Discrete responses of myenteric neurons to structural and functional damage by neurotoxins in vitro

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Lourenssen S, Miller KG, Blennerhassett MG. Discrete responses of myenteric neurons to structural and functional damage by neurotoxins in vitro. Am J Physiol Gastrointest Liver Physiol 297: G228–G239, 2009. First published April 30, 2009; doi:10.1152/ajpgi.90705.2008.—Damage to the enteric nervous system is implicated in human disease and animal models of inflammatory bowel disease, diabetes, and Parkinson’s disease, but the mechanism of death and the response of surviving neurons are poorly understood. We explored this in a coculture model of myenteric neurons, glia, and smooth muscle during exposure to the established or potential neurotoxins botulinum A, hydrogen peroxide, and acrylamide. Neuronal survival, axonal degeneration and regeneration, and neurotransmitter release were assessed during acute exposure (0–24 h) to neurotoxin and subsequent recovery (96–144 h). Unique and selective responses to each neurotoxin were found with acrylamide (0.5–2.0 mM) causing a 30% decrease in axon number without neuronal loss, whereas hydrogen peroxide (1–200 μM) caused a parallel loss in both axon and neuron number. Immunoblotting identified the loss of synaptic vesicle proteins that paralleled axon damage and was associated with marked suppression of depolarization-induced release of acetylcholine (ACh). The caspase inhibitor zVAD, but not DEVD, significantly prevented neuronal death, implying a largely caspase-3/7-independent mechanism of apoptotic death that was supported by staining for annexin V and cleaved caspase-3. In contrast, botulinum A (2 μg/ml) caused a 40% decrease in ACh release without effect on neuronal survival or axon structure. By 96 h after exposure to acrylamide or hydrogen peroxide, axon number was restored to or even surpassed the level of time-matched controls, regardless of partial neuronal loss, but ACh release remained markedly suppressed. Neural responses to toxic factors are initially unique but then converge upon robust axonal regeneration, whereas neurotransmitter release is both vulnerable to damage and slow to recover.

annexin V; botulinum toxin; acrylamide; hydrogen peroxide; axon regeneration; apoptosis

CHALLENGES TO THE ENTERIC NERVOUS SYSTEM (ENS) disrupt normal gastrointestinal function because neurons within the ENS regulate intestinal absorption, secretion, and motility. Studies of diverse pathologies such as inflammation, the effects of peripheral neurotoxins, and diabetes have all identified the ENS as a target for reversible or permanent damage, which emphasizes both its sensitive nature as well as its capacity for regeneration. For example, structural damage to axons has been observed in the inflamed human intestine in episodes of inflammatory bowel disease (IBD) (10, 25). In animal models, significant neuronal death occurs early in the chemically induced model of colitis in the rat and in infectious colitis in the mouse (4, 22). In trinitrobenzene sulfonic acid (TNBS)-induced colitis in the rat, this neurotoxic insult was then followed by the rapid regeneration of axons from the surviving neurons (18).

Extensive evidence in animal models and in IBD shows that functional changes to intestinal innervation, such as altered neurotransmitter release, are a prevalent and important aspect of intestinal inflammation (e.g., Refs. 7 and 25). In the rodent intestine, inflammation markedly affects the cholinergic neurons that comprise the major excitatory phenotype of the ENS (19), causing decreased release of acetylcholine (ACh) (8). This may be derived from changes in expression of the synaptic vesicle proteins that are necessary for excitation-secretion coupling and neurotransmitter release, such as the selective decrease of the synaptic vesicle protein neuronal calcium sensor 1 during TNBS-induced colitis (17). Overall, considerable evidence shows that the ENS is a sensitive target of intestinal inflammation and that the disturbed motility and related symptoms of disease may derive from these alterations.

Much of the process of ENS damage and recovery during inflammation or other neurotoxic challenge is unknown. Although there is evidence for extensive regeneration of axons within intestinal smooth muscle following inflammatory damage, the restoration of both neuronal structure and function is largely unexplored. These questions are difficult to study in vivo because complex interactions between resident intestinal and invading immune cells are present and because the role of individual components cannot easily be determined. To address this, we developed a model to study damage to the ENS in vitro using a coculture model of intestinal neurons, smooth muscle cells, and glia (3).

We employed this model to investigate the actions of established or potential neurotoxins on the ENS. Using either the neurotoxin botulinum neurotoxin A (BtxA), the peripheral nervous system toxin acrylamide, or the general oxidizing agent hydrogen peroxide (H2O2), we evaluated the consequences to both structural and functional aspects of myenteric innervation in vitro. The abilities of these agents to target myenteric neuron and axon number, synaptic vesicle protein expression, and neurotransmitter release were assessed both in the initial phase of acute damage as well as following repair and regeneration. The outcome establishes a model for the study of enteric neuron damage and repair and identifies phases of structural damage and recovery that are discrete from functional changes to neurotransmitter release.

