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An Ultrastructural Classification of the Neuronal Cell Bodies of the Rat Dorsal Root Ganglion Using Zinc Iodide-Osmium Impregnation

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Summary. Zinc iodide-osmium (ZIO) impregnation of rat dorsal root ganglia differentially stained various elements in the neuronal cells, particularly their Golgi bodies. On the basis of this differential ZIO staining dorsal root ganglion neurones have been classified into seven types. The ultrastructure of these is described and the numbers of each type in the L4 dorsal root ganglion have been determined. Prolonged nerve stimulation did not change the relative numbers of the different cell types suggesting that none of the differences between cell types represents differences in their state of activity. The possibility is discussed that differences in morphology may reflect differences in neurotransmitter function.

Key words: Spinal ganglia – Golgi apparatus – Afferent neurones – Spinal nerve roots – Synaptic vesicles.

Introduction

There is general agreement that when the neuronal cell bodies in dorsal root ganglia (DRG) are examined by either light or electron microscopy they may be divided into two types: (1) 'large light' cells and (2) 'small dark' cells (see Lieberman, 1976, for review). A number of authors (e.g. Clark, 1926; Scharf, 1958; Andres, 1961) have further sub-classified light and dark cells by light microscopy. In particular Andres (1961) described six types of neuronal cell body in rat lumbar DRG and measured their relative frequencies. He termed his three types of light cell A_1–A_3, and his dark cells B_1–B_3. Andres' detailed classification was based on phase-contrast microscopy although he does show an electron micrograph of the cytoplasm of an A cell and of a B cell. Jacobs et al. (1975) describe the ultrastructural appearance of A_1, A_2 and B_1 cells but no full classification at the ultrastructural level has been presented.

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There is currently a great deal of interest in determining whether the morphological appearance of the different types of primary afferent neurone can be correlated either with the modality which they subserve (e.g. proprioception, nociception), or with the neurotransmitter which they utilise. Suggested transmitters include glutamate (Curtis and Johnston, 1974) and the peptides substance P and somatostatin (Hökfelt et al., 1976). Differences between cells utilising different neurotransmitters may be reflected in the ultrastructure of the organelles involved in the synthesis, packaging and transport of the various neurotransmitters and their associated molecules, i.e. the Golgi apparatus, endoplasmic reticulum and mitochondria. We have found that Zinc Iodide-Osmium (ZIO) impregnation, often held to be specific for transmitter-related organelles, stains a variety of structures in DRG neuronal cell bodies and thereby emphasises differences between sub-types of A and B cells.

This work presents an ultrastructural classification of the different types of neurone in ZIO-impregnated DRG. The possibility existed that differences between cell types might reflect different states of activity and so we have also examined the effect of prolonged electrical stimulation on the morphology of these cells.

Materials and Methods

Six female Wistar rats (200–250 g) were anaesthetised with urethane (1.38 g/Kg s.c.) and the nerves of the left hind limb were electrically stimulated (40 v, 0.2 Hz. square wave pulses, 1 ms duration) via two electrodes, one in the foot pad and the other subcutaneously in the thigh. Stimulation was continued for 24 h after which time these animals and six control rats were killed by transcardiac perfusion with 6.25% glutaraldehyde in 0.1 M phosphate buffer. The left L4 DRG were removed and fixed for a further two hours in the same fixative. The tissue was processed by the method of Akert and Sandri (1975) as follows: DRG were washed 3 times in a 1:1 dilution of the following buffer: 3.3 g NaCl, 0.06 g $CaCl_2$, 0.31 g $MgCl_2$ 0.605 g tris, distilled water to 50 mls, brought to pH 7.4 with HCl. Tissues were impregnated overnight in ZIO solution prepared as follows: 6 g of zinc metal and 2 g of iodine were added to 40 mls of distilled water and the mixture was shaken, cooled and filtered. Four mls of this solution was added to 4 mls of Tris HCl buffer and 2 mls of 2% aqueous osmium tetroxide solution. The ganglia were then rapidly dehydrated in a graded ethanol series, embedded in Spurr resin and sectioned on a Porter Blum MT2B ultramicrotome using a diamond knife. Sections were picked up on uncoated grids and stained with lead citrate (Reynolds, 1963). All cell types were classified by direct examination in a Hitachi HU12A electron microscope at a wide range of magnifications. The cell types were counted on sections cut at 3 levels through DRG L4 of control and electrically stimulated animals. All cells visible in each section were classified by the method above and were counted.

Results

Direct electron microscopic examination of ZIO-impregnated DRG from normal rats suggested that the neuronal cell bodies could be divided into seven types. The classification into large light (A) cells and small dark (B) cells is generally accepted and so this nomenclature was retained, but subdivision of these types was initially made entirely on ultrastructural grounds and without reference to the classification adopted by other authors. Where there subsequently proved to be an obvious correlation between any of our classes and those of Andres (1961) the latter author's nomenclature has been retained.

Large Light (A) Cells

Our examination of the fine structure of the L4 DRG confirmed that a population of large light cells was clearly distinguishable. The light appearance of these cells was due to the fact their cytoplasm contained islands of ribosomes separated by strands of neurofilaments (Figs. 1b, 2b, 3b).

