Expression of α_2-adrenergic receptors in rat primary afferent neurones after peripheral nerve injury or inflammation

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1. Immunocytochemistry with polyclonal antibodies directed against specific fragments of intracellular loops of α_{2A}- and α_{2C}-adrenergic receptors (α_{2A}-AR, α_{2C}-AR) was used to explore the possibility that expression of these receptors in dorsal root ganglion (DRG) neurones of rat alters as a result of peripheral nerve injury or localized inflammation.
2. Small numbers of neurones with positive α_{2A}-AR immunoreactivity (α_{2A}-AR-IR) were detected in DRG from normal animals or contralateral to nerve lesions. In contrast, after complete or partial sciatic nerve transection the numbers of ipsilateral L_4 and L_5 DRG somata expressing α_{2A}-AR-IR sharply increased (> 5-fold). There was no discernible change in the number of DRG neurones exhibiting α_{2A}-AR-IR innervating a region in association with localized chemically induced inflammation.
3. After nerve injury, double labelling with Fluoro-Gold, a marker of retrograde transport from transected fibres, or by immunoreactivity for c-jun protein, an indicator of injury and regeneration, suggested that many of the neurones expressing α_{2A}-AR-IR were uninjured by the sciatic lesions.
4. In general the largest proportionate increase in numbers of neurones labelled by α_{2A}-AR-IR after nerve lesions appeared in the medium–large diameter range (31–40 μm), a group principally composed of cell bodies of low threshold mechanoreceptors. The number of small diameter DRG neurones labelled by α_{2A}-AR-IR, a category likely to include somata of nociceptors, also increased but proportionately less.
5. Relatively few DRG neurones exhibited α_{2C}-AR-IR; this population did not appear to change after either nerve lesions or inflammation.
6. These observations are considered in relation to effects of nerve injury on excitation of primary afferent neurones by sympathetic activity or adrenergic agents, sympathetically related neuropathy and reports of sprouting of sympathetic fibres in DRG.

After nerve injury some primary afferent neurones, particularly nociceptors, develop a novel excitatory response to sympathetic efferent activity or exogenously applied adrenergic agonists (Sato & Perl, 1991; Bossut & Perl, 1995; O'Halloran & Perl, 1997; Abdulla & Smith, 1997). Similarly, afferent fibres terminating in a neuroma formed after transection of a peripheral nerve become responsive to adrenergic agents (Wall & Gutnick, 1974). A partial loss of sympathetic innervation also induces an adrenergically produced excitation of cutaneous nociceptors (Bossut *et al.* 1996). The sympathetic adrenergic excitation takes place at the peripheral terminals of nociceptors or the cell body of dorsal root ganglion (DRG) neurones and is reversibly blocked by α_2-adrenergic antagonists. Excitation of nociceptors by adrenergic agents and sympathetic stimuli has also been reported to be associated with inflammation (Hu & Zhu, 1989; Sato *et al.* 1993). It is possible that denervation and inflammation serve as stimuli for alterations in expression of adrenergic receptors by DRG neurones. Increased catecholaminergic innervation of DRG after peripheral nerve injury (McLachlan *et al.* 1993) also is possibly related to altered expression of adrenergic receptors.

Causalgia and related dystrophies are syndromes in which injury to regional innervation leads to a spontaneous burning pain accompanied by allodynia (pain resulting from non-noxious stimuli) and hyperalgesia in a partially denervated area (Nathan, 1947; Janig, 1992). These symptoms are often relieved or modified, at least temporarily, by sympathectomy (see review by Gybels & Sweet, 1989). Several proposals have been put forth to explain the relationship of the sympathetic

system to pain after nerve injury or denervation, including the possibility that primary afferent neurones develop novel adrenergic receptors (Nathan, 1947; Devor & Janig, 1981; Sato & Perl, 1991; Perl, 1994).

Two α_2-adrenergic receptor subtypes (A and C) have wide distribution in the central nervous system (Nicholas *et al.* 1993; Scheinin *et al.* 1994; Rosin *et al.* 1996; Talley *et al.* 1996; Gold *et al.* 1997). Our experiments were directed at determining whether the number and spectrum of primary afferent neurones expressing these adrenergic receptors alters as a consequence of either injury to a mixed somatosensory nerve or an artificially induced inflammation. These observations have been reported in a preliminary fashion (Birder & Perl, 1996).

METHODS

A total of 42 adult Wistar rats (150–250 g) of both sexes were used; eight served as unoperated controls. All procedures on living rats were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of North Carolina, Chapel Hill.

Peripheral nerve lesions

Twenty animals were deeply anaesthetized by a combination of ketamine ($10\ \mathrm{mg\ kg^{-1}}$ i.p.) and xylazine ($10\ \mathrm{mg\ kg^{-1}}$ i.p.). Anaesthesia was established as being adequate for surgery by periodically testing for the absence of a withdrawal reflex to a strong pinch of a hindpaw and absence of an eye blink reflex to contact with the cornea. Additional anaesthetic was given to maintain the areflexic state until surgery was completed. Usual surgical aseptic precautions were employed to expose the sciatic nerve of one leg through an incision over the sciatic notch and to either partially ($n = 7$) or totally ($n = 13$) transect it using fine sharp scissors. The aim of the partial transection was to divide the same one-half of the nerve each time; however, variability in exact exposure and orientation of the sciatic nerve made it probable that the partial lesions varied from animal to animal. In five animals, after total transection of the sciatic nerve, the central cut end was dipped into Fluoro-Gold (3% w/v), a retrogradely transported substance taken up by cut/damaged nerve fibres of all sizes (Schmued & Fallon, 1986; Baranowski *et al.* 1992), to identify DRG somata with transected peripheral fibres. Eighteen of the 20 animals were studied 7–14 days after the sciatic lesions; the survival period for two animals was 72 days. We made no systematic attempt to test for hyperalgesia, although the lesioned animals' behaviour was unremarkable during their survival with no visible signs of distress other than relatively minor favouring of the affected limb. The rare animal exhibiting autotomy after complete sciatic transection was killed immediately by an overdose of pentobarbital anaesthetic and was not included in the analyses.