MATERIALS AND METHODS

Cell culture. To obtain cultures of intestinal smooth muscle cells and neurons from the ENS, the small intestine of 4- to 11-day-old Sprague-Dawley rats (Charles River, Montreal, Quebec, Canada) was isolated and the mesentery completely removed. All experimental procedures were reviewed and approved by the Queen’s University...
Animal Care Committee. The mucosa with the attached muscularis mucosa was separated from the smooth muscle layers and discarded. The remaining longitudinal and circular smooth muscle layers and the enclosed myenteric plexus were dissociated using 0.25% trypsin II (Sigma, St. Louis, MO) in HEPES-buffered Hanks saline (pH 7.35) as described previously (3). Cell suspensions were plated onto glass-bottomed culture plates (13-mm diameter) previously coated with Matrigel (Collaborative Research, Belmont, CA). Medium (DMEM) containing 5% fetal calf serum and 2.5% rat serum was then added and replaced 48 h later at the time of toxin addition.

BtxA was added to the cocultures at a final dose of 0.2 or 2 μg/ml followed by incubation for 24 h. The ability of BtxA to cleave the synaptic vesicle protein S-nitroso-N-acetyl penicillamine (SNAP)-25 was measured using Western blot analysis. Alternatively, cocultures were processed for immunocytochemistry or ACh metabolism as described below. Acrylamide was added to the cocultures in doses ranging from 0.01 mM to 12 mM, followed by incubation for 24, 96, or 144 h. The cells were then processed for immunocytochemistry, Western blot analysis, or 3H-ACh release. H2O2 was added to the cocultures in doses ranging from 1 μM to 200 μM, followed by further incubation for 24 or 96 h. The cells were then processed similarly to the acrylamide-treated cultures.

Determination of cell viability. To assess viability, cultures were incubated with propidium iodide (10–7 M) and calcine-AM (1 μM; Invitrogen, Carlsbad, CA) for 30 min at 37°C, washed in PBS, and assessed by fluorescence microscopy. In some cases, these were further processed for immunocytochemistry using anti-Hu/C/D (HuD) antibodies as described below. The presence of apoptotic cell death was determined by immunocytochemistry for cleaved caspase-3 (Cell Signaling, Beverly, MA) or staining for annexin V according to manufacturer’s instructions (Miltenyi Biotec, Auburn, CA) in combination with propidium iodide used as a vital stain (10–7 M), with staining visualized by fluorescence microscopy. To determine the role of apoptotic cell death, some cultures were pretreated for 1 h with either DEVD-fmk (20 or 100 μM; Calbiochem, La Jolla, CA) or zVAD-fmk (50 μM; Calbiochem) before toxin addition.

Immunoblotting. Cells were lysed in buffer containing 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM EDTA pH 8.0, 2% Triton X-100, 0.5% sodium deoxycholate, 10 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride and were stored at −70°C. Aliquots (20 μg per lane) were resolved by 12% SDS-PAGE and transferred to PVDF membrane with the use of a semidy transfer apparatus (Bio-Rad, Hercules, CA). The membrane was blocked in 5% non-fat milk in Tris-buffered saline containing 0.2% Tween-20 and then incubated with antibodies to SNAP-25 (1:5,000; Sigma), syntaxin (1:1,000; Sigma), protein gene product 9.5 (PGP 9.5) (1:5,000; Ultraclone, Wellow, Isle of Wight, UK), or β-actin (1:5,000; Sigma). This was followed by incubation with appropriate horseradish peroxidase-linked secondary antibodies (1:20,000; Pierce, Rockford, IL) and visualization using a chemiluminescent substrate (Super Signal, Pierce).

Immunocytochemistry. To detect neurons and axons, cells were fixed using 4% neutral-buffered formalin and incubated with antibodies to SNAP-25 (1:2,000) and HuD (1:500; Molecular Probes, Eugene, OR), PGP (1:1,000; Ultraclone), βII tubulin (1:500; Chemicon, Temecula, CA), neuronal nicotinic acetylcholine synthesize (nNOS) (1:500; En- rodagnostica, Hornby, Ontario, Canada), or vesicular ACh trans- porter (VACHT) (1:500; Chemicon) diluted in antibody diluting buffer (DAKO, Carpinteria, CA). This was followed by incubation in appropriate secondary antibodies linked to Alexa 555 (1:20,000; Molecular Probes) or Alexa 488 (1:1,000; Molecular Probes) and visualized with a fluorescent microscope (Olympus BX51). Some cocultu- res were treated with antibodies to α-smooth muscle actin (1:500; DAKO), desmin (1:500; Chemicon), or glial fibrillary acidic protein (GFAP; 1:500; Sigma) followed by the appropriate secondary antibodies to confirm the presence of smooth muscle cells and glia, respectively. The nature of the smooth muscle cells was further verified by detection of positive staining for desmin (mAB3430; Chemicon). Nuclei were identified using Hoechst 33352 (10–7 M).

Determination of neuron and axon number. Neuron number was determined following immunocytochemistry for HuD by counting all of the positively labeled neurons in the field of view of a strip spanning the dish diameter, using a ×40 objective, and this was then repeated in the perpendicular axis. The area analyzed was 1.8 × 105 μm² per culture, which was 10.6% of the total area. For axon counts in the same culture dish, adjacent images of SNAP-25-labeled axons were acquired using a ×40 objective along a diameter of the culture dish and then repeated along the perpendicular diameter. A transpar- ency with three perpendicular black lines (transsects) placed equidis- tantly was then mounted onto the computer screen, and the number of axon intersections with a transect was determined for each image. This was summed across fields to give a number representative of the axon number per culture well.

