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# TARGET-DEPENDENCE OF SENSORY NEURONS: AN ULTRASTRUCTURAL COMPARISON OF AXOTOMISED DORSAL ROOT GANGLION NEURONS WITH ALLOWED OR DENIED REINNERVATION OF PERIPHERAL TARGETS

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**Abstract—Evidence is emerging for a role of rough endoplasmic reticulum (RER) in the form of stress granules, the unfolded protein response and protein bodies in the response of neurons to injury and in neurodegenerative diseases. Here, we have studied the role of the peripheral target in regulating the RER and polyribosomes of Nissl bodies in axotomised adult cat dorsal root ganglion (DRG) neurons where axonal regeneration and peripheral target reinnervation was either allowed or denied. Retrograde labelling with horseradish peroxidise was used as an independent marker to enable selection of only those DRG neuronal cell bodies with axons in the injured intercostal nerves. Indications of polyribosomal dispersal were seen by 6 h following axotomy, and by 24 h the normal orderly arrangement of lamellae of RER in Nissl bodies had become disorganised. These ultrastructural changes preceded light microscopical chromatolysis by 1–3 d. The retrograde response was maximal 8–32 d after axotomy. Clusters of debris-laden satellite cells/macrophages were present at this time but no ultrastructural evidence of neuronal apoptosis or necrosis was seen and there were no differences in the initial retrograde response according to the type of injury. By 64 d following axotomy with reinnervation, approximately half the labelled DRG neurons showed restoration of the orderly arrangement of RER and polyribosomes in their Nissl bodies. This was not seen after axotomy with reinnervation denied. We propose that the target-dependent changes in Nissl body ultrastructure described here are part of a continuum that can modify neuronal protein synthesis directed towards growth, maintenance or death of the neuron. This represents a possible structural basis for mediating the varied effects of neurotrophic interactions.**


**Key words: axotomy, dorsal root ganglion, ultrastructure, Nissl body, peripheral target.**

## INTRODUCTION

Changes in the endoplasmic reticulum (ER), particularly the rough endoplasmic reticulum (RER) and its light microscopical correlates, the Nissl bodies, are classically associated with the retrograde response of neurons to axotomy (Nissl, 1894; Lieberman, 1971; Sears, 1987). Recently, ER shape and its local distribution within cells have been found to be of fundamental importance to the cell, enabling local regulation of protein synthesis and adaptive responses to cellular stress. This appears to be a property that is highly conserved and present in plant and animals cells, including neurons (Shibata et al., 2010; Lin et al., 2012). A major role in neuronal survival after injury has also been proposed for RER in the form of stress granules and protein bodies (Chakrabarti et al., 2011; Thomas et al., 2011; Hu et al., 2012), and in neurodegenerative diseases (Doyle et al., 2011). The RER of these structures is similar to the disorganised and fragmented Nissl at the height of the axon reaction and this may reflect common underlying processes. Local changes in the RER associated with the unusual C-type synapse are also characteristic of the response of motoneurons to partial central deafferentation, leading to the suggestion that this may represent the structural basis by which neurotrophic interactions with local interneurons affect protein synthesis at the synapse (Pullen and Sears, 1983).

We previously showed that the nature of the retrograde response to axotomy of adult motoneurons, and in particular the response of their Nissl bodies, varies according to whether or not peripheral target contact is re-established and whether they innervate intrafusal muscle or extrafusal muscle targets. (Johnson et al., 1985, 1993; Johnson, 1996). In those studies retrograde labelling with horseradish peroxidise (HRP) was used as an independent marker of axotomised motoneurons, discriminating them from (i) non-axotomised motoneurons, (ii) motoneurons injured in unknown ways

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*Abbreviations:* DRG, dorsal root ganglion; EM, electron microscopy; EMG, electromyographic; ER, endoplasmic reticulum; HRP, horseradish peroxidase; IM, intramuscular; mTOR, mammalian target of rapamycin; RER, rough endoplasmic reticulum; RSE, relative standard error of the mean; RTA, restoration of target allowed; RTD, restoration of target denied; UK, United Kingdom.

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during the surgical exposure of the intended peripheral nerves and (iii) nearby interneurons. It also allowed sampling to be independent of any of the features of the retrograde response measured. Here we have used the same sampling method and tissue from many of the same animals that were used for the motoneuron studies to compare the ultrastructural features of axotomised adult dorsal root ganglion (DRG) neurons under conditions where peripheral target reinnervation was either allowed or denied. A preliminary report has been published (Johnson and Sears, 2004).

## EXPERIMENTAL PROCEDURES

### Animals

Twenty-one adult (1–2-year old) cats of both sexes were obtained from a Medical Research Council-accredited dealer in the United Kingdom (UK). All studies were carried out in strict compliance with the requirements of the UK Home Office for research involving the use of animals (Animals (scientific procedures) Act 1986).

### Axotomy

Under sodium pentobarbitone anaesthesia (45 mg/kg), up to 2 non-adjacent intercostal nerves in the seventh to tenth intercostal spaces were exposed unilaterally at the level of the levator costae muscle. As illustrated in Fig. 1, the nerves were either crushed ('restoration of target allowed' – RTA); or were transected, followed by ligation of the proximal stump and removal of 3–5 mm of the distal stump ('restoration of target denied' – RTD). In most cats, RTA-axotomy involved the 7th intercostal nerve and RTD-axotomy involved the 9th intercostal nerve of the same side. In five cats, the intercostal spaces were misidentified at surgery and found at dissection to be one segment caudal i.e. RTA-axotomy involved the 8th intercostal nerve instead of the 7th intercostal nerve and RTD-axotomy involved the 10th intercostal nerve instead of the 9th intercostal nerve. Depending on the intercostal nerve actually injured, therefore, DRG from T7 and T8 (RTA-axotomy), and T9 and T10 (RTD-axotomy) from different cats were pooled.