Induced inflammation

Twelve other animals anaesthetized with ketamine and xylazine to areflexia had 0·1 ml of one of the following injected subcutaneously into the plantar part of one hindpaw through a 30 gauge needle: carrageenan (4 mg in 0·9% NaCl), formalin (4% in 0·9% NaCl) or Freund's complete adjuvant. These agents cause inflammation and hyperalgesia, which, in some cases, can persist for up to 2 weeks (Iadarola *et al.* 1988). After these injections the animals developed localized oedema and guarded the affected limb; otherwise their behaviour was unremarkable. Subcutaneous plantar injections of 0·1 ml of Fluoro-Gold (3% w/v) were made in two additional anaesthetized rats to identify DRG neurones with fibres innervating the foot. Eight of this group of 14 animals, including two injected with Fluoro-Gold, survived for 1–5 days; the remaining six were killed 2 weeks after the plantar injections.

Immunohistochemistry

Terminally, the animals were deeply anaesthetized to areflexia with the ketamine–xylazine mixture and killed by intracardiac perfusion with ice-cold 0·9% NaCl followed by a zinc formalin fixative (Anatech, Inc., Battle Creek, MI, USA), a solution of formaldehyde and zinc sulphate used at a working concentration of 4% formaldehyde. Good perfusion fixation proved essential for consistent immunocytochemical staining and low background. The DRGs contributing to the innervated region were removed, postfixed for 1–2 h in the zinc formalin fixative and then cryoprotected in 25% sucrose. Serial frozen (10 μm) sections were mounted on slides (superfrost). Non-specific binding was blocked by 3% normal goat serum.

The primary polyclonal antibody against the presumed rat α_{2A}-adrenergic receptor (α_{2A}-AR) was developed by immunizing rabbits with a recombinant fusion protein prepared from the cloned rat gene; the protein consisted of glutathione-S-transferase (GST) and a 47 amino acid fragment from the third intracellular loop (Rosin *et al.* 1993). The targeted fragment of the receptor differed substantially from equivalent regions of other α_2-AR subtypes. Receptor selectivity of the affinity purified antisera was established by Western blot analysis of COS cells (a transformed fibroblast-like cell line derived from African green monkey kidney) expressing only the α_{2A} receptor subtype. It was established that this α_{2A}-AR antibody lacked cross-reactivity with other α_2-receptor subtypes and recognized native receptor as well as receptor in fixed tissue (Rosin *et al.* 1993). The polyclonal antibody against the α_{2C}-AR was similarly produced using a recombinant fusion protein consisting of GST and a 70 amino acid fragment of the 3rd intracellular loop and characterized in the same fashion (Rosin *et al.* 1996).

DRG sections were first incubated with affinity purified α_{2A}-AR or α_{2C}-AR antibodies ($2·5\ \mu\mathrm{g\ ml^{-1}}$ for 48 h) which had been preabsorbed for 2–3 h with a 5- to 10-fold excess (w/w) of GST (Rosin *et al.* 1993) and then, after rinsing, with a cyanine fluorescent probe (cy-2 or cy-3 conjugated anti-rabbit IgG).

The c-jun marker for DRG neuronal injury (Jenkins *et al.* 1993) was compared with α_2-AR labelling using a cocktail for the initial incubation containing both the α_{2A}-AR primary antibody and a c-jun antibody (mouse monoclonal). After rinsing, these sections were further incubated in a second cocktail containing cyanine fluorescent probes (cy-2 conjugated anti-rabbit IgG, cy-3 conjugated anti-mouse IgG).

Data analysis

The processed DRG sections were examined by epifluorescence and transmitted light microscopy; images were captured using a high resolution video camera (Optronics DE1-470T) and an MS DOS computer fitted with an image analysis program (Image-Pro Plus, v. 2.0 or 3.0, Media Cybernetics, Silver Spring, MD, USA). The diameter of labelled neurones was determined by the imaging program from a tracing of the perimeter of each counted profile containing a nucleus to give a mean diameter for each cell. The number of labelled neurones in a ganglion was estimated from counts at moderate to high magnification (× 250–630) of positively stained profiles containing nuclei in 10 sequential sections separated by 100 μm. All neurones in each counted section were examined. All cell counts (either α_{2A}-AR-IR or α_{2C}-AR-IR) are presented as

Table 1. Average number of ipsilateral or contralateral neurones in DRG expressing α_{2A}-AR (L_4, L_5) or α_{2C}-AR (L_4 only) in unoperated animals and animals with partial or complete sciatic transection or inflammation