ACh release. Determination of 3H-ACh release was carried out in 24-well tissue culture plates (Sarstedt, Nuremburg, Germany). At specified times, media was removed from the wells, and the cells were washed with Krebs buffered saline (in mM: 25 NaHCO3, 118 NaCl, 4.7 KCl, 1 NaH2PO4, 1.2 MgSO4, 11 glucose, 2.5 CaCl2) (bubbled with 95% O2-5% CO2). A sample (250 μL) of Krebs saline containing 3H-labeled choline (1,500 dilution of 1 μCi per ml; Amersham, Uppsala, Sweden) was added to the cocultures and incubated for 1 h at 37°C, followed by three washes in Krebs saline. Krebs saline containing 40 mM K+ was then added for 5 min (a cycle that was found to increase subsequent specific release in initial studies; data not shown). The cells were then washed with standard Krebs saline, and labeled choline in Krebs was again added for 1 h at 37°C. For each condition tested, half of the cultures were then treated with Krebs buffer containing 80 mM K+ for evaluation of stimulated release, with the other half used to determine the baseline or unstimulated release by treatment with control saline. In each case, the supernatants were taken for measurement of 3H-acetylcholine release by aqueous scintillation counting. Residual cellular radioactivity was determined by addition of 400 μl of 10% SDS per well and incubation for 15 min at room temperature. Cell solubilization was verified by microscopy, and the solution was placed into scintillation vials for analysis of residual 3H. Specificity of the method was tested by addition of hemicholinium (100 μM) before measurement of stimulated 3H-ACh release.

Reagents. All chemicals were from Sigma unless specified other- wise.

Statistics. Values are expressed as the means ± SE, where n = number of animals. Statistical significance was assumed when P ≤ 0.05 (t-test or ANOVA).

RESULTS

Characterization of enteric neurons in coculture. Cells dis- persed from the smooth muscle/myenteric plexus of postnatal rats by enzymatic dissociation attached rapidly to the culture surface and formed a confluent, uneven layer 1–2 cells thick by 2 days in vitro, with smooth muscle cells as the predominant cell type. Numerous myenteric neurons were present in these cocultures, identified by immunocytochemistry using antibodies to the panneuronal marker HuD (Fig. 1A). Enteric glia were identified by positive staining with anti-GFAP antibodies and were generally localized to the perineuronal regions (not shown).

To identify axons, cocultures were labeled with antibodies to the v-SNARE synaptic vesicle protein SNAP-25 (Fig. 1B), which showed the presence of a few axon extensions as early as 1 h after plating, with significant numbers detectable by 18 h in vitro and increasing thereafter. The number of branch points...
per 100-μm axon length was 2.1 ± 0.5 (n = 37) at day 3 in vitro and increased to 4.3 ± 0.5 (n = 50) by day 6, indicating that the increase in axon number involves significant branch formation, whereas the neuron number was constant over this period (Fig. 1F). This shows that neurons in enteric cocultures continue to display dynamic structural changes throughout the culture period.

To evaluate the formation of functional synapses in culture, we studied the synthesis and release of ACh. ACh is the principal excitatory neurotransmitter in the intestine, found in close to 50% of myenteric neurons in vivo (19) (unpublished observations) and similarly represented in these cocultures (Table 1). For this, we incubated cocultures with 3H-choline first using a preliminary cycle of K+–induced depolarization to
Following BtxA treatment, measurement of syntaxin (Fig. 2A) showed a concentration-dependent cleavage of the v-SNARE SNAP-25, without effect on the related v-SNARE proteins. Western blot analysis of cocultures exposed to BtxA for 24 h showed a concentration-dependent cleavage of the v-SNARE SNAP-25, but the possibility of its selective targeting by BtxA can occur without detectable consequences to myenteric neuronal structure.

Botulinum toxin affects neuronal function but not structure in vitro. BtxA causes long-lasting impairment of cholinergic nerve function in several neural systems through its action on the synaptic vesicle protein SNAP-25, but the possibility of its similar action on intestinal myenteric neurons is so far untested. Western blot analysis of cocultures exposed to BtxA for 24 h showed a concentration-dependent cleavage of the v-SNARE SNAP-25, without effect on the related v-SNARE syntaxin (Fig. 2A). Following BtxA treatment, measurement of K⁺-stimulated [³H]-ACh release showed a sharp reduction to less than 60% of control (Fig. 2A). However, there were no significant differences in neuron number, axon number, or the density of varicosities with BtxA treatment compared with control (Fig. 2B). We conclude that SNAP-25 may be essential for cholinergic neurotransmission in myenteric neurons, but its selective targeting by BtxA can occur without detectable consequences to myenteric neuronal structure.

