None in antrum &amp; corpus at 24m, late 38% loss in forestomach. 17–31% losses in SI, 38–39% in LI</td>
<td>Phillips and Powley (2001)</td>
</tr>
<tr>
<td>Fischer 344 ♂</td>
<td>LI regions Stomach SI regions</td>
<td>3, 24m</td>
<td>Total (Cuprolinic Blue) Nitrigeric (NADPHd) /area</td>
<td>AL</td>
<td>Total 3–7% loss in stomach, 10–27% loss in SI regions, 37–41% loss in LI</td>
<td>Phillips et al. (2003)</td>
</tr>
<tr>
<td>Sprague-Dawley &amp; Wistar</td>
<td>Oesophagus</td>
<td>3–4.5, 18–20m</td>
<td>Total (PGP 9.5)</td>
<td>AL</td>
<td>Total neuronal loss ~27%; Strain difference in nitrigeric changes, reduction in Sprague-Dawley, no change in Wistar [analysis independent of intestinal dimensions]</td>
<td>Wu et al. (2003)</td>
</tr>
<tr>
<td>Fischer 344 ♂</td>
<td>SI regions</td>
<td>5–6, 26m</td>
<td>Total neurons (HuC/D)</td>
<td>AL</td>
<td>7–37% neuronal loss / gang area; 18–48% loss /ganglion, dependent on GI region (glial losses also observed, see text)</td>
<td>Phillips et al. (2004b)</td>
</tr>
<tr>
<td>Sprague-Dawley ♂</td>
<td>Ileum</td>
<td>6, 12, 13, 17, 24m</td>
<td>Total (CP9.5, Hu C/D) Sub pop's: Calretinin, calbindin /area</td>
<td>AL, CR</td>
<td>PGP9.5 (24m); AL 51% loss, CR 22% loss Hu C/D (17m); AL 50% loss, CR no data Calretinin (17m) AL&amp; CR show similar non-significant loss (~30%) Calbindin (17m) AL; 62% loss, CR no loss</td>
<td>Thrasivoulou et al. (2006)</td>
</tr>
<tr>
<td>Wistar ♂</td>
<td>Duodenum</td>
<td>21, 60, 90, 210, 345 &amp; 428d</td>
<td>Total (Geimsa &amp; Myosin V-IR)/area</td>
<td>AL</td>
<td>Reduction in neuronal density in younger animals because of gut growth, in older animals because of neuronal loss (and see text) [Changes in intestinal dimensions not corrected for]</td>
<td>Mares et al. (2007)</td>
</tr>
<tr>
<td>Wistar ♂</td>
<td>Duodenum</td>
<td>6, 18m</td>
<td>Total (Geimsa) Nitrigeric (NADPHd)/area</td>
<td>AL, CR*</td>
<td>AL 29% total loss, CR 17% loss Increase (20%) in NADPHd neuronal numbers in AL-fed ageing animals, smaller increase in CR</td>
<td>da Silva Porto et al. (2012)</td>
</tr>
<tr>
<td>Species &amp; strain</td>
<td>Regions studied</td>
<td>Ages studied</td>
<td>Populations studied</td>
<td>Diet</td>
<td>Loss (notes)</td>
<td>Ref.</td>
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<tr>
<td><strong>GUINEA-PIG</strong></td>
<td>Small intestine</td>
<td>3–4, 26–30m</td>
<td>Total (NADH diaphorase)</td>
<td></td>
<td>40–60% loss total</td>
<td>Gabella (1989)</td>
</tr>
<tr>
<td></td>
<td>N/S Ileum</td>
<td>1–3, 8–10, 22–26m</td>
<td>Total (HuC/D)</td>
<td>N/S</td>
<td>~30% loss total, ~50% loss calretinin [Changes in intestinal dimensions not corrected for]</td>
<td>Abalo et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>Dunkin-Hartley ♀</td>
<td>2–3, 8–10, 22–26m</td>
<td>Total HuC/D Sub pop*: nNOS, calbindin, calretinin, neurofilament (double labelling) /ganglionic area</td>
<td></td>
<td>Total – decreased in 22–26m cf 8–10m Calbindin, Calretinin and NFT alone, no change at 22–26m, Calretinin + NFT (co-localised) and NOS alone, loss in 22–26m, [Changes in intestinal dimensions not corrected for]</td>
<td>Abalo et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>Tri-colour strain</td>
<td>Mid -colon</td>
<td>2w, 6–8m, 24–32m</td>
<td>Total (HuC/D)</td>
<td>19% (non-significant) loss</td>
<td>Peck et al. (2009)</td>
</tr>
<tr>
<td><strong>MOUSE</strong> NMRI/Bom</td>
<td>Stomach (antrum)</td>
<td>1, 3, 12, 24m</td>
<td>Total (PGP 9.5)</td>
<td></td>
<td>~40% loss between 3 and 12m, no change 12–24m</td>
<td>El-Salhy et al. (1999)</td>
</tr>
<tr>
<td></td>
<td>Bom Duodenum</td>
<td>/ganglion</td>
<td>Total (PGP 9.5)</td>
<td></td>
<td>[Changes in intestinal dimensions not corrected for]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Colon Sections</td>
<td>3–4, 12–13, 18–19, 24–25m</td>
<td>Total (HuC/D) Calbindin-, nNOS-IR /area</td>
<td>AL</td>
<td>No change in any population after individual correction for change in LI dimensions, and stretch</td>
<td>Gamage et al. (in press)</td>
</tr>
<tr>
<td><strong>GERBIL</strong></td>
<td>Duodenum</td>
<td>1.5, 3, 6, 12, 24m</td>
<td>Total (Geimsa)</td>
<td>AL</td>
<td>17% loss total neurons, 5% loss calbindin [Changes in intestinal dimensions not corrected for]</td>
<td>Choi et al. (2008)</td>
</tr>
<tr>
<td><strong>HUMAN</strong></td>
<td>Oesophagus</td>
<td>20–40, &gt; 70y</td>
<td>Total (Geimsa stain) / area</td>
<td>N/A</td>
<td>22–62% loss [Changes in intestinal dimensions not corrected for]</td>
<td>Meciano Filho et al. (1995)</td>
</tr>
<tr>
<td>♂♀</td>
<td>Small intestine</td>
<td>20–40, 69–76 yr</td>
<td>Total (Geimsa stain) / area</td>
<td>N/A</td>
<td>34% loss [corrected for changes in gut diameter]</td>
<td>de Souza et al. (1993)</td>
</tr>
<tr>
<td>♂♀</td>
<td>Colon</td>
<td>20–35, &gt; 65 yr</td>
<td>Total (Geimsa stain) / area</td>
<td>N/A</td>
<td>37% loss [Changes in intestinal dimensions not corrected for]</td>
<td>Gomes et al. (1997)</td>
</tr>
<tr>
<td></td>
<td>Ileum</td>
<td>42–71, 78–86 yr</td>
<td>Nitricergic (NADPHd)&amp; calretinin proportion of total PGP9.5-IR</td>
<td>N/A</td>
<td>Nitricergic and calretinin-IR increase as proportion of total in aged samples. [analysis independent of intestinal dimensions]</td>
<td>Belai and Burnstock (1999)</td>
</tr>
<tr>
<td>♂♂</td>
<td>Ileum Colon</td>
<td>30d – 92 yr (n= 168)</td>
<td>Vital dye-4-Di-2-ASP* Nitricergic (NADPHd) /ganglion</td>
<td>Total not counted (ganglion morphology studied). Non-significant increase in proportion NADPHd/ganglion with age</td>
<td>Hanani et al. (2004)</td>
<td></td>
</tr>
<tr>
<td>♂♂</td>
<td>Colon</td>
<td>33–99 yr</td>
<td>Sections Total (HuC/D, PGP9.5), ChAT, NOS /ganglion and /unit length</td>
<td>N/A</td>
<td>HuC/D &amp; ChAT neuronal number negatively correlated with increasing age. Non-significant NOS-IR decrease neurons ~38% total loss between ages of 30 and 60 yr [Changes in intestinal dimensions not corrected for]</td>
<td>Bernard et al. (2009)</td>
</tr>
</tbody>
</table>

