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Reactive oxygen species, dietary restriction and neurotrophic factors in age-related loss of myenteric neurons

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Summary

We have studied the mechanisms underlying nonpathological age-related neuronal cell death. Fifty per cent of neurons in the rat enteric nervous system are lost between 12 and 18 months of age in *ad libitum* (AL) fed rats. Caloric restriction (CR) protects almost entirely against this neuron loss. Using the ROS-sensitive dyes, dihydrorhodamine (DHR) and 2-[6-(4′-hydroxy)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (HPF) *in vitro*, we show that the onset of cell death is linked with elevated intraneuronal levels of reactive oxygen species (ROS). Treatment with the neurotrophic factors NT3 and GDNF enhances neuronal antioxidant defence in CR rats at 12–15 months and 24 months but not in adult or aged AL-fed animals. To examine the link between elevated ROS and neuronal cell death, we assessed apoptotic cell death following *in vitro* treatment with the redox-cycling drug, menadione. Menadione fails to increase apoptosis in 6-month neurons. However, in 12–15mAL fed rats, when age-related cell death begins, menadione induces a 7- to 15-fold increase in the proportion of apoptotic neurons. CR protects age-matched neurons against ROS-induced apoptosis. Treatment with neurotrophic factors, in particular GDNF, rescues neurons from menadione-induced cell death, but only in 12–15mCR animals. We hypothesize that CR enhances antioxidant defence through neurotrophic factor signalling, thereby reducing age-related increases in neuronal ROS levels and in ROS-induced cell death.

Key words: aging; antioxidant defence; apoptosis; cell death; dietary restriction; myenteric neurons; neurotrophic factors; reactive oxygen species.

Introduction

Relatively little is known about the mechanisms which underlie nonpathological age-related neurodegeneration. Neither the nature nor extent of this phenomenon is well established. Studies of mammalian central (Morrison & Hof, 1997; Turlejski & Djavadian, 2002; Baquet *et al*., 2004) and peripheral (Cowen & Gavazzi, 1998; Cowen *et al*., 2003) neurons demonstrate that age-associated degenerative changes only occur in select groups of neurons such as the cholinergic neurons of the basal forebrain and the sympathetic neurons projecting to cerebral blood vessels and pineal gland. Previous studies have shown that around 50% of the neurons of the myenteric plexus in the gut of rodents (Johnson *et al*., 1998; Cowen *et al*., 2000), guinea pigs (Gabella, 1989) and humans (Porter *et al*., 1996) are lost during aging. While in rats neurons expressing choline acetyl transferase are vulnerable while those expressing nitric oxide synthase are protected (Cowen *et al*., 2000), the reasons for this selective vulnerability in different regions of the nervous system are poorly understood.

Age is the biggest risk factor for several of the more common neurodegenerative diseases, including Alzheimer, Parkinson and motor neuron diseases. Damage by reactive oxygen species (ROS) is implicated in nonpathological brain aging processes (Poon *et al*., 2004; Lau *et al*., 2005) as well as in all of these chronic disorders (Schapira, 1998; Jung *et al*., 2001; Nunomura *et al*., 2001; Huang *et al*., 2004). Although there is little concrete evidence, the implication of these observations is that common mechanisms involving ROS may underpin pathological as well as nonpathological age-related neurodegeneration.

Neurons are considered particularly vulnerable to free radical damage because of their large size, high level of metabolic activity and relatively poor antioxidant defence (Sohal & Weindruch, 1996). Survival of adult and aged neurons appears to be in some way related to the capacity to resist ROS-induced damage (Finkel & Holbrook, 2000). Previous evidence of age-related vulnerability of enteric neurons (Phillips *et al*., 2003) does not provide a link with ROS sensitivity. Calcium dyshomeostasis is a key event in neuronal aging (Bu *et al*., 2003) and in Alzheimer

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disease (LaFerla, 2002) which have been linked to altered mitochondrial ROS generation in old age (Mattson & Liu, 2002). The calcium-binding proteins, calretinin and calbindin, present in many central and peripheral neurons including neurons of the myenteric plexus (Abalo *et al*., 2005), are thought to protect neurons against calcium dyshomeostasis and may therefore allow the identification of neurons that are selectively protected against ROS-induced damage.

Caloric restriction (CR) extends lifespan in organisms as diverse as yeasts, worms, flies and mammals by enhancing the capacity for stress resistance (Sohal & Weindruch, 1996). Furthermore, CR enhances plasticity in the aging brain (Mattson *et al*., 2002), increases expression of an anti-apoptotic repressor in the cerebral cortex of aging rats (Shelke & Leeuwenburgh, 2003), and rescues neurons of the rat enteric nervous system (ENS) from age-related cell death (Cowen *et al*., 2000). We hypothesize that the effects of diet on neuronal survival are mediated by altered capacity to regulate ROS.

Neurotrophic factor signalling is crucial to neuronal survival and differentiation during development (Miller & Kaplan, 2001). A novel role for neurotrophins in regulating ROS has recently been demonstrated in developing sympathetic neurons (Dugan *et al*., 1999), in PC12 cells (Mills *et al*., 1998) and in an animal model of Parkinson's disease (Salinas *et al*., 2003). Because CR has been shown to influence neurotrophin-mediated plasticity in the CNS (Prolla & Mattson, 2001; Nicoletti *et al*., 2005), we hypothesize that CR might enable neurons to resist age-related cell death by enhancing neurotrophic factor-mediated antioxidant defence.

Our aims were therefore (i) to confirm protection by dietary restriction against age-related loss of myenteric neurons and to determine whether neurons expressing calcium-binding proteins were selectively protected against age-related cell death; (ii) to find out if age- and diet-related alterations in intracellular ROS levels are associated with neuronal cell death in the ENS; (iii) to discover whether elevated ROS levels might induce apoptotic cell death in enteric neurons; and (iv) to demonstrate a possible role for neurotrophic factors in regulating antioxidant defence and resistance to ROS-induced neuronal cell death.

