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Target-dependence of sensory neurones: An ultrastructural comparison of axotomised dorsal root ganglion neurones with allowed or denied reinnervation of peripheral targets

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LIST OF ABBREVIATIONS

DRG: Dorsal root ganglion

EM: Electron microscopy

ER: Endoplasmic reticulum

HRP: Horseradish peroxidase

IM: Intramuscular

RER: Rough endoplasmic reticulum

RSE: Relative standard error of the mean

RTA: Restoration of target allowed

RTD: Restoration of target denied

UK: United Kingdom

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 neurones 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) neurones 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 6h following axotomy, and by 24h the normal orderly arrangement of lamellae of rough endoplasmic reticulum in Nissl bodies had become disorganised. These ultrastructural changes preceded light microscopical chromatolysis by 1-3d. The retrograde response was maximal 8-32d 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 64d following axotomy with reinnervation, approximately half the labelled DRG neurones 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 toward growth, maintenance or death of the neurone. 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

1.0 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 neurones 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 neurones (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 motoneurones to partial central deafferentation, leading to the suggestion that this may represent the structural basis by which neurotrophic interactions with local interneurones affect protein synthesis at the synapse(Pullen and Sears, 1983).

We previously showed that the nature of the retrograde response to axotomy of adult motoneurones, 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, Johnson et al., 1993, Johnson, 1996). In those studies retrograde labelling with horseradish peroxidise (HRP) was used as an independent marker of axotomised motoneurones, discriminating them from (i)

non-axotomised motoneurones, (ii) motoneurones injured in unknown ways during the surgical exposure of the intended peripheral nerves and (iii) nearby interneurones. 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 motoneurone studies to compare the ultrastructural features of axotomised adult dorsal root ganglion neurones under conditions where peripheral target reinnervation was either allowed or denied. A preliminary report has been published (Johnson and Sears, 2004).

2.0 METHODS

2.1 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).

2.2 Axotomy

Under sodium pentobarbitone anaesthesia (45mg/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 5 cats, the intercostal spaces were misidentified at surgery and found at dissection to be 1 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.

2.3 Retrograde labelling

To ensure that analysis was restricted to the previously axotomised neurons, their cell bodies were labelled by the retrograde axonal transport of horseradish peroxidise (HRP). With the

exception of 4 cats used for the study of the early (6-48h) response to axotomy, HRP (see below) was applied to the proximal ends of non-adjacent T7-T10 intercostal nerves which were newly sectioned 1d before perfusion(Johnson, 1986). Briefly, the nerves were freed from surrounding tissue 1d 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 10–20µl 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 neurones had been labelled by the intramuscular (IM) injection of approximately 100µl of 40% HRP into the external and internal intercostal muscles 1d before perfusion (IM HRP). Our previous studies of cat thoracic motoneurones 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 neurone 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 64d 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

200ml/min, using 1.5m hydrostatic pressure). To analyse the early response to axotomy in more detail, intercostal nerves in a further 4 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 48h later (n=1 per time point). To analyse the possible role of diffusible substances from muscle, the proximal 5mm of intercostal nerves in a further 2 cats were drawn up into 10mm 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 1mm of the tube was crushed and the tubes placed in denervated intercostal muscle. After 63d, neurones 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 1d later as described above.

Fixed DRG were sectioned longitudinally at 70µm 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, 5-10mm lengths of the fixed intercostal nerves were also removed approximately 40mm from the intervertebral foramen which corresponded to 20mm 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.

2.4 Sampling of DRG cell bodies

Guided by an initial low power light microscopic examination of the DRG sections (FIG.2), small 1mm^2 portions of DRG containing labelled neuronal cell bodies were cut out and glued with cyanoacrylate glue onto polished Araldite stubs. The light-microscopic images of individual labelled neurons in the thick section of DRG were correlated with the same cell bodies in semithin toluidine blue-stained sections obtained from the block and subsequently with the DRG cell bodies seen by electron microscopy in ultrathin sections stained with uranyl acetate and lead citrate (FIG 3).

To avoid sampling bias, DRG cell bodies were selected for ultrastructural examination on the basis of their HRP labelling and order of appearance in sections taken from the mesa, and not on the basis of size or features of the retrograde response. Analysis of 2-3 random Vibratome sections from each of the animals indicated that 20-30% of all DRG neurones were labelled by HRP. In these sections, unlabelled DRG neurones with clearly eccentric nuclei were often seen adjacent to HRP labelled neurones indicating that unlabelled axotomised DRG neurones were also present (FIG 2). Approximately 5-10 HRP-labelled DRG cell bodies would be present at various depths in a typical mesa trimmed for ultrathin sectioning. As 2-3 mesas were normally prepared for each cat, approximately 10-30 labelled DRG cell bodies of all sizes per cat were examined in semithin sections. Of these, 5-10 randomly-selected HRP-labelled DRG cell bodies per cat were analysed in the electron microscope.

2.5 Quantifying chromatolysis and nuclear eccentricity

The proportion of DRG neurones, showing chromatolysis or eccentric nuclei was determined for the first 100 HRP-labelled DRG neurones per stage. Semithin toluidine blue stained sections used as part of the ultrastructural analysis described above were used, as well as

sections cut from large mesas before they were trimmed to a size suitable for ultrathin sectioning. A chromatolytic DRG cell body was taken as one where discrete patches of cytoplasmic basophilia (Nissl bodies) could no longer be resolved in the light microscope over more than half the area of the cell body cytoplasm. An eccentric nucleus was taken to be one where the distance from the centre of the nucleus to the cell body periphery was less than the distance from the centre of the nucleus to the centre of the cell body (Barr and Hamilton, 1948).

2.6 Quantifying polyribosomal dispersal within Nissl bodies

A Nissl body was defined ultrastructurally as any aggregate of RER and/or polyribosomes that could be resolved in the light microscope as a basophilic patch in the cytoplasm of toluidine blue-stained sections(Johnson, 1996). A 4μm^2 transparent overlay was placed randomly over such Nissl bodies at 29,000 times magnification and within this area the mean distance to the nearest polyribosome was measured according to the nearest neighbour method (Clarke, 1954). The mean distance between polyribosomes of approximately 10-15 ‘runs’ per Nissl body was calculated. Five Nissl bodies were examined per neurone in 5 neurones per cat. This approach gave a relative standard error of the mean (RSE) between 1.2-3.6% for all time points studied except 1d after IM- labelling with HRP where a slightly larger RSE (5.27%) was obtained, but this was still considered a reliable measure. Nissl bodies were quantified in non-operated and control (IM HRP) cats and also in cats from 6h to 4d after axotomy. Only 1 cat was examined at each stage between 6h and 2d after axotomy and so no statistical analysis was done.

3.0 RESULTS

3.1 Normal DRG

Except for the increased electron density of their lysosomes, DRG neurones labelled by IM HRP were indistinguishable from non-operated and unlabelled DRG neurones. Retrogradely labelled cell bodies had a wide range of diameters (20-80 µm) with a mean of 40µm (see also Larnicol et al, 1988). The light and electron microscopic features were similar to those described previously (Lieberman, 1976, Jones and Cavanagh, 1981), although the light microscopic distinction between large light and small dark DRG neurones was not clear cut (FIG 4). In all neurones, nuclei were circular and centrally-placed within the cell body; nucleoli were compact and prominent; Nissl substance was mostly evenly distributed throughout the cell body. The largest Nissl bodies were found peripherally within the cytoplasm of neurones of all sizes. In a typical semithin section, approximately 2-3 satellite cells were present adjacent to large- and medium-sized DRG cell bodies and 0-1 satellite cells were adjacent to small cell bodies. Occasional (4-7% of all cell bodies) eccentric nuclei were seen in non-operated and control DRG cell bodies (FIG. 6).

Ultrastructurally, Nissl bodies were composed of a mixture of RER and free polyribosomes (see FIG. 13). In small Nissl bodies, these organelles were clustered in a disorganised manner, while in the larger Nissl bodies, the RER was arranged in regular arrays or lamellae similar to the Nissl bodies of motoneurones.

3.2 Response to axotomy

All observations refer to HRP-labelled axotomised DRG neurones unless stated.

As colour cannot be detected by electron microscopy, we restrict the use of the term 'chromatolysis' here to observations made using the light microscope whereby a reduction in the size of Nissl bodies results in an apparent overall loss of basophilic cytoplasmic staining(Nissl, 1894, Marinesco, 1898). By 2d, such chromatolysis, together with nuclear eccentricity with occasional nucleolar vacuolation, was clearly seen. By 4d, chromatolysis was marked, especially in small diameter DRG neurones, and the previously round eccentric nuclei had become elongated and crenated (Fig.5).