	Normal	Partial	Complete	Inflammation
α_{2A}-like-IR				
Ipsilateral L_4	19·2 ± 5·8	135·2 ± 46·5	194·3 ± 57·3	12·8 ± 3·4
Contralateral L_4	20·3 ± 5·7	21·4 ± 8·7	27·7 ± 9·4	16·1 ± 7·4
α_{2A}-like-IR				
Ipsilateral L_5	16·3 ± 1·0	238·3 ± 65·1	125·5 ± 52·3	—
Contralateral L_5	15·2 ± 1·1	34·4 ± 14·7	21·4 ± 9·2	—
α_{2C}-like-IR				
Ipsilateral L_4	17·4 ± 5·4	13·6 ± 5·7	16·2 ± 7·6	17·2 ± 5·1
Contralateral L_4	17·1 ± 5·2	11·8 ± 3·2	18·4 ± 5·4	19·5 ± 5·4

Normal, unoperated animals ($n = 8$); Partial, 7–14 days after partial sciatic transection ($n = 7$); Complete, complete sciatic transection ($n = 11$); Inflammation, 1–14 days after injection of Freund's adjuvant ($n = 4$). Data are means ± S.D. The range of the number of positive cells in 10 sections in different animals was as follows. α_{2A}-AR-IR normal: ipsilateral (IL), L_4, 12·8–27·1; contralateral (CL), L_4, 11·4–24·7; IL, L_5, 14·5–17·5; CL, L_5, 14·0–16·5. Partial sciatic transection: IL, L_4, 82·1–184·2; CL, L_4, 12·2–32·3; IL, L_5, 118·5–315·0; CL, L_5, 20·1–61·2. Complete sciatic transection: IL, L_4, 71·5–257·0; CL, L_4, 8·8–35·4; IL, L_5, 31·2–195·6; CL, L_5, 10·2–35·6. Inflammation: IL, L_4, 8·7–17·4; CL, L_4, 8·1–26·8. For α_{2C}-AR-IR, as follows. Normal: IL, L_4, 11·8–23·2; CL, L_4, 12·5–24·1. Partial sciatic transection: IL, L_4, 4·2–20·5; CL, L_4, 3·5–25·3. Complete sciatic transection: IL, L_4, 8·5–28·2; CL, L_4, 9·2–29·1. Inflammation: IL, L_4, 10·3–22·4; CL, L_4, 10·7–25·2.

the mean number of positive neurones per 10 sections ± standard deviation (S.D.) from each ganglion. For the size distributions shown in Fig. 7, the values reported are the percentage of α_{2A}- or α_{2C}-AR-IR neurones counted. Student's t test was used to estimate the probability that differences between means of the experimental groups could have occurred by chance.

Materials

Chemicals were obtained as follows: Fluoro-Gold from Fluorochrome, Inc., Eaglewood, CO, USA; cyanine conjugated anti-rabbit IgG from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA or Rockland, Inc., Gilbertsville, PA, USA; normal goat serum from Vector Laboratories, Inc., Burlingame, CA, USA. Other chemical compounds were obtained from Sigma. Slides (superfrost) used to mount the tissue sections were obtained from Fisher Scientific, Pittsburgh, PA, USA.

RESULTS

α_{2A}-IR and α_{2C}-IR expression after partial sciatic nerve lesions

Examples of the α_{2A}-IR-like reaction product in DRG neurones are shown in Fig. 1*A* and *C*. As described by Rosin *et al.* (1993), the punctate bodies varied in both size and number (mean 12, range 8–16, for each positive neuronal profile) and often circled the soma. The α_{2C} reaction product differed; it appeared as a more typical diffuse label distributed throughout the cytoplasm (Fig. 1*E*). Neither the punctate stained bodies nor the diffuse label was present when the primary antibodies were preabsorbed with the respective α_{2A} or α_{2C} fusion protein (5- to 10-fold excess; Fig. 1*B*, *D* and *F*) or if the primary antisera were omitted.

As Table 1 and Figs 2 and 3 indicate, in unoperated animals few DRG neurones (< 25 neurones per 10 sections) exhibited either α_{2A} or α_{2C} immunoreactivity (IR). Seven to 14 days after either partial or complete transection of the sciatic nerve, the number of neurones in the ipsilateral L_4 and L_5 DRG exhibiting α_{2A}-AR-IR was sharply increased. Most of the observations were from animals surviving 14 days after the sciatic lesion; however, there was considerable variation between animals at all time points. No significant differences were detected between animals killed at 7 days and those surviving up to 14 days. Therefore, data were pooled for the animals studied 7–14 days after the sciatic transections.

Figure 4*A* and *B* and Table 1 show that after a partial transection of the sciatic nerve ($n = 7$) significantly more (> 6-fold, $P < 0·05$) ipsilateral L_4 and L_5 DRG neurones demonstrated α_{2A}-AR-IR than in normal or equivalent contralateral ganglia. Complete transection of the sciatic nerve resulted in similar marked increases in the number of neurones demonstrating α_{2A}-positive IR in L_4 and L_5 ganglia of the lesioned side relative to normal ganglia (Fig. 5*A* and *B* and Table 1). Some ganglia contralateral to the sciatic lesions had apparent small increases in numbers of α_{2A}-AR-IR neurones (Table 1) compared with normal animals but the variation from animal to animal was large enough for these differences to have occurred by chance ($P > 0·05$). To estimate the proportion of neurones in a ganglion expressing α_{2A}-AR-IR, 500 L_4 neurones were counted in 10 sections spaced by 100 μm. After a partial sciatic transection, 14·8% of this count exhibited α_{2A}-AR-

IR. After complete sciatic transection in another animal, a somewhat larger proportion, 21·1% of 500 neurones, showed α_{2A}-AR-IR. Considerable variation occurred from animal to animal as is documented by the range of values in the legend to Table 1, although even the smallest number of labelled neurones in the experimental group was substantially greater than was found in normal ganglia. Few α_{2A}-labelled neurones (<20 neurones per 10 sections) were found in adjacent segments (L_1–L_3, L_6) after sciatic lesions. Seventy-two days after complete sciatic nerve transection