Type A_1 (Fig. 1)

The cytoplasm of A_1 cells contained a high proportion of neurofilaments giving it a particularly light appearance. Some profiles of smooth endoplasmic reticulum were impregnated with ZIO. The Golgi bodies were small, simple and scattered throughout the cytoplasm. They were straight and contained three or four lamellae, the inner of which stained with ZIO. In cells from stimulated rats the inner lamellae were intensely stained with ZIO and there was darkening of the outer lamellae. A_1 cells contained two types of mitochondria: the conventional type with transverse cristae and also numerous smaller 'type I' mitochondria which in both longitudinal and transverse section consisted of two concentric membranes separated by electron-dense material and have previously been described as accumulating in the large myelinated axons of sectioned dorsal roots (Duce and Keen, 1976).

Type A_2 (Fig. 2)

In A_2 cells the neurofilaments were restricted to strands running between the islands of ribosomes, these islands being larger than in A_1 cells. The principal feature which distinguished A_2 cells from their A_1 counterparts was their Golgi apparatus. A_2 Golgi profiles were very much larger, more complex and fewer in number. They were long and strongly curved, often in the form of dictyosomes. They contained more lamellae (6–8) than those of A_1 cells and the inner lamellae stained with ZIO. Following nerve stimulation the outer lamellae also stained with ZIO and the inner lamellae were fragmented. The mitochondria appeared denser and more elongated than those of A_1 cells and in some cases were curved.

Type A_3 (Fig. 3)

A_3 cells were classed as light cells because their cytoplasm contained strands of neurofilaments although these were less abundant than in A_1 or A_2 cells. A high proportion of the ribosomal material was in the form of rough endoplasmic reticulum. The Golgi bodies were long and curved. The inner lamellae were characteristically fragmented into short segments and vesicles which stained with ZIO. Following nerve stimulation the inner lamellae were more fragmented and ZIO-positive vesicles were more numerous. The cytoplasm contained a large number of dense bodies, many of which stained with ZIO. A further characteristic of these cells was their numerous round or ovoid mitochondria.

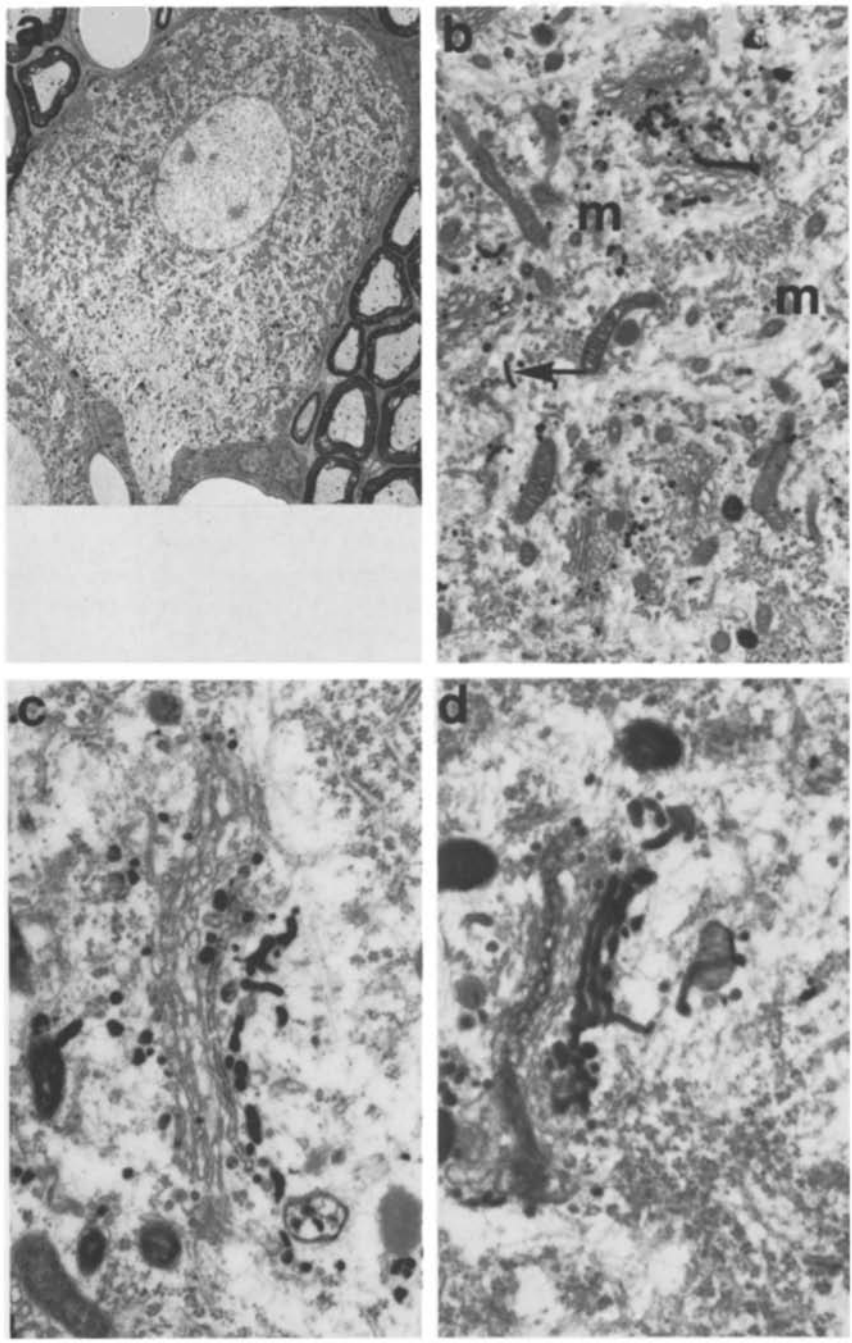

Fig. 1a–d. Electron micrographs of cell type A_1.**a** Cell body; ×1150. **b** Cytoplasm containing simple Golgi bodies, type I mitochondria (*m*) and ZIO stained endoplasmic reticulum (→); ×10,690. **c** Golgi body from an A_1 cell of a normal rat; ×27,450. **d** Golgi body from an A_1 cell of an electrically stimulated rat; ×27,450.