Key: AL = Ad libitum; ChAT = choline acetyltransferase; CR = calorically restricted; ENK = enkephalin; IR = immunoreactive; PGP = protein gene product; N/A = not applicable (human study); NADH = nicotinamide adenine dinucleotide; NADPH = nicotinamide adenine dinucleotide phosphate; N/S = not stated; NOS = nitric oxide synthase; NPY = neuropeptide Y; SOM = somatostatin; SP = substance P; VIP = vasoactive intestinal polypeptide.
neurons have also been described in ageing human myenteric ganglia (Hananai et al., 2004) and Abalo et al. (2005) have also reported an increased occurrence of extraganglionic neurons in the myenteric plexus of ageing guinea-pig ileum.

Changes in the number of enteric neurons during ageing

Myenteric neurons

The first work to quantify possible changes in myenteric neuronal numbers during ageing was by Santer and Baker (1988) on rats, and Gabella (1989), on guinea-pigs. Since then, analysis of changes in myenteric neuronal numbers has been performed in several additional species. Most studies have been performed on rats, but guinea-pigs, mice, gerbils and humans have also been investigated. The majority of research has been carried out on the small and large intestines, but some groups have investigated age-related changes in myenteric neuronal numbers in the oesophagus, stomach and rectum. The results of studies of changes in myenteric neuronal numbers are summarised in Table 1.

A reduction in the number of myenteric neurons during ageing has been described in most, but not in all studies. Moreover, the proportion of the total population of neurons that are reported to be lost varies considerably, for example in ad libitum (AL) fed rat ileum, losses of between 17% (Phillips and Powley, 2001) and 50% (Cowen et al., 2000) have been described. Similar ranges of neuronal losses are reported in three different strains of rats (Wistar, Sprague Dawley and Fischer 344, see Table 1). Myenteric neuronal loss has been found to vary in different regions of the rat gut, being greater in the large intestine than in the small intestine (Phillips et al., 2003; Phillips and Powley, 2001, 2007; Santer and Baker, 1988). Losses in rat stomach have also been found to vary according to region; in the antrum and corpus of 24 month old animals losses were either very low (3–7%, Phillips et al., 2003) or absent (Phillips and Powley, 2001) but in the forestomach, although no losses were detected at 24 months, a 38% loss was reached by 27 months, but no such late loss was seen in the antrum or corpus (Phillips and Powley, 2001).

The time course of myenteric neuronal loss during ageing has also been investigated, most studies showing that it begins at ‘middle-age’, but continues for variable periods thereafter. For example, Phillips and Powley (2001) found only a relatively small reduction in AL-fed 12 month-old Fischer 344 rats compared to 3 month-old controls (10–15%, depending upon the GI region studied), but that losses continued, in a linear manner in most gut regions, at least until 21 months of age. In contrast others, working on AL-fed Sprague-Dawley rats, found that neuronal losses began at about 13 months, but were complete by 16 months of age (Cowen et al., 2000; Thrasivoulou et al., 2006). Thus the onset and rate of neuronal loss may be variable in different cohorts of ageing animals, and may, of course be affected by differences in animal husbandry in the different studies (see Section Challenges in the analysis of changes of neuronal numbers in the ageing gut, below).

In this context, it is important to note that caloric restriction has been found to have a marked effect on age-associated neuronal losses in the ENS. The loss of myenteric neurons observed in AL-fed rats has been reported to be eliminated (Cowen et al., 2000; Johnson et al., 1998) or significantly reduced (da Silva Porto et al., 2012; Thrasivoulou et al., 2006) by caloric restriction introduced at 6 months of age.