A semi quantitative assessment of the frequency of occurrence of four classical features of the retrograde response (chromatolysis, nuclear eccentricity, peripheral clumping of Nissl substance and nucleolar vacuolation) revealed that only the first two features occurred with sufficient frequency for any patterns associated with axotomy to be determined. Chromatolytic DRG neurones peaked at 8-16, following both RTA and RTD axotomy. Chromatolysis was still evident at 64d, but had reduced to approximately 50% and 25% of peak values following RTA and RTD axotomy, respectively (FIG 6). Nuclear eccentricity peaked between 4-16d following both types of axotomy. A reduction to approximately 50% peak values was seen 64d after RTA axotomy, but not after RTD axotomy (FIG. 6).

3.3 Nissl body ultrastructure: Although chromatolysis was not evident in the light microscope at 1d, correlative light- and electron- microscopical analyses showed that ultrastructural disorganisation of Nissl bodies was always present at this time (FIG. 7).

Slight dispersal of the polyribosomes within the Nissl bodies of axotomised DRG cell bodies was detected qualitatively 12-24h after axotomy. Closer examination of the distances to the

nearest neighbour polyribosomes in ‘runs’, where this distance is always decreasing, indicated that polyribosomal dispersal began as early as 6h after nerve transaction (FIG. 8). The nearest neighbour interval ranged from 0.021 to 0.028 in all groups which indicated unsurprisingly that the polyribosomes in both organised and disorganised Nissl bodies were clustered (0= highly clustered, 1= random distribution, 2.15 uniform distribution) (Clarke, 1954).

3.4 Degenerative changes

By 4-8d following axotomy, marked chromatolysis and nuclear eccentricity was seen, with no clear difference according to the type of nerve injury (FIG. 9). Occasional clusters of cells resembling satellite cells (Nageotte nodules) were seen from 8d following both types of axotomy (FIG. 9). Nageotte nodules are generally taken to indicate the sites of DRG degeneration(Nageotte, 1907, Thomas, 1992), although we found no qualitative evidence of DRG neuronal loss. The extent, if any, of DRG neurone loss remains unknown however as the tissue did not lend itself to stereological analyses. By 32d, approximately 5-6 satellite cells were seen per DRG neurone irrespective of cell body size. These cells often contained electron dense inclusions, suggestive of a phagocytic role (FIG. 10). No clear evidence of apoptotic or necrotic cell bodies was found, although large swollen (unlabelled) neurites resembling axonal spheroids were seen (FIG. 11). No degenerating HRP-labelled DRG neurones or processes were seen.

3.5 Target-dependent features: Sixty four days after RTA and RTD, light microscopic chromatolysis persisted in small cell bodies, but was rarely seen in large and medium cell

bodies (FIG. 12). Ultrastructurally, about half the cell bodies of all sizes showed reformation of orderly RER in their Nissl bodies after RTA axotomy, but none showed this after RTD axotomy (FIG. 13).

Analysis of the intercostal nerves 20mm distal to the site of axotomy (FIG. 14) revealed no axonal regeneration 64d after RTD, whereas axonal regeneration was seen 64d following RTA. In contrast to uninjured nerves, however, axons in nerves 64d following RTA were smaller and had thinner myelin sheaths. Both motor and sensory axons would be regenerating in these nerves. While we did not attempt to obtain direct evidence of restoration of sensory function, we did obtain evidence of successful functional regeneration distal to the original nerve lesion from electromyographic (EMG) recordings obtained just prior to the second lesion for retrograde labelling with HRP (FIG 15). The middle trace of FIG 15 shows the inspiratory-phased activity of the levator costae muscle whose innervation, via the posterior spinal ramus(Hilaire et al., 1983), remained intact. The left side of the lower trace shows the restored inspiratory-phased activity recorded through a pair of small ball electrodes on the surface of the external intercostal muscle, and this local activity greatly diminished and came later in the breath when the electrodes were moved distally along the same segment. This pattern would be expected from the normal topographically distributed pattern of rib-cage inspiratory activity in eupnoea (Sears, 1964, Sears and Stagg, 1976).

Where the proximal stumps of crushed intercostal nerves had been placed in polysulphone microdialysis tubes (100,000 molecular weight cut off) and placed in the substance of denervated intercostal muscles (FIG 16), only the occasional small regenerating fascicle was seen, although the tubes were populated by many Schwann cells, fibroblasts and blood vessels. Retrogradely-labelled DRG neurones with peripheral processes in these tubes

showed no restoration of orderly Nissl body ultrastructure and were indistinguishable from DRG neurones 64d following RTD-axotomy (data not shown).

4.0 DISCUSSION

Using the retrograde axonal transport of HRP to identify axotomised DRG cell bodies we show that highly ordered Nissl body ultrastructure is only seen where neurones have the opportunity to establish peripheral target contact. We previously reported that restoration of Nissl body ultrastructure is seen with restoration of peripheral target contact for both α- and γ- motoneurones (Johnson et al., 1985, Johnson and Sears, 1989, Johnson et al., 1993). Taken together, the results of the present study indicate that the ultrastructural orderliness of Nissl bodies is a sensitive measure of the presence of neurone-target interaction for a range of neurone types and types of peripheral target.

4.1 Target-dependence of Nissl bodies

The general features of the axon reaction of cat thoracic dorsal root ganglion neurones seen here are similar to those reported previously for a variety of species (Lieberman, 1971, 1976). Here, we newly report that restoration of Nissl body orderliness after axotomy of DRG neurones occurred when peripheral target reinnervation was allowed (RTA), but this did not occur when peripheral target reinnervation was denied (RTD). This restoration of Nissl body orderliness was seen more often in large DRG neurones compared to small ones. We obtained similar findings for the restoration of Nissl body orderliness of large(α-) and small (γ-) motoneurones which have extrafusal- and intrafusal- muscle targets, respectively (Johnson et al., 1993). In DRG, small cell bodies (C and Aδ afferents) are primarily concerned with somatic noiciception (Cervero et al., 1984), whereas large DRG cell bodies (Aα and Aß afferents) are generally concerned with tactile function and proprioception (Fyffe, 1983, Harper and Lawson, 1985, Larnicol et al., 1988, Lawson, 2005, Parekh et al.,

2010). Similarly, for the motor system, large (α-) and small (γ-) motoneurones are concerned with generating bulk changes in muscle tension or to regulate the sensitivity of the intrafusal fibres of muscle spindles (Kuffler et al., 1951), as specifically established for intercostal motoneurones (Andersen and Sears, 1964). These different functional properties of both motor and sensory neurones are reflected by a wide variety of peripheral target types. This suggests that irrespective of their specific nature, contact with peripheral targets generally may be sufficient to restore Nissl body orderliness. Although we have EMG evidence for motor reinnervation following RTA-axotomy, we have no comparable information for the regenerating peripheral processes of the DRG examined. As we found no trophic ulcers or hair loss in any animals, peripheral innervation through existing overlap of sensory territories and collateral sprouting is probably sufficient for peripheral target maintenance, even with RTD-axotomy. Our analysis of the distal nerves after RTA-axotomy showed that axonal regeneration was by no means complete, yet Nissl body orderliness had returned. Since nerve crush preserves the endoneurial tubes that guide regenerating axons back to their original peripheral target structures (Fawcett and Keynes, 1990, Sunderland, 1990, Ide, 1996), we assume that precise anatomical axonal regeneration to the original peripheral targets has occurred in this study. This contrasts with other studies of sensory reinnervation in the cat hind limb where nerve transection was employed, leading to muscle afferents reinnervating inappropriate targets (Banks and Barker, 1989) and the failure of such fibres to recover normal function (Collins et al., 1986). In the present study, the predominance of small, thinly myelinated axons and the absence of large myelinated fibres in the crushed nerved indicates that axonal conduction will have been slowed overall. In such circumstances, precise functional recovery is unlikely for those aspects of sensory and motor function that require the conduction of nerve impulses to and from the CNS within specific velocity ranges. Our

use of HRP-labelling to restrict analysis to DRG neurones with peripheral processes in nerves injured in specific ways meant that we restricted our analysis to the 20-30% of DRG neurones that retained the capacity to transport HRP up to 64d following axotomy. It is possible that some DRG neurones will have been axotomised but failed to label with HRP. Equally, some DRG neurones with peripheral processes in unrelated segmental nerves, such as the dorsal rami, will also have been axotomised by nerve crush, transection or avulsion during surgery and these would also have been left unlabelled by HRP. Unlike the motor pools of the spinal cord (Romanes, 1946), however, there is no somatotopic organisation in the DRG(Ygge, 1984) that allows identification of particular DRG neurones as having peripheral processes in particular peripheral nerves. In the absence of a further independent label, therefore, we cannot tell if unlabelled DRG neurones exhibiting a retrograde response belong to RTA or RTD intercostal nerves, or to unknown nerves that have been injured in some non-specific fashion. In these circumstances, any disadvantages introduced by restricting our analysis to HRP-labelled DRG neurones are likely to be outweighed by the increased reliability with which our results can be equated to different types of nerve injury. Our observations that Nissl body ultrastructure is not restored when the proximal nerve stump is placed in a microdialysis tube within denervated muscle indicate that contact with Schwann cells and access to diffusible factors from muscle are insufficient. The molecular weight cut off of 100,000 used here is sufficient to allow all known neurotrophic and growth factors access across the tube to the proximal axons and our ultrastructural analyses show that direct contact with Schwann cells was maintained within the tubes. Taken together, these observations suggest that anatomical contact with the peripheral target is required for the restoration of Nissl body orderliness. Cross reinnervation studies will be required to clarify this point.