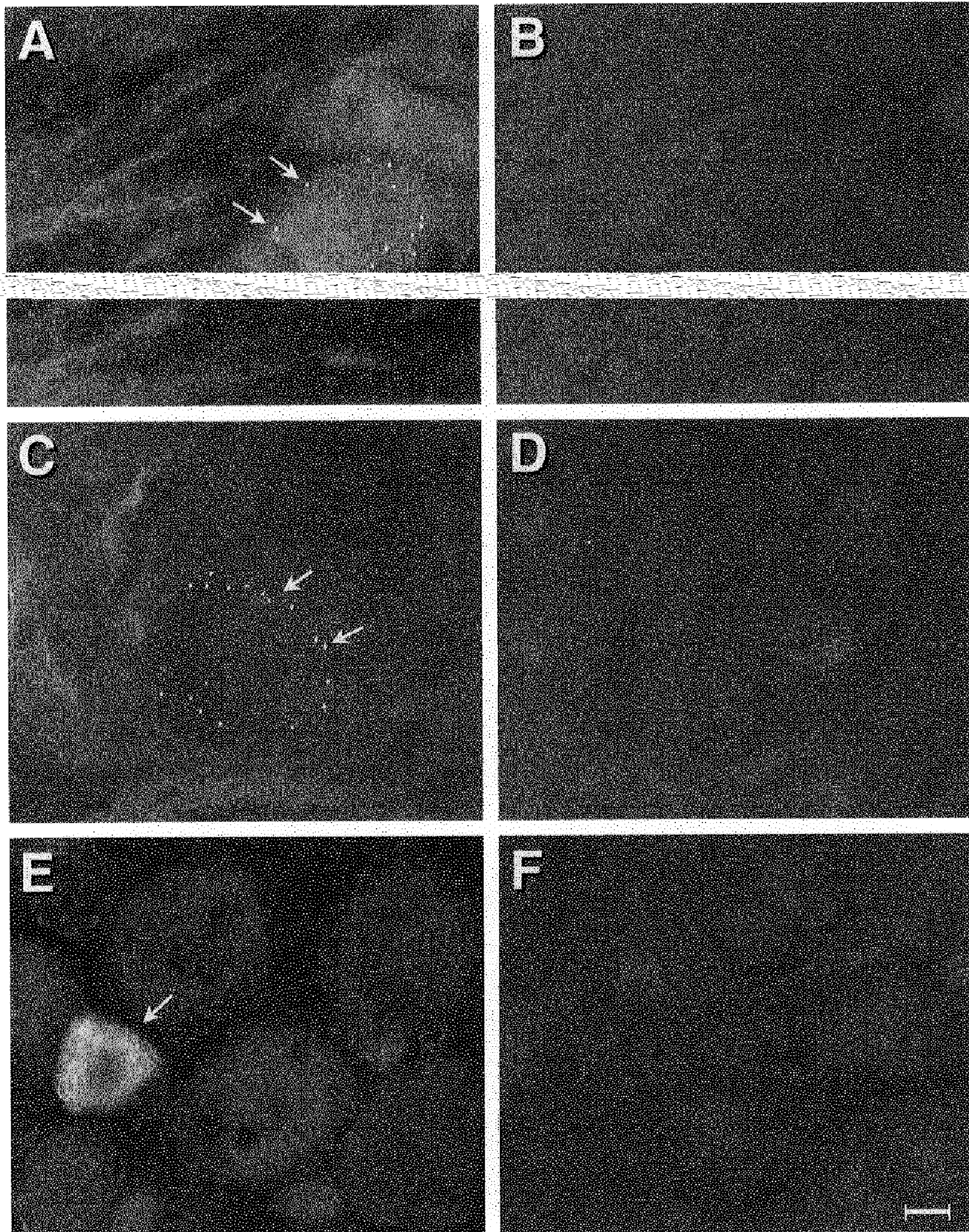

Figure 1. Epifluorescence image of sections from L_4 dorsal root ganglia depicting α_2-AR-IR

A and *C*, sections depicting α_{2A}-AR-IR after partial sciatic nerve transection (*A*) and complete sciatic nerve transection (*C*). *B* and *D*, adjacent sections to *A* and *C* showing block of reaction product by addition of α_{2A}-AR fusion protein. Arrows indicate positive reaction product. *E*, epifluorescence image of a section from L_4 dorsal root ganglion depicting α_{2C}-AR-IR (complete sciatic transection 14 days previously). Identical images were found in DRG of normal animals. *F*, block of reaction in a section adjacent to *E* by addition of α_{2C}-AR fusion protein. Arrow indicates neurone with positive reaction product. Calibration bar, 10 μm for each panel.

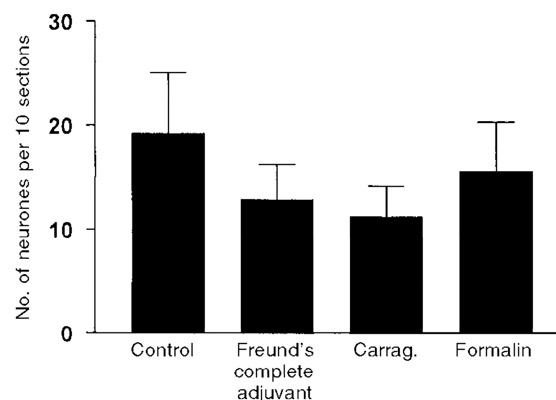

Figure 2. Number of L_4 DRG neurones expressing α_{2A}
AR-IR in 8 unoperated animals (Control) or after injection into the ipsilateral hindpaw of the inflammatory agents Freund's complete adjuvant ($n = 4$), carrageenan (Carrag., $n = 4$) and formalin ($n = 4$). Data are means per 10 sections ± S.D.