Subpopulations of myenteric neurons

The subpopulations of myenteric neurons that are lost during ageing have been investigated by a number of groups. Several studies have presented evidence that age-associated myenteric neuronal loss is specific to the cholinergic subpopulation in rats (e.g. Cowen et al., 2000; Phillips et al., 2003) and in humans (Bernard et al., 2009), and that nitrergic neurons are maintained (e.g. Belai et al., 1995; Cowen et al., 2000; Gamage et al., in press). In some cases the loss of cholinergic neurons has been indirectly deduced because the proportion of NADPH diaphorase (NADPHd) neurons have been found to increase in older animals (e.g. Phillips et al., 2003), although a direct reduction in ChAT-IR neurons has also been reported (Bernard et al., 2009; Cowen et al., 2000). Evidence about the fate of nitrergic neurons during ageing, however, is conflicting, because a loss of nitrergic neurons has been measured during ageing in some studies (Abalo et al., 2007; Santer, 1994; Takahashi et al., 2000). In one study of myenteric neurons in the oesophagus, numbers of nitrergic neurons were found to be reduced in Sprague Dawley rats, but maintained in Wistar rats (Wu et al., 2003). Swollen and dystrophic processes of both NADPHd (Phillips et al., 2003) and nNOS-IR (Gamage et al., in press) nerve fibres however, have been observed in ageing rat and mouse myenteric plexus wholemounts respectively, indicating that nitrergic neurons may undergo degenerative changes during ageing, even if they do not die in detectable numbers at the ages studied.

Few studies have looked in detail at other neuronal subpopulations, although no loss of any subpopulation of a range studied (including SP, VIP and somatostatin-IR neurons) was measured in ageing rats fed a calorically-restricted diet (Johnson et al., 1998, see Table 1). The possibility that a subpopulation of cholinergic myenteric neurons, such as intrinsic sensory, motoneurons or inter-neurons, is selectively vulnerable to age-related cell death has also not been studied in detail. Calbindin-IR myenteric neurons were found to be reduced in number in AL-fed ageing animals (Thrasivoulou et al., 2006), but no age-related change in calbindin-IR neurons was detected in guinea-pig ileum (Abalo et al., 2007) or mouse distal colon (Gamage et al., in press). Calretinin-IR neurons were reported to be lost in guinea-pig ileum (Abalo et al., 2007), but not in rat ileum (despite a non-significant 30% loss in this rat tissue Thrasivoulou et al., 2006). The possible reasons for discrepancy between results from different studies, and for possible selective vulnerability of particular neuronal populations to degeneration or are considered in later sections of this review.

Submucosal neurons

Changes in the numbers of submucous plexus neurons during ageing have been little studied in comparison to those of myenteric neurons. Changes in the total population (identified by Cuprolinic blue staining) of submucosal neurons during ageing have been studied in the rat proximal and distal colon (Phillips et al., 2007). In this study, which analysed total neurons in samples from 6, 12, 18, 24 and 27 month old animals, a linear loss of submucosal neurons was seen from 12 months of age. The loss was greater in the distal colon than the proximal colon (38% compared to 27% at 27 months), and the maximal loss was also reached later in the distal colon (27 months compared to 24 months). The mean number of neurons per ganglion also decreased during ageing. El-Salhy et al. (1999) also described a reduction in the number of neurons per submucosal ganglion in the antrum, duodenum and colon of 12 and 24 months compared to 3 month-old mice. Bernard et al. (2009); however, did not detect an age-associated reduction in the number of HuC/D-IR neurons per submucosal ganglion in human colon samples.

Age-related changes in enteric glial cells

Enteric glial cells have been little studied during ageing. Phillips et al. (2004b); however, compared glial cell numbers in different
regions of the small and large intestines in 5–6 and 26 month-old rats. These authors reported a significant loss of enteric glia (expressed as counts per ganglion and per ganglionic area) in all areas except the rectum, where loss was observed, but was not significant. The glial cell loss was also found to be proportional to the neuronal loss in this study.

Challenges in the analysis of changes of neuronal numbers in the ageing gut

The previous section (and Table 1) demonstrates that although age-related neuronal loss, particularly of cholinergic neurons, has been widely described, not all evidence supports the hypothesis that extensive neuronal death takes place in the ENS during ageing. Furthermore, there are large variations in the proportions of neurons that have reported to be lost in AL-fed animals. Since caloric restriction has been shown to reduce or eliminate neuronal loss, this variation raises an important question about possible differences in food intake by AL-fed animals, which could result in a range of body weights and variable levels of neuronal loss. It is also notable that, for example, reported losses of as much as 20% (total neurons, Peck et al., 2009) and 30% (calretinin-IR, Thrasivoulou et al., 2006) have been found to be non-significant, indicating that variation in the data may be large, even for major neuronal subpopulations; this is also evident in some other studies. Analysis of changes in smaller subpopulations of enteric neurons, distributed over a wide area is therefore inevitably more difficult.

There are a number of other possible reasons for the variation in the published data on neuronal losses in the ENS, and why the evidence is conflicting. Careful consideration of these reasons may not only inform future studies in this area, but provide valuable information about the nature, and possible causes, of neuronal ageing in the gut. The sources of variation and issues that contribute to complication of analysis of ageing ENS analysis, and which make comparison of data from different studies problematic, are numerous and complex. They are summarised in Table 2 and discussed here (also see Phillips et al., 2007).

The anatomy of the enteric plexuses poses difficulties for accurate neuronal quantification

As is well-known among enteric neurobiologists, the arrangement and structure of the enteric ganglionic networks lying within a non-uniform tube make quantification of enteric neurons challenging. In contrast to other peripheral nervous system ganglia, which are discrete structures in which it is possible to perform accurate quantification on an entire ganglion using unbiased stereology, myenteric ganglia have an irregular size and arrangement in most parts of the gut. Methods for the quantification of enteric neurons and comparison of the abundance of different neuronal populations using whole mount preparations are now well-established. However in ageing studies it is not always possible to obtain sufficient material for whole mount preparations, or the time needed for preparations of samples for whole mounts may not be available, so some studies have analysed changes in neuronal numbers using tissue sections.

