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SOMA NEUROFILAMENT IMMUNOREACTIVITY IS RELATED TO CELL SIZE AND FIBRE CONDUCTION VELOCITY IN RAT PRIMARY SENSORY NEURONS

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SUMMARY

1. Intracellular recordings were made in dorsal root ganglia *in vitro* at 37 °C. The L4, L5 and L6 ganglia from 46- to 51-day-old female Wistar rats were used. In each neuron conduction velocity (CV) was measured and fluorescent dye was injected. Later the intensity of the immunoreactivity to RT97 (a monoclonal antibody to the phosphorylated 200 kDa neurofilament subunit) as well as the cell size (cross-sectional area at the nuclear level) were measured in the dye-injected neurons. RT97 was used to distinguish between the L (light, neurofilament-rich) and the SD (small dark, neurofilament-poor) neuronal somata.

2. Neurons were classified as C neurons (CV < 1·3 m/s), C/Aδ neurons (1·3–2 m/s), Aδ neurons (2–12 m/s) or Aα/β neurons (> 12 m/s).

3. All A-fibre somata were RT97 positive (L) and all C-fibre somata were RT97 negative (SD), although in the C/Aδ group both positive and negative neurons were seen. Thus, RT97-negative somata had C (unmyelinated) or C/Aδ fibres, while RT97-positive somata had A (myelinated) or C/Aδ fibres.

4. The size distributions of A neurons and C neurons were consistent with their classification as L- and SD-cell neurons respectively. The size distribution of Aδ cells was skewed with a peak of small cells and a tail of medium-sized cells.

5. There was a loose positive correlation between cell size and fibre CV.

6. RT97 intensity was positively correlated with CV if all neurons were considered together, but no correlation was seen within the C, Aδ or Aα/β CV groups.

7. RT97 intensity was positively correlated with cell size when all neurons were considered together. Although no correlation was seen within the C or the Aδ CV groups, a clear positive correlation was seen for Aα/β neurons.

8. The relationship of RT97 intensity to cell size was not demonstrably altered by axotomy, time *in vitro* or the presence of intracellular dye in control experiments.

9. RT97-negative and -positive neurons could be seen in neonatal rat ganglia. Their size distributions resembled, respectively, the SD- and L-neuron populations at this age. RT97 immunoreactivity may therefore be a useful predictor of the cell type and myelinated state which a sensory cell is destined to reach in the adult rat.

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INTRODUCTION

It is well established that there are two main morphologically defined neuronal subtypes in rat and mouse dorsal root ganglia (DRGs) (Andres, 1961; Lawson, 1979). These two populations, which have been called the A or large light neurons and the B or small dark neurons (Andres, 1961; Lieberman, 1976; Lawson, 1979), can be distinguished on the basis of light and electron microscopic studies (e.g. Andres, 1961; Rambourg, Clermont & Beaudet, 1983). Here the term 'light' is used in preference to 'large light' as the latter has proved misleading by incorrectly implying that none of the neurons in this population is small. In this paper the names of the two populations are abbreviated to L (light) and SD (small dark). The populations each have a normal distribution of soma size (cross-sectional areas), and their distributions overlap. Sizes of SD neuronal somata are restricted to the lower end of the size range, while those of L neurons cover the entire range of neuronal sizes in the ganglion (Lawson, 1979; Lawson, Harper, Harper, Garson & Anderton, 1984).

Predictions about the fibre conduction velocity (CV) of L and SD neurons can be made from information relating CV to soma size in these neurons. DRG neurons with C-fibre CVs have smaller mean sizes than those with A-fibre CVs (Yoshida & Matsuda, 1979; Harper & Lawson, 1985; Cameron, Leah & Snow, 1986; Lee, Chung, Chung & Coggeshall, 1986) and C fibres are thus more likely to be associated with the SD neurons. Furthermore, there is indirect evidence that in rat the cell bodies of $A\alpha/\beta$ fibres are L neurons and those of C fibres are SD neurons, since neuronal somata with $A\alpha/\beta$ fibres have the same size range as L neurons, while somata with C fibres are restricted to the size range of SD neurons (Harper & Lawson, 1985). A similar pattern can also be seen for cat DRGs (Lee *et al.* 1986). However, the size distribution of neurons with $A\delta$ fibres in the rat was found to be intermediate between that of the L and SD neurons (Harper & Lawson, 1985) and the population to which these cells belonged was not clear.

In the present study we have been able to correlate CV directly with the DRG cell types by using a marker which distinguishes between L and SD cells. The anti-neurofilament antibody, RT97, specifically and exclusively labels the L-neuron population (Lawson *et al.* 1984), which correlates with the finding that L neurons have many neurofilaments while the SD neurons have very few neurofilaments (Yamadori, 1970; Duce & Keen, 1977; Lawson *et al.* 1984). RT97 is a mouse monoclonal antibody which binds predominantly to the 200 kDa neurofilament protein subunit; it also binds to a lesser extent to the 150 kDa but not to the 70 kDa subunits of bovine neurofilament (Wood & Anderton, 1981; Calvert & Anderton, 1982). It has recently been shown to bind to the phosphorylated and not to the dephosphorylated form (Haugh, Probst, Ulrich, Kahn & Anderton, 1986; Dahl, Labkovsky & Bignami, 1988).

Within the group of RT97-positive neurons, intensity of the RT97 reaction product varies widely and is weakly correlated with cell size (Lawson *et al.* 1984). Electron microscopic studies of rat sciatic nerve indicate that the numbers of neurofilament profiles in a nerve axon are approximately proportional to the axon diameter (Friede & Samorajski, 1970), but there is no evidence as yet that the neurofilament content of the soma is directly related to that of the axon. Thus we

have investigated whether the intensity of RT97 reaction product in the soma is more closely related to the soma size or to the CV of the axon, which is related to axon diameter (Hursh, 1939; Rushton, 1951).

Since neurofilament appears in some rat DRG neurons as early as 17 days of gestation (Ayer-LeLievre, Dahl, Björkland & Sieger, 1985), two categories of neurons are visible even before birth, the neurofilament-rich and the neurofilament-poor neurons. However, there is no quantitative evidence available to show whether the L-neuron population is neurofilament-rich by the time of birth. If it is, this would have very important implications for developmental studies, particularly when combined with information about the CV range of the L-neuron population in the adult rat. We have therefore examined the pattern of RT97 immunoreactivity in neonatal rat DRGs.

A report of some preliminary results has been published (Lawson & Waddell, 1985; Lawson, Waddell & McCarthy, 1988).

METHODS

Electrophysiological experiments

Preparation of tissues. An *in vitro* preparation was used for the electrophysiological aspects of this study because of the improved stability of recording compared with an *in vivo* preparation. This is essential for recording from C-fibre neurons.

Female Wistar rats (46–51 days old and 120–190 g weight) were anaesthetized with sodium pentobarbitone (60 mg/kg intraperitoneally) and maintained at 36·5 ± 1 °C, while the L4, L5 and L6 DRGs, each with a length of dorsal root and peripheral nerve attached, were removed from both sides. The rat was then killed with an overdose of anaesthetic.

The six ganglia were maintained at room temperature in oxygenated balanced salt solution (BSS) containing (in mM): NaCl, 140; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 2; glucose, 5; HEPES buffer, 5; pH 7·4 at 37 °C. For recording the ganglia were used one at a time and were warmed to 37 °C, but were not kept at this temperature for more than about 2 h. Recordings were mostly within 8 h after removal from the animal, and were all within 12 h. No consistent difference was found in the electrophysiology of the cells in the first and the last ganglion used in each experiment.

The DRG was pinned to Sylgard in a small chamber and its peripheral nerve and dorsal root led through notches, sealed with Vaseline, into side chambers. The peripheral nerve and dorsal root were laid over chlorided silver electrodes and covered with liquid paraffin, warmed to 36·5 ± 1 °C by a heater below the bath. The DRG was superfused at 1–2 ml/min with oxygenated BSS, pre-heated to 36·5 ± 1 °C.

The tips of the glass microelectrodes were filled with either 4% Lucifer Yellow CH in 0·1 M-LiCl and the electrode back-filled with 0·1 M-LiCl, or ethidium bromide and the electrodes back-filled with 3 M-KCl (see McCarthy & Lawson, 1988). Ethidium electrodes had DC resistances of 20–50 MΩ, similar to equivalent electrodes filled with 3 M-KCl, while resistances of the Lucifer Yellow electrodes were usually more than 100 MΩ. The reference electrode was an Ag–AgCl wire in an agar–BSS salt bridge at the side of the bath. An active bridge circuit allowed both recordings and current injections to be made through the microelectrode.