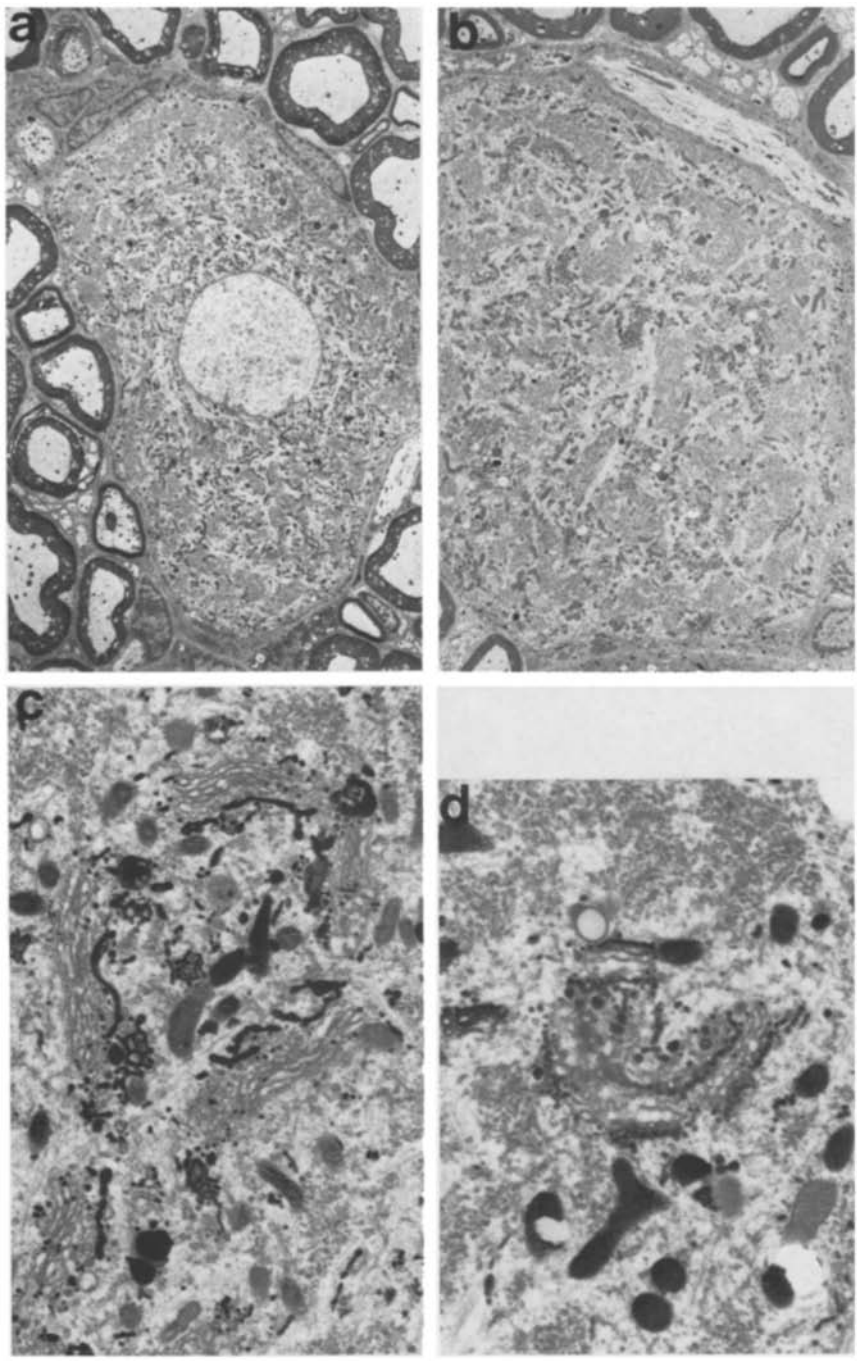

Fig. 2a–d. Electron micrographs of *cell type* A_2. **a** Cell body; ×1600. **b** Cytoplasm showing strands of neurofilaments; ×2520. **c** Golgi body from an A_2 cell of a normal rat; ×13,500. **d** Golgi body from an A_2 cell of an electrically stimulated rat; ×13,500