Changes in the dimensions of the gut and arrangement of the plexuses during the lifespan

The issue of gut growth is very important to consider during analysis of changes in the density of enteric neuronal cell bodies during ageing, since the same number of neuronal cell bodies in a larger sample will appear smaller when expressed per unit area (a ‘dilution’ effect, see Cowen et al., 2000; Peck et al., 2009; Phillips and Powley, 2007). Thus a false loss of neurons would be recorded. To avoid this dilution effect due to gut growth, many authors have

Table 2
Possible sources of variability in reports of age-related enteric neuronal loss.

<table>
<thead>
<tr>
<th>Sources of variability</th>
<th>Notes:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue sections/wholemounts</td>
<td>Most studies have used whole mounts, but some have used tissue sections.</td>
</tr>
<tr>
<td>Use of different markers for the total neuronal population</td>
<td>Wide range of general neuronal markers used, including histological (Giemsa, Cuprolinic blue), histochemical (NADH diaphorase) and immunolabelling for a range of general neuronal markers (e.g. PGP 9.5, Hu C/D).</td>
</tr>
<tr>
<td>Different counting methods</td>
<td>From images or directly from slides. For wholemounts with or without confocal analysis of z stacks (these are needed in regions where the plexus is thicker and neurons may lie on top of each other)</td>
</tr>
<tr>
<td>Random or systematic sampling methods used</td>
<td>A large range of sampling methods have been employed</td>
</tr>
<tr>
<td>Differing sample sizes</td>
<td>Differing total areas have been used for neuronal counts</td>
</tr>
<tr>
<td>Different methods to ensure standard distension of the sample at the time of fixation</td>
<td>Some studies use injection of fluid to maximise distension, others use nicardipine to ensure maximal smooth muscle relaxation</td>
</tr>
<tr>
<td>Gut growth corrected for or not</td>
<td>Many studies correct for changes in intestinal dimensions with age, but some do not. Clearly this is a difficult problem when analysing human samples. The intestinal dimensions of individuals within a cohort at the same age may not all be the same, so application of a mean correction factor may lead to inaccuracies.</td>
</tr>
<tr>
<td>Measurement of the degree of stretch of wholemount preparations</td>
<td>The degree of stretch of individual wholemount preparations is not usually taken into account. Possible changes in dimensions after fixation and processing have also not usually been considered</td>
</tr>
<tr>
<td>How neuronal numbers expressed</td>
<td>Neuronal density has been expressed per unit serosal area, per unit length of gut region, per ganglion and per ganglionic area</td>
</tr>
<tr>
<td>Ranges of ages studied</td>
<td>Ages compared include ‘old’ vs. ‘young’, or variable ranges of ages. The ages studied in relation to the maximum lifespan of the species/strain/cohort being used has not often been considered, in part because the typical maximal lifespan of the cohort being studied may not be known. Some studies do not use older than ‘middle-aged’ animals. The age of the ‘young’ control animals varies.</td>
</tr>
<tr>
<td>Animal husbandry</td>
<td>Particularly diet; differences between results from ad libitum-fed and caloric restriction have been demonstrated. Most studies have used AL feeding regimes, but the type of chow used in AL feeding may vary, for example a ‘breeding’ or a ‘maintenance’ diet may be used. This is often not detailed in publications, but may influence the rate of ageing. Other aspects of husbandry may also affect animal ageing and longevity.</td>
</tr>
</tbody>
</table>

* Some studies however, have used established colonies, such as the National Institute of Ageing (NIA) colony of Fischer 344 rats. See Phillips and Powley, 2007.
measured the size of the intestine in the animals that they have studied, and then applied a correction factor to compensate for the effect. However, not all studies have performed this correction, so some reports of neuronal loss may in fact be due to a dilution effect. This effect is a particular problem for analysis of human specimens, which may be biopsies, or post-mortem samples taken under conditions in which measurements of the gut cannot be taken.

There are four further complicating factors that affect the use even of this correction factor. First is that a change in the overall length of the small (or large) intestine may not be the result of a uniform increase along the length of the organ – growth may be different in different regions. Second is that the degree of change in dimensions may vary even within a cohort of animals of the same age, so application of a standard correction factor may result in inaccuracies (see Gamage et al., in press). Third is that the degree of distension of the gut at the time of sampling, but this is usually controlled for by the use of maximal distension or pharmacological relaxants. Finally, the degree of stretch during preparation of whole mounts may vary; this has not been controlled for in many studies (see Gamage et al., in press).

In a different approach to avoiding the problems of gut growth, some workers have expressed neuronal density per ganglion or per ganglionic area. While individual myenteric ganglia are regular in shape and easily distinguished in the ileum, in other areas, particularly in the large intestine, it is not possible to ascertain the borders of individual ganglia with certainty, so in this case expression of numbers per ganglionic area is more reliable and so preferable, but this measure has not been used in all studies.

**Issues of sampling**

A wide variation in the sampling methods has been used in the analysis of changes in neuronal number during ageing. This variation applies not only to the preparation of samples (e.g. wholemounts versus tissue sections and choice of fixative), but also to the actual area of the samples in which neurons are counted. The choice of the areas to be sampled also varies, some workers counting random fields or images taken randomly, others using a systematic sampling method to ensure, for example that there is no bias caused by disproportionate sampling of mesenteric and anti-mesenteric regions (which are known to have different densities of neurons). This type of problem is particularly difficult in GI regions in which the plexus is irregular, so that even some systematic sampling methods can introduce an element of bias (see Gamage et al., in press; Phillips and Powley, 2007).

**Choice of neuronal markers used**

A number of different pan-neuronal markers have been used to study enteric neuronal ageing (see Tables 1 and 2). The reliability of these different markers varies considerably. For example it has been reported that NADHd only labels approximately 80% of guinea-pig myenteric neurons, and PG9.5 also does not label all myenteric neuronal cell bodies (Phillips et al., 2004a; Phillips and Powley, 2007). Another difficulty in the use of immunolabelling to determine if total or particular subpopulations are reduced in number during ageing is that expression of the marker being studied may be down-regulated, so that the neurons in question may be present, but not detectable by the method being used. This problem has been described for HuC/D expression, which has been shown to be reduced in ageing rat myenteric neurons (Phillips et al., 2004b).