($n = 2$), the number of α_{2A}-AR-IR neurones in ipsilateral L_4 and L_5 DRG did not significantly differ from findings in unoperated controls (< 20 cells per 10 sections, $P > 0{\cdot}05$).

In contrast to the α_{2A} observations, after partial or complete sciatic transection no increase appeared in the number of ipsilateral neurones labelled by α_{2C}-AR-IR (Fig. 3 and Table 1).

Only limited and scattered α_{2A}-AR-IR appeared in the control spinal dorsal horn. No obvious increase in α_{2A}-AR-IR was noted with the antibody used in the ipsilateral L_4/L_5 spinal dorsal horn after nerve injury. Spinal cord α_{2C}-AR-IR also did not increase after either complete or partial sciatic transections.

After the sciatic nerve lesions, all diameter ranges of ipsilateral L_4 DRG neurones expressing α_{2A}-ARs increased in number, although the largest proportionate increase usually occurred in the medium–large diameter category (31–40 μm). After partial transection, 41·5% of labelled L_4 neurones were in the 31–40 μm category, with a mean diameter of 34 μm. Following complete sciatic transection 40·3% of the labelled L_4 cells were in the 31–40 μm range (mean diameter of 36 μm). This distribution contrasts with the diameter spectra of neuronal somata of comparable DRG from normal rat in which notably smaller cells predominate (Harper & Lawson, 1985; Tandrup, 1993). On the other hand, in mean values from L_5 ganglia after partial lesions, the greatest number of α_{2A}-AR-IR appeared in the 21–30 μm group, although the size distribution of labelled neurones remained skewed toward the larger diameters relative to that of the DRG population as a whole (Fig. 4). Counting only neurones in histological sections with nuclei theoretically creates a bias in favour of large neurones (Harper & Lawson, 1985; Tandrup, 1993); however, this shift should account for only a small part of these distributions.

α_{2A}-AR-IR and evidence of injury

An indication of which DRG neurones in the L_4/L_5 ganglia had divided peripheral fibres after complete sciatic transections was gained from experiments with Fluoro-Gold (FG). Seven to 14 days after complete sciatic nerve transection, the number of DRG neurones that were labelled by both α_{2A}-AR-IR and FG (72 neurones per 10 sections) was significantly greater ($P < 0{\cdot}05$) than the number of α_{2A}-AR-IR-positive cells in control or contralateral ganglia (Fig. 6*A*); however, more neurones showed only α_{2A}-AR-IR than those that were double-labelled ($P < 0{\cdot}05$, Fig. 6*A*). About 25% of FG-containing neurones were α_{2A}-AR-IR positive. For both FG-positive and FG-negative subsets most of the α_{2A}-AR-IR-positive neurones were of the medium–large diameter category (31–40 μm).

Transection of a peripheral nerve results in a massive increase in the number of DRG neurones expressing the c-jun proto-oncogene from the very low level present in the absence of injury (Jenkins & Hunt, 1991). The c-jun expression may be due to alterations in growth or neurotropic factors (Jenkins *et al.* 1993). The increase in c-jun labelling continues until regeneration of the nerve is complete (Herdegen *et al.* 1993). We used immunoreactivity to detect the presence of c-jun protein and thereby to identify

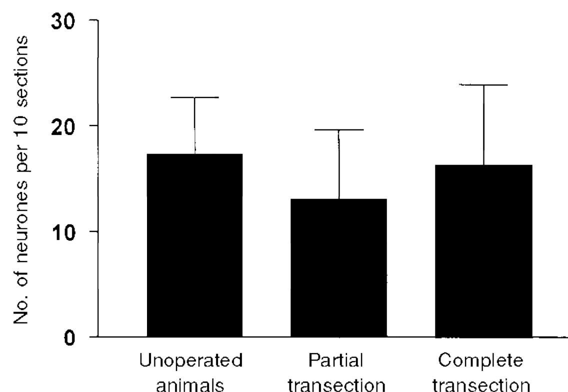

Figure 3. Number of L_4 DRG neurones expressing α_{2C}-AR-IR in unoperated animals ($n = 8$) or after partial ($n = 7$) or complete transection ($n = 11$) of the sciatic nerve
Data are shown as means per 10 sections ± S.D. No change of α_{2C}-AR expression was noted after injection of identical inflammatory agents as in Fig. 2.

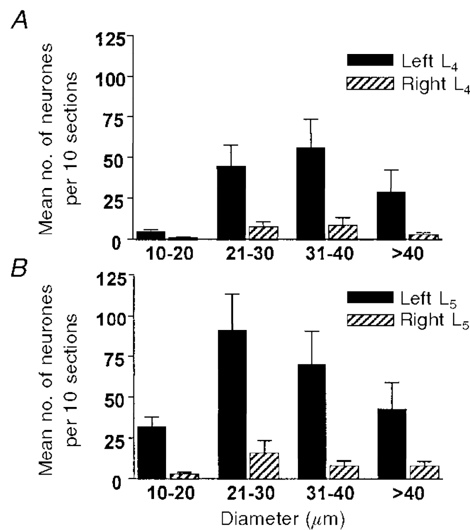

Figure 4. Diameter distribution of DRG neurones expressing α_{2A}-AR-IR after partial transection of the sciatic nerve 7–14 days previously

Data are means per 10 sections ± S.D. ($n = 7$). Filled bars, ipsilateral to lesion; hatched bars, contralateral to lesion. *A*, L_4 DRG; *B*, L_5 DRG. See Methods for additional details.