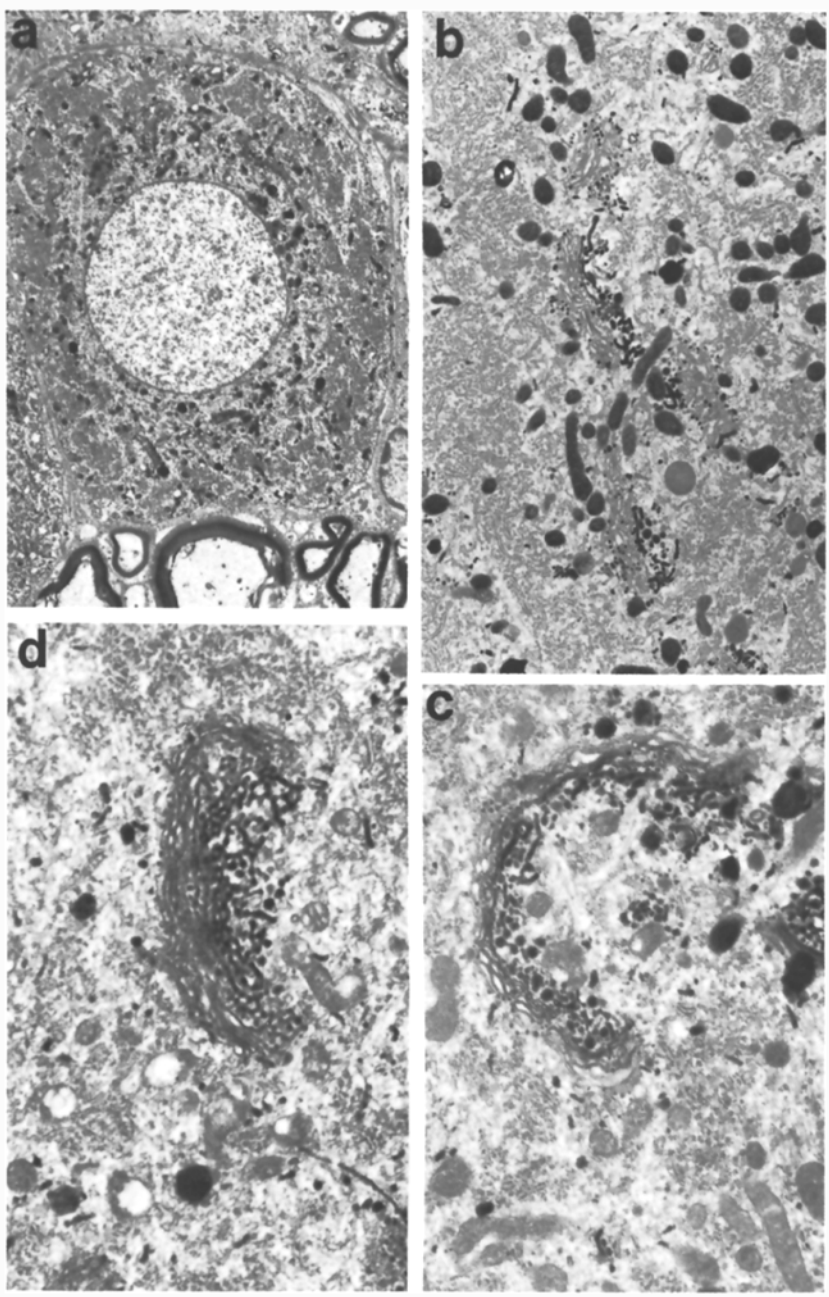

Fig. 3a–d. Electron micrographs of *cell type* A_3. **a** Cell body; ×2815. **b** Cytoplasm showing large Golgi bodies and dense bodies; ×9280. **c** Golgi body from an A_3 cell of a normal rat; ×12,870. **d** Golgi body from an A_3 cell of an electrically stimulated rat; ×12,870

Small Dark (B) Cells

The remaining neuronal cell bodies were classed as small dark cells on the grounds that strands of neurofilaments were absent from their cytoplasm which consequently had an even, dark appearance.

Type B_1 (Figs. 4 and 5)

In B_1 cells the peripheral cytoplasm was largely occupied by rough endoplasmic reticulum, much of it running parallel with the plasmalemma. In consequence the other organelles were concentrated in the central part of the cell. The Golgi bodies were large and numerous and, following nerve stimulation, became so osmiophilic that individual lamellae became almost indistinguishable. Following stimulation there was also a marked increase in the extent of the GERL complex. B_1 cells also contained dense-cored vesicles and numerous mitochondria and dense bodies. Cells having these general characteristics can be further subdivided on the basis of consistent ultrastructural differences which are emphasised by ZIO impregnation.

Sub-Type $B_{1\alpha}$ (Fig. 4). These cells had a dark osmiophilic cytoplasm and abundant peripheral rough endoplasmic reticulum. A striking feature was their large, complex and deeply-stained Golgi bodies which were not confined to the perinuclear cytoplasm. These Golgi bodies were long, frequently straight and usually stained with ZIO throughout. They consisted of approximately six lamellae with a prominent GERL complex. The outer lamellae were characteristically very thin and closely stacked.

Sub-Type $B_{1\beta}$ (Fig. 5). When compared with that of the α sub-type the cytoplasm of $B_{1\beta}$ cells was lighter and contained less rough endoplasmic reticulum. The Golgi bodies of $B_{1\beta}$ cells were clearly and consistently different from those of $B_{1\alpha}$ cells in the following respects: 1) they were more strongly curved and often formed dictyosomes 2) they had a greater tendency to be perinuclear 3) their outer lamellae were widely dilated 4) only the GERL complex stained with ZIO, the lamellae remained unstained. Nerve stimulation led to increased ZIO-staining of all $B_{1\beta}$ Golgi elements; however, the Golgi bodies were still clearly distinguishable from those of $B_{1\alpha}$ cells. The mitochondria of $B_{1\beta}$ cells were in general shorter, more rounded and less dense than those of the α sub-type.