Electrophysiology and dye injection. Impalements were made in cell bodies in the outer 200 μm of any surface of the DRG. Recordings were made of the latency to the start of the somatic action potential evoked by stimulation of the peripheral nerve and/or dorsal root at twice threshold voltage. The stimulus duration was 0·1 ms for A fibres and 1·0 ms for C fibres. At the end of the experiment the peripheral nerve and dorsal root were crushed over the cathodes of their stimulating electrodes, and the distances from these points to the centre of the ganglion were measured. Each conduction distance (usually about 10 mm in the dorsal root and 15 mm in the peripheral nerve) was divided by the latency to give the CV. Neurons with a CV from the peripheral nerve or the dorsal root were included, but where both were available, the former was used. The use of both peripheral nerve and dorsal root CVs in this study is justified by a previous study in which we have shown that although on average dorsal root fibres conduct more slowly than

peripheral nerve fibres in the rat, there are very few occasions in which this would lead to an inappropriate classification of CV group (Waddell, Lawson & McCarthy, 1989).

After the CV of a neuron had been measured, dye was injected from the electrode by passing 1 s current pulses of 1–5 nA at 0·5 Hz, or 750 ms current pulses at 1 HZ, for several minutes. Hyperpolarizing currents were used for Lucifer Yellow and depolarizing currents for ethidium bromide. Two cells could thus be injected in each ganglion, one with each dye.

Cell location and immunocytochemistry. The ganglion was fixed overnight at room temperature in Bouin's fixative, dehydrated, embedded in paraffin wax and then serial 7 μm sections were cut. After overnight drying the sections were dewaxed and examined at $\times 160$ and $\times 400$ magnification under a fluorescence microscope (Leitz Dialux 22). The filter blocks used were the N_2 for ethidium bromide (orange–red fluorescence) and the I_2 for Lucifer Yellow (yellow–green fluorescence). Both Lucifer Yellow- and ethidium bromide-filled cells could be seen under the FITC filter, but only the ethidium bromide-filled cells could be seen under the N_2 filter, so they could easily be distinguished from each other (McCarthy & Lawson, 1988). Drawings were made of every section through each located cell including other features of the ganglion necessary to relocate the cell later. These were made under interference contrast optics, with the aid of a camera lucida.

The sections which contained the labelled cell were then processed to show RT97 immunoreactivity, as described in detail previously (Lawson *et al.* 1984). Briefly, after washing the slides in phosphate-buffered saline (pH 7·5), the first antibody layer applied was RT97 (1:10000) and the second was rabbit anti-mouse peroxidase (1:100). The sections were washed in phosphate-buffered saline, then Tris-buffered saline pH (7·6). The peroxidase was then reacted by the addition of diaminobenzidine HCl in Tris-buffered saline with 1% v/v hydrogen peroxide. After further washing the sections were mounted in Permount. To control for non-specific peroxidase staining, RT97 was omitted from some sections in all runs, with all other procedures being the same.

After immunocytochemistry, the dye-labelled cell was relocated with the aid of the camera lucida drawing, as the fluorescence had usually faded. The cross-sectional area of the largest section containing a nucleus was measured under interference contrast optics with a Graf pen system interfaced to a BBC microcomputer.

Microdensitometry. The density of the RT97 staining in each cell was measured quantitatively with a Vickers M85 microdensitometer with the wavelength set at 485–510 nm (see Lawson *et al.* 1984). In each section the mean absorbance (log 1/transmission) from three spots of 4 μm diameter in the cytoplasm of the most darkly staining neuron was taken as 100% staining, while the mean absorbance of similar spots in the cytoplasm of three RT97-negative neurons was taken as 0%. The mean absorbance of three points in the cytoplasm of the nuclear section of the dye-filled cell was then expressed as a percentage on this range.

Criteria for acceptance of data. Initially only neurons with a known membrane potential more negative than -35 mV were included in the analysis ($n = 35$). However, two cells (both C-fibre cells) with potentials of 20–35 mV were later also included as there were no apparent differences between the parameters of these neurons and those of other neurons in the same CV group (see Table 1). In addition, one neuron with no record of membrane potential was included as its action potential height was 60 mV, indicating a membrane potential of > -40 mV. If more than one cell per ganglion was labelled inadvertently by one of the dyes, the cells in that ganglion labelled by that dye were not included. If a particular section or ganglion had weak RT97 immunoreactivity the data were discarded.

Several neurons had inadequate electrophysiological data for inclusion in the analysis. One of these had a C-fibre CV but a cross-sectional area of 750 μm^2 and was RT97 positive. Otherwise all the excluded data fitted with the patterns described in the results.

Immunocytochemical studies

Normal size distributions of RT97-positive and RT97-negative neurons. Sizes of RT97-positive and RT97-negative neurons were measured in an L4, L5 and L6 ganglion from a 50-day-old, 160 g female rat (i.e. within the age and weight range used for the electrophysiological experiments). Ganglia were removed under anaesthetic, as described above, fixed in Bouin's fixative overnight, processed and embedded in wax and 7 μm sections cut in series with a section taken every 200 or 400 μm throughout the ganglion. The whole series was immunostained, as described above, and the cross-sectional areas of all cells with a nuclear profile were recorded, along with a subjective grading of their RT97 staining intensity on a scale of 1–5.

Controls for effects of axotomy, time in vitro and intracellular dye. Neuronal sizes and RT97 staining were measured in control L4 and L5 ganglia removed with 10–20 mm of peripheral nerve from female rats in the same age and weight range as those in the electrophysiology experiments. They were treated as follows: (1) two L4 ganglia were placed in oxygenated BSS at room temperature for 4 h and then at 36·5 ± 1 °C for 2 h, to mimic a typical experiment; or (2) ganglia (one L4 and one L5) were superfused with oxygenated BSS for 12 h at room temperature and 2·5 h at 36·5 ± 1 °C with the nerve passing through a Vaseline seal and the cut end of the peripheral nerve immersed in a solution of the dye for the entire time. The Lucifer Yellow was a saturated solution in BSS and was applied to the L4 nerve. The ethidium bromide was a 5 mM solution in BSS and was applied to the L5 nerve. This was to control for the effect of intracellular dye (both Lucifer Yellow and ethidium bromide) on immunostaining, and the length of time was necessary to ensure that adequate dye had reached the cell bodies. It therefore also acted as a control for the effects of a much longer time *in vitro* after axotomy than was used in any experiment.

These control ganglia were fixed after the above procedures and processed for immunocytochemistry exactly as for the electrophysiology experiments, and size distributions were prepared as described above from series of wax sections at 200 or 400 μm intervals throughout the ganglia.

RT97 staining pattern in neonatal DRGs. RT97 staining was examined in L4 DRGs from two 1-day-old rats. These were killed by an overdose of sodium pentobarbitone (250 mg/kg), and the ganglia were removed and fixed for 1 h in Bouin's fixative. The procedures were exactly as above. Cells were measured from a series of sections 100 μm apart from one of these ganglia.

Sources of dyes and antibodies. Lucifer Yellow CH and ethidium bromide were obtained from Sigma, RT97 antibody was a gift from J. Wood and the rabbit anti-mouse peroxidase antibody was from Dako.

Statistical methods

Throughout the results, means plus and minus a value indicates the standard deviation. The statistical test used to compare means was the non-parametric Wilcoxon Ranking Test unless otherwise indicated. To test whether correlation coefficients were significantly different at the 5% level from zero, we used Student's t test (two tailed) with the degrees of freedom calculated as $n-2$, where n is the number of observations. The significance of all correlations was also checked with the non-parametric Spearman ranking test. The regression lines of correlations showing significance with both these tests are shown on the graphs.

To examine the distribution of the CV data, plots on probability paper were made of CV and $\log_{10}$ CV (for methods see Harding, 1949). A single normal distribution is seen as a single straight line, while n normal distributions are seen as n discontinuous straight lines.

Statistical tests were carried out with the 'FIRST' software package from Serious Statistical Software.