Results

Effects of dietary restriction on age-related loss of myenteric neurons

Counts of PGP 9.5-stained neurons confirm previous data (Cowen *et al*., 2000) by showing an age-related loss of 51% of neurons ($P < 0.001$) in 24mAL animals (Fig. 1A). CR animals show a much smaller loss of neurons over 24 m (22%; $P < 0.05$) indicating, like our previous data, substantial protection by dietary restriction. Examination of the timescale of neuron loss reveals that at 12 months, numbers of neurons are not significantly different from those in 6-month rats and there are no differences between AL and CR groups (Fig. 1A). However, by 13 months there are 24% ($P < 0.05$) fewer neurons in the AL

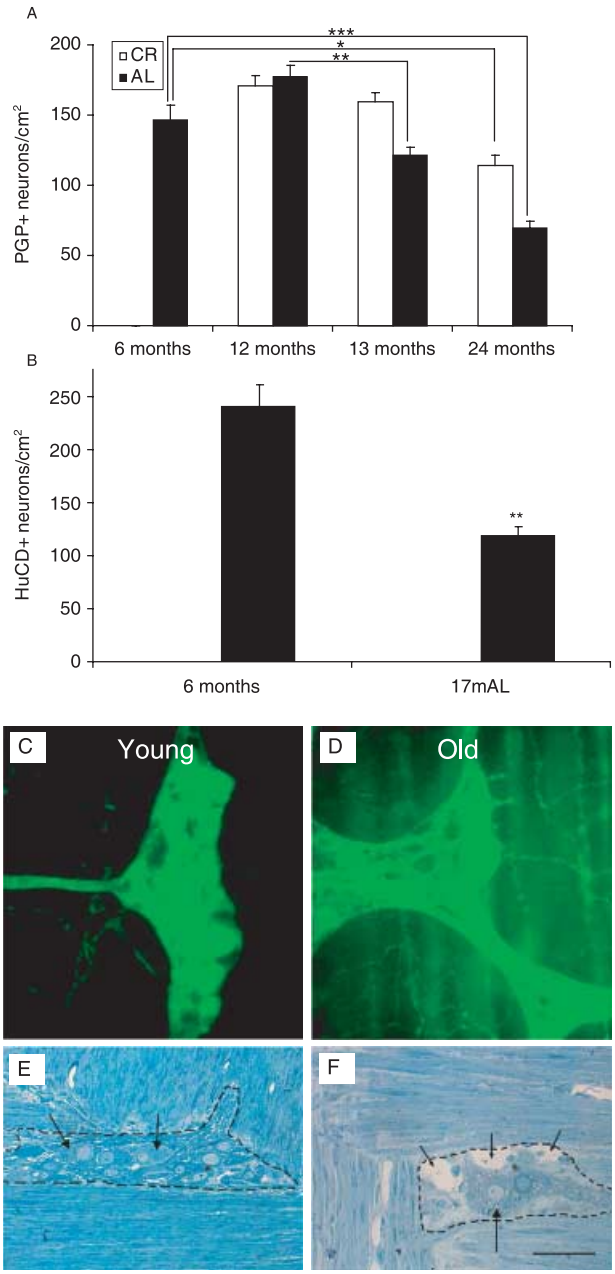

Fig. 1 Total numbers of myenteric neurons. (A) Counts of PGP-immunoreactive neurons. The histogram shows significant losses of neurons, commencing at 12–13 months of age in AL-fed animals and reaching a peak at 24 months (AL; 51%). There is a much smaller but statistically significant ($P < 0.05$) reduction seen in 24mCR samples, demonstrating substantial protection by CR against age-related cell death in myenteric neurons. (B) Counts of HuCD-immunoreactive neurons. The histogram shows loss of neurons in 17-month AL-fed, confirming that PGP 9.5 labelling gives a reliable estimate of age- and diet-related cell loss. However, total neuron numbers were significantly ($P < 0.01$) higher in the HuC/D-stained samples. (C–F) Photomicrographs of 6-month (C,E) and 24-month AL (D,F) PGP-immunostained whole-mounts (C,D) and toluidine blue-stained 1-μm resin sections (E,F). Neurons in myenteric ganglia (dotted lines) are indicated by large arrows. Note the large spaces in the ganglia from 24-month AL rats (small arrows) visible in D and F but absent in the 6-month samples (C,E), indicating where neuron cell loss has occurred. n numbers: A: 6 months ($n = 15$); 12mCR ($n = 4$); 12mAL ($n = 4$); 13mCR ($n = 8$); 13mAL ($n = 12$); 24mCR ($n = 15$); 24mAL ($n = 14$); B: 6 months ($n = 6$); 17 months ($n = 3$). Scale bar = 50 μm.

compared to CR groups and this number is significantly lower than either of the 12 months groups (13mAL vs. 12mCR, $P < 0.05$; 13mAL vs. 12mAL, $P < 0.01$). As reported previously (Cowen *et al.*, 2000), neuron loss is complete by 16–18 months (when corrected for gut size) in AL-fed rats. Whilst these data show similar levels of loss of neurons with HuC/D at 17mAL (~50%) with those shown in Cowen *et al*. (2000), the PGP 9.5 data only show a partial, but significant loss, at the 13mAL age group studied. Counts are corrected for changes in gut size to ensure that alterations are due to cell loss rather than redistribution of neurons. However, our measurements reveal no significant effect of diet or age on the overall dimensions of the small intestine (Table 1).

As a result of a recent study evaluating pan-neuronal markers in the rat myenteric plexus (Phillips *et al*., 2004), tissues were taken from small groups of 3-month and 17mAL animals and immunolabelled for HuC/D, which was shown to be one of the most reliable pan-neuronal markers for rodent myenteric neurons. Neuron counts made on HuC/D-labelled preparations demonstrated significantly ($P < 0.01$) higher neuron numbers compared to PGP 9.5, confirming previous reports that PGP 9.5 does not stain all neurons in the gut (Young *et al*., 2003). HuC/D, however, demonstrated a comparable loss of neurons (50%; $P < 0.01$; Fig. 1B) in 17mAL fed animals to that shown previously using PGP 9.5 at 16 and 24 months in AL-fed rats (Cowen *et al.*, 2000). The 50% loss of neurons seen with HuC/D at 17 months is similar to the extent of loss of PGP 9.5-positive neurons seen at 24 months (Fig. 1A,B), supporting the view that neuron loss in aging AL-fed rats is largely complete by 17–18 months.

Loss of immunolabelling may indicate reduced binding of the antibodies rather than neuron loss. To examine this possibility, we studied parallel samples from 24mAL and 6-month rats following PGP staining of whole mounts and toluidine blue staining of 1 μm resin sections (Fig. 1C–F). Both methods reveal clearly defined spaces surrounded by enteric glia in the ganglia of 24mAL samples which are not present in 6-month samples. Overall, these results confirm that age-related neuron loss has indeed taken place and that dietary restriction protects myenteric neurons against age-related loss.

Table 1 Age- and diet-related changes in areal dimensions (cm^2) of small intestine

6 months	148.1 ± 5.0
13mCR	144.8 ± 13.5
13mAL	128.7 ± 6.7
24mCR	134.2 ± 9.4
24mAL	158.4 ± 3.7

There are no statistically significant differences between any of these measurements (i.e. there is no significant change in gut dimensions with age or diet).