Acrylamide damages enteric neuron structure. Exposure to acrylamide by routes such as respiration results in autonomic neuropathy with axon degeneration in peripheral and central neurons, but little is known about its consequences to enteric neurons (2). Our initial determination showed that concentrations of acrylamide up to 0.5 mM had no effect on either neuron or axon number when examined 24 h later. However, higher levels of acrylamide (0.5–2.0 mM) were associated with a 30% reduction in SNAP-25-positive axon profiles (Fig. 3), which was completely uniform over these concentrations without change in neuron number (Fig. 3D; shaded region).

Cocultures treated with 0.5–2.0 mM acrylamide showed no cytotoxicity in any cell type compared with untreated control cultures, as assessed by retained fluorescence of calcein and exclusion of propidium iodide (not shown), suggesting a selective effect on axonal structure. Glial cell number remained unchanged relative to control at this dose of acrylamide (119 ± 7% of untreated control; n = 6; P > 0.05). However, an increased concentration of acrylamide (4 mM) caused the significant loss of HuD-positive neurons (58 ± 7% of control, n = 4–10; P < 0.05), and axon number was decreased to only 21 ± 5% of control (P < 0.05; Fig. 3, D and H). In some cases, neurons lost expression of SNAP-25 while maintaining expression of HuD (e.g., Fig. 3, A and B), suggesting that degradation of axonal structure preceded the loss of cell body integrity. Neuronal death was not selective among neuronal phenotypes because the proportion of nNOS and VAChT neurons remained constant (Table 1). Glial cell numbers remained similar to untreated controls in these cultures (102 ± 3%; P > 0.05). Adduct formation by acrylamide affects many proteins, including SNAP-25 among other synaptic vesicle proteins (15). To verify the reduction in axon number in the presence of acrylamide, cocultures were also labeled with antibodies to the major neurotransmitter phenotypes in cultured enteric neurons is not altered by 24 h after 4 mM acrylamide or 100 μM H₂O₂.

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<th>% nNOS</th>
<th>% VAChT</th>
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<td>34 ± 3</td>
<td>55 ± 5</td>
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<td>H₂O₂</td>
<td>42 ± 5</td>
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<td>32 ± 3</td>
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Data are expressed as percentage of neuronal nitric oxide synthase (nNOS) or percentage of vesicular acetylcholine transporter (VAChT)-expressing neurons as a proportion of the total HuCD-positive neurons (n = 4 animals per condition, P > 0.05 relative to control for all data), and values are means ± SE.

...maximize the releasable pool of radiolabeled ACh and then evaluating the outcome of a second K⁺-induced depolarization. This stimulation caused a significant release of radioactivity compared with the unstimulated time-matched control cocultures. The stimulated release of label was completely blocked by prior incubation with hemicholinium-3 (100 μM), a specific inhibitor of the neuronal high-affinity choline uptake mechanism (Fig. 1G), and was interpreted to represent the release of [³H]-ACh. These methods allowed reproducible quantification of neuronal structure and function, which were the foundations for the study of the actions of the peripheral neurotoxins BtxA and acrylamide on myenteric neurons.

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panneuronal marker PGP 9.5 as well as βIII-tubulin. In both cases, results showed a decrease in the number of axon profiles in response to acrylamide that was identical to that derived from SNAP-25 immunocytochemistry. With confirmation that SNAP-25 was an effective marker of axonal structure, this approach was used in all further experiments.

Figure 3E shows an example of the outcome of semiquantitative Western blot analysis used to examine the expression of synaptic vesicle proteins in control and acrylamide-treated cocultures. Although this showed no change in SNAP-25 protein with concentrations of acrylamide ≤0.5 mM, image analysis showed that there was a progressive decrease with exposure to 1 mM acrylamide and higher (P < 0.05; Fig. 3F). A parallel examination of syntaxin expression showed identical changes, with expression reduced to 65 ± 10% of control after treatment with 2 mM acrylamide (similar to SNAP-25; P > 0.05).

The reduction of expression of synaptic vesicle proteins by acrylamide at low concentrations where neuron number remained unchanged suggested that neuronal function might be impaired despite the absence of change to structure. This was confirmed by analysis of ACh release in cohort cocultures, where there was a sharp decrease in the stimulated release of $^3$H-ACh with acrylamide concentrations of 0.5 mM and greater (Fig. 3F). For example, $^3$H-ACh release was reduced to 64 ± 6% of control after treatment with 0.5 mM acrylamide, a concentration without effect on neuron number. The uptake of $^3$H-choline into existing neurons was not significantly altered by acrylamide treatment over this range (data not shown), further suggesting an effect at the level of synaptic vesicle