4.2 Nissl bodies and the cytoskeleton

We have found that disorganisation of Nissl body ultrastructure is an early event that precedes light microscopical changes and have preliminary evidence from individual cats examined up to 48h after axotomy that polyribosomal dispersion may be occurring as early as 6h following axonal injury. Image analysis in the light microscope revealed evidence of chromatolysis as early as 4hr in rats after facial and hypoglossal axotomy, with better recovery from chromatolysis when post operative conditions favoured peripheral target reinnervation than when they did not (Guntinas-Lichius et al., 1996). Changes in Nissl bodies, therefore, fall within the range of some of the earliest events reported following axotomy. The transcription factor c-jun is induced in axotomised adult rat spinal DRG as early as 10h and as early as 3h in neonatal rat spinal motoneurones (Casanovas et al., 2001), while increases in the synthesis of nuclear proteins occur within 5h of axotomy of giant R2 neurones of Aplysia (Buriani et al., 1990). The early changes in Nissl bodies observed in our study may in part be due to the relative proximity (~1cm) of the axonal lesion to the parent DRG cell bodies when compared with the total length of the intercostal nerves in adult cats (~10-15cm). The early dispersal of polyribosomes could reflect a change in their interaction with cytoskeletal elements in the cell body, since changes in the expression of neurofilament proteins have been found as early as 1d following axotomy of rat DRG (Wong and Oblinger, 1990) and recent immunocytochemical studies of retinal ganglion cells in pigs subjected to a very proximal axotomy show changes in neurofilament-light, microtubule associated protein and tubulin within 1h (Balaratnasingam et al., 2011). One possibility is that interruption of peripheral target contact first induces cytoskeletal changes which in turn facilitate a change of the spatial relationships of RER and polyribosomes, which is seen as the ultrastructural

disorganisation of Nissl bodies. We calculate that the transport rate of the 'signal' for polyribosomal dispersal (seen at 6h) in cat thoracic DRG being at least 60mm/d (assuming 15mm distance from the site of axotomy to the cell body). This is in the fast axonal transport range.

4.3 Functional significance of changes in Nissl body ultrastructure

A long-held view is that axotomy changes the neurone from a cell concerned primarily with secretion to one primarily concerned with growth (Watson, 1974). This is supported by observations that the retrograde response of neurones to axotomy is associated with the down regulation of proteins destined for export from the cell body such as neurotransmitter-associated enzymes and the up regulation of those associated with growth such as tubulin (Bisby and Tetzlaff, 1992, Fu and Gordon, 1997). It is tempting, therefore, to equate the uninjured neurone with a typical secretory cell, such as a pancreatic acinar cell, since both are characterised by the presence of lamellae of RER. However, the similarity is superficial. Most ribosomes in neuronal Nissl bodies exist as free polyribosomes arranged in linear arrays between the lamellae of RER (FIG. 17), unlike the heavily studded membrane-bound ribosomes of secretory cells. This suggests that the Nissl body represents a site of protein synthesis that is more specific than one simply concerned with the elaboration of secretory proteins.

Following axotomy, we show that chromatolysis is associated with fragmentation and disorganisation of the RER lamellae and disruption of their associated polyribosomes. The change in Nissl bodies following axotomy, therefore, comprises loss of orderliness, rather than loss of membrane-bound polyribosomes. As such, it may be inappropriate to adduce

information derived from studies of cell-free systems to deduce the types of protein known to be synthesised by membrane-bound and free-polyribosomes in neurones, since former takes no account of any ultrastructural orderliness. Nissl body orderliness appears dependent on the restoration of peripheral target contact in both sensory and motor neurones. For sensory neurones in particular, the range of peripheral targets is extensive, indicating that the nature of the signal for such orderliness is common to many peripheral targets. By contrast, in the central nervous system the motoneurone itself is both a target for synaptic inputs as well as itself depending on a peripheral target for its morphological and functional integrity. The unusual C-type synapse, formed presynaptically by the largest bouton on motoneurones is characterised by a large sub-synaptic lamellated Nissl body. After partial 'central deafferentation' of motoneurones following hemisection of the spinal cord, the C-type synapse, now believed to be a short cholinergic propriospinal neurone (Liu et al.2010), increases its coverage of cat thoracic α-motoneurones presynaptically, with a corresponding expansion postsynaptically of its Nissl body (Pullen and Sears, 1983). In contrast, Nissl bodies located generally within the cytoplasm of deafferentated rat lumbar motoneurones show loss of ultrastructural orderliness with upregulation of microtubule associated tubule-2 immunostaining and both these features can be ameliorated by progesterone (Gonzalez et al., 2009). These different responses of intracytoplasmic and subsynaptic Nissl bodies to deafferentation suggest the operation of precise local controls over regional protein synthesis in the neurone. A hypothesis, originally developed from the study of C-type synapses after partial central deafferentation(Pullen and Sears, 1983), was that changes in Nissl body ultrastructure may represent the structural basis by which neurotrophic interactions with local interneurones affect protein synthesis at the synapse. The present study provides evidence to support this hypothesis. Moreover, it suggests that this hypothesis applies more generally so

that it includes all Nissl bodies within the cytoplasm, motor and sensory neurones, and Nissl body changes induced by the loss of both peripheral as well as central synaptic connectivity. As this Nissl body response occurs in so many situations it would be reasonable to propose that it is regulated by a common molecular signal. Nissl bodies of axotomised motoneurones remained disorganised when the proximal nerve stumps were enclosed in a polysulphone dialysis tubes with a molecular weight cut off of 100,000KD that was placed in the substance of innervated- or denervated- muscle, indicating that synaptic contact is required for Nissl body orderliness, regardless of whether a soluble factor is secreted by the peripheral target(Johnson, 1996). In this regard, it would be interesting to determine if DRG neurones encouraged to regenerate their central processes by the application of neurotrophic factors (Smith et al., 2012) also reform their Nissl bodies. Our preliminary results on entubulated nerves indicate that access to diffusible factors without direct contact with the periphery is insufficient for Nissl body reformation.

While much has been learned about the process of protein synthesis from studies of homogenised tissue lysates, the ultrastructural orderliness and topographical distribution of the protein synthetic machinery is a dimension that is lost in such cell-free systems. This may be an important determinant of the amount and types of proteins synthesised and it is a feature highlighted here by our observations on the ultrastructural orderliness of Nissl bodies. A role for local cytoplasmic factors in maintaining such orderliness is indicated by observations that RER isolated from rat hepatocytes and injected into Xenopus oocytes initially had a fragmented, disorganised appearance similar to the rough microsomes of cell-free models of protein synthesis, but within 2 hours had started to reorganise itself into parallel lamellae of RER, similar to the small Nissl bodies shown in the present study. (Paiement et al., 1988, Lavoie et al., 2011). More recent evidence shows that RER is a labile

structure that alternates between a sheet-like form and a tubular form and that the tubular form can move within the cytoplasm under the influence of various molecules such as atlastin which is an integral membrane guanosine triphosphatase that can drive membrane fusion (Pendin et al., 2011). Overexpression of the atlastin-1 gene in drosophila and the plant homologue Root Hair Defective 3 in tobacco root hair cells results in the development of aberrant sheets of ER (Zheng and Chen, 2011), while mutations in atlastin are associated with motoneurone degeneration(Moss et al., 2011). Whether atlastin or changes in other proteins, such as reticulons (Fergani et al., 2005, Lin et al., 2012), drives the RER changes seen after axotomy is unknown. What may be more important than RER fragmentation and reformation, however, is the topographical segregation of protein synthesis that is represented by Nissl bodies. Such segregation is known to occur in neurones, particularly at the synapse (Steward et al., 1988, Bramham, 2008, Wang et al., 2010). More recently, ribosomal binding proteins have been shown to play a key role causing the aggregation of large amounts of messenger ribonucleic acid and regulating its translation at topographically discrete sites within the cell (Liu-Yesucevitz et al., 2011). One molecule in particular that has been implicated both in synaptically driven local protein synthesis (Kindler and Kreienkamp, 2012)and the action of a variety of neurotrophic factors, is the serine/threonine protein kinase termed mammalian target of rapamycin (mTOR)(Santos et al., 2010, Bove et al., 2011). It has been reported that mTOR is upregulated by axotomy in DRG neurones and this increases axonal regeneration by an effect on Growth Associated Protein-43 (Abe et al., 2010). Conversely Inhibition of mTOR causes cell death (Asnaghi et al., 2004). This protein kinase which is implicated in regeneration, degeneration, neurotrophism and changes in local protein synthesis therefore presents as candidate molecule that could be involved in the regulation of Nissl body ultrastructure.