Selective vulnerability in subpopulations of myenteric neurons

Calcium dyshomeostasis is a key event in neuronal aging (Bu *et al.*, 2003). We therefore investigated whether neurons containing the calcium-binding proteins, calretinin and calbindin, were particularly protected compared to the general neuron population in aging rats. Calretinin-positive neurons represented a significant (25%) proportion of the total population of myenteric neurons and exhibited similar small but nonsignificant losses at 17 months of age in preparations from both AL- or CR-fed animals (Fig. 2A).

Calbindin-positive neurons formed a much smaller proportion of the total (2.5%) (compare Fig. 2A,B). Numbers were reduced by 62% ($P < 0.01$) in the 17mAL fed group indicating that these neurons were selectively vulnerable during aging, while CR at the same age demonstrated almost complete protection of calbindin-positive neurons (Fig. 2B). These results suggest that while the calcium-buffering capacity of calretinin may be a factor in protecting these neurons against age-related calcium dyshomeostasis and death, calbindin is not associated with a similar protective effect. Dietary restriction is shown to be strongly neuroprotective in the case of the calbindin-positive population of myenteric neurons.

ROS levels in myenteric neurons

Initial comparisons revealed a visually obvious elevation of ROS levels in 24-month compared to 6-month myenteric neurons using the ROS-sensing dye, DHR (Fig. 3A,B). We therefore made systematic comparisons of neuronal ROS levels between the different experimental groups. *In vitro* preparations of 6-month rat myenteric neurons exhibit different rates of increase in DHR fluorescence during the period of incubation, which peaks around 2 h (data not shown). This timescale was therefore used in all subsequent experiments. Comparison of ROS levels using DHR fluorescence in preparations from 6-month and from 12- to

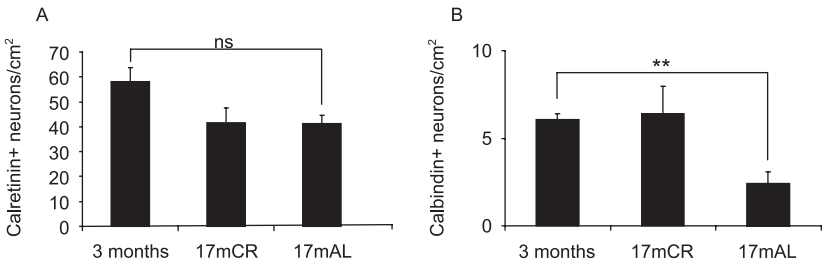

Fig. 2 Numbers of calretinin- (A) and calbindin- (B) immunoreactive myenteric neurons. Note the lack of significant change in calretinin + neurons between 6 and 17 months of age or with AL feeding which contrasts with the age-related loss of over 50% of calbindin + neurons in the 17mAL group ($P < 0.01$) which is fully rescued in age-matched CR animals. n numbers: A: 6 months ($n = 4$); 17mCR ($n = 3$); 17mAL ($n = 4$); B: 6 months ($n = 5$); 17mCR ($n = 3$); 17mAL ($n = 6$).

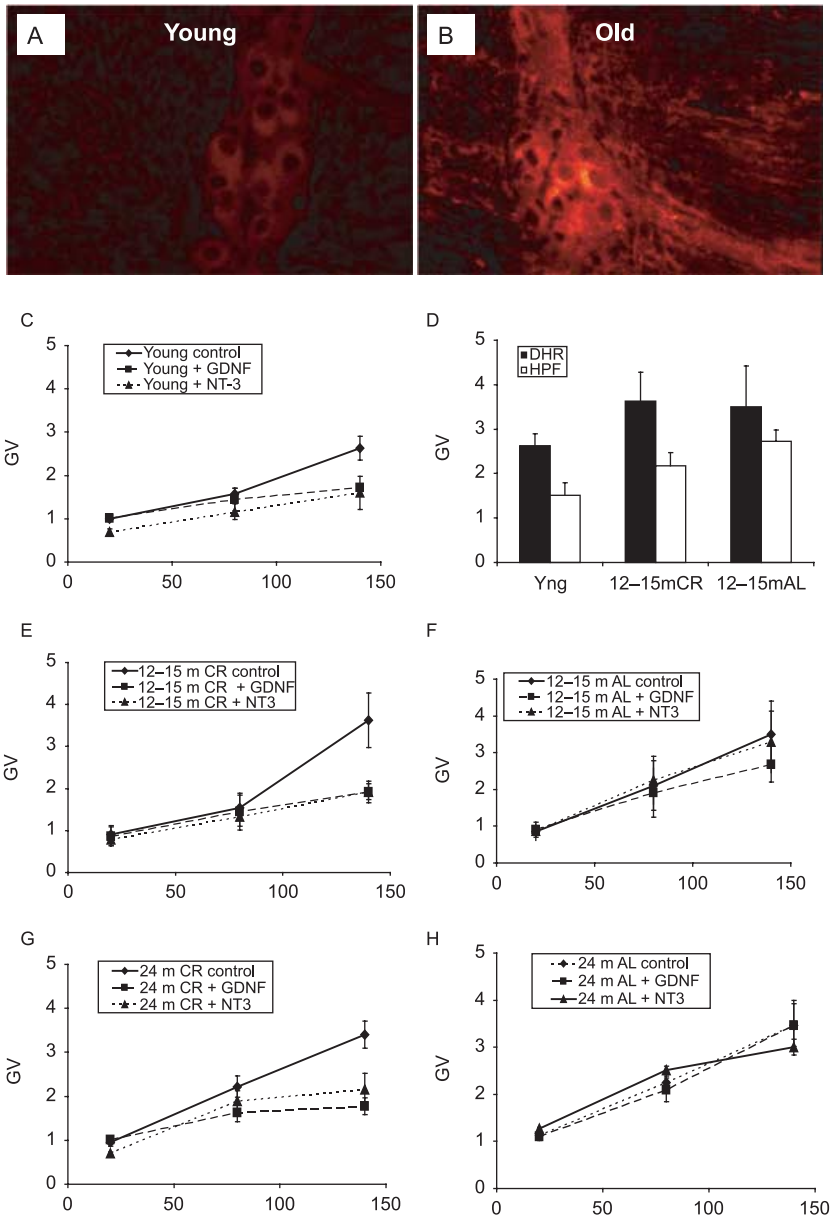

Fig. 3 ROS generation in myenteric neurons. Photomicrographs of 6-month (A) and 24mAL (B) whole mount preparations of myenteric neurons showing ROS-induced DHR fluorescence generated following 2 h of incubation. Note the substantial increase in brightness of cytoplasmic fluorescence in the 24mAL preparation. (C–H) Graphs showing extent and timescale of free radical generation (GV = mean grey value of DHR fluorescence intensity) in enteric neurons, with and without treatment with the neurotrophic factors, NT3 and GDNF, from 6 months (C; $n = 10$), 12–15mCR (E; $n = 4$) and AL (F; $n = 4$) and 24mCR (G; $n = 4$) and AL (H; $n = 12$) rats over 2 h of incubation. (D) Graph showing fluorescence intensity (mean GV) in 6 months, 12–15mCR and 12–15mAL preparations following 2 h of incubation in DHR (as C) or in the OH-sensitive probe, HPF. Note the similar age-related increase in HPF fluorescence compared to that shown by DHR. Both groups, ($n = 3$).