**Selection and number of time points in ageing studies**

Another key variable that is important in ageing studies is the time-points that are used. Studies that include larger numbers of time points are increasingly favoured, because the time at which age-associated changes begin, and the rate at which changes occur are important aspects in understanding both the ageing process itself and the development of interventions that may alleviate or delay detrimental changes. Detailed ageing studies thus require a significant time commitment and are expensive, so it is perhaps not surprising that some studies report differences between ‘old’ and ‘young’ ages, or are confined to younger ages that do not approach the maximum lifespan of the species involved. In this respect, it is essential to know the typical survival of the animals being studied; not only do different laboratory animal species, such as rats, mice and guinea-pigs have different lifespans, but the lifespan of different strains varies, and is dependent upon husbandry (e.g. see Merry, 2005; Nadon, 2006). Thus ‘old’ animals in some studies may be equivalent to ‘middle-aged’ animals in others.

**Effects of diet and environmental enrichment on enteric neuronal survival during ageing**

The issue of animal husbandry is very important in the study of ageing. Myenteric neuronal loss has been reported to be reduced (da Silva Porto et al., 2012; Thrasivoulo et al., 2006) or eliminated (Cowan et al., 2000; Johnson et al., 1998) by caloric restriction. The widespread effects of caloric restriction on ageing are now well-characterised in a number of species and the intervention is known not only to decrease morbidity but to increase life-span. These effects raise an interesting question in the interpretation of differential changes in AL-fed and calorically restricted animals; the effective physiological age of the latter may in fact be younger than that of the former, so direct comparison of the two groups may not be strictly valid (e.g. see Hoyle and Saffrey, 2012; Merry, 2005). Another factor that has not usually been considered is the type of diet used (e.g. breeding versus maintenance diet) and dietary supplements. The amount of food intake by AL-fed animals is also an issue; this could vary considerably, some ageing rodents may gain a lot of weight and it is possible that they may be obese, thus confounding data believed to be due to ageing. Not all studies have provided detailed information about food intake and body weight of the animals studied. The effects of diet are also not confined to post-weaning period. Prenatal nutrition may also influence neuronal numbers in the adult gut (Santer and Conboy, 1990).

It is also possible that other aspects of husbandry, such as provision of an enriched environment that encourages increased exercise, may influence ageing of enteric neurons. Total myenteric neuronal loss in 12 month old rats has been reported to be reduced by exercise, although nitergic neuronal numbers were reduced (Martinez Gagliardo et al., 2008). Lack of mobility of humans is known to have a negative effect on frequency of bowel movements (e.g. see Rao and Go, 2010).

Nevertheless, despite all the issues described above, the presence of spaces in the myenteric ganglia of ageing humans (Hanani et al., 2004) and rats (Thrasivoulo et al., 2006) supports the evidence that some enteric neurons are lost during ageing, at least in some animals. Further evidence for enteric neurodegeneration in ageing is described in the following section.

**Degenerative changes in the ENS**

Although the extent of neuronal cell death that may occur in the ENS during ageing is not established, there is clear evidence that neurodegeneration of enteric neurons does occur in older
animals. Most of this evidence comes from studies at the light microscope level; very few studies have described ultrastructural changes in the ENS during ageing.

Several groups have demonstrated the presence of swollen and dystrophic nerve fibres in ageing gut. NADPHd positive dystrophic fibres have been described in the myenteric plexus of rats (Phillips et al., 2003), and similar nNOS-IR fibres are seen in ageing mice (Gamage et al., in press). In the rat myenteric plexus, degenerating NADPHd fibres (Phillips et al., 2003) have been detected in rats at 24 months of age. In the mouse distal colon, dystrophic nNOS-IR and calbindin-IR nerve fibres were seen occasionally in 12 month animals, but were frequent in animals at 18 and 24 months of age (Gamage et al., in press). Degenerating SP-, VIP- and somatostatin-immunoreactive nerve fibres have also been described in aged rat small intestine at the ultrastructural level (Feher and Penzes, 1987).

Ageing is associated with the accumulation of lipofuscin in postmitotic cells (see Grey and Woulfe, 2005). Accumulations of lipofuscin in ageing enteric neurons have been reported in rats (Corns et al., 2002) (Phillips et al., 2004a) and guinea-pigs (Abalo et al., 2005, 2007).

Although ultrastructural changes in the ageing ENS have not been studied in detail, some analysis of the ENS at the electron microscope level has been performed. Gabella (1989) has reported that degenerating myenteric neurons are sometimes seen in the ageing guinea-pig myenteric plexus, but that the fine structure of the surrounding ganglion appears intact. Feher and Penzes (1987) have described the presence of degenerating SP-VIP and somatostatin-IR fibres in the aged rat small intestine. In preliminary studies of ageing myenteric plexus ganglia in the mouse terminal colon, we have observed increased numbers of inclusions, indicative of neurodegeneration, in ageing mouse myenteric neurons (unpublished results).

**Alpha-synuclein and enteric neurodegeneration**

Recent evidence has shown that alpha-synuclein is expressed by enteric neurons (Phillips and Powley, 2007; Phillips et al., 2008). This protein is also expressed in many CNS neurons and its aggregation in Lewy bodies is a hallmark of Parkinson’s disease. Phillips et al. (2008) have demonstrated that alpha-synuclein is expressed by 3% of myenteric neurons in the stomach and 22% of myenteric neurons in the duodenum of Fischer 344 rats, and that it colocalises with nNOS, calbindin and calretinin in myenteric subpopulations. Abundant alpha-synuclein-IR nerve fibres were also found in these tissues. In 18 month- and in 24–25 month-old Fischer 344 rats, alpha-synuclein positive aggregates were observed in dystrophic axons that were also immunoreactive for nNOS, calbindin and calretinin (Phillips et al., 2009), although not all dystrophic axons expressing these markers also expressed alpha-synuclein. Hyperphosphorylated Tau aggregates were also observed in the ageing myenteric plexus, but in a limited area, the proximal small intestine (Phillips et al., 2009). The expression and aggregation of alpha-synuclein in enteric neurons is not only of interest in the development of neurodegeneration during ageing of the ENS, but also in Parkinson’s disease. Lewy bodies have been described in the ENS of Parkinson’s disease patients, and it has been suggested that the ENS may be one possible site of onset of the disease caused by an ingested pathogen, and that the pathology may spread to the CNS from the intestine (e.g. see Hawkes et al., 2007).