Type B_2 (Fig. 6a, b)

In contrast to B_1 cells, in which peripheral rough endoplasmic reticulum displaced other organelles to the centre, the organelles of B_2 cells were distributed evenly throughout the cytoplasm. B_2 cells were characteristically small and elongated. In transverse section (Fig. 6b) the nucleus was centrally placed, whilst in longitudinal section (Fig. 6b) it was displaced to one end. The Golgi bodies were small, simple and few in number. They consisted of five or six short lamellae only the inner of which stained with ZIO. Mitochondria were ovoid with transverse cristae.

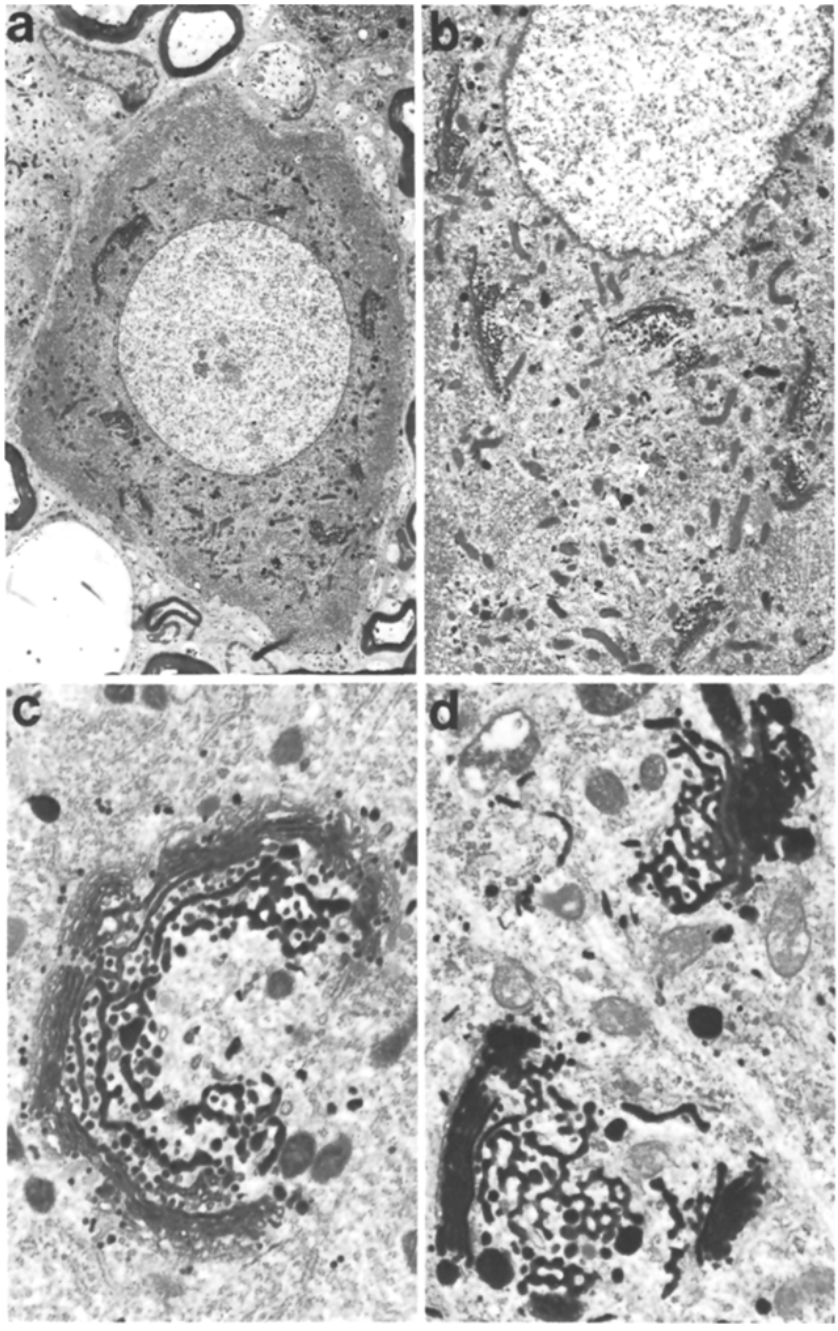

Fig. 4a–d. Electron micrographs of *cell type* $B_{1\alpha}$. **a** Cell body; ×2125. **b** Cytoplasm showing straight ZIO stained Golgi bodies; ×4950. **c** Golgi body from a $B_{1\alpha}$ cell of a normal rat. Note the flat outer lamellae and inner GERL complex; ×19,260. **d** Golgi body from a $B_{1\alpha}$ cell of an electrically stimulated rat showing increased ZIO staining and extensive GERL complex; ×19,260