Fig. 3. The peripheral neurotoxin acrylamide affects both structure and function of myenteric neurons in vitro. Axon numbers were reduced at lower concentrations of acrylamide without affecting survival, whereas higher concentrations reduced both axon number and neuron survival. A–C: dual-label immunofluorescence micrographs of a coculture treated with 4 mM acrylamide 24 h previously showing neuronal damage with axonal segmentation. A: anti-HuD labeling of neuronal cell bodies (green). B: same field with labeling for SNAP-25 (red). Arrow represents a HuD-positive neuron that expresses little or no SNAP-25. Discontinuous axon segments (e.g., arrowheads) suggested fragmentation and degeneration. Insert: control time-matched coculture labeled for SNAP-25. C: composite image of HuD and SNAP-25 expression. Scale bar = 50 μm. D: comparison of neuron number and axon number at 24 h after treatment with acrylamide. The average number of axons was significantly decreased after incubation with 0.5–2.0 mM acrylamide but without significant neuronal loss (shaded bars). Both neuronal loss and severe axon damage occurred with 4 mM acrylamide. E: representative Western blot showing decreased expression of the synaptic vesicle protein SNAP-25 at 24 h after treatment with acrylamide. β-actin was used as a loading control to enable comparison. F: structural and functional outcomes of acrylamide treatment on myenteric neurons. Bars represent Western blot data showing that acrylamide caused the concentration-dependent decrease in SNAP-25 expression by 24 h after treatment (≥1 mM; P < 0.05). Line plot represents average K$^+$-stimulated release of $^3$H-ACh showing that acrylamide-induced decrease occurred in parallel with a decrease in SNAP-25 expression (≥0.5 mM; P < 0.05). IOD, integrated optical density.
synthesis and function. Therefore, impairment of neural function was the most sensitive sign of acrylamide exposure of myenteric neurons in vitro and correlated well with a concomitant decrease in expression of synaptic vesicle proteins.

**Apoptosis in acrylamide-induced neuronal death.** Analysis of cocultures early after addition of 4 mM acrylamide showed that no significant decrease in neuron number occurred before 8 h posttreatment. However, close examination showed the appearance of condensed HuD-positive (cHuD) neuronal cell bodies (e.g., Fig. 4A) by 4 h that suggested neuron degeneration and accounted for 4 ± 2% (n = 4–8) of the total neuron number (Fig. 4). In contrast, cHuD-positive neurons were not observed in untreated cultures.

To determine whether neuronal loss involved an apoptotic mechanism, cultures were pretreated with the general caspase inhibitor zVAD-fmk or the caspase-3/7 inhibitor DEVD-fmk, followed by toxin addition, and neuron counts were determined 24 h later. Pretreatment with zVAD-fmk at 50 μM (maximally effective elsewhere, Ref. 1) resulted in a 20 ± 10% increase in neuron number compared with cultures treated with toxin alone, a significant increase in survival but still significantly lower than untreated control (P < 0.05). However, there was no sparing of neurons after pretreatment with DEVD-fmk at either 20 or 100 μM, concentrations shown effective elsewhere (5) (Fig. 4).

The occurrence of apoptotic neuronal cell death was pursued further through immunocytochemistry for cleaved caspase-3, but no colabeling of HuD with cleaved caspase-3 was detected at 4, 8, or 24 h after addition of 4 mM toxin (n = 4 per time point). In addition, there was no evidence of HuD colocalization with fragmented Hoechst-labeled nuclei in these cultures, further suggesting that apoptosis is not the primary method of neuronal death. Overall, we conclude that the majority of death of myenteric neurons attributable to acrylamide toxicity occurs by a necrotic mechanism. A low frequency of noncaspase-3-mediated apoptotic death is also present.

**Axonal regeneration of myenteric neurons in vitro.** Earlier, we described the rapid regeneration of myenteric axons in vivo following inflammation-induced damage (18), and, therefore, we investigated the ability of myenteric neurons to recover from the effects of neurotoxins in vitro. Cocultures of myenteric neurons were analyzed at 96 h after acrylamide addition to determine the extent of neuronal survival and axonal regrowth. For concentrations up to 2 mM, neuron numbers remained similar to those of untreated cohort cultures (Fig. 5). In contrast, prior treatment with 4 mM acrylamide further reduced neuron number by 96 h, to 67 ± 7% of the time-matched control or a further 15% decrease from the value at 24 h (P < 0.05). Glial cell numbers were not changed relative to control-treated cultures at this time point (77 ± 14% of untreated control, n = 4, P > 0.05).

In control cultures at 96 h, axon number increased to 153 ± 17.5% of the values observed at 24 h, evidence of continued axonal proliferation. However, cocultures previously treated with acrylamide (0.05–2.0 mM) showed even more dramatic evidence of axonal proliferation by 96 h because these values were also similar to the untreated control (Fig. 5C). For example, cultures treated with 1 mM acrylamide showed an increase in axon number to 209 ± 30% of the 24 h value. This indicates that axon growth in acrylamide-treated cultures occurred more rapidly than control, first compensating for the...
axon loss present at 24 h and then increasing further to attain the time-matched control level.

Determination of the number of branches per axon length after treatment with acrylamide 1 mM for either 24 or 96 h showed that this was similar to time-matched controls (2.3 ± 0.7 vs. 2.7 ± 0.6 vs. 2.1 ± 0.5; n = 3 animals) in control cultures, indicating that most of the axonal regrowth occurred by linear extension. Thus accelerated axonal proliferation in regenerating cultures provides the structural basis for efficient compensation for the initial damaging insult.