**Changes in nerve fibre density in the ageing gut: evidence for regeneration?**

As discussed above, an increase in the length and circumference of the gut during adulthood and ageing would be expected to result in a dilution of nerve fibres, unless there is growth and/or sprouting of nerves to compensate for increases in the volume of the smooth muscle, for example.

In addition to the possible dilution of nerve fibres due to GI growth, a significant loss of enteric neurons in aged animals, such as the 40–60% losses reported in some studies (see Table 1) would be expected to result in a clearly-detectable loss of nerve fibres. Several studies have investigated changes in nerve fibre density in the ageing gut, although unfortunately no such analysis has been performed in cohorts reported to have major losses of neuronal cell bodies.

Santer (1994) observed that the density of NADPHd positive nerve fibres appeared to be greater in the myenteric plexus and muscularis externa of ageing rat small intestine, but did not quantify the changes. Despite a decrease in density of fibres due to smooth muscle growth, an increase in the total innervation of the guinea-pig taeniæ coli in aged guinea-pigs has been described (Gabella, 2001). Peck et al. (2009), who studied changes in the density of different types of nerve fibre in the circular muscle of the guinea-pig colon during ageing, found an apparent reduction in density of nNOS and SP-IR fibres in samples from older animals. However, once increase in muscle thickness was taken into account, no change in total area of these nerve fibres was measured during ageing, indicating that reduced fibre density was due to gut growth rather than neuronal loss. In human sigmoid colon, a similar result was obtained; regression analysis showed a decrease in the density of nNOS-, VIP- and SP-IR nerve fibres in the circular muscle during ageing (Southwell et al., 2010), but this was accompanied by an increase in circular muscle thickness, indicating that there was no age-related loss of nerve fibres. Bernard et al. (2009) have also reported no change in the density of nerve fibres, in this case despite neuronal loss (see Table 1), and suggest a compensatory mechanism of neuronal growth may occur during ageing.

Changes in the density of nerve fibres in the tertiary plexus have also been examined. Abalo et al. (2007) investigated changes in calretinin-IR nerve fibre density in the tertiary plexus of the guinea-pig ileum during ageing, and found no significant change, again despite a loss of myenteric neurons expressing calretinin in the same animals. Santer (1994) however, observed that the density of NADPHd positive fibres in the tertiary plexus appeared greater in the small intestine of aged rats and an increase in the density of nNOS-IR fibres has been described in the tertiary plexus of the mouse distal colon (Gamage et al., in press).

A different approach has been used in the study of changes in neuronal subpopulations in ageing mice by Van Ginneken et al. (2011). These authors performed volume density measurements of labelled neurons in tissue sections from wild type and transgenic (see below) mice. No reduction in the density of nervous structures labelled for βIII-tubulin, substance P or nNOS were observed between 18 month old (the oldest animals studied) and 6 month old animals, although some reduction at 12 months was observed.

While more work clearly needs to be done, taken together these data all indicate that if major neuronal losses occur in the ageing gut, they do not necessarily lead to loss of nerve fibres. It is possible, and perhaps even likely, that compensatory mechanisms occur, such as sprouting and or growth of remaining neurites from surviving neurons. In this context it is of interest that extensive neurite regeneration occurs after chemical ablation of the myenteric plexus in adult mice (Hanani et al., 2003), indicating that mechanisms for regeneration exist in the adult ENS.

**Neurogenesis in the ageing ENS**

It is now established that enteric neural stem cells are present in the adult ENS (Kruger et al., 2002) and also that adult enteric
glia have the potential to form neurons in vitro (Joseph et al., 2011; Laranjeira et al., 2011 and see Gershon, 2011; Metzger, 2010). Studies to investigate if neurogenesis is ongoing in adulthood however, have not detected formation of new neurons except in culture, or under conditions in which damage or stress has occurred (Joseph et al., 2011; Laranjeira et al., 2011 and see Gershon, 2011). Among a range of other paradigms studied, Joseph et al. (2011) investigated if neurogenesis occurred in ageing rats, but found no evidence for the formation of new neurons in 21 month old animals. Thus it appears as if neurogenesis does not typically occur in the ageing ENS. However it is possible that small local regions of damage or stress occur during ageing, which may stimulate generation of new neurons. In this context the appearance of occasional single neurons at the edge of some ganglia in old samples (Abalo et al., 2005; Gabella, 1989; Hanani et al., 2004) is perhaps interesting, as this is a site of neural precursors. Differences in restorative neurogenesis in the samples used for analysis of age-related changes in neuronal number could possibly account for the different degrees of neuronal loss obtained in different studies.

Importance and implications of age-related enteric neuronal loss: are enteric neurons more vulnerable than other neurons to age-related degeneration?

Age-related loss of enteric neurons is significant for two very different reasons; first are the obvious implications for age-related changes in gut function (described in the next section), second is that in other parts of the nervous system neuronal loss during ageing does not occur. Although neuronal loss in the CNS was at one time considered to be typical, relatively recent developments in the techniques of unbiased stereology revealed that such loss is limited to neurodegenerative diseases. Enteric neurons may therefore be more vulnerable to ageing than neurons of the CNS and other peripheral ganglia. Thus it is both of local and broader interest if neuronal losses do indeed occur in the ageing ENS.

There are several reasons why enteric neurons may be more vulnerable to age-related loss than neurons in other parts of the nervous system. In addition to the continual physical changes (which are also a feature of some other autonomic neurons, such as those that innervate other organs that regularly undergo distension, the heart and bladder), the intestine is exposed to continual changes in its contents, including the intestinal microbiota. As already touched upon, the possible exposure of enteric neurons to pathogens has been suggested as one site for the development of neuropathies such as those seen in PD, which may spread to the CNS via the vagal nerve (Hawkes et al., 2007; Phillips et al., 2009, 2008). This may be just one example of a pathology resulting from exposure to ingested pathogens. However, changes in the balance of non-pathogenic microbial populations in the gut during ageing may also influence enteric neurons.