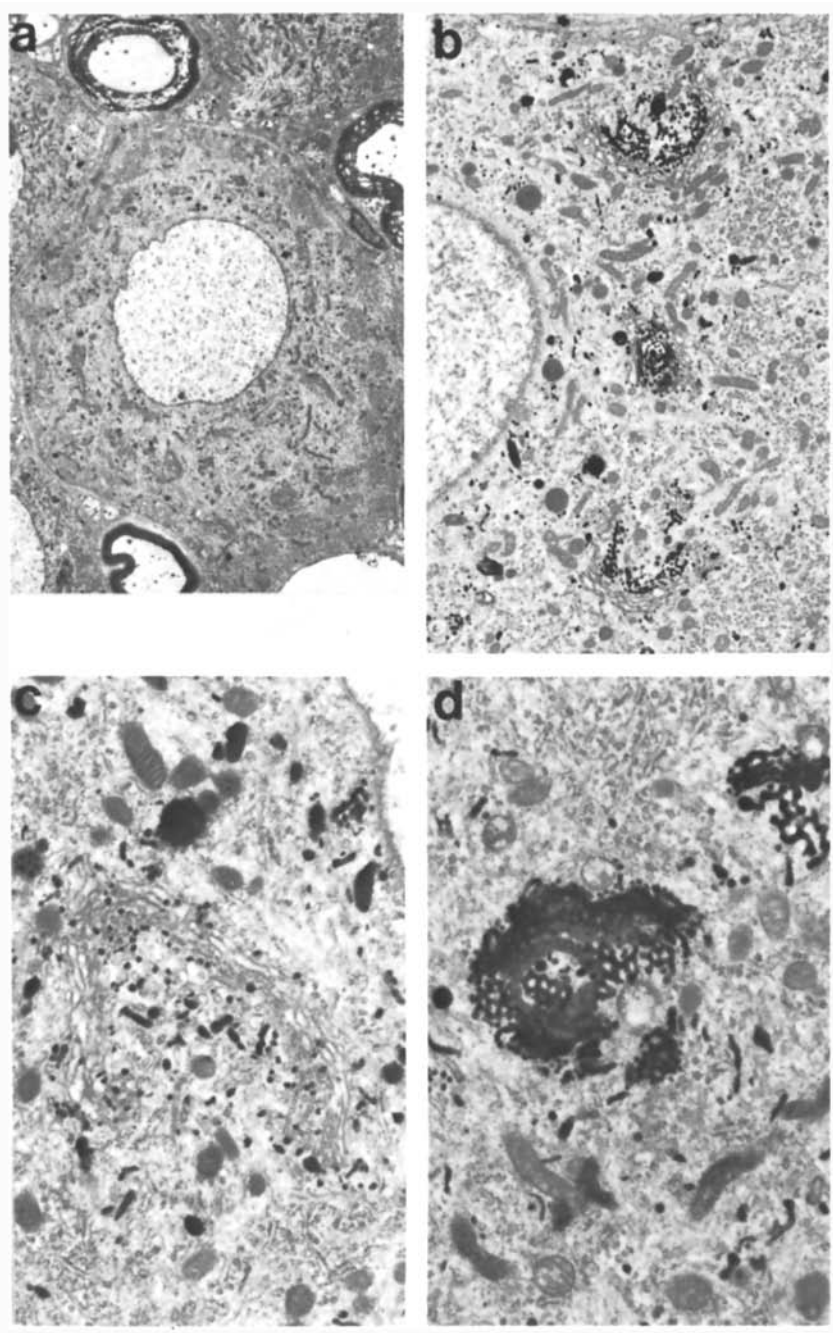

Fig. 5a–d. Electron micrographs of *cell type* $B_{1\beta}$. **a** Cell body; ×2010. **b** Cytoplasm showing curved Golgi bodies with dilated outer lamellae and ZIO stained GERL complex; ×6890. **c** Golgi body from $B_{1\beta}$ cell of a normal rat. Note the dilated unstained lamellae; ×15,210. **d** Golgi body from $B_{1\beta}$ cell of an electrically stimulated rat showing increased ZIO staining and extensive GERL complex; × 15,210