15-month CR rats shows increased levels of free radical generation with age (Fig. 3C,E). Samples from 12- to 15-month AL rats, where neuron loss has begun (see Fig. 1), show a slightly sharper rate of increase of ROS levels assessed after 1 h of incubation compared to CR specimens of the same age (Fig. 3E,F), suggesting that CR may partially protect neurons against diet-induced elevation of free radical generation. The (nonsignificant) increases in ROS after 2 h of incubation are summarized in Fig. 3D.

Comparison of DHR fluorescence levels in preparations from 24-month animals indicates a significant effect of age on fluorescence levels compared to 6-month neurons after 1 h of incubation [Fig. 3; comparing C (6 months; 1.58 ± 0.169) and G (24mCR; 2.22 ± 0.066), and C (6 months) and H (24mAL; 2.24 ± 0.148); (both $P < 0.02$)], suggesting that increasing age correlates with higher levels of intrinsic neuronal ROS. There is no significant difference between the extent or rate of increase in free radical levels in 24mAL compared to 24mCR groups indicating that the neuroprotective effect of dietary restriction is exhausted by 24 months (the mean lifespan of Sprague Dawley rats).

DHR fluorescence generated by the oxidation of dihydrorhodamine to rhodamine 123 may be influenced by mitochondrial membrane potential (Hempel *et al*., 1999) as well as by other age-associated variables such as ferric iron and cytochrome *c*. In order to confirm the effects of age and diet on neuronal ROS levels, we assessed neuronal levels of fluorescence using the ROS-sensing dye, HPF (2-[6-(4′-hydroxy)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid) (Setsukinai *et al*., 2003), in myenteric neurons from young and old AL-fed animals (Fig. 3D). HPF is specifically activated by OH, derived principally from superoxide (Setsukinai *et al*., 2003; Ooe *et al*. 2005). HPF fluorescence, like

DHR, increases with incubation time, peaking after 2–3 h (data not shown). HPF demonstrates an age-related increase in neuronal ROS in the 12- to 15-month samples which is slightly, but not significantly, greater in the AL group. These differences are similar to those seen with DHR, confirming that DHR fluorescence provides a reliable measure of ROS levels in myenteric neurons. Because HPF fluorescence is specifically induced by superoxide-derived $OH^{\bullet}$ it is likely that mitochondrial ROS provide the majority of the free radicals assessed in our experiments.

Neurotrophic factors in antioxidant defence

Because of evidence that neurotrophic factors contribute to antioxidant defence in early postnatal neurons (Dugan *et al*., 1999), we tested the capacity of GDNF and NT3, with known survival-promoting roles in myenteric neurons (Chalazonitis *et al*., 2001; Busciglio *et al*., 2002), to reduce ROS levels in these neurons from adult rats. Treatment of *in vitro* preparations of enteric neurons from 6-month rats with 100 ng mL^{-1} of GDNF or NT3 results in significantly lower levels of neuronal ROS compared to untreated preparations after 2 h of incubation (Fig. 3C; GDNF: $P < 0.01$; NT3: $P < 0.05$). In contrast, treatment with NGF or IGF-1 [with known antioxidant effects in other groups of neurons (Dugan *et al*., 1999) and cell types (Jallali *et al*., 2005)], has no effect on ROS levels (data not shown).

Comparisons of the effects of neurotrophic factors on ROS levels in 12- to 15-month animals (Fig. 3E,F) show that in CR preparations, ROS are significantly reduced at the 2-h time point after treatment with NT3 and GDNF (both, $P < 0.05$). In contrast, neither factor has a significant effect on ROS levels in preparations from 12- to 15-month AL rats. Thus CR appears to enhance neurotrophin-mediated antioxidant mechanisms around the age at which cell loss occurs *in vivo*. In the 24mCR group, treatment with neurotrophic factors suppresses ROS levels significantly (Fig. 3G,H; GDNF: $P < 0.001$; NT3: $P < 0.05$) but not in the AL preparations, resembling the effects seen at 12–15 months. It is worth noting that these differential effects, like the onset of neuron cell death, are already established at the relatively young age of 12–15 months (i.e. well before the onset of senescence). Our results therefore show that age enhances levels of ROS generation, while age and diet, respectively, inhibit and enhance neurotrophic factor-mediated antioxidant defence mechanisms, in adult and aged neurons.

ROS-induced neuronal cell death

In order to discover whether ROS contribute to age- and diet-induced neuronal cell death, we examined the effect of treatment with menadione (a drug which accelerates mitochondrial ROS generation by redox cycling) on the incidence of apoptotic cell death in myenteric neurons. We chose to focus on the period up to and including the early stages of age-related neuron loss in the myenteric plexus (i.e. 12–15 months of age). The increase in the TUNEL response to menadione was time-dependent, reaching a maximum after 4 h of incubation (data not shown). Menadione treatment for 4 h *in vitro* produced a visually obvious increase in TUNEL-positive neurons in preparations from 12- to 15-month AL animals compared to those from 6-month animals (Fig. 4A,B). In addition, other cells including smooth muscle, endothelial and glial cells can be seen to exhibit significant vulnerability to ROS-induced apoptosis. Counts of TUNEL-positive neurons and visual examination of nuclear morphology reveal that after 4 h of incubation in culture medium without menadione, sparse and unaltered numbers of apoptotic profiles were present in all experimental groups (Fig. 4C). Treatment with menadione induces a small but nonsignificant increase in apoptotic profiles in 6 months preparations compared to untreated controls (Fig. 4C). In contrast, the proportion of apoptotic neurons after menadione treatment increases 10-fold ($P < 0.001$) in the 12–15mAL group; a similar increase in apoptotic neurons after menadione was seen in samples from 24mAL animals (data not shown). Interestingly, CR protects 12- to 15-month neurons from menadione-induced cell death, reducing the proportion of apoptotic neurons significantly compared to the AL group (Fig. 4C; $P < 0.001$). Menadione-induced apoptosis is apparently higher in the 12–15mCR group compared to 6-month samples, but the difference is not statistically significant. Examination of nuclear morphology revealed an increase of fragmented and crenellated nuclei in both of the 12- to 15-month groups, suggesting strongly that at least a proportion of these neurons