The gut microbiota are now known to play a major role in human health (e.g. see Grenham et al., 2011; Nicholson et al., 2012) including gastrointestinal health (see Aziz et al., 2013) Changes in the in the gut microbiota have been described during ageing (e.g. Makivuukko et al., 2010 and see Aziz et al., 2013; Makivuukko et al., 2010; Tiitonen et al., 2010), and these age-associated changes can be associated with diet, and with negative indicators of health (Claesson et al., 2012). Interactions between the microbiota, the cells of the gut and the rest of the organism are complex, and only now beginning to be understood. In addition to effects within the gut itself, including effects on enteric neurons, the influence of the microbiota exerted via the brain-gut axis is also now an area of great interest (e.g. see Rhee et al., 2009). Here, the effects of the microbiota on enteric neurons and glia are briefly considered, in order to highlight possible areas that may impact upon ageing of the ENS. Once again, these effects are complex, and involve interactions with other intestinal cells, notably enteric glia and the cells of the mucosal immune system.

One key aspect of exposure of enteric neurons to microbes is the permeability of the intestinal epithelium, which has an important barrier role (see Neunlist et al., 2013). The intestinal epithelial barrier (IEB) is not fully developed at birth and some permeability during the postnatal period has been suggested to be important for the appropriate development of the immune system. Changes in IEB permeability are associated with several disease states and can lead to intestinal inflammation, which in turn may lead to neuronal damage (see below). Of particular interest are recent reports suggesting that cells of the ENS play an important role in the maintenance of IEB function (Neunlist et al., 2013; Savidge et al., 2007).

Once microbes cross the IEB, they can have a number of effects, and may cause an inflammatory response. Inflammatory diseases of the gut are a major cause of morbidity and have been studied intensively. Interaction between enteric neurons, glia and the cells of the mucosal immune system is an area that is of increasing interest in the context of these diseases. Evidence demonstrates close interactions of both enteric neurons and glia with cells of the mucosal immune system, and inflammation may affect both the structure and function of the ENS (see Lomax et al., 2005; Mawe et al., 2009). For example, a loss of enteric neurons has been described in some experimental models of inflammation (see Lomax et al., 2005) and hyperexcitability of AH neurons (IPANs) occurs in inflammation (Mawe et al., 2009). Changes in the microbiota during ageing, which may be related to diet, may therefore increase intestinal inflammation, which may, in turn influence cells of the ENS, possibly resulting in their damage or death. It is also possible that relatively small localised areas of inflammation may occur during ageing, leading to small areas of neuronal loss.

Recent studies have also indicated that the microbiota may also have an important influence on the physiological and/or neurochemical properties of enteric neurons. For example, in recent work, McVey Neufeld et al. (2013) found that excitability of intrinsic primary afferent neurons (IPANS) was reduced in germ-free mice. di Giancamillo et al. (2010) presented evidence that there was an increase in the number of galanin- and calcitonin gene-related peptide immunopositive neurons in the ileal submucous plexuses of piglets fed a diet supplemented with probiotics. The total number of neurons, however, was not affected, suggesting an effect on existing neurons. An increase in the number of glial cells in the submucosa was also detected, in the same regions. Whether these changes result from direct or indirect effect of the bacteria used, however, is not clear. Thus it is possible that changes in the microbiota during ageing could impact upon the properties of enteric neurons and glial cells. Clearly this is an important area for future study.

Thus there is a complex interrelationship between the gut microbiota, epithelium, mucosal immune system and the ENS, which may not only make enteric neurons particularly vulnerable during ageing, but may also play an important role in the aetiology of other diseases.

Age-associated changes in the microbiota pose an interesting question of cause or effect. For example age-associated changes in effector cells may affect gut physiology resulting in delayed transit, which may lead to bacterial overgrowth, in turn leading to a change in the balance of microbiota in the gut. Alternatively, changes in microbiota could influence intestinal cells, including neurons, thus leading to physiological changes including delayed transit. It is perhaps, likely, that ageing involves both these possibilities.

Finally, if contact with gut microbes and/or mucosal inflammation is indeed linked to enteric neural damage and degeneration
during ageing, one would expect submucosal neurons and myenteric neurons that project to the mucosa, IPANs, might be preferentially affected. Extrinsic nerves that innervate the mucosa may also be affected.

Does enteric neuronal loss during ageing lead to age-related GI dysfunction?

The potential significance of age-related neuronal loss to the changes in gut function that are common during ageing is clear. However in most studies in which high percentages of neuronal loss have been reported, the issue of possible GI dysfunction in the experimental animals used has not been addressed. It would be expected that a loss of 40–60% of the total myenteric neuronal population would have a marked effect on function, which could be manifest as decreased frequency of defeacation; constipation is common in elderly humans. Observations on the frequency, size and composition of stools in older animals however, have not often been reported in studies that have investigated age-related changes in neuronal number. Gabella (1989); however, reported that there were no obvious changes in feeding habits or faeces production of the guinea-pigs in his study, which exhibited a loss of 40–60% of myenteric neurons in the small intestine.

Most physiological and pharmacological studies of GI tissue from ageing animals have not investigated neuronal numbers in the same tissues, and the converse is also the case; few studies have investigated physiological changes in animals in which neuronal numbers have also been studied. However, in a comparison of cholinergic neuromuscular transmission in ileal longitudinal muscle of young and aged ad libitum fed and calorically-restricted animals no age-associated impairment of the cholinergic system was observed, and diet did not affect responses to field stimulation (Hoyle and Saffrey, 2012). This work was carried on animals in which a neuronal loss had been demonstrated in AL-fed animals (Thrasivoulou et al., 2006), so it appears as if some cholinergic function at least was unimpaired in animals that exhibited a major neuronal loss. Abalo et al. (2005) found an increase in the force of contraction induced by excitatory stimuli in guinea-pig ileum, which paralleled an age-related decrease in density of excitatory motor neurons, and suggested a compensatory mechanism might be involved.