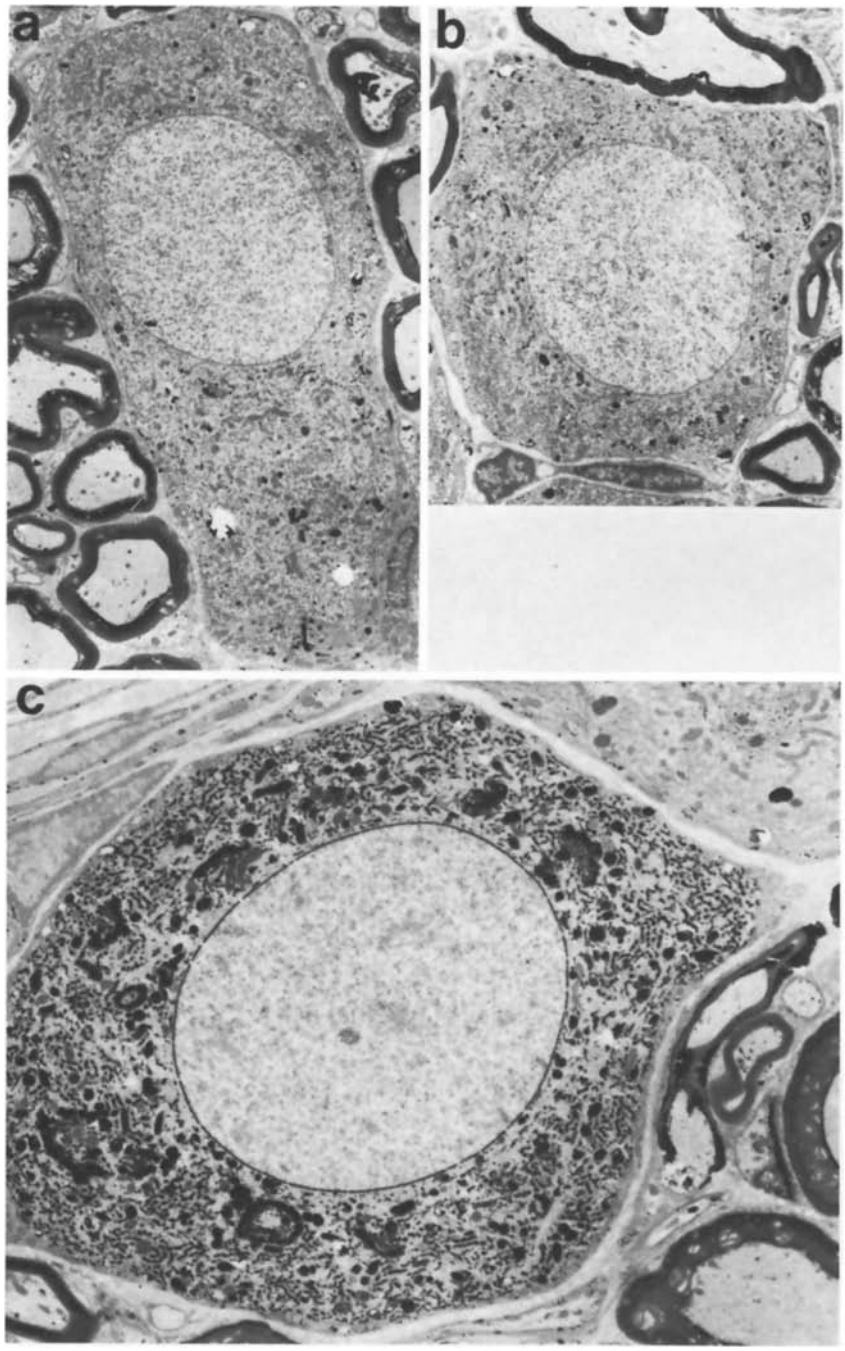

Fig. 6 a and b. Electron micrographs of *cell type* B_2. **a** Longitudinal section through a B_2 cell showing the displaced nucleus and organelles evenly distributed throughout the cytoplasm; ×2925. **b** Transverse section through a B_2 cell; ×2925. **c** Electron micrograph of a B_3 *cell* showing many dense bodies and ZIO staining of the Golgi bodies, nuclear membrane and extensive endoplasmic reticulum; ×3825

Table 1. Percentage distribution of different cell types in L4 DRG of electrically-stimulated and control rats.

	A_1	A_2	A_3	$B_{1\alpha}$	$B_{1\beta}$	B_2	B_3
Control ($n=358$)	44	9	4	22	15	6	<1
Stimulated ($n=120$)	42	9	10	20	11	5	3

Type B_3 (Fig. 6c)

These were quite distinct from all other cell types in that they were small, polygonal in shape and deeply stained with ZIO throughout. The endoplasmic reticulum, which was entirely impregnated with ZIO, extended throughout the cytoplasm. The nucleus was relatively large and had a ZIO-positive membrane. The Golgi bodies were large with six flattened lamellae, all of which stained strongly with ZIO.

Counting of Cell Types and the Effect of Nerve Stimulation

Table 1 shows the percentages of the different cell types in L4 DRG. The cell types were mapped and found to be randomly distributed throughout the ganglia. It seemed possible that the differences between certain of the cell types described above might represent differences in the state of activity of a single cell type. To investigate this possibility the nerves of one hind-limb were subjected to prolonged electrical stimulation and the effect of this on the morphology of the cells in the ipsilateral L4 DRG was determined. As described in detail above, electrical stimulation affected the morphological appearance of each of the cell types. However, despite their changed morphology, the different cell types were still easily recognisable and their relative numbers had not been significantly changed by the stimulation (Table 1).

Discussion

It was initially suggested that the ZIO technique specifically stained neurotransmitter vesicles (Akert and Sandri, 1968; Pellegrino de Iraldi and Gueudet, 1968). Although it has since been reported that other neuronal organelles may react with ZIO (Osborne and Thornhill, 1974) we thought the application of this technique to dorsal root ganglia (DRG) neurones might be worthwhile as part of a study of their neurotransmitter mechanisms. We report here that ZIO consistently gives differential staining of the Golgi bodies and other elements in the cell bodies of DRG neurones. It was suggested that ZIO may react with tissue-SH groups (Pellegrino de Iraldi, 1976) or alternatively that ZIO staining may correlate with phosphatase activity (Griffith and Bondareff, 1972). Our own studies (Wilks et al., in preparation) suggest a strong correlation between ZIO staining and acid phosphatase activity in DRG cells.

Table 2. Percentage distribution of different cell types in rat DRG

Cell type	Andres (1961)		This study	
Cell type	uncorrected	corrected	uncorrected	corrected
A_1	37	25	44	31
A_2	2	2	9	8
A_3	7	8	4	5
$B_{1\alpha}$	47	56	22	27
$B_{1\beta}$			15	19
B_2	6	10	6	10
B_3	1	1	<1	–

Corrected values obtained as described in text

Differential ZIO staining has enabled us to describe seven types of neurone in the L4 DRG of the rat. Some types of cell cannot immediately be distinguished in low-power electron micrographs but they are clearly identifiable if individual cells are examined at a range of magnifications. For this reason the classification and counting of neurones was carried out by direct examination in the electron microscope. Prolonged electrical stimulation did not change the relative numbers of the different cell types, suggesting that morphological differences did not reflect different states of activity.