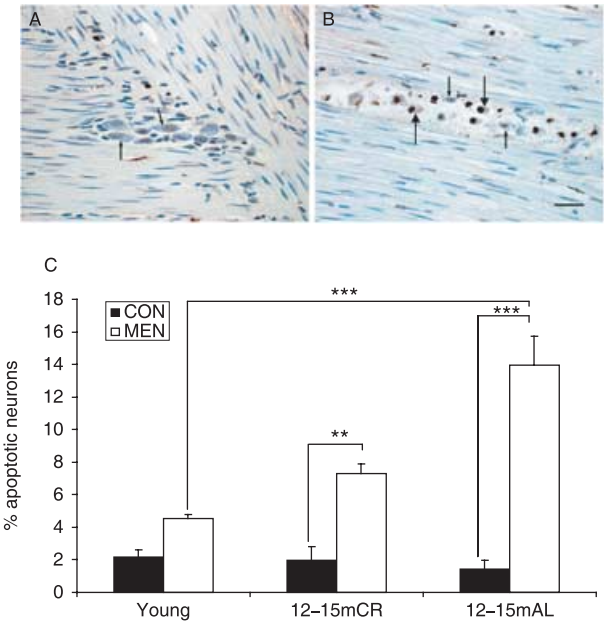

Fig. 4 ROS-induced apoptotic neuron cell death: TUNEL-stained cryosections of 6-month (A) and 12–15mAL (B) myenteric neurons following 4 h incubation with menadione. Toluidine blue stained neurons (small arrows, A and B) are healthy; neurons showing brown TUNEL-positive nuclei (large arrows, B only), some of which are fragmented or with crenellated outlines, are undergoing apoptosis. The histogram (C) shows the proportion of TUNEL-positive neurons from the different experimental groups following treatment with menadione. Note the neuroprotective effect of CR after treatment with menadione at 12–15 months. Six months groups ($n = 10$); 12–15mCR groups ($n = 6$); 12–15mAL con ($n = 4$); 12–15mAL + men ($n = 8$).

were dying apoptotically. Thus, age and *ad libitum* feeding enhance vulnerability to ROS-induced death of myenteric neurons.

Next we wanted to find out whether menadione-induced death of neurons could be ameliorated by *in vitro* treatment with antioxidants and by neurotrophic factors. Preparations from 6-month and from 12- to 15-month AL and CR animals were treated with menadione as before, following pretreatment with the synthetic antioxidant carboxyfullerene also known as C3 (Dugan *et al*., 1997) or with the neurotrophic factors GDNF or NT3 (Fig. 5A–C). Fullerene compounds react avidly with free radicals and are regarded as 'radical sponges'. The trimalonic acid derivative of fullerene, C3, is a synthetic water-soluble compound that has been found to be an effective antioxidant both *in vivo* and *in vitro* (Tsao *et al*., 2002). Preliminary experiments using a 'Live-Dead' assay (Molecular Probes Inc., Eugene, OR, USA) demonstrated improved imaging and sampling compared to the TUNEL method and this was therefore used for these experiments. The effects of menadione on cell death are qualitatively similar using this assay compared with TUNEL staining in the young and 12–15mAL groups. However, menadione induces a greater extent of cell death as measured by this method compared with TUNEL staining in the 12–15mCR group. We speculate that this is because the Live-Dead assay demonstrates ethidium staining in compromised cells not yet committed to apoptosis, i.e. still capable of being rescued (see below).

C3 reduces the extent of cell death very markedly in all groups (Fig. 5). Interestingly, the rescue effect of C3 is greatest in the 12–15mCR group (96% and $P < 0.001$, compared to 80% and $P < 0.01$ for 12mAL). GDNF pretreatment produced no significant effect on cell death in young or in 12–15mAL preparations. However, GDNF exerted significant protection (45%; $P < 0.01$) in the 12–15mCR group. Pretreatment with NT3 gives small but nonsignificant rescue from menadione-induced cell death in all groups, but with no difference between 12 and 15mAL and CR animals. Because of the greater rescue effects of C3 and GDNF in the 12–15mCR group, we speculate that the Live-Dead assay demonstrates ethidium staining in compromised cells not yet committed to apoptosis, i.e. still capable of being rescued. The presence of small numbers of cells staining for both calcein and ethidium supports this hypothesis. Our results suggest that at the stage of life when neurons become vulnerable to age-related cell death, caloric restriction enhances the protective effect of neurotrophic factors against ROS-induced cell death. The lack of protective effect of GDNF at the young stage may indicate that the young enteric neurons already obtain a maximal supply of the endogenous growth factor, or have other ways of inducing maximal antioxidant defence. Alternatively it is possible that the antioxidant defensive role of GDNF increases during adult life. Ongoing studies are examining these alternatives.

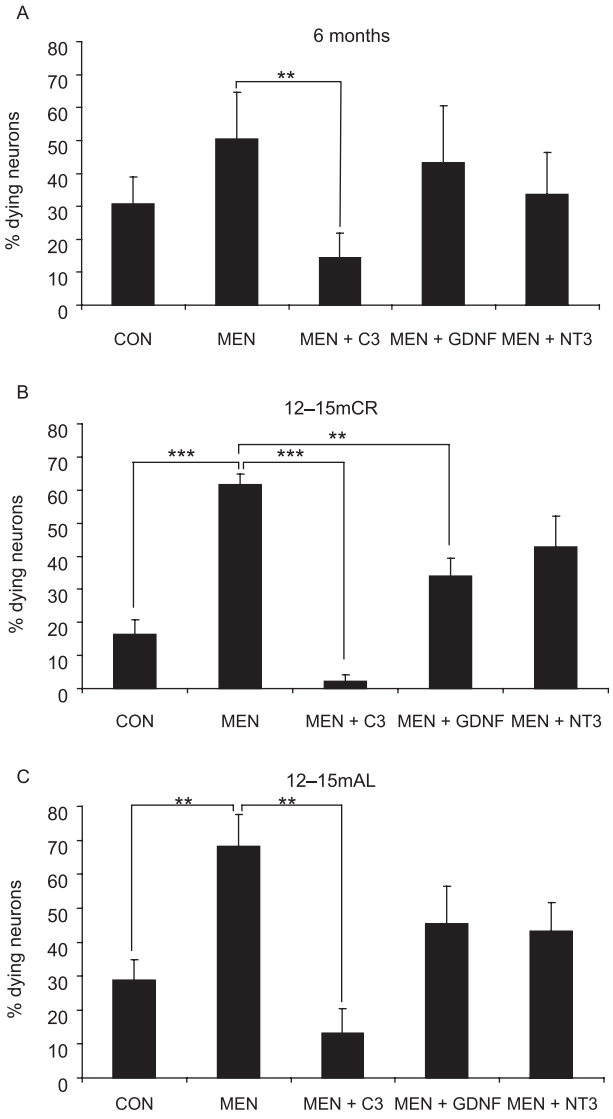

Fig. 5 Rescue by antioxidants and neurotrophic factors of ROS-induced cell death. Bar charts showing effects of menadione, with and without pretreatment with antioxidants (C3) and neurotrophic factors (GDNF, NT3), on death of myenteric neurons from 6-month (A), 12–15mCR (B) and 12–15mAL (C) animals. Note that C3 reduces cell death at all ages very substantially, confirming that menadione induces cell death by raising intracellular ROS. NT3 has no significant effect on ROS levels in any of the experimental groups. In contrast, GDNF produces a significant (50%, $P < 0.01$) reduction of menadione induced cell death in 12–15mCR preparations, but not in age-matched AL preparations. All groups, ($n = 4$), except 6 months MEN + GDNF, and 12–15mCR MEN + C3, ($n = 3$).