In recent work, we have been performing physiological and pharmacological analyses of the terminal bowel of ageing mice in parallel with morphological and immunohistochemical studies. We observe changes in motility and stool numbers (Patel et al., 2012), but no significant changes in neuronal numbers in the distal colon in mice up to 24 months of age (Gamage et al., in press).

What are the mechanisms of enteric neuronal loss during ageing?

Many different mechanisms of cellular ageing have been proposed and it is of course possible, or indeed likely, that ageing involves more than a single mechanism. Some cell types may be more vulnerable than others to particular types of change or damage, however. Several potential mechanisms of neuronal loss have been investigated in the ageing ENS; these are considered below.

Oxidative and free radical damage have been proposed to play a major role in neuronal ageing, because neurons are highly metabolically active cells. Such damage may be exacerbated for enteric neurons because of their environment in close proximity to the gut contents, microbiota and local immune system, as described above. In support of the suggestion that oxidative stress may play a role in enteric neurodegeneration, ageing myenteric neurons have been shown to have elevated levels of reactive oxygen species (ROS) (Thrasivoulou et al., 2006). Furthermore the rate of ROS generation in 15 month-old rats was found to be reduced by caloric restriction (Thrasivoulou et al., 2006). Neurons from middle-aged (15 month) AL-fed rats were also more vulnerable to menadione –induced apoptosis than neurons from CR-fed animals at the same age, or 6 month-old AL fed rats (Thrasivoulou et al., 2006).

There is evidence that neurotrophic factors protect neurons from oxidative stress (Hardingham and Lipton, 2011; Numakawa et al., 2011) and the neurotrophic factors GDNF and NT-3 have been shown to reduce ROS generation by myenteric neurons in samples from both 12–15 month and 24 month old CR-fed, but not AL-fed rats at the same ages (Thrasivoulou et al., 2006). Neurotrophin-3, but not GDNF, also reduces neuronal death in dissociated myenteric plexus in response to hydrogen peroxide treatment (Korsak et al., 2012b). Thus it has been suggested that neurotrophic factors may play a role in protection of enteric neurons throughout life, and that a breakdown in neurotrophic factor support leading to increased susceptibility to free radical damage may contribute to enteric neuronal degeneration, and eventually neuronal death (e.g. see Camilleri et al., 2008 Thrasivoulou et al., 2006). The neurotrophic factors glial cell line-derived neurotrophic factor (GDNF) and neurturin are known to promote the survival and development of enteric neurons during embryonic and early postnatal development (see Saffrey, 2009). Analysis of the expression of these factors and their receptors in the ileum of AL- and calorically-restricted rats during ageing show that expression continues in ageing animals, and the levels GDNF and its receptor GFRA-1 are elevated in ageing AL-fed, but not CR animals. (Korsak et al., 2012a). Such an increase could represent a compensatory upregulation in response to elevated stress and/or neurodegeneration. In contrast the expression of the neurturin receptor, GFRA-2b, was reduced in ageing AL-fed animals (Korsak et al., 2012a).

Calcium dysregulation has also been proposed to be a factor that contributes to neuronal ageing, and the presence of calcium-binding proteins in some neurons has been suggested to be protective. Reduction in the number of enteric neurons expressing calcium binding proteins such as calbindin (Thrasivoulou et al., 2006), calretinin (Abalo et al., 2005; Thrasivoulou et al., 2006) and neurocalcin (Comas et al., 2002) in ageing animals could be the result of changes in the expression of these proteins, indicative of calcium dysregulation. The development of alpha-synuclein immunopositive aggregates in calbindin- and calretinin-IR myenteric neurons during ageing (Phillips et al., 2009) suggests that these neurons may in fact be vulnerable to age-related degeneration.

Cellular senescence occurs in many cell types during ageing. In addition to well-known phenomenon of replicative senescence, exhibited by mitotic cells, recent evidence shows that post-mitotic neurons exhibit a p21-dependent senescence-associated phenotype, induced by a DNA damage response (Jurk et al., 2012). Recent evidence indicates that this senescence-associated phenotype is also exhibited by a population of myenteric neurons in the ageing mouse small intestine (Jurk et al., 2012). The proportion of neurons affected was reported to be between 20 and 40%, in 22–24 month-old animals. The subpopulation/s of neurons exhibiting the senescence-associated phenotype has not yet been established, but it of interest that recent evidence indicates that an element of the phenotype is secretory, and that in culture at least, senescent fibroblasts can induce damage in adjacent cells (Nelson et al., 2012). This last observation raises the interesting possibility that ageing of enteric neurons could also be affected by ageing of surrounding replicative cells.

Transgenic animal models and the study of ENS ageing

A number of transgenic models of ageing and neurodegeneration are now available. Analysis of these animals may provide
valuable information about the mechanism of ageing in wild-type animals. ENS changes however, have been studied in only very few of these models. Klotho is an ‘anti-ageing’ peptide that occurs both bound to membranes and in a soluble state, is a co-receptor for FGF and also regulates vitamin D and phosphate metabolism (see Asuzu et al., 2011). Transgenic mice expressing low levels of Klotho exhibit premature ageing phenotypes and have a shortened lifespan (60–70 days), over-expressers have an extended lifespan, 20–30% longer than their wild-type littermates (see Asuzu et al., 2011). Analysis of the ENS in mice with reduced Klotho expression have shown that myenteric neuronal numbers were reduced in some regions of the gut, but that gut lengths were also reduced, possibly because of reduced food intake by these animals. These results serve to indicate that caution is necessary in interpretation of the data from transgenic animals, as observations may be due to secondary or compensatory changes, rather than a primary effect of the under-or over-expression of the gene involved.

Another recent study has investigated changes in the ENS in Thy1-APP23 transgenic mice, which have been used as a model for Alzheimer’s disease and oger-express a form of amyloid precursor protein (Van Ginneken et al., 2011). Although amyloid deposits were observed in the smooth muscle and mucosa of the intestines of the under-or over-expression of the gene involved.

References


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