Morphological features that are being increasingly described in relation to axotomy-induced neuronal death and in neurodegenerative diseases are the presence of stress granules and protein bodies. These are small cytoplasmic aggregates of ribonucleic acid and protein not unlike the disorganised and fragmented Nissl at the height of the axon reaction. These aggregates are seen universally in stressed cells where the unfolded protein response is initiated(Thomas et al., 2011). This response, initially aimed at helping the cell survive, is characterised by an overall decrease in protein synthesis and an increase in activities associated with removing misfolded proteins, although if the stress is severe cell death can follow (Chakrabarti et al., 2011). Stress granules have been found in association with a degenerative response to axotomy by retinal ganglion cells (Hu et al., 2012) and in some neurodegenerative diseases (Doyle et al., 2011). One possibility, therefore, is that disorganised Nissl bodies and stress granules are part of a continuum of RER and polyribosomal changes associated with a survival response or a degenerative response to the initial stress of axotomy and the longer term effects that are driven by the restoration or prevention of peripheral target contact.

4.4 Degenerative changes

From 8d after axotomy, we noted an increase in satellite cells, which often formed clusters corresponding to the 'nodules of Nageotte' that are classically associated with neuronal loss and their subsequent phagocytosis (Nageotte, 1907, DeFilipe, 1991). Ultrastructurally, these cells had the appearance of phagocytes and often contained electron-dense debris. While both satellite cells and haematogenous macrophages are known to contribute to this increase in non-neuronal cells in DRG after peripheral nerve injury (Gehrmann et al., 1991, Hu and

McLachlan, 2002), both cell types have a similar morphological appearance(Lu and Richardson, 1993). We also noted the presence of enlarged neurites which were packed with filaments and electron dense lamellar bodies from 8d onwards irrespective of the type of peripheral nerve injury. Such features are commonly seen in the axonal spheroids that develop with Wallerian degeneration (Beirowski et al., 2010). They have also been described in association with transganglionic degenerative changes in the rat substantia gelatinosa 3-9d after sciatic nerve transaction(Knyihar and Csillik, 1976). While all this points towards DRG degeneration, we did not see any evidence of this at any of the time-points studied. This contrasts with studies in rats where approximately 35-40% of rat lumbar DRG neurones are lost several months after sciatic nerve transaction (Himes and Tessler, 1989, Tandrup et al., 2000), with evidence that most of this loss is of neurones supplying skin rather than muscle (Welin et al., 2008). Most of this DRG loss appears to be due to apoptosis (Groves et al., 1997). There is also evidence that adult DRG survival after axotomy is regulated by peripheral target contact, since greater neuronal loss occurs if peripheral target reinnervation is prevented (Vestergaard et al., 1997, Jivan et al., 2006) and nerve repair can ameliorate this (McKay Hart et al., 2002). We have found satellite cell/macrophage clusters and debris-laden macrophages, but no direct evidence of neuronal loss. This may be due to insufficient sampling. Thus, we estimate that the total number (HRP-labelled and unlabelled) of DRG cell bodies examined per cat we would be approximately 200-300 while an estimate of the total number of DRG cell bodies of around 15000 can be made based on the ranges reported for rat lumbosacral DRG after stereological counting (Hart and Terenghi, 2004). This indicates that fewer than 5% of the DRG would have been sampled in the present study. It is also likely that many neuronal cell bodies may have escaped axotomy. Up to one third of thoracic DRG have axons in the (uninjured) dorsal spinal ramus (Ygge, 1984) and other DRG cell bodies can

have peripheral processes in spinal nerves that do not correspond to the segmental number of the ganglion at all (Devor et al., 1985, Himes and Tessler, 1989, Swett et al., 1991) which probably represent the more dispersed somato-visceral afferent fibres (Cervero et al., 1984, Lechner et al., 2011) This, together with the narrow time-window available to catch degenerating cell bodies before they are phagocytosed could explain why degenerating neurones have not been found in adult cat DRGs. Additional stereological studies will be required to clarify this point.

5.0 CONCLUSIONS

We report that Nissl body ultrastructure is target-dependent across the whole size spectrum of DRG cell bodies that supply a variety of peripheral targets. We have reported similar finding for α- and γ- motoneurones. This leads us to conclude that Nissl body ultrastructure is a sensitive measure of peripheral target contact that is independent of neurone type. We also extend to sensory neurones the hypothesis previously based on motoneurone C-type synapses that changes in Nissl body ultrastructure may represent the structural basis by which neurotrophic interactions affect neuronal protein synthesis.

6.0 ACKNOWLEDGEMENTS

Multiple Sclerosis Society of Great Britain

7.0 REFERENCES

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8. 0 LEGENDS TO FIGURES

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 3-5mm of the distal nerves.

FIG.2 Seventy micron section of the T9 DRG 32d after application of HRP to the proximal stumps of the internal and external intercostal nerves. (a) HRP-labelled cell bodies of various sizes are seen. (b) Plot of the position of all labelled cell bodies in this section by through focus analysis showing that the labelled cell bodies were randomly distributed throughout the DRG. Inset in (a): Unlabelled cell body with an eccentric nucleus (arrow) adjacent to HRP-labelled cell bodies. Only HRP-labelled cell bodies were analysed.

Fig. 3 Large and small DRG neurones retrogradely labelled after applying with HRP to the freshly cut proximal nerve stumps. (a) 70µm Arladite-embedded section mounted and trimmed for sectioning. (b) Enlargement of (a) showing the labelled large and small (arrow) DRG neuronal cell bodies. (c) Semithin toluidine blue section showing the same labelled DRG neurones. (d) Enlargement of (c).

FIG. 4 Non-operated cat. Light microscopic appearance of large (a), medium (b) and small(c) T7-9 DRG cell bodies. 2-3 satellite cells (arrow) are typically found adjacent to large- and medium- cell bodies.

FIG 5. Light microscopical features of axotomised DRG neurones. Semithin toluidine blue-stained sections. (a) Non-operated neuronal cell bodies are characterised by predominantly central nuclei and the presence of granular Nissl. (b) 1d following axotomy, slight nuclear eccentricity is occasionally seen (arrow), but otherwise there is little change from the non-injured DRG. (c) 2d following axotomy, nuclear eccentricity is more marked and frequent and some neurones show mild chromatolysis. Occasional neurones (inset) show clear areas within the normally compact nucleolus (nucleolar vacuolation) (d) 4d following axotomy marked nuclear eccentricity, often with nuclear elongation or crenation as well as chromatolysis (arrows) is seen.

FIG. 6. Percentage of DRG neurones showing chromatolysis or eccentric nuclei. See text for further details.

FIG. 7. DRG neurone 1d following nerve transaction. (a) HRP-labelled neurone (arrow) in 70µm section (b) same neurone (arrow) in semithin toluidine blue-stained section. Small Nissl bodies are visible. (c) Same neurone seen by EM (d) enlargement of (c) to show small patches of randomly-arranged polyribosomes and short fragments of RER.

FIG. 8. Mean ‘run’ distance for polyribosomes in Nissl bodies of DRG neurones after nerve transaction. The mean distance to nearest neighbour polyribosomes increased significantly by approximately 20% 6h after axotomy and remained so until 2d. (mean ± SEM)

FIG. 9. (a-c) DRG neurones 8d after RTD- axotomy showing marked chromatolysis, nuclear eccentricity and crenation. Panels b & c show clusters of satellite cells which may mark the sites of neuronal loss. (d-f) 32d after RTA-axotomy. Features of the axon reaction similar to that found at 8d are seen in small (d), medium (e) and large (f) neurones. Very occasionally, a peripheral rim of Nissl substance is seen giving the cell body a classical ‘central chromatolytic’ appearance.

FIG. 10. Clusters of satellite cells 33d after RTA-axotomy.

FIG. 11 (a) Swollen neurite packed with intermediate filaments and dense lamellar bodies 32d after RTD-axotomy. (b) enlargement of (a) showing lamellar bodies.

FIG. 12. Small DRG neurone with persisting retrograde response 64d after RTA-axotomy. (a) HRP cell body in 70μm section (b) same cell body in a semithin (b) and ultrathin (c) section showing eccentric nucleus and fragmented Nissl. (d & e) Small disorganised patches of RER and polyribosomes are seen amongst increased numbers of tubules and intermediate filaments.