Discussion

In this study, the mechanisms underlying age-related loss and cell death of rat myenteric neurons have been examined. At the outset, we wished to establish more accurately the timescale of neuron loss in order that examination of vulnerability to cell death could be studied before cell death had occurred, and also to consider the relation of age-related neuron cell loss to other aspects of the aging process. Previously, neuron loss has been shown to occur around 16 months of age (Cowen *et al*., 2000); here we demonstrate that it develops rapidly between 12 and 13 months and seems to be complete by 17 months in AL-fed rats as demonstrated by the HuC/D results. Although rats and

mice remain relatively healthy at 12 months of age, it is thought that these rodents rarely live longer than 12 months in the wild (Phelan & Austad, 1989). Selective pressures on neuroprotective mechanisms are therefore likely to be reduced or nonexistent by this age, providing a possible explanation for this apparently early and substantial neuron loss. Through the use of alternative markers (PGP 9.5, HuC/D) and staining methods (immunofluorescence, toluidine blue staining of resin-embedded sections) we confirm that PGP 9.5 labelling provides an accurate indication of age-related neuron loss. Resin-embedded sections exhibited substantial 'holes' surrounded by enteric glial cells in ganglia from AL-fed, aged rats. This arrangement resembles the nodules of Nageotte seen in dorsal root ganglia following pathological neuron loss (Thomas *et al*., 1997).

Significant neuron loss is only seen in AL-fed animals at the 12- to 15-month age stage, confirming previous observations that diet is a key coregulator of age-related neuron cell death; however, a smaller but significant ($P < 0.05$) loss of neurons is seen in CR animals at 24 months indicating the dietary neuroprotection is not complete. CR is known to increase the expression of neuroprotective genes including those encoding protein chaperones, neurotrophic factors (Prolla & Mattson, 2001), mitochondrial gene expression (Nicoletti *et al*., 2005) and repressors of apoptosis in the aging rat brain (Shelke & Leeuwenburgh, 2003). Our data confirm the protective effect of dietary restriction against age-related neuron cell death. Furthermore, the observation that cellular vulnerability to aging occurs relatively early in life supports predictions based on the evolutionary theory of aging (Kirkwood & Austad, 2000) that dietary restriction will enhance investment in cellular maintenance and repair during adult, reproductive life.

We examined the possibility that myenteric neurons expressing the calcium-binding proteins, calretinin and calbindin (Abalo *et al*., 2005), might be particularly vulnerable to age-related neurodegeneration as a result of calcium dyshomeostasis (Bu *et al*., 2003), which may be linked to altered mitochondrial ROS generation in old age (Mattson & Liu, 2002). Calretinin-positive neurons form a far larger (25%) proportion of myenteric neurons compared to calbindin (2.5%) but exhibited no age-related loss. Calbindin-positive neurons, in contrast, exhibited a substantial 50% loss which was fully rescued by CR. This is an interesting addition to the literature on selective vulnerability of subpopulations of neurons in the enteric nervous system (Wade & Cowen, 2004), but does not further our understanding of the contribution of calcium dyshomeostasis in this process, nor does it tell us more about the phenotype of the large majority of myenteric neurons which are vulnerable during aging.

Evidence is growing of an association between ROS and age-related neurodegenerative disease (for review, see (Butterfield & Lauderback, 2002; Poon *et al*., 2004). However, there are few direct demonstrations of increased ROS levels in the non-diseased aging nervous system, or of the protective effects of CR against a possible age-related increase in ROS (Poon *et al*., 2004). Indeed, in *Drosophila* a lack of correlation between mitochondrial ROS and lifespan was recently demonstrated (Miwa *et al*., 2004). Our data show a significant increase in ROS levels in 12–15 months in both AL and CR groups compared to 6-month myenteric neurons *in vitro* which is maintained in 24-month neurons. ROS levels were not markedly influenced by diet although the rate of increase in ROS levels at 1 h of incubation (following 20 min of loading with DHR, see Experimental procedures) was reduced in 12–15mCR neurons compared to the parallel AL group. ROS levels showed similar trends in both 24-month groups indicating that even after 50% of neurons had died in the AL group, the remainder continue to exhibit elevated ROS levels compared to young neurons. This suggests that while some neurons are more vulnerable than others to age-related neurodegeneration, all are exposed to the damaging effects of elevated ROS.

The free radical-sensing dyes have advantages and disadvantages (for review see Tarpey *et al*., 2004). An important advantage of DHR for the present study is its capacity to provide a clear, neuron-specific signal which allows us to separate the confounding effects of changes in ROS in smooth muscle, enteric glial and vascular endothelial cells, all of which contribute to the ROS signal. These considerations rule out the use of more widely accepted biochemical assays for ROS in our preparations. Our laboratory has recently published further methodology for the use of DHR in cell-specific ROS assays, including effects of age and growth factor treatments, which support the view that with appropriate controls this method can provide reliable information on superoxide generation (Jallali *et al*., 2005). Here, the possibility that the DHR signal is affected by alterations in ROS from sources other than superoxide (e.g. reactive nitrogen species) or by alterations in mitochondrial membrane potential (Hempel *et al*., 1999) was examined using the recently developed dye, HPF (Setsukinai *et al*., 2003; Ooe *et al*., 2005), which reacts specifically with hydroxyl radicals. We confirmed the principal DHR results (i.e. the effects of age and diet on ROS) by showing a significant age-related increase of over 50% in ROS levels in 12- to 15-month neurons which is partially reduced by CR. Because HPF responds primarily to $OH^{\bullet}$, derived principally from superoxide, this experiment indicates that DHR in this model is probably measuring a ROS signal predominantly originating in mitochondrial superoxide.