RESULTS

Electrophysiological experiments

A total of thirty-eight cells were studied (see Methods) from seventeen rats, from thirty-three of which data were obtained on all of the following: CV, RT97 staining intensity and cell cross-sectional area. Six out of thirty-eight cells were injected with ethidium bromide, and the rest with Lucifer Yellow. Table 1 gives the data and shows that the dye used and the level of the membrane potential had no noticeable influence on the data.

Conduction velocity classification

These neurons were classified as follows: 10/38 were C-fibre cells (< 1·3 m/s), 3/38 were borderline C/Aδ neurons (1·5–2 m/s), 10/38 were Aδ cells (2–12 m/s) and 15/38 were Aα/β cells (> 12 m/s). The mean velocities in each group (m/s, ± S.D.) were:

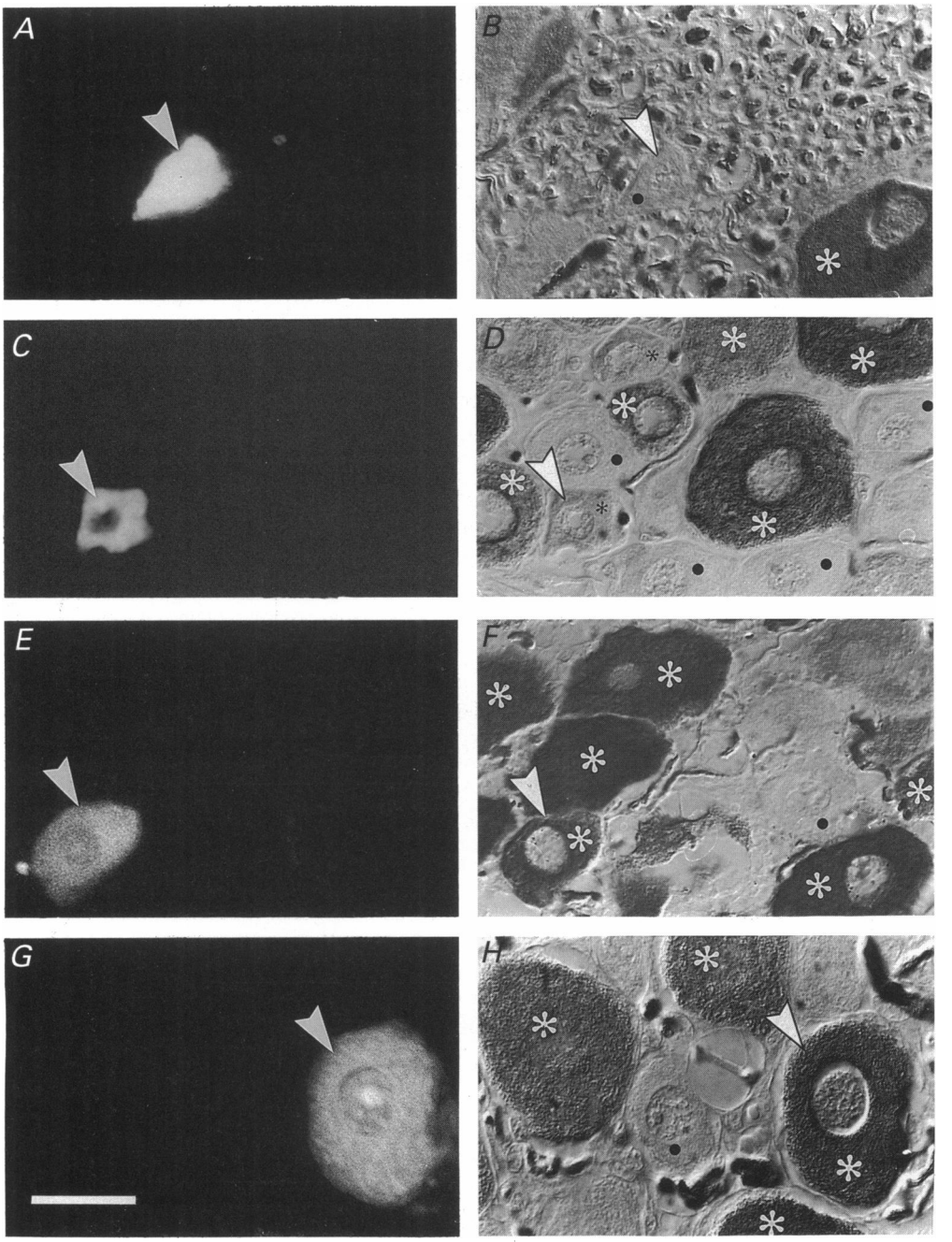

Fig. 1. Appearance and immunocytochemistry of dye-injected cells. Left (*A*, *C*, *E* and *G*): fluorescence photomicrographs of neurons, injected with Lucifer Yellow dye, before immunocytochemistry. Right (*B*, *D*, *F* and *H*): interference contrast photomicrographs of the same neurons after immunocytochemistry to show RT97 immunoreactivity with the indirect immunoperoxidase technique. In each case the dye-injected neuron is indicated with an arrow-head. The CV and percentage absorbance of the RT97 reaction product for

C cells, $0{\cdot}75 \pm 0{\cdot}2$; C/Aδ cells, $1{\cdot}5 \pm 0$; Aδ cells, $6{\cdot}2 \pm 1{\cdot}8$; and Aα/β cells, $18{\cdot}3 \pm 6{\cdot}8$. The CV ranges are lower than for adult rats because fibre diameter is still increasing at this age. The borderlines of these groups in 6- to 8-week-old female rats have previously been discussed (Harper & Lawson, 1985; Waddell *et al.* 1989).

The probability plots (not shown) made on the present data showed three discontinuous straight lines confirming discontinuities in the distribution of CVs between C and C/Aδ, between C/Aδ and Aδ and between Aδ and Aα/β groups, although the latter discontinuity occurred below 10 m/s rather than between 10 and 12 m/s, which was the boundary chosen. This indicates that boundaries chosen were correct, except that the 10 m/s value should perhaps have been included in the Aα/β group.

Appearance of RT97 immunoreactive neurons

Examples of Lucifer Yellow-filled cells from each of the CV ranges can be seen in Fig. 1 with their appearance after RT97 immunostaining. It can be seen that RT97-negative neurons (indicated by black dots) are easily distinguishable by eye and that there is a wide range of RT97 staining intensities. When the staining intensities of the dye-filled neurons were measured with the microdensitometer, relative to the darkest (100% absorbance) and the lightest (0%) staining neurons in the section, all cells which were thought to be positive by eye had an absorbance of 16–100% of the maximum, while cells assessed as clearly RT97 negative by eye usually had absorbances of $< 2\%$ of the maximum. We have taken $\leqslant 15\%$ as negative and $\geqslant 16\%$ as positive in the subsequent analyses. In Fig. 1 examples of RT97-negative C and C/Aδ cells and RT97-positive Aδ and Aα/β cells are shown.

Cell sizes of RT97-positive and RT97-negative neurons

The size distributions of RT97-positive and RT97-negative neurons from DRGs of a female rat within the age and weight range of the experimental rats were compared for L4, L5 and L6 DRGs. The percentages of positive neurons were 41, 37 and 40% respectively; their size distributions appeared to be normally distributed and the mean cell sizes of the positive neurons were 927, 937 and 664 μm^2 respectively. Positive neurons in the L6 DRG therefore had a lower mean size than those in L4 and L5. Areas of RT97-negative neurons also appeared to be normally distributed with means of 344, 315 and 327 μm^2 respectively. The size distributions from the three ganglia were summed and are displayed in Fig. 2*A*.

The size distributions of RT97-negative and RT97-positive dye-injected neurons from all the electrophysiological experiments are shown in Fig. 2*B* and *C*. These include neurons from L4, L5 and L6 ganglia. The mean sizes are slightly smaller than the distributions in *A*, which may be due in part to the age of the rat in *A* being towards the top of the age range for *B* and *C*. The patterns of the RT97-positive and

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each cell are: *A* (C-fibre cell), 0·9 m/s, 0%; *B* (C/Aδ), 1·5 m/s, 15%; *C* (Aδ), 7·1 m/s, 69%; *D* (Aα/β), 14·7 m/s, 79%. RT97-negative cells are indicated by filled dots ($< 5\%$ absorbance), or by small black asterisks (5–15% absorbance). RT97-positive neurons are indicated by large white asterisks. Scale bar 25 μm.