FIG. 13. Peripheral portions of the cytoplasm of medium DRG cell bodies 64 days following RTA-axotomy (a) or RTD-axotomy (b). Lamellae of RER with orderly array of polyribosomes are only seen after TRA axotomy.

FIG. 14. Intercostal nerves (a) Normal, uninjured nerve approximately 40mm from the intervertebral foramen exhibiting a wide range of axon diameters, including several large-diameter, thickly-myelinated axons. (b) 64d following RTA-axotomy, approximately 20mm distal to the axotomy site and 40mm distal to the intervertebral foramen. Axonal regeneration is occurring, but the axons are small and thinly-myelinated. (c) 64d following RTD-axotomy, approximately 20mm distal to the axotomy site and 40mm distal to the intervertebral foramen. No axonal regeneration is seen.

FIG. 15. Evidence of functional reinnervation of external intercostal muscle 64d following RTA-axotomy. Upper trace: integrated inspiratory air flow. Middle trace: control EMG from the intact T7 levator costae muscle. Lower trace: EMG from a reinnervated portion of the T7 external intercostal muscle. See text for details.

FIG. 16. Intercostal nerve 64d following crush and placement in a dialysis tube (t) (MW cut off 100,000). (a) The tube lumen is filled with Schwann cells, fibroblasts and blood vessels. Occasional small regenerating nerve fascicles (arrows) are seen. Semithin section. (b) By EM the axons in the nerve fascicles seen in (a) are shown to be thinly myelinated or unmyelinated and the fascicle is surrounded by much collagen.

FIG. 17. Comparison of the RER and polyribosomes in a Nissl body (a) and a pancreatic acinar cell (b). Nissl bodies have far fewer membrane bound ribosomes and many more free polyribosomes than the aggregates of RER seen in typical secretory cells.

9.0 HIGHLIGHTS

- Local endoplasmic reticulum changes play a critical role in the response of most cells to stress.
- We show that the RER of Nissl bodies in cat DRG neurones was disrupted as early as 6h after axotomy.
- DRG Nissl body ultrastructure was peripheral target-dependent.
- This may represent peripheral target regulation of local protein synthesis.

Fig. 1

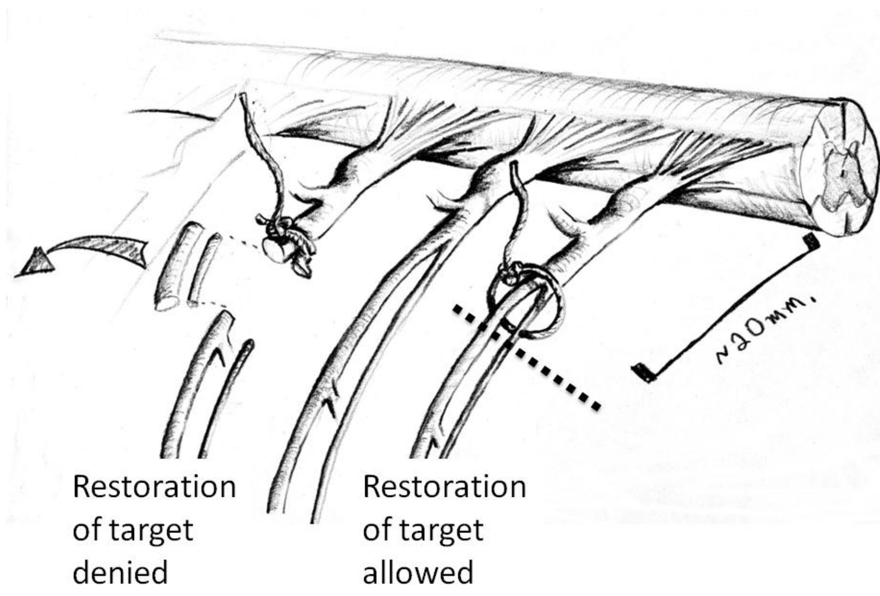


Fig. 2

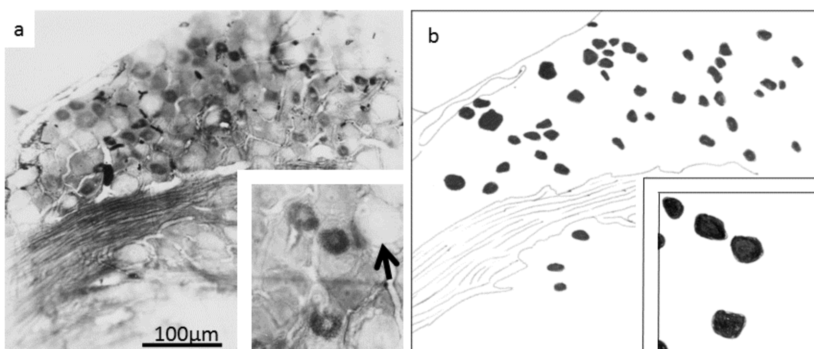
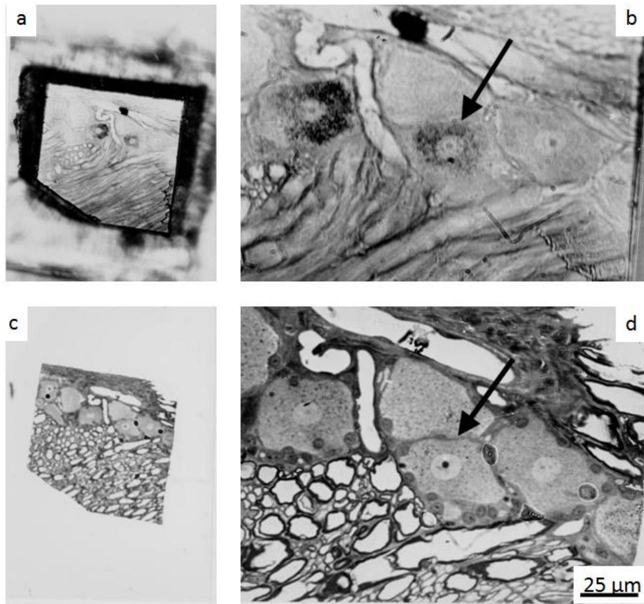


Fig. 3



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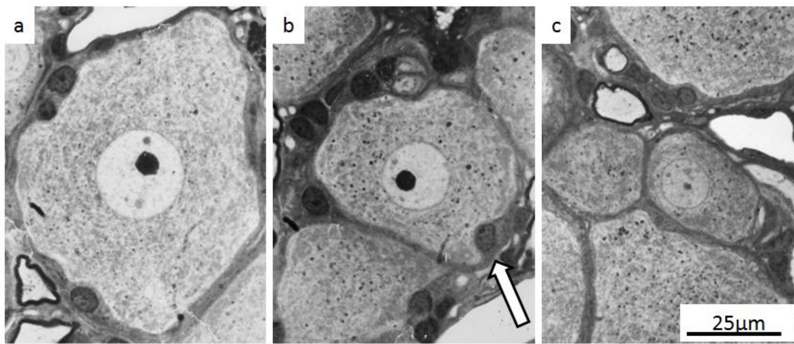
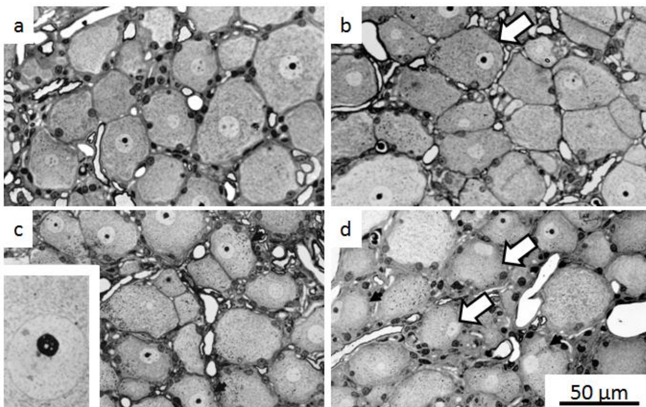