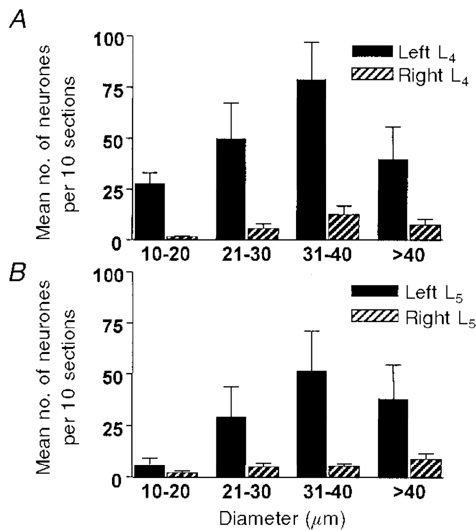

Figure 5. Diameter distribution of DRG neurones expressing α_{2A}-AR-IR after complete transection of the sciatic nerve 7–14 days previously

Data are means per 10 sections ± S.D. ($n = 11$ animals). Filled bars, ipsilateral to the lesion; hatched bars, contralateral. *A*, L_4 DRG; *B*, L_5 DRG.

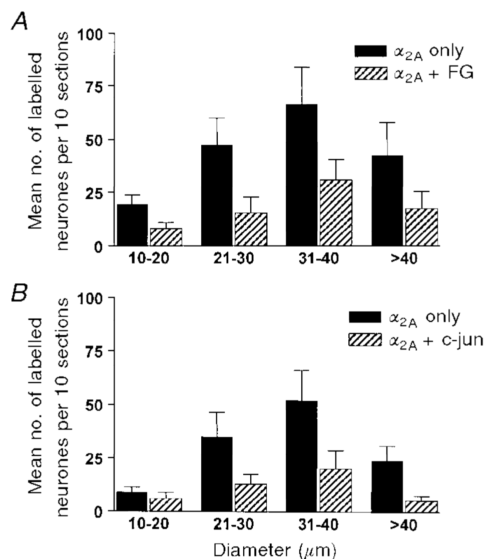

Figure 6. Diameter distribution of DRG neurones expressing α_{2A}-AR-IR in relation to markers for transection or injury of peripheral fibres

A, mean number (± S.D.) of α_{2A}-AR-IR neurones in 10 sections of the ipsilateral L_4 DRG from 5 animals after complete sciatic nerve transection 7–14 days previously. Filled bars, α_{2A}-AR-IR only; hatched bars, α_{2A}-AR-IR and retrogradely transported Fluoro-Gold (FG). *B*, mean number (± S.D.) of α_{2A}-AR-IR neurones in 10 sections of the ipsilateral L_4 DRG from 6 animals after partial transection of the ipsilateral sciatic nerve. Filled bars, α_{2A}-AR-IR only; hatched bars, α_{2A}-AR-IR and c-jun-IR. See Methods and Results for additional details.

neurones with peripheral fibres putatively transected or otherwise injured by the nerve lesion. The proportion of neurones in an L_4 DRG exhibiting c-jun-IR after the complete sciatic nerve transections (36%) was roughly comparable to those marked by FG in similar experiments (28%; difference $P > 0{\cdot}05$). The number of neurones in the ipsilateral L_4 DRG expressing the α_{2A}-AR-IR alone or in combination with c-jun-IR increased significantly relative to the contralateral side ($P < 0{\cdot}05$); however, as with the FG marker, the profiles with only α_{2A}-AR-IR (presumably uninjured) were more numerous (Fig. 6*B*). As depicted in Fig. 7, after partial or complete sciatic transection, the c-jun protein appeared in more small diameter than large diameter DRG neurones (44% in the smallest diameter category, 10–20 μm), as might be anticipated from the relative proportion of small neurones in DRG. The distribution of injury marked cells in Fig. 7 contrasts with the size distribution of the largest category staining with both labels (e.g. Fig. 6), those of medium–large diameter (31–40 μm). Nonetheless, some smaller diameter neurones (21–30 μm) had both α_{2A}-AR-IR and c-jun-IR. It is noteworthy that not all FG or c-jun-labelled neurones showed α_{2A}-AR-IR. While there is no certainty that either the retrograde and the c-jun tags label all and only injured neurones, the similarity of results with the two techniques suggests that after sciatic lesions more uninjured neurones than injured ones develop α_{2A}-AR-IR.

Effects of local inflammation

The three agents injected into the plantar footpad to induce local inflammation – carrageenan, formalin and Freund's adjuvant – produced variable degrees of oedema and guarding of the hindlimb. Formalin and Freund's adjuvant caused the most marked local reactions. After induction of the local inflammation, the number of neurones labelled by either the α_{2A} or the α_{2C} antibodies in the L_4/L_5 ganglia did not significantly differ from controls ($P > 0{\cdot}05$) either ipsilaterally or contralaterally to the injected paw (Table 1 and Fig. 2).