Several authors (Clark, 1926; Scharf, 1958; Andres, 1961) have classified DRG neurones using light microscopy and have summarised attempts to ascribe to the various cell types a specific sensory modality. The present ultrastructural classification was initially made quite independently of these light microscope classifications. When our classification was completed, however, it was obvious that certain of our classes corresponded closely with those which Andres (1961) described using phase-contrast light microscopy. For groups where such a correspondence existed Andres' nomenclature has been retained.

Table 2 shows the percentage distribution of the different cell types in Andres' (1961) study of rat L5, L6 DRG compared with the values obtained in the present study. Section thickness and cell size must be taken into account when calculating the actual numbers of cells present in the tissue. The corrected percentages in Table 2 were obtained using the relationship given by Elias et al. (1971): $N = n/(D + t)$ where N = the corrected number, n = number of profiles counted, D = average diameter of the cells and t = section thickness (assumed to be 70 nm and 500 nm for the present study and that of Andres (1961) respectively).

We have followed Andres (1961) in classifying all light cells, i.e. those having strands of neurofilaments in their cytoplasm, as *A cells.*

A_1 cells

These are the most common of the large light cells and on grounds of morphology and numbers are almost certainly the same as the A_1 cells of Andres (1961). Jacobs et al. (1975) show an electron micrograph of an A_2 cell which appears from the

distribution of neurofilaments and ribosomes in the cytoplasm to be an A_1 cell on our classification. Our criteria, however, include a number of other features, particularly the form of the Golgi apparatus.

A_1 cells have small, simple Golgi bodies and relatively fewer ribosomes than other cell types, suggesting that they are unlikely to produce a peptide neurotransmitter. Large light cells are thought to give rise to the large myelinated axons which carry proprioceptive impulses (Warrington and Griffith, 1904) and which, it is thought, may release glutamate as neurotransmitter (Curtis and Johnston, 1974). Now it has been suggested that neurotransmitter glutamate may be synthesised in a specific metabolic compartment residing in a separate population of mitochondria (Neidle et al., 1969; van den Berg et al., 1969) and so it may be relevant to note that A_1 cells contain large numbers of an unusual type of mitochondrion. These mitochondria accumulated in large myelinated axons of sectioned dorsal roots (Duce and Keen, 1976) and could possibly be those involved in the metabolism of neurotransmitter glutamate.

Cell Types A_2 and A_3

We distinguish two further types of light cell, A_2 and A_3, both of which are much less frequent in occurrence than A_1. It is uncertain whether they correspond to types A_2 and A_3 of Andres (1961) because the latter based his light microscope classification largely on the distribution of Nissl substance in cytoplasm. The present study on the other hand is based on ultrastructural examination which makes it possible to differentiate between the different types of A cell on the basis of the form of their Golgi apparatus. The larger Golgi bodies of A_2 and A_3 cells, and the frequency of dense bodies in A_3 cells may indicate that their neurotransmitter mechanisms are different from those of A_1 cells.

Following the nomenclature of Andres (1961) we have classified small dark cells as *type B*. Electron microscopy shows that their dark appearance is due to the fact that they have no bands of neurofilaments in their cytoplasm.

B_1 Cells

B_1 cells are equivalent to the B_1 cells of Andres (1961) in both morphology and frequency of occurrence. These cells have large and complex Golgi bodies, abundant rough endoplasmic reticulum and dense bodies and contain large dense-cored vesicles in their cytoplasm. These features all suggest that these cells may produce and package proteins or polypeptides.

We clearly distinguish two subtypes of B_1 cell. Since the proportions of these remain unchanged following prolonged electrical stimulation we are confident that they are in fact different types and so have classified them as $B_{1\alpha}$ and $B_{1\beta}$. It is possible that the morphological differences between these sub-types, particularly with respect to their Golgi bodies, may be attributable to differences in their neurotransmitter mechanisms. The Golgi bodies of the α-subtype stain throughout with ZIO. As discussed above the significance of ZIO staining is uncertain but could

indicate the presence either of -SH groups or of phosphatase activity. Carrier molecules for polypeptide hormones are known to be rich in -SH groups and so the ZIO reaction in the Golgi bodies of $B_{1\alpha}$ cells may indicate that these are packaging such a carrier molecule. Alternatively Knyihar (1971) has shown that the small dark cells involved in nociception exhibit fluoride-resistant acid phosphatase activity and, if ZIO-staining is correlated with phosphatase activity, this raises the possibility that the $B_{1\alpha}$ neurones may be nociceptive. Recent evidence suggest that nociceptive afferents may release substance P (Henry, 1976). Using immunocytochemistry Hökfelt et al. (1976) have described two populations of B cells in DRG which contain respectively substance P and somatostatin. These two populations could correspond to our $B_{1\alpha}$ and $B_{1\beta}$ cells; alternatively, on the basis of the estimates of numbers given by Hökfelt et al. (1976) both populations could be accommodated within our $B_{1\alpha}$ subgroup.

B_2 and B_3 cells

These are morphologically, and presumably therefore functionally, quite distinct from B_1 cells. They correspond to the B_2 and B_3 cells of Andres (1961).

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