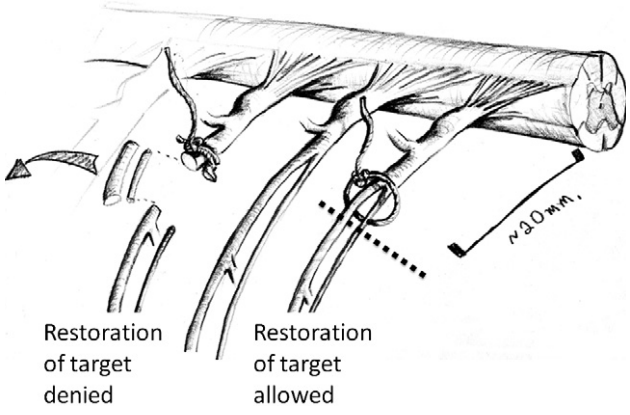


**Fig. 1.** Diagram of the thoracic spinal cord and spinal nerves. The ventral primary ramus is seen dividing into a larger internal intercostal nerve and a smaller external intercostal nerve. RTA-axotomy was produced by crushing both nerves at the site indicated by the dashes. A loose ligature was placed around the nerves to enable identification of the crush site up to 2 months later for application of HRP to the proximal nerves. RTD-axotomy was achieved by nerve transaction, proximal ligation and removal of 3–5 mm of the distal nerves.

### Retrograde labelling

To ensure that analysis was restricted to the previously axotomised neurons, their cell bodies were labelled by the retrograde axonal transport of HRP. With the exception of four cats used for the study of the early (6–48 h) response to axotomy, HRP (see below) was applied to the proximal ends of non-adjacent T7–T10 intercostal nerves which were newly sectioned 1 d before perfusion (Johnson, 1986). Briefly, the nerves were freed from surrounding tissue 1 d prior to perfusion, sectioned and isolated from surrounding tissue either by covering them with cotton wool soaked in Vaseline, or by drawing them into a short upright polyethylene cannula, the base of which was secured with 5% Agar. The newly lesioned proximal nerves were then immersed in 10–20 μl of 40% HRP (Sigma type VI) in saline. Control neurons were examined in non-operated cats ($n = 2$) and in cats ($n = 3$) where DRG neurons had been labelled by the intramuscular (IM) injection of approximately 100 μl of 40% HRP into the external and internal intercostal muscles 1 d before perfusion (IM HRP). Our previous studies of cat thoracic motoneurons labelled by IM HRP, revealed that this did not alter the ultrastructural appearance of the labelled cell bodies, save for an increase in the electron density of their lysosomes (Johnson, 1986). On this basis, we have assumed that retrogradely labelled muscle afferents will similarly escape damage by the very minor mechanical disruption caused by the IM injection. In this way, IM HRP served as a control for any possible effects of the retrograde transport of a foreign protein on DRG neuron ultrastructure. We did not use DRG from the contralateral side of operated animals as controls, as the midline incision and retraction of skin used to expose the intercostal spaces on one side would inevitably damage sensory axons running in branches of the dorsal rami of the spinal nerves of both sides and possibly damage axons running in the lateral cutaneous branches of the intercostal nerves as these were put on stretch during skin retraction.

Four, 8, 16, 32, or 64 d after axotomy, cats ($n = 2$–3 per time point) were perfused via the abdominal aorta with approximately 300-ml saline followed by 1.5 l fixative (2% glutaraldehyde–1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4; flow rate 200 ml/min, using 1.5-m hydrostatic pressure). To analyse the early response to axotomy in more detail, intercostal nerves in a further four cats were transected and the proximal nerve stumps immediately surrounded by 40% HRP. These cats were perfused with fixative as described above 6, 12, 24 or 48 h later ($n = 1$ per time point). To analyse the possible role of diffusible substances from muscle, the proximal 5 mm of intercostal nerves in a further two cats were drawn up into 10-mm lengths of polysulphone microdialysis tubes with a molecular weight cut off of 100,000 (Harvard Apparatus, MA). The portion of the nerve in the proximal 1 mm of the tube was crushed and the tubes placed in denervated intercostal muscle. After 63 d, neurons with axons in the most proximal part of the tube were retrogradely labelled by exposing the nerve to HRP as described above. Cats were perfused with fixative 1 d later as described above.

Fixed DRG were sectioned longitudinally at 70 μm using a Vibratome and the sections processed to demonstrate peroxidase activity by using 3,3′-diaminobenzidine as the enzyme substrate, with cobalt intensification of the reaction product (Adams, 1977). After osmication and dehydration, sections were flat-embedded in Araldite between polytetrafluoroethylene-coated glass microscope slides.

Small, 5–10-mm lengths of the fixed intercostal nerves were also removed approximately 40 mm from the intervertebral foramen which corresponded to 20 mm distal to the site of axotomy for those nerves that had been injured. These portions of nerves were affixed by fine sutures to small cardboard frames to ensure the nerves stayed straight during processing and they were then osmicated, dehydrated and embedded in Araldite as described above.