Neurotrophic factors have been recently shown to exert antioxidant effects in developing sympathetic neurons (Dugan *et al*., 1999); however, their role in the adult and aging nervous system has not been investigated. We demonstrate that two of the principal endogenously expressed neurotrophic factors in the gut, GDNF and NT3, reduce ROS levels significantly in 6 months myenteric neurons showing that neurotrophic factors have antioxidant effects in adult myenteric neurons. This neuroprotective role was abolished in older (12–15 months and 24 months) neurons by AL feeding but retained with CR, showing that diet influences the capacity of neurotrophic factors to affect antioxidant defence in the adult nervous system. Interestingly, the effects of both GDNF and NT3 are maximal in young and CR-fed groups during the first hour of treatment and then plateau out between 1 and 2 h. This result suggests that neurotrophic factors

have a finite effect on ROS homeostasis, but does not indicate whether they influence ROS generation by direct effects on mitochondrial function or through the up-regulation of antioxidants. Further experiments are required to examine this question.

ROS are increasingly understood to be associated with neurodegeneration, including neuron cell death, in a number of pathological conditions (see Introduction). However, while age is generally the strongest predictor of neurodegenerative disease, there is little evidence that nonpathological aging is accompanied by ROS-induced neuronal cell death. Recent data (Cowen *et al*., 2000) demonstrated that rat myenteric neurons are exceptionally vulnerable to cell death with over 50% of cells dying in animals at a relatively early stage in the aging process (16 m). Because cell death under these circumstances, even in relatively short-lived animals, occurs over weeks and months it is not possible to observe cell death directly *in vivo* or in *ex vivo* preparations. Stimulating the mitochondrial electron transport chain using the redox-cycling drug, menadione, causes oxidative stress and, in some cells, apoptotic cell death (Laux & Nel, 2001; Gerasimenko *et al*., 2002). We therefore exposed myenteric neurons to menadione *in vitro* in order to examine their vulnerability to ROS-induced apoptotic cell death. TUNEL-positive neurons increased significantly following menadione treatment in 12- to 15-month and 24-month neurons from AL-fed animals in comparison with neurons from 6-month animals. The proportional increases in apoptotic neurons were 21% and 43%, respectively, while in contrast only 3% of 6-month neurons responded in the same way. Interestingly, CR very largely protected neurons against ROS-induced apoptotic cell death since the difference between the proportions of apoptotic neurons in 12mCR and 6-month was not statistically significant. In addition to the characteristic brown nuclear staining characteristic of the TUNEL reaction, many dying neurons exhibited fragmented and pyknotic nuclei, indicating that they were indeed undergoing apoptotic cell death. In addition, other cells including smooth muscle, endothelial and glial cells exhibited widespread vulnerability to ROS-induced apoptosis.

Stimulation of ROS formation from the mitochondrial electron transport chain therefore provokes apoptotic cell death in older adult and aged neurons. Furthermore, like the ROS levels seen *in vitro* in these neurons, ROS-induced apoptotic neuron cell death is sensitive to diet, being largely prevented by CR at 12–15 months (Fig. 4 and above) as well as at 24 months (data not shown). This is the first demonstration that, during nonpathological aging, neurons become vulnerable to ROS-induced apoptosis and that CR plays a key role in protecting neurons against this form of stress-induced cell death.

Adult neurons become relatively independent of neurotrophic factors for their survival *in vitro*, as has been demonstrated by studies of adult sensory (Vogelbaum *et al*., 1998) and sympathetic (Easton *et al*., 1997; Orike *et al*., 2001b) neurons. NGF-independent survival of adult sympathetic neurons appears to be the result of an intrinsic up-regulation of PI3-kinase signalling (Orike *et al*., 2001a), which may be set by early life exposure to varying levels of neurotrophic factors (Gatzinsky *et al*., 2004) (T. Cowen & M. Campioni-Noack, unpublished observations). However, this observation does not rule out the possibility that ongoing neurotrophin signalling influences in an unknown way the responses of adult and aging neurons to oxidative stress, hence protecting them against ROS-induced cell death. We therefore attempted to discover whether menadione-induced death of neurons could be ameliorated by *in vitro* treatment with antioxidants and by neurotrophic factors. The synthetic antioxidant carboxyfullerene, C3, reduced the extent of cell death very significantly in young and 12- to 15-month groups, with greatest effect in the 12–15mCR group, indicating that menadione-induced cell death was indeed the result of elevated ROS. Pretreatment with GDNF resulted in significant protection from menadione-induced cell death in the 12–15mCR group only, confirming our hypothesis from the ROS experiments that CR enhances antioxidant defence by neurotrophic factors. In contrast, NT3 effected a small but nonsignificant rescue of 12–15mCR neurons, despite the fact that it significantly depressed ROS levels in similar samples. The lack of protective effect of GDNF on menadione-induced cell death in young neurons, combined with the significant effect of C3, may be explained by the presence of optimal levels of intrinsic GDNF and saturated neuronal responses in the young myenteric plexus. Further studies of the relation between specific neurotrophic factors, antioxidant enzyme activity and cell death pathways are underway in attempts to resolve these apparent anomalies.

In summary, our results confirm the selective loss of rat myenteric neurons during aging and suggest a plausible mechanism whereby age-related neuron cell death is induced by elevated intraneuronal levels of ROS, which are accentuated by *ad libitum* feeding. In contrast, dietary restriction protects against ROS-induced neuronal cell death through mechanisms which include enhancement of a novel, antioxidant defensive role of neurotrophic factors.

Experimental procedures

Animals

All experiments were carried out on male Sprague Dawley rats from a colony maintained at Royal Free and University College Medical School (RFUCMS). All animals were reared up to 6 months of age with *ad libitum* (AL) access to BEEKAY Rat and Mouse standard 1 diet (B&K Universal Ltd, Hull, UK), composed of 19% crude protein, 5% oil, 4% fibre and 72% dry matter. The caloric content is 16.16 mJ/kg and the rats consumed an average of 50–60 g per day (i.e. 0.80–0.96 mJ per day). From 6 months of age, some animals were transferred to a calorie-restricted diet (CR) of 25 g per day (0.40 mJ), whereas others remained on the AL dietary regime. Numbers of samples used in each experiment are included in the figure legends.

Animals between the ages of 6 and 24 months were killed by carbon dioxide inhalation. Caloric restriction and all other conditions were undertaken in compliance with UK Home Office legislation under the Animals (Scientific Procedures) Act 1986.

Tissue preparation

Immediately after respiration and heart beat ceased, the abdominal cavity was opened and the small intestine removed *in toto*.

Dimensions of small intestine

After the animals were killed, gut contents were flushed out with Hanks balanced salt solution (HBSS; pH 7.4). The gut was then inflated with HBSS to a pressure at which the gut began to curl (Johnson *et al*., 1998) and placed on a sheet of transparent plastic. Marks showing length and diameter were made at several points along the gut and transferred to graph paper allowing the total area of gut wall to be calculated.