DISCUSSION

Following partial and complete transection of the sciatic nerve, the antibody we used against the α_{2A} adrenergic receptor fragment labels a sharply increased number of DRG neurones. The increase is confined to ipsilateral ganglia supplying large numbers of afferent fibres to the injured nerve (L_4–L_5). The proportion of α_{2A}-AR-IR neurones in ipsilateral ganglia adjacent to L_4 and L_5 remains comparable to that of control animals or contralateral ganglia. On the other hand, we noted no increase in the number of neurones expressing α_{2C}-AR-IR after the sciatic injuries, an indication that the α_{2A}-AR-IR changes were specific. Increases in α_{2A}-AR-IR also did not appear in the inflammatory models.

There are considerable data favouring the concept that immunoreactivity to the antibody we employed marks an α_{2A}-type of adrenergic receptor. The antibody has been extensively characterized in other studies and shown to label selectively neurones established to express the α_{2A}-type of adrenergic receptor (Rosin *et al.* 1993; Talley *et al.* 1996). This characterization includes *in situ* hybridization studies demonstrating the presence of α_{2A}-AR-like mRNA (Zeng & Lynch, 1991; Nicholas *et al.* 1993). However, other immunocytochemical studies analysing the presence of α_2-adrenergic receptors in DRG neurones have reported varying results, including some differing quantitatively from ours (e.g. Roberts & Connick, 1997; Gold *et al.* 1997). We can only speculate about the basis of these differences. In part, it could reflect differences in experimental protocols (e.g. Gold *et al.* 1997). These could represent different experimental procedures, varying immunocytochemical methods, cross-reactivity with other proteins and/or problems in

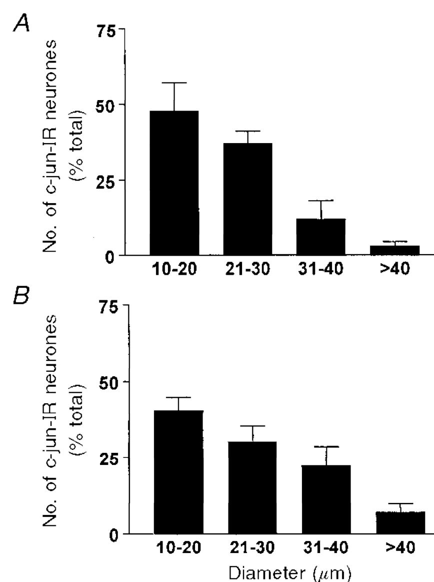

Figure 7. Diameter distribution of neurones displaying c-jun-IR after nerve injury

Note that the ordinate is the proportion (percentage) of the total counted in L_4 ganglia ($\pm$ S.D.). *A*, 788 counted positive neurones from 2 animals following complete ipsilateral sciatic transection. *B*, 658 counted positive neurones from 2 animals after partial ipsilateral sciatic transection.

interpretation due to high levels of background reactivity. From their published illustration, the latter could contribute to the larger number of α_{2A}- and α_{2C}-immunoreactive DRG neurones reported by Gold *et al.* (1997). We took special care to keep the background in our analyses very low. Our judgements were based on the presence of the punctate reaction product, at least partially distributed around profiles containing a nucleus. These criteria made for ready identification of positive labelling. In our experiments, the appearance of the reaction product and α_{2A}-AR-IR staining of neurones was regularly blocked by preabsorbance with the fusion protein or omission of the primary antibody.

Uninjured as well as injured neurones participate in the increase in α_{2A}-AR-IR, as evidenced by the absence of labels for transection/injury (presence of Fluoro-Gold and c-jun expression). Admittedly, the completeness of marking of injured neurones can be questioned. The FG marker was used only for complete transection and in this situation, it has been argued to label retrogradely most, if not all, neurones whose fibres are transected (Baranowski *et al.* 1992). Similarly, the c-jun protein or mRNA is reported to appear in many, if not all, injured and regrowing neurones after axotomy (Jenkins *et al.* 1993; Herdegren *et al.* 1993). While it is possible that some neurones with injured fibres were unmarked by either the FG or the c-jun technique, it is notable that substantial numbers of neurones with α_{2A}-AR-IR but lacking the other marker appeared in both variations of the double-labelling experiments. Moreover, the distribution of sizes of neurones labelled for the c-jun protein compares well with the established distribution of neuronal size in normal ganglia but differs distinctly from the size distribution of the DRG neurones exhibiting the α_{2A}-IR after the partial or complete transections (cf. Figs 4, 5, 6 and 7). Therefore, the conclusion that substantial numbers of uninjured neurones develop increased α_{2A}-immunoreactivity after nerve injury seems tenable.

The proportion of neurones in a DRG exhibiting the increased α_{2A}-AR-IR presence after sciatic transection is considerable. We estimate it to be about 15% or more. That many neurones changing phenotype after nerve injury have the capability to produce significant changes in afferent signalling and sensory function. It is important to note that not every injured neurone exhibited α_{2A}-AR-IR, implying a form of selectivity in the process.

A particularly puzzling part of our data is that the number of L_5 DRG neurones in seven animals showing the α_{2A}-AR-IR after partial sciatic lesions was greater than either the mean for L_4 or L_5 after complete transection. It is possible that this apparent anomaly is the result of the large proportion of apparently uninjured neurones that develop the immunoreactivity. Whatever combination of factors links the lesion of the nerve to the change in adrenergic receptor expression, the partial lesions in our sample may have selected more of those triggering the change in the L_5 distribution. A possibility is the relative proportion of sympathetic efferent fibres divided by the lesion (Bossut *et al.* 1996). Perhaps interuption of both sets of fibres, primary sensory and sympathetic postganglionic, combines to influence the increased α_{2A}-AR-IR. In any case, the number of peripheral fibres divided does not appear to be directly related to the number of DRG neurones showing increased appearance of α_{2A}-AR-IR.