Fig. 5



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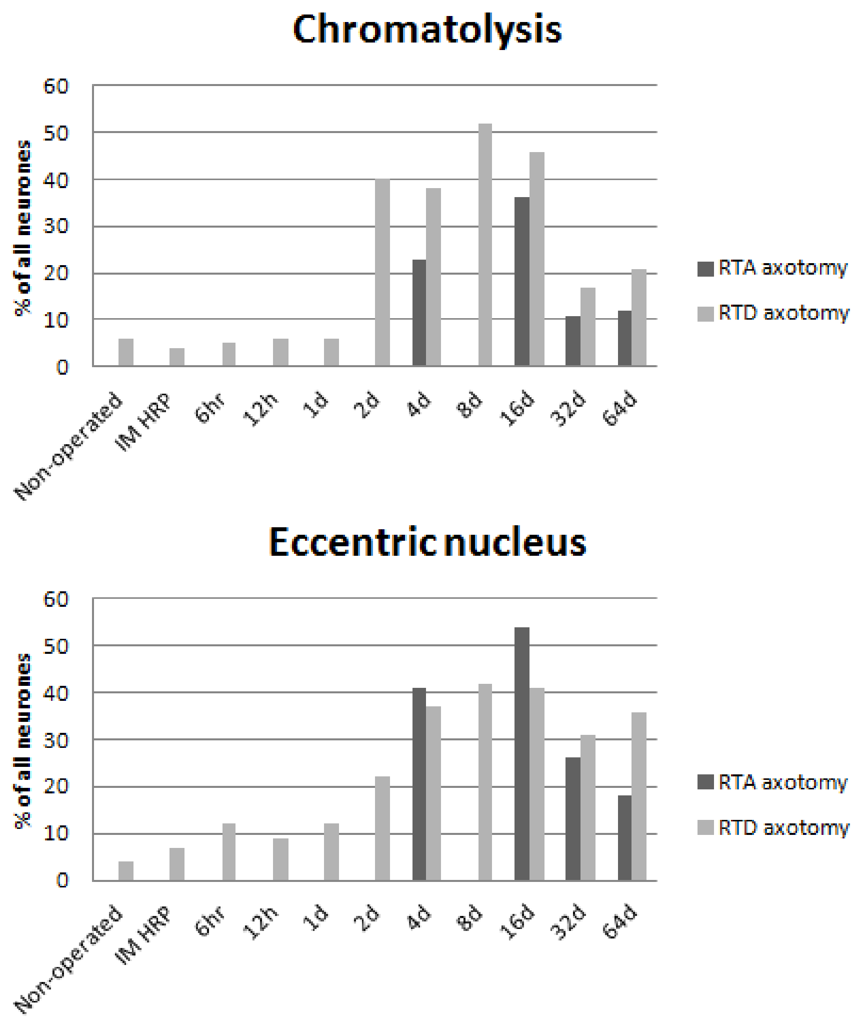

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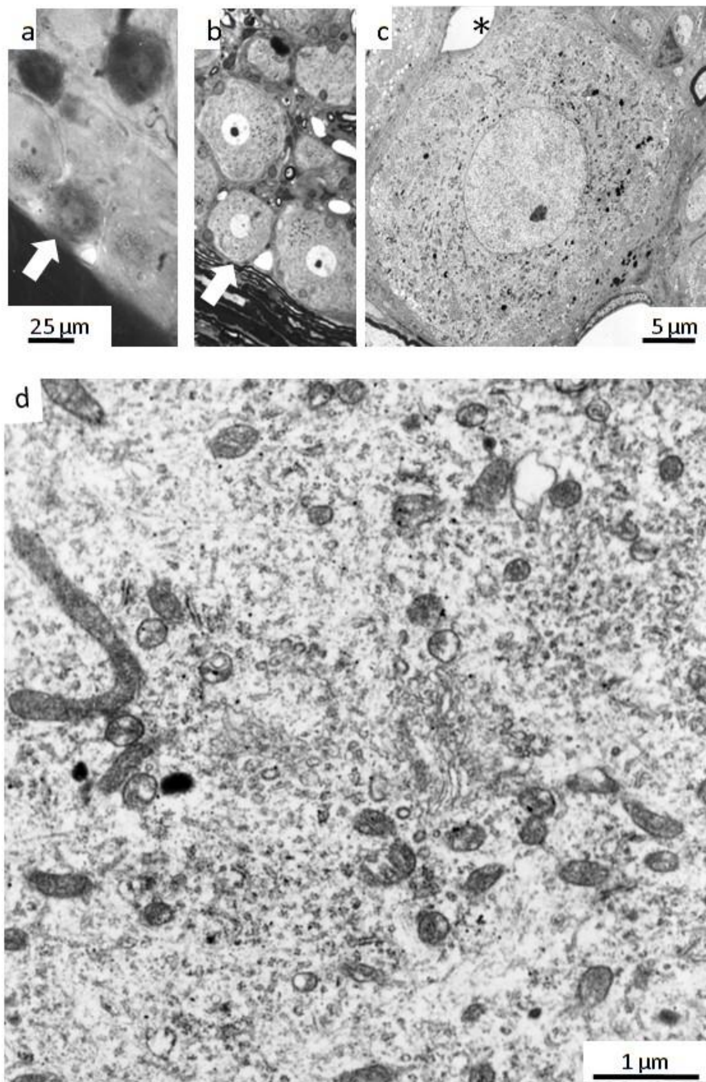

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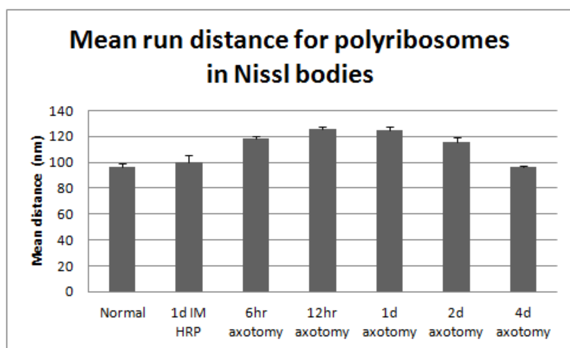