RT97-negative size distributions are similar to those in normal ganglia (Fig. 2*A*), indicating that the dye injection is unlikely to have altered the immunoreactivity of the cells to RT97.

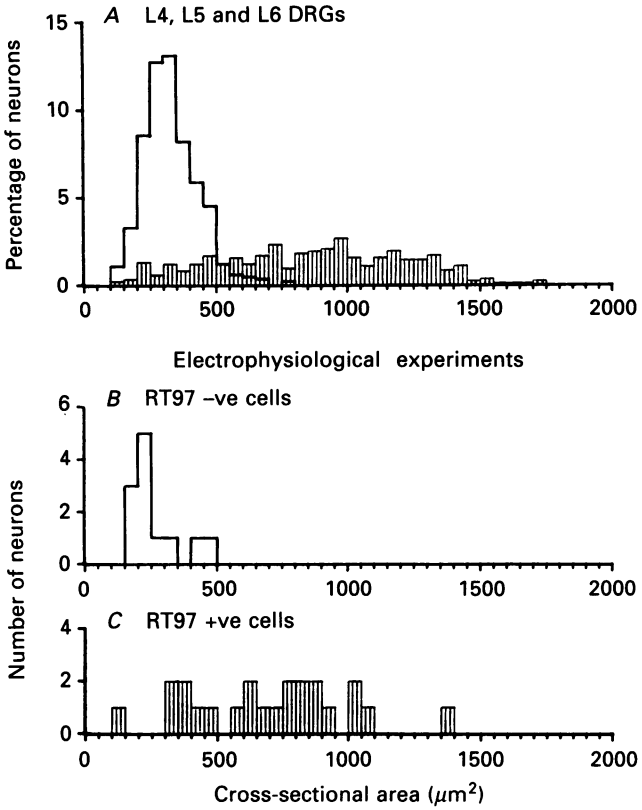

Fig. 2. Distributions of cell areas of RT97-positive and RT97-negative neurons. Vertical hatching shows RT97-positive neurons ($\geqslant 16\%$ absorbance). Open histograms show RT97-negative neurons ($\leqslant 15\%$ absorbance). *A*, combined data ($n = 816$) from an L4, an L5 and an L6 DRG from a female 50-day-old, 160 g rat. *B* and *C*, sizes of the dye-injected and electrophysiologically characterized L4, L5 and L6 neurons listed in Table 1; *B*, RT97 negative ($n = 12$); *C*, RT97 positive ($n = 23$).

Conduction velocities of RT97-positive and RT97-negative cells

A clear relationship between RT97 immunoreactivity and CV emerged (see Table 1 and Fig. 3). All ten C-fibre cells were RT97 negative (i.e. $\leqslant 15\%$ absorbance). Of these, nine had absorbances of 0–3% and one had an absorbance of 13%. Of the three C/Aδ cells, two were negative (0·3 and 15%) and one was positive (20%). All (10/10) Aδ neurons and all (15/15) Aα/β neurons were RT97 positive (i.e. $\geqslant 16\%$ absorbance). The mean percentage values for absorbance for each group were: C cells, $1{\cdot}6 \pm 4{\cdot}1$; C/Aδ cells, $11{\cdot}8 \pm 10$; Aδ cells $45{\cdot}5 \pm 19{\cdot}8$; and A$\alpha/\beta$ cells $60{\cdot}6 \pm 20{\cdot}2$.

The mean cell areas increased with the CV group as follows (in μm^2): C cells, 248 ± 92; C/Aδ cells, 349 ± 123; Aδ cells, 443 ± 214; and Aα/β cells, 877 ± 196. This pattern can be seen in Fig. 3. The lower graph shows that the A cells taken together span the entire size range of the DRGs shown in Fig. 2*A*, although the Aδ cells

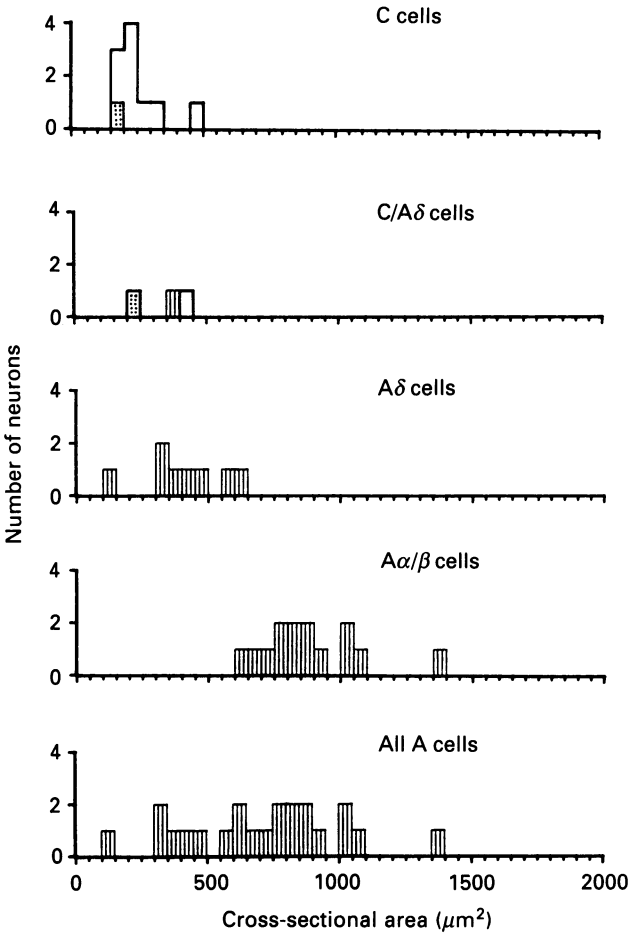

Fig. 3. Distributions of cell areas of neurons in different conduction velocity groups. Hatching indicates $\geqslant 16\%$ absorbance (RT97 positive); stippling indicates 5–15% absorbance (RT97 negative) and open histograms indicate $< 5\%$ absorbance (RT97 negative). Histograms for each CV group plotted separately, and in the lower histogram for all A cells combined.

predominate in the lower end of this distribution while the faster conducting Aα/β cells predominate in the upper end.

Intensity of RT97 immunoreactivity versus conduction velocity

There was a significant positive correlation between RT97 staining intensity and CV if all cells were considered but no correlation within individual CV groups (see Fig. 4).

RT97 staining intensity was positively correlated with CV in the peripheral nerve ($r = 0{\cdot}64$, $P < 0{\cdot}001$, $n = 25$) and in the dorsal root ($r = 0{\cdot}51$, $P = 0{\cdot}05$–$0{\cdot}025$, $n = 17$) when cells of all CVs were considered together. The stronger correlation in the peripheral nerve may result from the greater amount of data available (see Table 1).

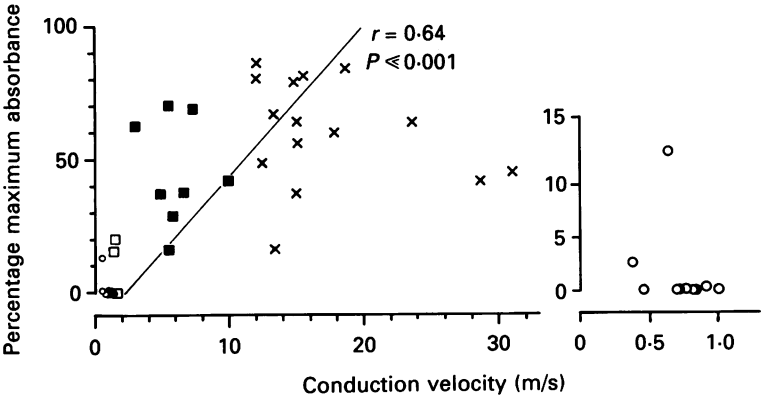

Fig. 4. Soma RT97 intensity against fibre conduction velocity. Data from Table 1 are plotted. Symbols are: C cells (○), C/Aδ cells (□), Aδ cells (■) and Aα/β cells (×). Left, data from cells of all CVs. Right, data for C neurons on expanded axes. There was a significant positive correlation for cells of all CVs ($r = 0{\cdot}64$, $P \ll 0{\cdot}001$, regression line shown on graph) but no significant correlation was found within the CV groups: Aα/β cells, $r = 0{\cdot}25$, $P > 0{\cdot}3$; Aδ cells (excluding C/Aδ cells), $r = 0{\cdot}12$, $P > 0{\cdot}7$; C cells, $r = 0{\cdot}28$, $P > 0{\cdot}4$.