Western blot analysis of untreated control cultures showed that the amount of SNAP-25 was also greatly increased by 96 h vs. 24 h (170 ± 33% increase; n = 8; Fig. 5D). In contrast to the decrease seen in cocultures treated with acrylamide for 24 h, the profile of expression of SNAP-25 at 96 h was again similar to time-matched controls. Therefore, these values were all more than 50% greater than the matching values at 24 h (0.5 to 4 mM; Fig. 5E). The levels of SNAP-25 and syntaxin expression changed in identical fashion over this time course. For example, the mean integrated optical density of syntaxin was 99 ± 7% of the time-matched control at 96 h after addition of 2 mM acrylamide vs. that of SNAP-25 at 89 ± 14%.

3H-ACh release was used to evaluate neuronal function at 96 h after acrylamide treatment, in light of the restoration of structure and increased synaptic vesicle protein expression. Surprisingly, this showed the persistent suppression of 3H-ACh release at 96 h compared with the value at 24 h postacrylamide (P < 0.05), which continued to be suppressed by even 144 h after acrylamide exposure (Fig. 5F).

Therefore, the process of neuronal regeneration in vitro accomplished both the restoration of axonal structure and the increased expression of key synaptic vesicle proteins. However, the full recovery of function as reflected by neurotransmitter release lagged behind this by at least several days.

H$_2$O$_2$ is cytotoxic to enteric neurons. To model oxidative stress to enteric neurons in vitro, cocultures were exposed to...
hydrogen peroxide (H₂O₂) over a wide range of concentrations (1–200 μM) for 24 h (Fig. 6). Although calcein-AM labeling showed that the appearance and viability of smooth muscle and glial cells remained unchanged with doses up to 100 μM (not shown), there was a concentration-dependent, parallel decrease of neuron and axon number in the presence of increasing doses of H₂O₂, with the ratio of axon to neuron number remaining constant throughout (Fig. 6C). H₂O₂-induced neuronal death was nonselective among neurons because nitrergic and cholinergic phenotypes were equally reduced in number by 100 μM of H₂O₂ (Table 1).

Western blot analysis showed a strong concentration-dependent decrease in expression of SNAP-25 with increasing amounts of H₂O₂, reaching low levels of 25 ± 13% of control.

Fig. 6. Damage to structure and function of myenteric neurons by hydrogen peroxide (H₂O₂) in vitro. A and B: micrographs showing coculture labeled with anti-HuD (A) and anti-SNAP-25 antibodies (B) 8 h after treatment with 100 μM H₂O₂, showing both the normal appearance of cytoplasmic HuD within neurons (e.g., arrow) as well as degenerating neurons with nuclear HuD (arrowheads). In some cases, axons were discontinuous, indicative of damage (*). Scale bar = 50 μm. C: parallel decrease in both neuron and axon number at 24 h after addition of H₂O₂ (P < 0.05 vs. control for ≥25 μM H₂O₂). D: comparison of SNAP-25 protein expression and ³H-ACh release at 24 h after exposure to H₂O₂. Top: representative Western blot showing decreasing SNAP-25 expression in cocultures exposed to H₂O₂, with loading controls of β-actin. Bottom: combined graph showing significant decrease of SNAP-25 by 24 h after addition of 50 μM H₂O₂ or higher (left axis), and parallel decrease in stimulated ³H-ACh release (P < 0.05 vs. control for values ≥25 μM H₂O₂).
after treatment with 100 μM H₂O₂ (Fig. 6D). The stimulated release of [³H]-ACh at 24 h after H₂O₂ addition showed a close parallel to the changes in both axon number and SNAP-25 expression (Fig. 6D), in contrast to effects of acrylamide, which caused a selective decrease in function while structure remained intact.

H₂O₂ rapidly induces neuronal death by necrosis and apoptosis. Neuron number was rapidly reduced by H₂O₂, reaching 64 ± 15% of control by 6 h after exposure to 100 μM H₂O₂, thus attaining nearly 50% of the total loss seen with this concentration. Many cHuD-positive neurons were detected soon after treatment with a maximum proportion of 15 ± 7% cHuD-positive neurons observed at 8 h (Fig. 7A). As with the acrylamide-treated wells, there was no evidence of fragmented neuronal nuclei at 4, 8, or 24 h post-H₂O₂, as identified by Hoechst colabeling with anti-HuD antibodies (n = 12). Whereas pretreatment with the inhibitor of apoptosis zVAD-fmk resulted in a significant sparing of neuron number by 25 ± 6% (P < 0.05), there was no effect of pretreatment with DEVD-fmk at 20 or 100 μM (P > 0.05; Fig. 7B). Immunocytochemistry using cleaved caspase-3 antibodies showed no neuronal colabeling post-H₂O₂. However, live cell imaging of the cocultures using annexin V showed rare positive staining for this apoptotic marker following challenge; the onset of selective annexin V labeling of axons and cell bodies by 55 min after challenge (Fig. 7C, i–iii) occurred in the absence of propidium iodide uptake (Fig. 7C, iv–vi) and was interpreted as evidence for selective apoptotic neuronal death in vitro. However, simultaneous labeling with both annexin V and propidium iodide (Fig. 7C, vii) was more common, which is interpreted as potential evidence for nonapoptotic cell death. We conclude that the majority of neuronal death caused by H₂O₂ exposure occurred by a necrotic mechanism, with a lesser involvement of noncaspase-3-mediated apoptotic cell death, possibly distributed over the time of exposure.