Fig. 9

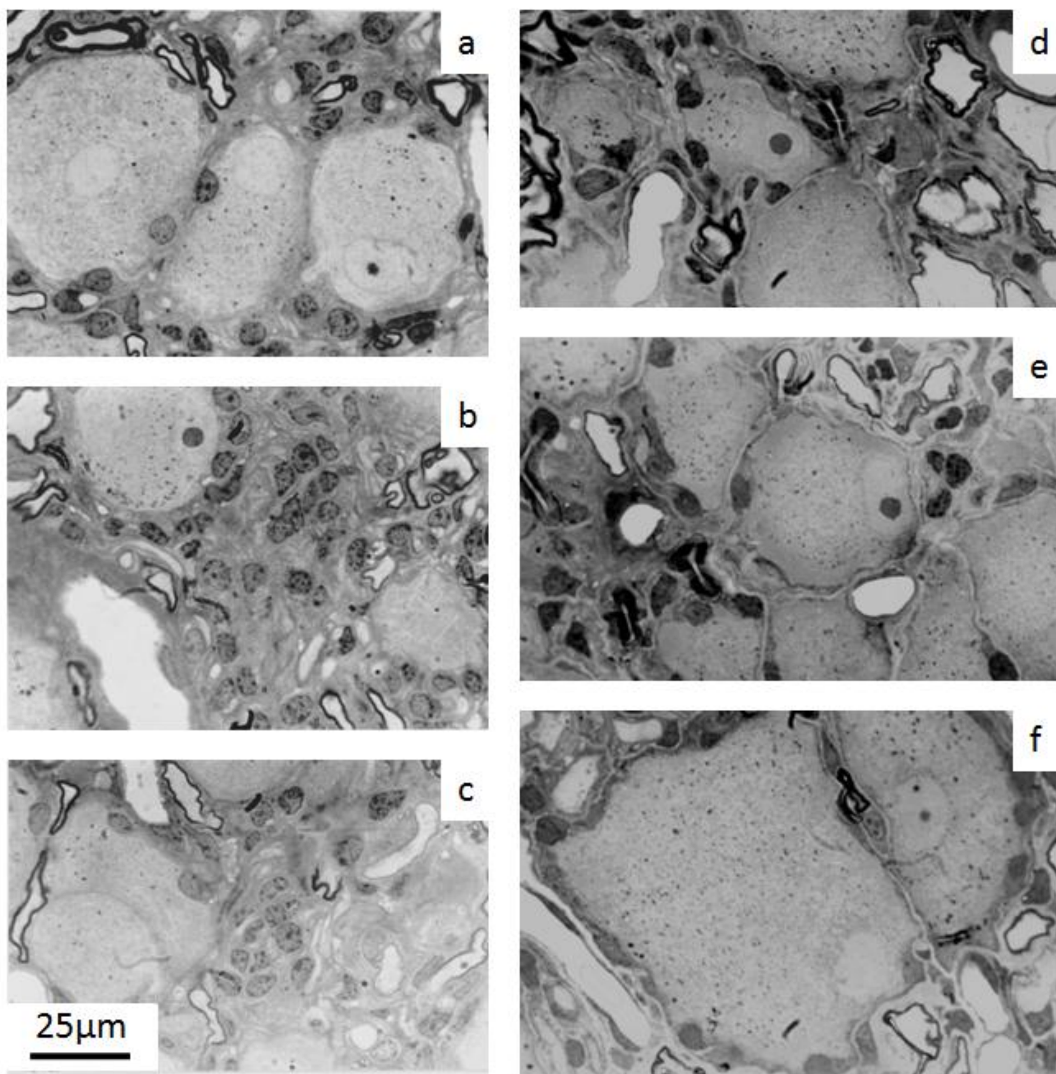

Fig. 10

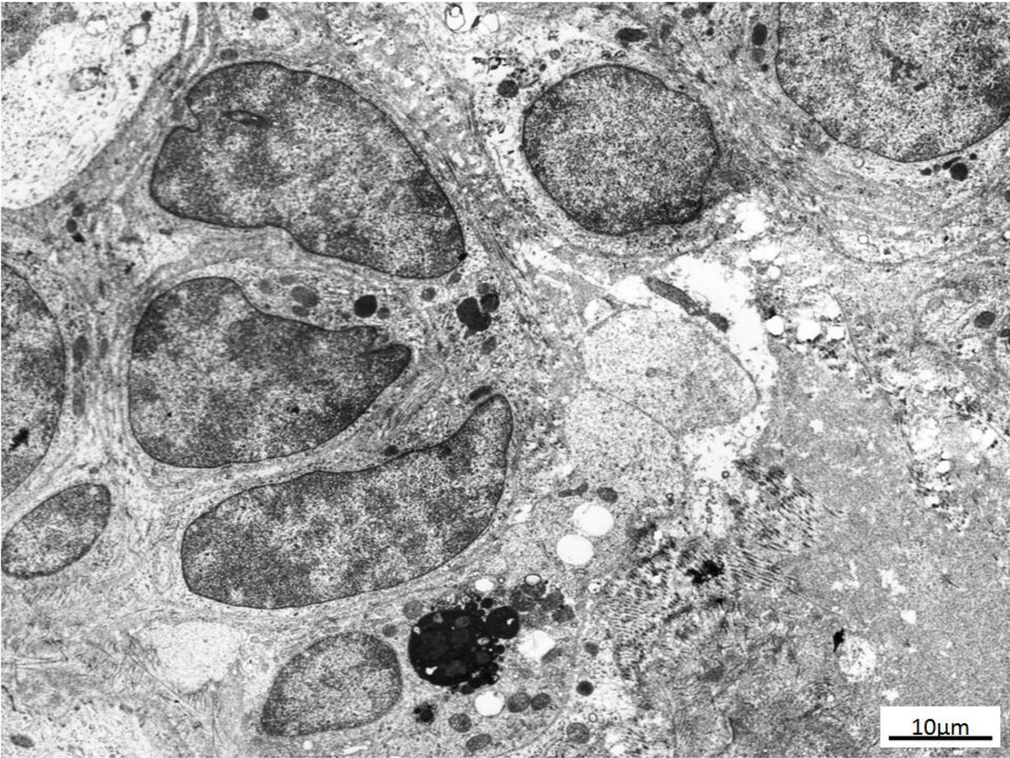

Fig.11

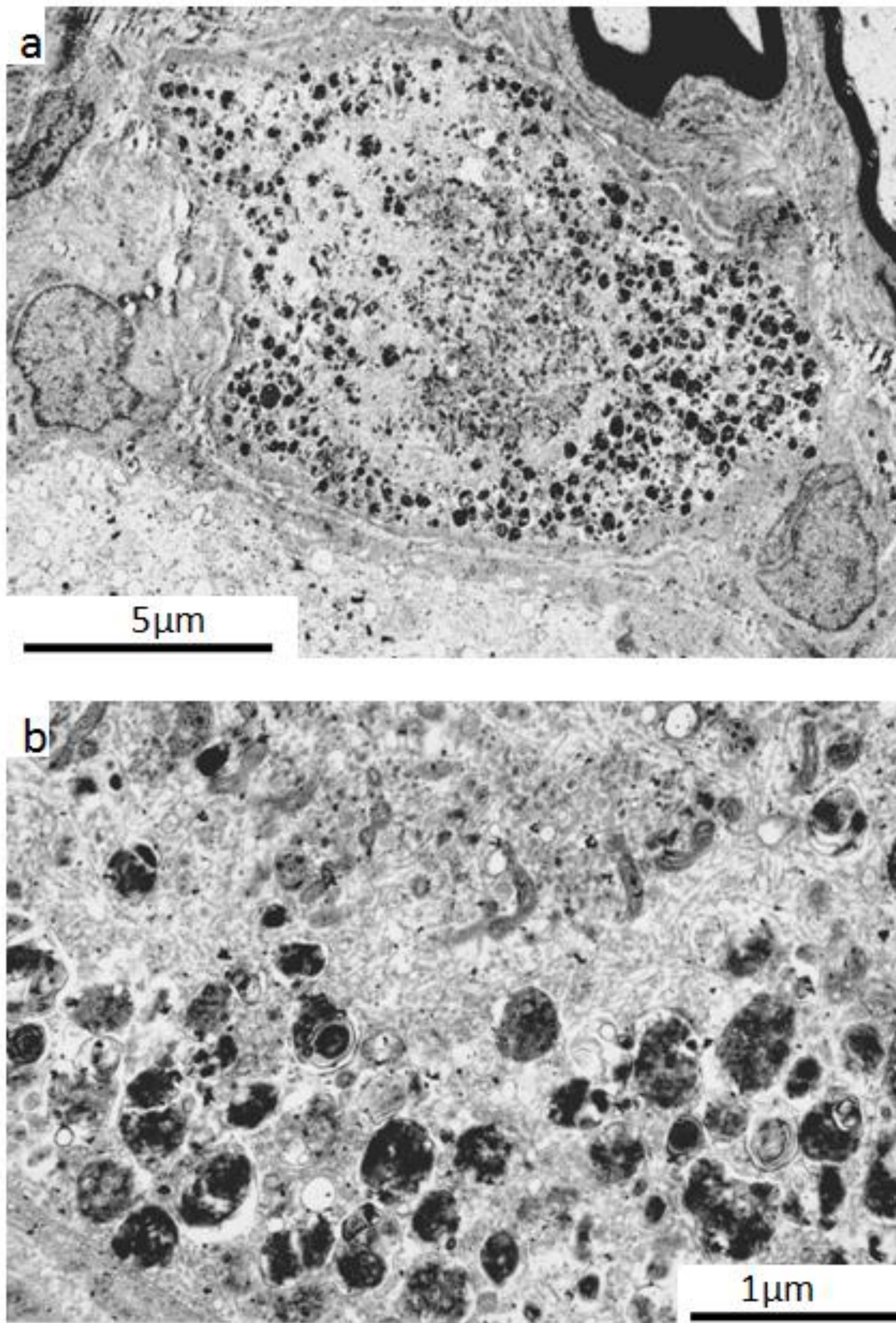

Fig. 12

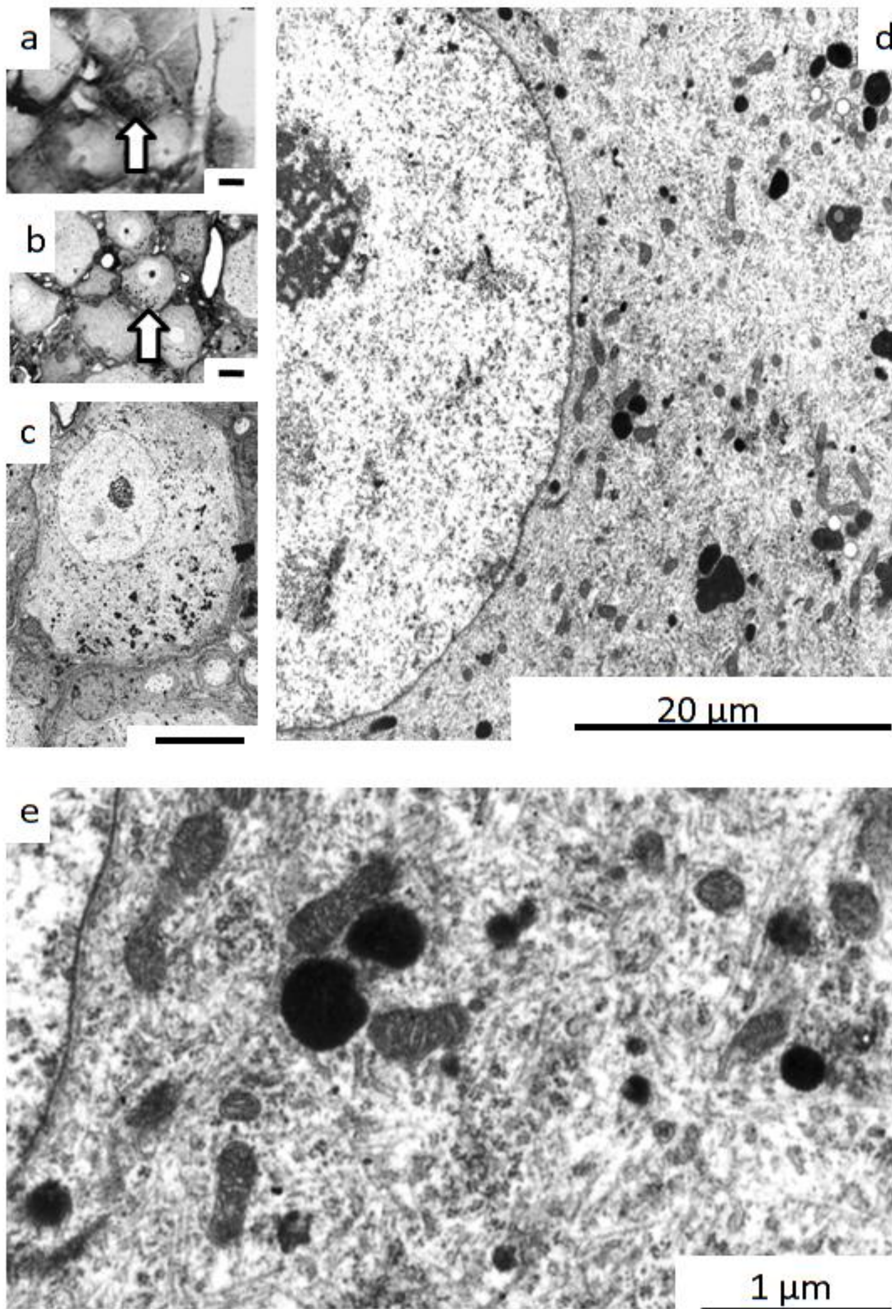