Intensity of RT97 immunoreactivity versus cell size

When the neurons were separated into CV groups (Fig. 5) there was no significant correlation within either the C or the Aδ groups and there were too few C/Aδ cells to carry out statistics. In contrast, there was a clear and significant correlation between staining intensity and cell area in the Aα/β neurons (Fig. 5, continuous line, $r = 0{\cdot}71$, $P < 0{\cdot}005$). However, a positive and significant correlation was found between staining intensity and the cross-sectional area of the neuron when cells of all CVs were considered together (regression line shown on Fig. 5, dashed line). A comparison of the two regression lines suggests that the correlation seen for all cells together might result largely from the correlation within the Aα/β group and the lack of staining in C cells.

Conduction velocity versus cell size

Since the RT97 intensity shows some correlation with both CV and cross-sectional area, the correlation between these two latter parameters was examined. Figure 6 shows that there is a significant positive correlation when all cells were considered together. However, no significant correlation was found within any of the CV groups.

Differences between electrophysiological data from L4, L5 and L6 DRGs

Data from the three ganglia are generally similar in the different CV groups (see Table 1), with the following exceptions. For both C and Aδ cells the mean sizes were smallest for L6 and largest for L4. The mean sizes for C cells were (in μm^2): L6,

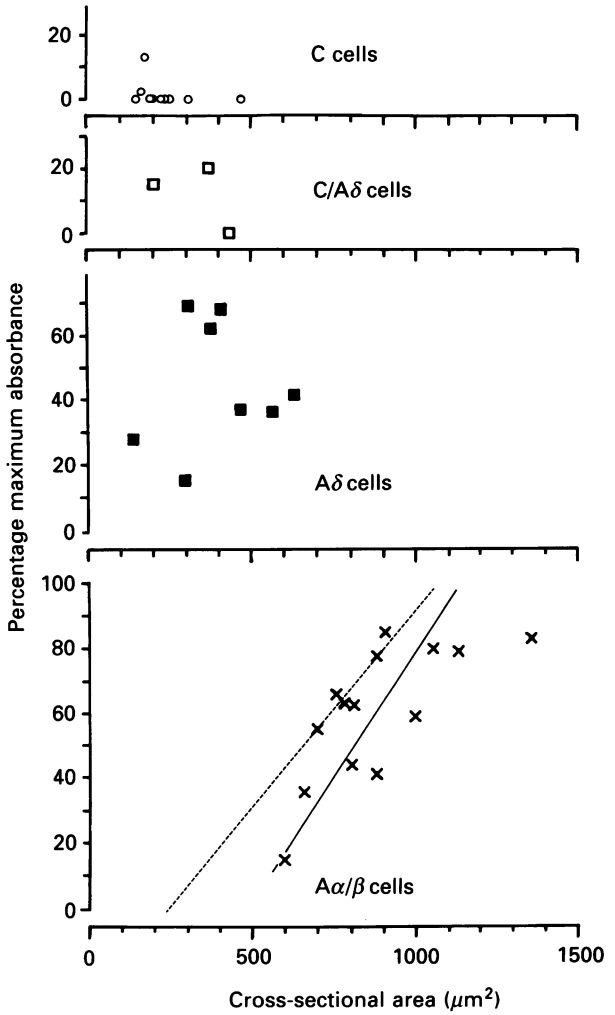

Fig. 5. RT97 absorbance against cell area for different conduction velocity groups. Data from Table 1 are plotted. Symbols as for Fig. 4. The only group of cells which showed a significant regression coefficient were the Aα/β cells ($r = 0{\cdot}71$, $P < 0{\cdot}005$) shown as a continuous line. For the Aδ cells (excluding C/Aδ cells) $r = 0{\cdot}09$ and $P > 0{\cdot}8$, and for the C cells $r = 0{\cdot}33$ and $P > 0{\cdot}3$. The regression line for cells of all the CV groups taken together is shown on the lower graph for comparison as a dashed line ($r = 0{\cdot}78$, $P \ll 0{\cdot}001$).

203 ± 42; L5, 248 ± 86; L4, 340 ± 140; and for Aδ cells: L6, 304 ± 136; L5, 506 ± 178; L4, 564 ± 284. It is interesting that all three C/Aδ cells were from L6.

Controls for effects of axotomy, time in vitro and intracellular dye on immunostaining

(1) For two L4 DRGs kept *in vitro* for 4 h at room temperature and 2 h at 37 °C, the mean sizes of RT97-positive neurons were 965 and 806 μm^2 and of RT97-negative neurons were 384 and 339 μm^2. The labelling appeared to be normal.

(2) In the DRGs which were *in vitro* for 14·5 h the retrograde labelling with the

fluorescent dyes, Lucifer Yellow or ethidium bromide, was successful: large areas of sections through the ganglia showed intense fluorescence which was equal to or greater than the intensity of the dye-injected cells from the electrophysiological

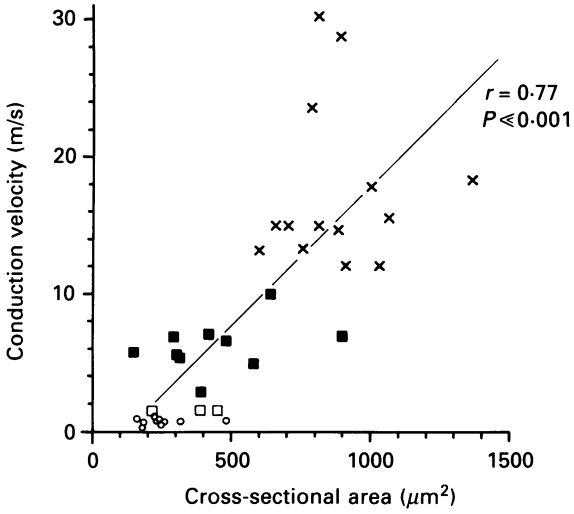

Fig. 6. Graph of conduction velocity against cell cross-sectional area. Symbols as for Fig. 4. The CV of the peripheral nerve or dorsal root is plotted against the cross-sectional area of the largest nuclear section through the neuron. There is a significant correlation between these parameters when all cells are considered together ($r = 0{\cdot}77$, $P \ll 0{\cdot}001$). No significant correlation was found within the separate groups; for the separate groups: Aα/**B** cells, $r = 0{\cdot}31$, $P > 0{\cdot}8$; Aδ cells (excluding C/Aδ), $r = 0{\cdot}36$, $P > 0{\cdot}3$; and C cells, $r = 0{\cdot}16$, $P > 0{\cdot}6$.

experiments. In these areas the RT97 immunoreactivity appeared to be normal (Fig. 7). The cell size distribution for RT97-positive and RT97-negative neurons showed the usual pattern of two overlapping, apparently normal distributions of cell size (Fig. 8*A* and *B*). The DRG labelled with Lucifer Yellow had a mean area for RT97-positive neurons of 732 μm^2 and for RT97-negative neurons of 355 μm^2. The DRG labelled with ethidium had mean cell areas for RT97-positive neurons of 920 μm^2 and for RT97-negative neurons of 390 μm^2. The RT97-negative populations of both ganglia and the RT97-positive populations of the ethidium bromide-labelled L5 DRG all had similar means to those of the control L4 and L5 DRGs included in Fig. 2*A*. The mean size of the RT97-positive neurons in the Lucifer Yellow-labelled L4 DRG was low, but still within the range of 557–883 μm^2 described in a previous study (Lawson *et al.* 1984) of L4 ganglia with similar histological processing, from 8- to 10-week-old rats.