Minimal restoration of neuronal structure and function after H₂O₂-induced damage. Cocultures showed some further reduction in neuron number by 96 h after addition of 50 μM H₂O₂, but not 100 μM H₂O₂, reaching 52 ± 10% and 33 ± 4% of time-matched controls, respectively (not significant, P > 0.05). Although there was some axon regrowth after the initial damage at 24 h of H₂O₂ treatment, this was much less pronounced than that achieved after acrylamide treatment (Fig. 8). For example, axon number was 73 ± 9% of the

Fig. 7. Contribution of necrotic and apoptotic cell death to neuronal loss caused by H₂O₂ in vitro. A: early contribution of cHuD-positive myenteric neurons to H₂O₂-induced loss of neuron number (*P < 0.05). B: pretreatment with zVAD-fmk but not the caspase-3/7 inhibitor DEVD partly reversed H₂O₂-induced death (*similar within group and significantly different from control; **significantly different from H₂O₂). C: fluorescence micrographs showing the onset of selective labeling of neuronal membranes with annexin V, a marker of apoptosis, following exposure to 50 μM H₂O₂. i–iii: Sequential views of a single field showing initial lack of labeling at 20 min followed by development of axonal and cell body staining at 55 min. Arrows indicate the axon in fluorescent and bright field images; asterisk represents neuronal cell body. iv–vi: Separate experiment showing that annexin V labeling occurred at 60 min after H₂O₂ challenge (iv) without uptake of propidium iodide (i.e., lack of fluorescent labeling of cell body in v). Arrows indicate the axon in each image. Scale bar = 20 μm. vii: High magnification view of typical outcome at 60 min after H₂O₂ challenge, with merged images showing onset of both annexin V (AV) labeling (green) and propidium iodide uptake (PI, red) into neuronal nuclei. A labeled axon with varicosities (arrow) is detectable. Scale bar = 50 μm.
time-matched control after treatment with 50 μM of H₂O₂ and 51 ± 10% after addition of 100 μM of toxin, still significantly less than the time-matched control values (Fig. 8D). Because axonal proliferation occurred in control cultures during this time as described above, our data suggest that growth of axons in the treated cultures occurred at a similar rate as the untreated cohort cultures. This contrasted with the acrylamide paradigm, where robust sprouting caused axon number to equal that observed in cohort cultures at 96 h. Western blot analysis of SNAP-25 expression showed significant impairment in H₂O₂-treated cultures (Fig. 8E), with a decrease to 45 ± 3% of untreated time-matched controls in cultures treated with 50 μM H₂O₂ 96 h earlier (P < 0.05). As well, ³H-ACh release at 96 h post-H₂O₂ showed continued suppression in cultures treated with 50 μM H₂O₂ and above.

**DISCUSSION**

We developed a model of myenteric neurons cocultured with smooth muscle and glial cells in which virtually all neurons survive the transition to tissue culture and show a distribution of nitrergic and cholinergic neurons that matches that seen in vivo. Application to the study of potential neurotoxins in vitro has given insight into neuronal plasticity in the ENS. Cytotoxicity and neuronal death. Activation of immune cells and the release of reactive oxygen species such as H₂O₂ are prominent early events in pathogenesis and could affect the ENS. There is a close parallel between the loss of neurons and their axons after exposure to H₂O₂ that we observed in vitro here, and the damage to the ENS that was seen in the acute phase of initiation of colitis in vivo where a significant loss of neurons occurred by 24 h (22). Although not yet known in vivo, our coculture model suggests this to be an important
mechanism of damage because H$_2$O$_2$ preferentially targeted neurons at concentrations up to 200 µM without effect on other cell types present (principally smooth muscle cells). Elsewhere, addition of catalase to smooth muscle from the inflamed human esophagus reduced the tone to control levels (6), implying that these cells remained viable despite responding to increased levels of H$_2$O$_2$.

Neither H$_2$O$_2$ nor acrylamide targeted a specific neuronal phenotype because the death of myenteric neurons was distributed equally among nNOS- and VACHT-expressing neurons. The neurotoxic events of inflammation in vivo may similarly be nonselective because neuron loss in TNBS-induced colitis in the guinea pig caused a matching reduction in the proportion of nitricergic and cholinergic neurons (13). The dextran sodium sulfate model of colitis in the rat was reported to cause the selective loss of nNOS neurons by day 7 of inflammation, but there was no overall loss of neurons, making it possible that a phenotypic switch occurred in this model (20).