Fig. 13

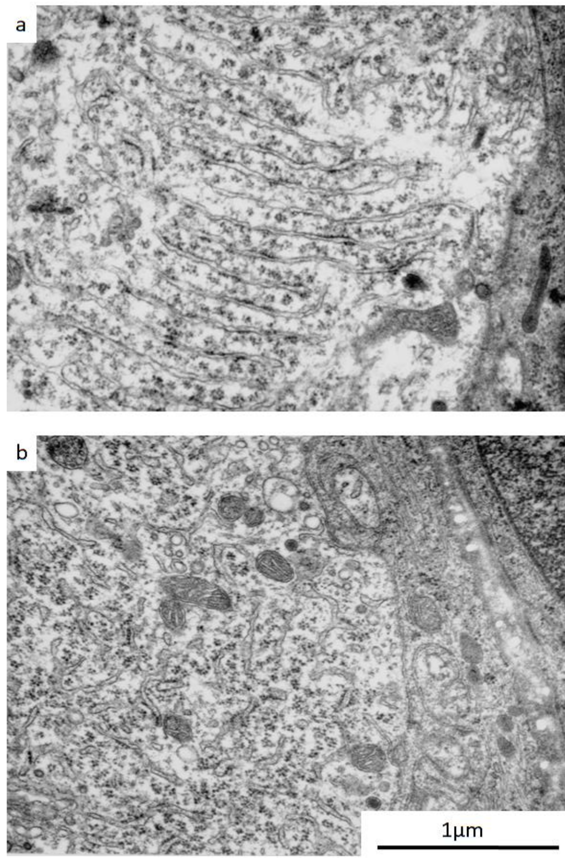

Fig. 14

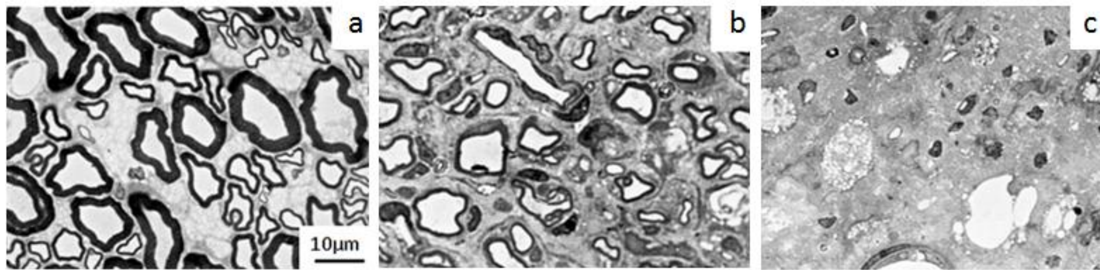

Fig.15

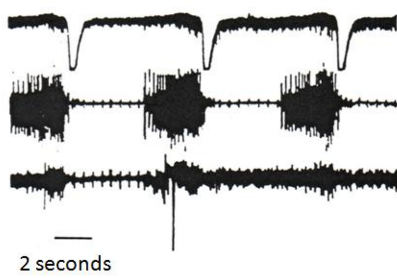

Fig 16.

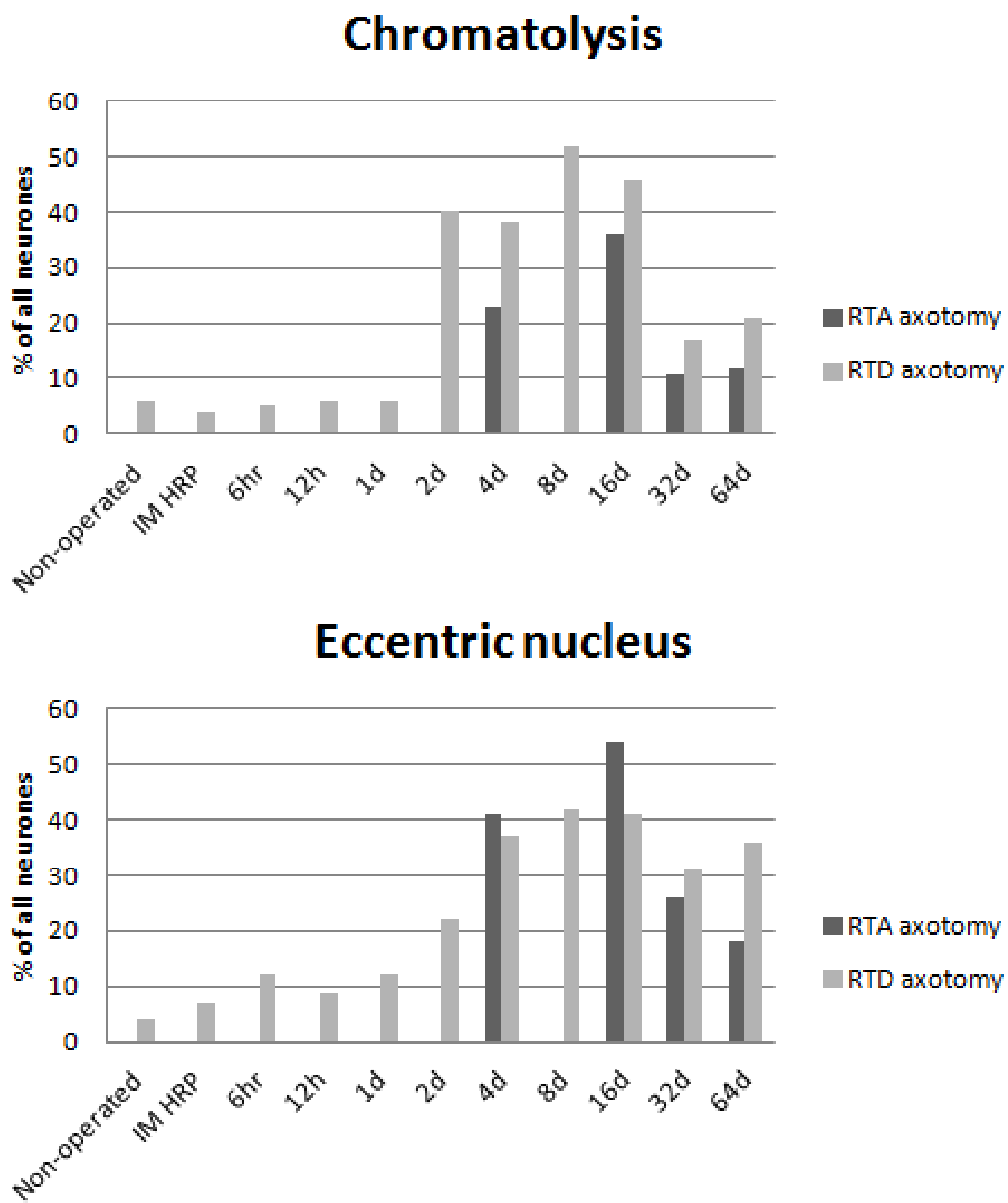

Fig. 17