It is also unclear how the increase in adrenergic receptor expression after nerve lesions relates to reports of inflammation-induced appearance of adrenergic excitation of nociceptive afferent units. The absence of an increase in the number of DRG neurones exhibiting α_{2A}-AR-IR (or α_{2C}-AR-IR) in association with inflammation emphasizes differences in the factors mediating its actions and those of nerve injury.

A number of functional changes take place in primary afferent neurones after a peripheral nerve injury including the appearance of abnormal spontaneous activity (Wall & Gutnick, 1974; Devor *et al.* 1994) and adrenergic excitation of nociceptors (Sato & Perl, 1991; Bossut & Perl, 1995; O'Halloran & Perl, 1997). It seems reasonable to hypothesize that the increased number of DRG somata expressing an α_2-adrenergic receptor marker is related to the induction of adrenergic excitation of these afferent neurones at their peripheral terminals (Sato & Perl, 1991; O'Halloran & Perl, 1997; Abdulla & Smith, 1997). This latter is consistent with the apparent peripheral origin of some symptoms in human cases of sympathetically related neuralgias (Nathan, 1947; Wallin *et al.* 1976; Torebjork *et al.* 1995). A moderate increase was detected in the number of small diameter neurones with α_{2A}-AR-IR. This size range of somata is likely to be associated with C fibre receptors and therefore with nociceptors (Harper & Lawson, 1985). However, the greatest increase in α_{2A}-AR-IR appeared in the medium–large diameter group of DRG neurones, a category primarily related to myelinated fibre, low threshold mechanoreceptors (Harper & Lawson, 1985). Some types of low threshold mechanoreceptors, muscle spindles, cutaneous slowly adapting type I and Pacinian corpuscles in the normal animal manifest enhanced activity after sympathetic stimulation (e.g. Hunt, 1960; Akoev, 1981; Roberts *et al.* 1985; Barasi & Lynn, 1986). Our observations suggest that the number of the latter types of sensory neurones with the potential for excitement by adrenergic agents markedly increases after partial denervation and that this increase takes place in both injured and uninjured neurones. The cellular mechanisms by which activation of an α_2-adrenergic receptor leads to excitation of a neurone is not well understood. Abdulla & Smith (1997) have provided evidence indicating that after sciatic transection, nor-adrenaline acting through an α_2-adrenergic receptor suppresses N-type Ca^{2+} channel current in dissociated DRG neurones and possibly thereby reduces Ca^{2+}-mediated K^+ conductances.

The latter could be expected to increase excitability of neurones. In their work noradrenaline actions were particularly evident in the smaller DRG somata. The α_2-AR-mediated effect was not found for DRG neurones dissociated from normal animals.

The development of sympathetic adrenergic excitation of nociceptors conceptually is readily linked to neuropathic pain (Sato & Perl, 1991; O'Halloran & Perl, 1997). On the other hand, a connection between a possible sympathetic excitatory action on low threshold mechanoreceptors and the symptom of pain requires a more involved explanation (e.g. Roberts, 1986). At this stage, the relationship of the increased α_{2A}-AR presence to neuropathic states is a matter of conjecture (Devor, 1983; Sato & Perl, 1991; Perl, 1994).

Removal or loss of the peripheral sympathetic innervation has been reported to be associated with increased adrenergic receptor binding by some DRG somata (Nishiyama *et al.* 1993) as well as by cells in other tissues (Davies *et al.* 1982). The present increased expression of α_2-AR-IR corresponds in time (1–3 weeks) to expected expression of adrenergic denervation supersensitivity (Cannon & Rosenblueth, 1949). The suggestion has been made that sympathetically associated neuropathies may be related to mechanisms underlying sympathetic denervation supersensitivity (Perl, 1994). In this concept the presence of sympathetic innervation and mediators would suppress expression of the α_2-adrenergic receptors by DRG neurones.

The augmented α_{2A}-adrenergic receptor-like expression we observed may be a factor in the increase reported in adrenergic connections from sympathetic postganglionic fibres to dorsal root ganglia after peripheral nerve injury (McLachlan *et al.* 1993; Chung *et al.* 1996). The ingrowth of fibres staining for sympathetic markers is described by McLachlan *et al.* (1993) to surround medium and large DRG neurones at a time (2–3 months) when our observations on two animals showed the α_{2A}-AR-IR no longer to be elevated. Although there may be a causal relationship between these two phenomena, the nature of that linkage and the significance of the increased sympathetic innervation remains unclear.

The large number of apparently uninjured neurones developing the α_{2A}-receptor expression suggests a possible basis could be alteration in the environment of DRG neurones. The increased adrenergic receptor expression conceivably could be triggered by processes related to growth of transected fibres or expansion of the peripheral terminal field (of uninjured fibres) following division of other peripheral nerve fibres, afferent or sympathetic. Peripheral nerve injury has been shown to result in a number of changes in DRG neurones including induction of immediate-early genes (e.g. c-jun) and trophic factors (Jenkins *et al.* 1993; Zhou *et al.* 1996). It is possible that whatever factors (e.g. leukemia inhibitory factor, Thompson & Majithia, 1998) and processes stimulate DRG neurones with transected fibres to respond with growth may spread to adjacent uninjured neurones and cause them to undergo similar alterations.

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