In our model, neuronal death from neurotoxic challenge was largely independent of caspase-mediated apoptosis, suggesting that the primary mechanism of enteric neuron death in vitro was necrotic. This is similar to the outcome of treatment of SHSY5Y neuroblastoma cells with 3 mM acrylamide, where more than 50% of cells died, with only a small proportion of those being rescued by caspase inhibition (23). In addition, we could not detect TUNEL-positive or cleaved caspase-3-labeled neurons in the rat colon following TNBS administration during the period when neuron number decreased (S. Lourenssen, unpublished observations). In contrast, an apoptotic mechanism of neuronal death was present in mouse colitis, where cleaved caspase-3 and cleaved poly(ADP-ribose) polymerase were identified in myenteric neurons (4). As well, a caspase-3-independent death mechanism may also contribute to apoptotic death in some systems. For example, zVAD was not able to prevent neuronal death in a model of retinal degeneration characterized by activation of apoptosis-inducing factor and caspase-12, molecules known to be activated during caspase-independent apoptosis (21). Further work is needed to clarify the presence and relative contributions of these mechanisms of cell death in vivo.

**Damage and regeneration in vitro.** Addition of acrylamide caused damage to cultures of myenteric neurons, which was predicted from its actions as a peripheral neurotoxin elsewhere. However, the findings that significant axon loss occurred without neuron death and was followed by rapid and effective regeneration are novel to the field of ENS research. In the rebound phase of axon regrowth, axon number in previously damaged cultures actually increased sufficiently to surpass the time-matched normal controls by 96 h. This capacity for regeneration in vitro is a striking match to that seen in vivo, where TNBS colitis caused an initial decrease in axon number, which was then followed by extensive regrowth of axons far in excess of the initial damage (18). Interestingly, the recovery of axon number after damage caused by H$_2$O$_2$ failed to approach the level seen in the time-matched controls, and this was a strong contrast to the outcome of acrylamide exposure. It appears that the nature, not the amount, of neuronal damage is a primary determinant of the extent of regeneration.

Little is known of the effect of acrylamide on the gastrointestinal tract, but the single experimental study reported changes to the ENS in acrylamide-treated rats that were similar to those of streptozotocin-induced diabetes, with alterations to catecholaminergic content, a decrease in the amount of calcitonin gene-related peptide, and a corresponding increase in the levels of vasodepressor intestinal peptide (2). However, it is not known whether these changes were associated with neuron loss, axonal degeneration, or altered function. Two other molecules in the type-2 alkene family, acrolein and hydroxynonenal, have been shown to be elevated in the central nervous system of patients with Alzheimer’s, Parkinson’s, and other neurodegenerative diseases (16). The presence of these molecules in the intestine has not been well studied, but the proximity of the ENS to luminal contents suggests that these molecules may also have a role in age and disease-related neuronal dysfunction.

**Repair of axonal structure and function are distinct.** ACh release was suppressed by 24 h after exposure to BtxA, H$_2$O$_2$, or acrylamide, which was associated with decreased expression of the synaptic vesicle marker SNAP-25 even when axonal number was constant (e.g., acrylamide 0.5–2.0 mM). This suggests that decreased expression of proteins needed for vesicular exocytosis may be a sensitive marker for a transition from the normal state to one emphasizing structural repair and regeneration that occurs at the cost of neural function (i.e., ACh release). It was remarkable that we could not identify a return to normal function even after axon number was restored. Instead, both $^3$H-ACh and SNAP-25 expression remained suppressed, further supporting a link between these functions. The dichotomy between structure and function in vitro was very clear in the recovery from acrylamide treatment, where $^3$H-ACh release remained suppressed at 96 and 144 h despite exuberant axon growth. The factors and time period needed for full recovery require further research.

In vivo, colitis causes impaired neurotransmitter function in both inflamed and noninflamed regions (12), and altered neuronal signaling can persist up to 8 wk after the initiation of colitis, long after inflammation is apparently resolved (14). Other animal models also show that neural function can remain affected by prior damage for very long periods, such as the impaired ACh metabolism seen at least 6 mo after *Trichinella spiralis*-induced intestinal inflammation (9). Therefore, our culture model of myenteric neuronal damage recapitulates a pattern of change to structure and function of the ENS that occurs in vivo, providing a valuable opportunity for the study of the mechanisms involved.

BtxA injection is presently used in the gastrointestinal tract for purposes such as the treatment of achalasia, hypercontractile esophageal motility disorders, and anal fissures, where it is assumed to cause a decrease in efferent activity of cholinergic nerves, as it does elsewhere in the nervous system (24). The mechanism of action on enteric nerve function has not been studied, but is thought to involve entry of BtxA into neurons after binding to the synaptic vesicle protein SV2 during its exposure to the external medium. The principal functional outcome of the cleavage of SNAP-25 through endopeptidase activity then prevents the exocytosis of neurotransmitter vesicles. Other actions of BtxA may contribute to this, such as degradation of the GTPase RhoB in PC12 cells, preventing the reorganization of actin that is required for ACh release from these cells (11). The marked drop in $^3$H-ACh release with BtxA treatment occurred in the absence of structural damage,
emphasizing the independence of the responses to functional impairment from those that stem from structural damage. In vivo, clinically significant responses can last from several months to more than a year, so there may be a broad inhibition of myenteric function for a similarly lengthy period, a close parallel to the delayed return to normal neural function seen with inflammation in animal models.

REFERENCES