For all DRGs in (1) and (2) above, there was good staining for RT97 immunoreactivity. As usual, cells could easily be classified as RT97 positive or negative, and the percentages of RT97-positive cells (47–53%) were within the normal range. The relationship between cell size and the subjectively graded RT97

TABLE 1. Data from individual neurons from which intracellular recordings yielded CV measurements, which were injected with dye and for which intensities of staining with RT97 were measured and which met the criteria set

CV (m/s)	RT97 (%)	Area (μm^2)	Dye	DRG	V_m (mV)
C-fibre neurons					
0·4*	2·7	172	LY	L5	−44
0·48	0	248	LY	L6	−36
0·66*	13	180	LY	L6	−54
0·74	0·1	314	LY	L5	−50
0·74	0	228	LY	L6	−42
0·8	0	250	LY	L4	−49
0·85	0	475	LY	L4	−35
0·87	0	231	LY	L5	−46
0·94*	0·6	156	LY	L6	−22
1·03	0	218	LY	L4	−26
C/Aδ-fibre neurons					
1·5	0·3	448	LY	L6	−57
1·5	15	211	LY	L6	−40
1·5	20	388	LY	L6	−41
Aδ-fibre neurons					
2·9	63	389	LY	L4	−56
4·9	37	573	LY	L5	−54
5·4*	70	310	EB	L6	>35
5·5	16	305	LY	L5	−45
5·8	29	144	LY	L6	−46
6·6	38	475	LY	L6	−44
6·9*	—	892	EB	L4	−60
6·9	—	286	LY	L6	−43
7·1	69	412	LY	L4	−37
10*	42	641	LY	L5	−46
Aα/β-fibre neurons					
12*	86	910	LY	L4	−58
12*	80	1033	LY	L5	−48
12·2*	48	—	LY	L4	−42
13·2	16	600	LY	L5	−40
13·3	67	753	EB	L5	−64
14·7*	79	898	LY	L5	−52
15*	56	705	LY	L4	−45
15	64	811	LY	L4	−56
15	37	659	EB	L5	−50
15·5	81	1062	EB	L4	−45
17·8	60	1006	LY	L4	−54
18·3*	84	1363	LY	L4	−47
23·5	64	790	EB	L6	−68
28·6	42	888	LY	L5	−54
31	45	811	LY	L4	−48

Column 1, CV from stimulating electrode to DRG cell body; asterisks indicate values from the dorsal root, the rest being from peripheral nerve. Column 2, absorbance of cytoplasm as a measure of intensity of RT97 staining. Column 3, cross-sectional area of nuclear section through cell. Column 4, intracellular dye used, Lucifer Yellow (LY) or ethidium bromide (EB). Column 5, rostrocaudal level of DRG. Column 6, membrane potential.

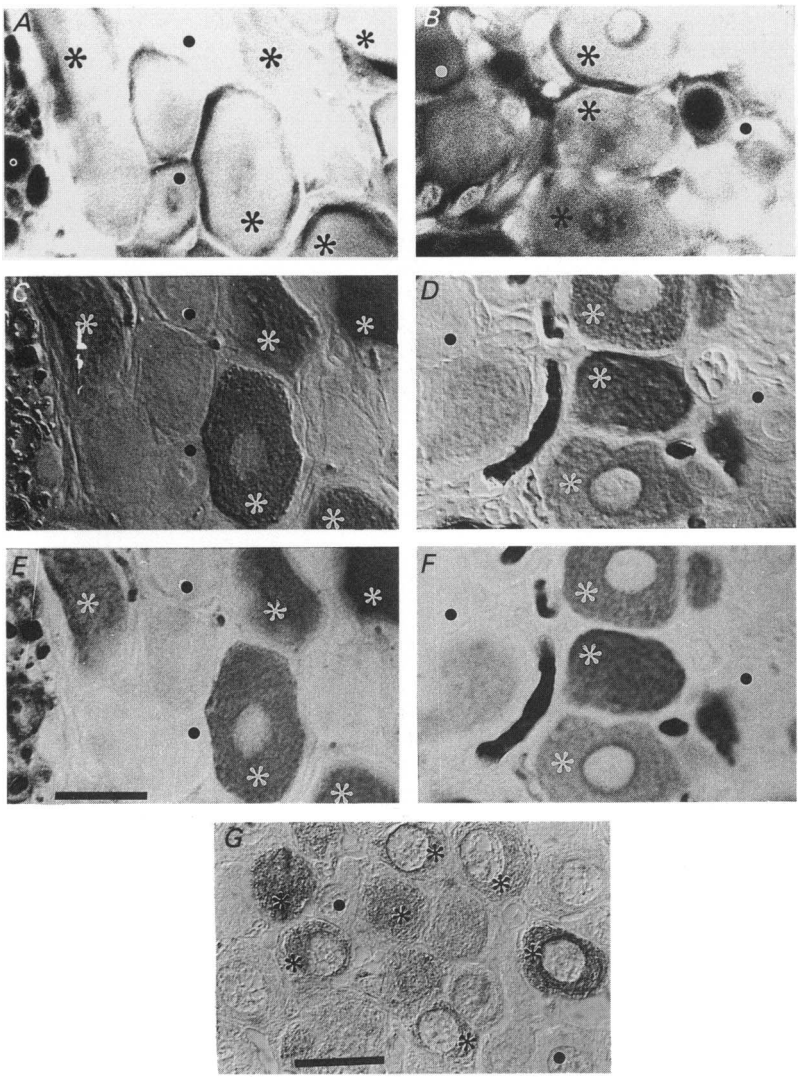

Fig. 7. Photomicrographs to show the effects of retrogradely transported Lucifer Yellow (*A*, *C* and *E*) and ethidium bromide (*B*, *D* and *F*) on RT97 immunoreactivity. *A* and *B*, fluorescence due to dye in sections prior to dewaxing and immunocytochemistry. Black regions indicate no fluorescence and light areas intense fluorescence. *C* and *D*, interference contrast of same areas as *A* and *B* after immunocytochemistry, to show the RT97 reaction product. *E* and *F*, bright field of same areas. Examples of RT97-positive and RT97-negative neurons are shown by asterisks and filled dots respectively. Note the lack of correspondence between intensity of fluorescent dye and intensity of RT97 reaction product, indicating no effect of dye on immunocytochemistry; also note that RT97 staining appears normal. Scale bar 25 μm. *G*, interference contrast photomicrograph to show RT97 immunoperoxidase reaction product in an L4 DRG from a 1-day-old rat. Asterisks and black dots as above. This field was chosen for the large number of RT97-positive neurons but in most areas RT97-negative neurons predominate. Scale bar 20 μm.

staining intensity was plotted for each ganglion (not shown) and appeared to be unaffected by the control procedures and retrograde filling with either of the dyes. Thus, despite up to 14·5 h post-axotomy *in vitro* and despite retrograde filling of the

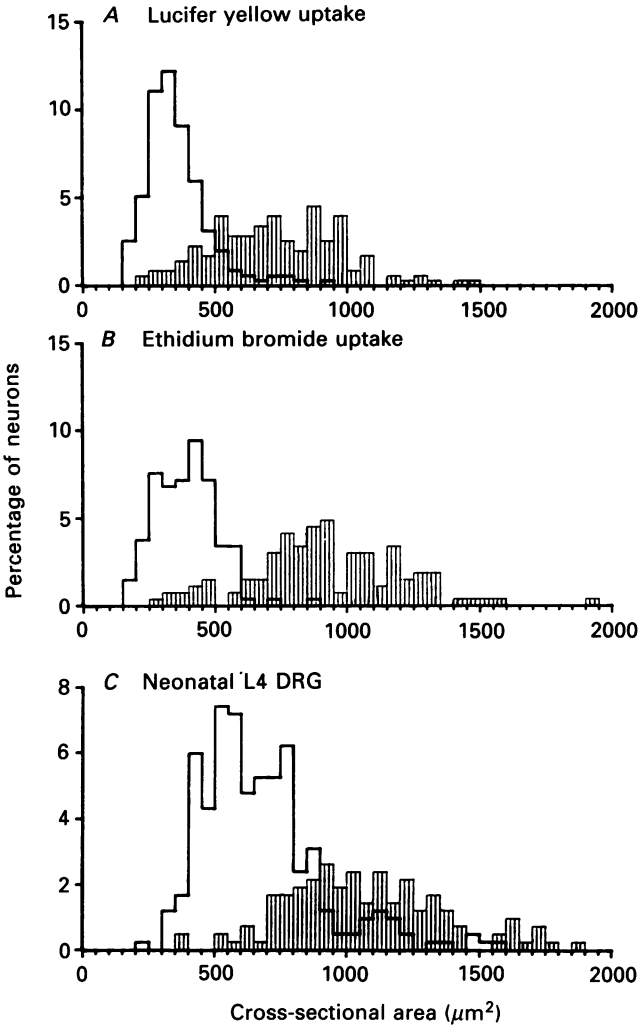

Fig. 8. Cell areas in adult control DRGs and newborn DRGs. *A* and *B*, DRGs *in vitro* for 14·5 h, 12 h at room temperature and 2 h at 37 °C. *A*, an L4 DRG retrogradely labelled with Lucifer Yellow ($n = 352$). *B*, an L5 DRG retrogradely labelled with ethidium bromide ($n = 264$). *C*, an L4 DRG from a 1-day-old rat, fixed immediately after removal ($n = 416$).

cells with the fluorescent dyes, the RT97 staining intensities and cell size distributions were within the range found for normal ganglia from rats of the same age, providing the usual clear differentiation between the L-cell population and the SD-cell population.