Numbers of neurons

Ileum was removed from AL and CR rats at 6, 12, 13, 15 and 24 months of age, inflated with HBSS, fixed in 4% paraformaldehyde for 2 h, washed in PBS, and treated overnight with PBS + 20% sucrose. Strip preparations of the muscularis externa containing the myenteric plexus of neurons were dissected off, opened and sections taken from 10 to 20 cm of distal ileum. Samples were processed for immunocytochemistry using PGP 9.5 (UltraClone, Isle of Wight, UK), HuC/D (Molecular Probes) and calretinin (Swant Antibodies, Bellinzona, Switzerland) polyclonal antibodies and a monoclonal antibody against calbindin (Swant), with appropriate Alexafluor (Molecular Probes) secondary antibodies. PGP 9.5 and HuC/D are established as pan-neuronal markers for rodent myenteric neurons (Johnson *et al*., 1998; Phillips *et al*., 2004). Neurons were counted using a fluorescence microscope, expressed as numbers of neurons per mm^2 and corrected for any age- or diet-related changes in size of the gut wall. All procedures have been described previously in detail (Johnson *et al*., 1998). To confirm that loss of PGP-immunoreactive neuron numbers coincided with neuron loss, some specimens were processed for resin embedding. One-micrometre sections were cut on an ultramicrotome, stained for toluidine blue and examined by light microscopy using a ×40 objective.

ROS levels in neurons

An *in vitro* assay of ROS levels in myenteric neurons has been developed in our laboratory using the fluorescent probe, dihydrorhodamine (DHR; Molecular Probes) (Soubeyre *et al*., 2001; Jallali *et al*., 2005). Comparisons of DHR with another ROS-sensitive probe, dichlorofluorescein diacetate (DCF-DA) (Hempel *et al*., 1999), demonstrated that the latter was not retained adequately in cells (Jallali *et al*., 2005). It should be borne in mind that these preparations contain many different cell types (muscle, connective tissue cells, endothelium) as well as neurons, hence more conventional biochemical assays for ROS are inappropriate.

Strip preparations of the myenteric plexus were prepared as before and transferred to F14 culture medium in 12-well culture plates maintained at 37 °C in a CO_2 incubator. Samples were treated with 10 μM DHR for 20 min then maintained under the same conditions for a further 1–4 h. Graphs show the timescale starting from the point of incubation of the tissues in DHR (Fig. 3C–H). The first measurement was taken at 20 min when DHR loading was complete, termed time zero. Eighty- and 140-min time points indicate 1 and 2 h of incubation post-probe loading, respectively. Preliminary experiments showed that responses peaked at 2 h (data not shown). To ensure that the changes in fluorescence signal obtained using DHR were not confounded by alterations in mitochondrial membrane potential, ferric iron or other age-associated variables (Hempel *et al*., 1999), comparisons were made between 6-month and 24-month AL preparations using the novel ROS-sensing dye, HPF (10 μM; Alexis Biochemicals, Nottingham, UK) (Setsukinai *et al*., 2003), in samples pre-incubated for 20–30 min (as for DHR) then maintained for up to 3 h in F14 medium containing HPF following preliminary experiments showing that fluorescence was maximal at 2–3 h (data not shown).

Free radical regulation

In order to examine the role of neurotrophic factors in reducing free radical levels in enteric neurons from animals of different ages and subjected to different dietary regimes, duplicate samples were pretreated for 2 h with those neurotrophic factors with established roles in the developing and adult ENS (Chalazonitis *et al*., 2001; Busciglio *et al*., 2002), namely NT3, GDNF and IGF-1 at 100 ng mL^{-1}. NGF was used at the same concentration as a control since it has no known role in the adult ENS. ROS-sensing dyes were added for the last 20–30 min of the pre-incubation.

Imaging and image analysis

At 0, 1 and 2 h after pre-incubations, samples were removed, mounted in antifade mountant (Citifluor, UK) and imaged using confocal microscopy. Single 2-μm optical slices were obtained at a constant depth within the myenteric ganglia under strictly standardized conditions. Laser current was monitored and neutral density, aperture, black level, Kalman filtering and all other settings were maintained constant. A minimum of six images of non-overlapping ganglia containing a total of at least 50 neurons from each sample were stored and subjected to image analysis using a Zeiss Kontron image analysis system (KS400 V2/3, Carl Zeiss Ltd, Welwyn Garden City, UK). Nucleated profiles of neurons were selected and the neuronal perimeter traced interactively and filled to generate a mask. The masks of all traced neurons within a ganglion were then used to measure mean grey value (intensity) (GV/μm^2) and integrated grey value per cell (GV/cell), as well as cell size.

Apoptotic neuronal cell death

Preparations similar to those used previously were treated with the redox-cycling drug, menadione (50 μM) (Iyanagi, 1990), for 4 h. After treatment, samples were fixed in 4% paraformaldehyde for 2 h, washed in PBS, followed by overnight treatment with 20% sucrose in PBS, then frozen in cryoprotectant and cryosectioned at 7 μm. TUNEL staining of the sections was carried out using a kit (NeuroTACS II, TA900, R&D Systems, UK)

to demonstrate apoptotic cell profiles. Numbers of apoptotic neurons were scored on at least three sections per sample using a light microscope and ×40 objective. Brown-stained neuronal nuclei, often exhibiting fragmentation, were scored as apoptotic, compared with blue-stained oval nuclei which were scored as normal. Visual analysis of neuronal nuclear morphology was carried out in parallel. Apoptotic neuron numbers were expressed as a percentage of normal neuron numbers.

To ensure that TUNEL staining provided an accurate indication of cell death, and because of the relatively limited sampling in cryosections of myenteric ganglia, similar preparations, but without fixation, were examined directly in the confocal microscope after treatment for 20 min using the Live-Dead assay following manufacturer's instructions (Molecular Probes). Neurons were distinguished from glial cells by clear differences of size and shape. Green, calcein-stained live neurons and red, ethidium-stained nuclei of dead neurons were counted in the same preparations and numbers of dead cells were expressed as a proportion of the total (live + dead).

Protection by neurotrophic factors against menadione-induced cell death

Because the Live-Dead assay gave better sampling and as a result more reliable quantification compared to TUNEL staining, it was used to study the protective effects of neurotrophic factors on menadione-induced cell death. Similar preparations to those used previously were exposed to GDNF and NT3 (100 ng mL^{-1}, as before) and then to menadione (50 μM, as before). After 4 h, cells were treated with the reagents of the Live-Dead assay, imaged and the proportion of dead cells counted, as previously described.

Data analysis

Mean, standard deviations and standard errors of mean were calculated using MS Excel. Statistical analysis of variance was carried out using two-way analysis of variance (ANOVA) followed by post-hoc LSD or Bonnferoni tests of significant differences between data pairs with $P < 0.05$ assigned as statistically significant (Unistat or Graphpad software).

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