RT97 immunoreactivity in neonatal ganglia

RT97 staining of neurons in L4 DRGs from 1-day-old rats was weaker than that seen in adult rat DRGs (Fig. 7*C*). In the L4 ganglion which was measured, 32% of the neurones were labelled. The labelled neurons had an apparently normal size distribution covering the entire size range of the cells in the ganglion, while the RT97-negative cells had a smaller mean size with a peak at the lower end of the size distribution (see Fig. 8*C*).

DISCUSSION

Size distributions in L4, L5 and L6 ganglia

Neurons of the L4, L5 and L6 DRGs have similar size distributions, although the RT97-positive population had a lower mean size in L6 than in L4 or L5 ganglia. This may result from the higher proportion of L6 neurons which have fibres in the Aδ range of fibre diameter (Suh, Chung & Coggeshall, 1984) and in the Aδ range of CV (Waddell *et al.* 1989), since Aδ neurons have a smaller mean size than Aα/β neurons in the rat DRG (this paper, Harper & Lawson, 1985). However, there is some indication in the present paper that L6 A neurons, including Aδ neurons, are smaller than those in L5 and L4. This could be explained if soma size is related to the length of the peripheral fibre, which may be shorter in many L6 neurons innervating pelvic viscera than in L5 and L4 neurons innervating the hindlimb. It could, however, also be explained if soma size is related to other factors such as the sensory receptor type of the neuron or the type of target tissue to which it projects, since these are somewhat different for L6 and L4/5.

Effects of histological procedures on cell size

Cell sizes in sections of wax-embedded ganglia are reduced compared with those in sections of Epon-embedded ganglia (e.g. Harper & Lawson, 1985) or in frozen sections (McCarthy & Lawson, 1990), although the overall patterns of cell size are unaffected by this (Lawson & Harper, 1985). This highlights the problem with quoting actual cell sizes as evidence of cells being 'small' or 'large', since actual size is only useful if taken in the context of the overall pattern of size distributions in ganglia from animals of a particular age and for a particular method of histological processing. Since all ganglia in this paper had the same histological processing the reduction of cell size does not affect our interpretation of the data or our conclusions.

Effects of experimental procedures on immunocytochemistry and cell size

Before discussing the relationships found in this study between CV, cell size and neurofilament content of the soma, it is necessary to establish that the experimental procedures did not alter the immunoreactivity and to consider what the RT97 staining is likely to mean in terms of neurofilament content.

The possibility that axotomy and time *in vitro* might have affected the neurofilament content of the soma is raised by recent studies which have indicated that changes in the somal content of neurofilament or its messenger RNA can occur after axotomy in rat DRGs. For instance a decrease in somal content of messenger

RNA for the 68 kDa neurofilament subunit has been shown in large neurons by 24 h post-axotomy (Wong & Oblinger, 1987), which would presumably lead to an increase in this subunit some hours later. However, the number of DRG somata stained with a variety of anti-neurofilament antibodies was also altered after 24 h (Rosenfeld, Dorman, Griffin, Ludwig, Sternberger & Price, 1987). Our controls showed that even after axotomy, 14·5 h *in vitro* and retrograde labelling with the dye, RT97 immunoreactivity could still be used to distinguish between the L and SD cell types; the usual neuronal size distributions of the two populations were seen; the relationship between cell size and RT97 immunoreactivity appeared the same as usual and the mean sizes of the two populations did not appear to vary more than between normal ganglia fixed *in situ* from different rats. Thus cell sizes and intensity of RT97 labelling in the electrophysiological experiments (usually within 8 h after axotomy) should have been little affected by axotomy and being *in vitro*.

It also seemed possible that the intracellular injection of dye might affect the immunoreactivity of the neurons. However, the intracellularly labelled neurons from the electrophysiological experiments showed patterns of size distributions for RT97-negative and RT97-positive neurons similar to those seen in normal ganglia. Furthermore, as described above, retrograde labelling of the neurons with dye did not noticeably affect the pattern either of the RT97 immunoreactivity or of the size distributions of positive and negative cells, even though the dye was probably in the cells for longer before fixation than was usual in the electrophysiological experiments.

Variability in immunocytochemistry

With all immunocytochemical procedures there is variability in intensity of the reaction product from one run to another. This did not affect the identification of positive and negative neurons. To overcome the problem of quantifying the intensity of reaction product (absorbance) the microdensitometric measurements were related to the range of staining intensities in that particular section. This should have cancelled out any variability in the immunocytochemical technique.

Boundaries between conduction velocity groups

The boundaries between CV groups used in this and in previous studies (e.g. Waddell *et al.* 1989) were further validated by the probability plots. Discontinuities were seen exactly at the borderlines chosen, with one exception: there was a discontinuity slightly lower than the borderline chosen between Aδ and Aα/β ranges, indicating that the cell conducting at 10 m/s might have been more appropriately included in the Aα/β group. This borderline is not easy to define and we have previously described it in a similar preparation as being at about 10–12 m/s (Waddell *et al.* 1989).

RT97 immunoreactivity in A-fibre and C-fibre neurons

The data presented in this paper show somata with A fibres were all RT97 positive whereas those cells with C fibres were RT97 negative and staining of the C/Aδ-fibre cells was variable. Since RT97 labels only the L-cell population (Lawson *et al.* 1984), the present data confirm the predictions of Harper & Lawson (1985) that C fibres arise from SD neurons (RT97 negative) and that Aα/β fibres arise from L

neurons (RT97 positive). Furthermore, it shows that Aδ fibres also arise from L cells, a point which could not be clarified previously without a specific marker such as RT97 for L cells. It also shows that the borderline between C and Aδ neurons seems to coincide with the borderline between L (RT97 positive) and SD (RT97 negative) neurons, judging from the variability in staining in the C/Aδ group. However, the numbers of cells in this study are small and exceptions to these patterns may well emerge with further studies. For instance, in another study (P. W. McCarthy & S. N. Lawson, in preparation) results obtained using a different immunocytochemical technique, namely immunofluorescence on frozen sections, fit exactly with the above patterns except that 2/16 C-fibre cells were RT97 positive.

Possible interpretations

This correlation between C- and A-fibre CV and classification as RT97 negative or positive is remarkably good, although exceptions can occur. It is usually assumed that C fibres are unmyelinated and that all A fibres are myelinated. There does, therefore, seem to be a clear link between the RT97 immunoreactivity of the soma and the state of myelination of the axon. However, even a perfect correlation between RT97-positive cell bodies and myelination of the axon could be marred by the occasional very fine myelinated axon conducting in the C-fibre range causing occasional RT97-positive C-fibre cells, although it is also possible that the RT97-positive C cells seen by McCarthy and Lawson indicate that a small proportion of unmyelinated fibres arise from neurofilament-rich neurons. If this is the case, the possibility should be raised that occasionally a neurofilament-rich cell might support a main myelinated fibre as well as a fine fibre or fibre branch, possibly a sprouting fibre, but this is very difficult to examine experimentally.

RT97 immunoreactivity in relation to neurofilament content of the cell

What does RT97 immunoreactivity indicate about the neurofilament content of the DRG somata? The RT97 antibody binds predominantly to the phosphorylated 200 kDa neurofilament subunit (see Introduction). It has been shown that all newborn rat DRG neurons which have electron microscopically identified neurofilament profiles show immunoreactivity to antibodies against all three neurofilament subunits (Sharp, Shaw & Weber, 1982). Since the antibodies against the 200 kDa neurofilament subunit used in that study have subsequently been shown to bind to the phosphorylated subunit (Shaw, Osborn & Weber, 1986), it seems probable that it is the amount of neurofilament in the soma that is diagnostic of the L-neuron population, rather than this diagnostic ability being limited to RT97. This supposition is supported by recent experiments in this laboratory with antibodies against all three subunits and against both phosphorylated and non-phosphorylated forms of these subunits. With the exception of antibodies specifically against the non-phosphorylated form of the 200 kDa subunit we have found that the antibodies against the other neurofilament subunits appear to be equally good at distinguishing between the L- and SD-cell populations and furthermore that intensity of fluorescent labelling with the different antibodies is similar to that with RT97 (M. Perry & S. N. Lawson, in preparation). The situation clearly differs in central nervous system neurons such as spinal cord neurons since these have been shown to have

predominantly non-phosphorylated neurofilament protein (Sternberger & Sternberger, 1983; Shaw *et al.* 1986; Dahl *et al.* 1988; Mansour, Bignami, Labkovsky & Dahl, 1989) and are not labelled by RT97 (see Lawson *et al.* 1984) although they are labelled by other anti-neurofilament antibodies (M. Perry, personal communication). Thus for primary afferent neurons only, the intensity of RT97 immunoreactivity appears to give an indication of the amount of neurofilament present. It should also be made clear that SD, RT97-negative neurons probably do have small quantities of neurofilament in their cytoplasm and are more accurately described as having neurofilament-poor than as having neurofilament-free somata (e.g. Yamadori, 1970; Sharp *et al.* 1982).

The next question to consider is how the variability in intensity of RT97 reaction product, as a measure of the density of cytoplasmic neurofilament, is related to CV and cell size.

Relative RT97 intensity in relation to conduction velocity

It has previously been shown that the number of neurofilament profiles in a fibre is approximately proportional to the fibre diameter in rat sciatic nerve (Friede & Samorajski, 1970) and the present data indicate that there is a correlation between soma RT97 immunoreactivity and CV and thus presumably (see Hursh, 1939; Rushton, 1951) with axon diameter. However, since our data show no correlation between RT97 immunoreactivity and velocity within the C-, the Aδ- or the Aα/β-fibre groups it seems that the soma neurofilament content is not solely dependent upon axon diameter.

Conduction velocity in relation to cell size

Significant correlations between velocity and soma size have previously been reported for rat DRG neurons (Harper & Lawson, 1985) with correlation coefficients of 0·67 for C cells, 0·57 for Aδ cells and 0·26 for Aα/β cells. A correlation has also been described for all A-fibre neurons together but not for C-fibre neurons in cat L5-S3 DRGs (Lee *et al.* 1986). In the present paper a clearly significant correlation existed when cells of all CVs were considered together, but no significant correlations were found within any of the CV groups. There are several possible reasons for the differences between these studies. There were many fewer Aα/β cells in the present study ($n = 14$) than in that of Harper and Lawson ($n = 45$) for which only a loose correlation was seen. It is therefore not surprising that no significant correlation was seen in this smaller group. Added to this are differences in the preparation. Harper and Lawson impaled only the neurons in the top 200 μm of the dorsolateral surface of the L4 ganglion whereas in this study cells from all surfaces of L4, L5 and L6 were included, adding variability between ganglia and between different areas of the same ganglion. This variability is the most likely explanation for the lack of correlation in C and Aδ neurons in the present study, and may also explain the lack of correlation in C cells seen by Lee *et al.* (1986).

Relative RT97 staining intensity in relation to cell size

It is interesting that the intensity of RT97 staining is better correlated with cell size than with CV for cells of all velocities grouped together. Despite no correlation

within either the C- or the Aδ-fibre groups, there was a remarkably good correlation between RT97 intensity and cell size in the Aα/β-fibre group considering the variability inherent in the immunocytochemical techniques and between animals. At present we can offer no explanation for this difference between the two groups of A cells. In considering the implications of the above correlations, the relationship of CV to soma size becomes important. The correlation between the RT97 intensity and cross-sectional area for Aα/β cells is not apparently secondary to a correlation with axon diameter/CV, since a significant correlation does not exist between either intensity and CV or between CV and cell size for this group in the present study. It therefore seems that soma neurofilament density is related not only to axon diameter but even more strongly to soma size when all cells are considered together and that the relationship to soma size is particularly strong for Aα/β cells.

Possible significance of soma neurofilament content

The developmental significance of soma neurofilament is worth considering. The L/SD cell classification of the neuron appears to be laid down as early as the neuronal birthday, or final cell division (Lawson, Caddy & Biscoe, 1974; Lawson & Biscoe, 1979; Lawson, 1979), since the cells which, in the adult, have the size distribution of the L-cell population are the first to stop dividing. Thus, since all L cells are RT97 positive in the adult (Lawson *et al.* 1984), the earliest cells to stop dividing must be destined to become the neurofilament-rich cells. It would be interesting to establish whether, within the neurofilament-rich population, the cytoplasmic neurofilament density is related to the timing of the final cell division. Neurofilament can be detected in some DRG neurons of the rat as early as 17 days of gestation (Ayer-LeLievre *et al.* 1985) and it could perhaps have an effect on axonal outgrowth. One possibility is that the amount of neurofilament in the soma might affect, or be affected by, the axon diameter. It might therefore be indirectly related to the state of myelination and thus the CV of the axon. The present data are compatible with this, but they indicate that the density of neurofilament is also related to the size of the neuronal soma especially in the fast-conducting A-fibre cells where this appears to be a much stronger relationship than with CV. One interpretation of these data is that both the soma size and the RT97 staining intensity are more closely related to the total axon size (volume or surface area) than to axon diameter in Aα/β cells. Other factors may also influence the amount of neurofilament, such as the target site, or sensory receptor type of the neuron, but further data are needed to establish whether this is the case.

Predictions possible from RT97 intensity and cell size

Certain predictions about rat DRG neurons can be made from the present data if they are extrapolated to all DRG neurons. Firstly taking cell size alone, small cells can have CVs in any range but are most likely to be in the C or Aδ range, medium-sized cells are likely to be Aδ or Aα/β cells and large cells are probably Aα/β cells. Secondly, taking both RT97 immunoreactivity and cell size together, the following predictions can be made: that RT97-negative cells have C fibres, that small neurons which are strongly RT97 positive have Aδ fibres, that small neurons which are weakly RT97 positive could have either Aδ or Aα/β fibres and that large neurons

which are strongly RT97 positive have Aα/β fibres. Thus, bearing in mind that the data in this study are based on small numbers and that exceptions may be found such as occasional C-fibre cells with RT97-positive somata, it would seem that in rat DRG neurons combined information about cell size relative to the overall size distributions in the ganglion, plus the intensity of RT97 immunoreactivity in the soma, can be used to predict a probable range of CVs for the fibre.

RT97 immunoreactivity in neonatal ganglia

The appearance of the cell size distributions of RT97-positive and RT97-negative neurons in neonatal ganglia is similar to that of the two overlapping normal distributions of L and SD neurons seen in DRGs of 1-day-old mice (Lawson, 1979); also the percentage of neurons labelled by RT97 (32%) was just at the lower end of the range found for the L-neuron population in that study (32–44%). The RT97-positive population appears to be normally distributed as is the L-cell population in the neonatal mouse, and is therefore considered to represent most of the cells in the L-cell population. The slightly lower percentage for RT97-positive cells in neonatal than in adult ganglia is partly due a different sampling bias. In the adult sampling is biased towards the larger cells but in the neonatal ganglia there is less bias due to a much lower size differential between the two cell types. However, the weakness of the immunoreactivity compared to that in the adult, and the tail of RT97-negative neurons extending into the larger end of the size range, make it appear that perhaps not all the neurons which will comprise the L population in the adult are being classed as RT97 positive in this study, and that this may also contribute to a small extent to the lower percentage of RT97-positive neurons counted.

Conclusions

In conclusion, it seems that neurofilament-rich DRG neuronal somata (L neurons) in the adult rat have A fibres, presumably myelinated, while neurofilament-poor somata (SD neurons) have C fibres which are presumed to be unmyelinated. The density of neurofilament is related to the neuronal size, particularly in fast-conducting A cells, and it is also related to CV. Since this antibody can distinguish between two cell populations in neonatal rats before myelination occurs, it seems possible that the density of neurofilament in the soma could be a useful predictor of the cell type and myelinated state to be reached in the normal adult rat.

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