Nerve growth factor depletion reduces collateral sprouting of cutaneous mechanoreceptive and tooth-pulp axons in ferrets

B. Doubleday and P. P. Robinson

Departments of Oral and Maxillofacial Surgery and Biomedical Science, University of Sheffield, UK

1. Electrophysiological experiments were carried out to determine whether or not collateral sprouting of cutaneous low-threshold mechanoreceptive fibres could be detected and to investigate the effect of nerve growth factor (NGF) deprivation on the sprouting of these fibres and the fibres innervating tooth pulps.

2. In twenty-one ferrets (eleven of which had been autoimmunized against NGF) the right inferior alveolar nerve (IAN) was sectioned and prevented from regenerating. After 12 weeks, transmedian innervation from the left IAN was determined by stimulating the nerve whilst recording from electrodes implanted in the contralateral anterior teeth and also by single unit recordings from the nerve whilst mechanically and electrically stimulating the skin. The results were compared with those from ten control animals.

3. Transmedian innervation of contralateral teeth was found in none of the control animals; in all ten of the animals which had undergone denervation without immunization (4/10 canines, 17/20 incisors); but in only six of the eleven immunized and denervated animals (0/11 canines, 7/22 incisors).

4. Of 270 cutaneous mechanoreceptive units sampled in the controls, only four units had transmedian receptive fields, extending a maximum of 1 mm across the mid-line. After denervation, significantly more units (42 of 274) crossed the mid-line and extended up to 4 mm. After immunization and denervation only eleven of 305 units crossed the mid-line by a maximum of 1 mm.

5. These data show that cutaneous low-threshold mechanoreceptive Aβ and Aδ fibres, as well as Aδ tooth pulp fibres, are able to undergo collateral sprouting. This sprouting is partially blocked by NGF depletion, suggesting that NGF plays an essential role in the process.

After peripheral nerve injury, reinnervation of the denervated tissue may occur either by regeneration of the injured nerve or by ingrowth of adjacent intact nerves, termed collateral reinnervation. Although many previous workers have shown that collateral reinnervation occurs in adult mammals (Devor, Schonfeld, Seltzer & Wall, 1979; Robinson, 1981; Kinnman, 1987) the types of nerve fibre which are capable of forming collateral sprouts remain to be resolved. Histological and electrophysiological techniques have been used to demonstrate collateral sprouting of unmyelinated C fibres (Diamond, Holmes & Coughlin, 1992; Doubleday & Robinson, 1992) and high-threshold, nociceptive Aδ fibres (Owen, Logan & Robinson, 1989; Diamond *et al.* 1992) but there is some debate about whether or not the low-threshold mechanoreceptive Aβ fibres also contribute to collateral reinnervation. Following animal studies it has been reported that low-threshold cutaneous mechanoreceptive axons do not form functional collateral sprouts in the adult (Devor *et al.* 1979; Horch, 1981; Jackson & Diamond, 1984). In humans, however, several investigators have described the return of sensitivity (including light touch sensation) to denervated skin by what appears to be collateral reinnervation (Inbal, Rousso, Ashur, Wall & Devor, 1987; Robinson, 1988). Previous studies from this laboratory have investigated collateral reinnervation from high-threshold fibres in branches of the trigeminal nerve, by sectioning and preventing regeneration of the inferior alveolar nerve (IAN) on one side and monitoring transmedian collateral reinnervation from the contralateral side (Robinson, 1981). We demonstrated that collateral reinnervation is initiated by a peripheral stimulus (Robinson, 1986) and that ingrowth of high-threshold fibres does not occur after nerve growth factor (NGF) depletion (Owen *et al.*, 1989; Doubleday & Robinson, 1992). This suggests that NGF plays a crucial role in this process and may provide the initiating stimulus. The present study was designed to investigate whether or not

collateral sprouting of low-threshold cutaneous mechanoreceptive axons in the inferior alveolar nerve could be detected and to determine the effect of NGF depletion on the sprouting of these fibres and the fibres innervating the tooth pulp.

METHODS

The extent of transmedian innervation of the pulps of the lower anterior teeth and the skin overlying the chin by fibres from the left IAN was measured in thirty-one adult female ferrets (approx. wt. 800 g). These were divided into three experimental groups: a denervated group, in which the right (contralateral) IAN was sectioned 3 months prior to the terminal experiment (ten animals); an immunized and denervated group, which were autoimmunized against NGF and then denervated as above (eleven animals); and a control group, which did not undergo either immunization or prior denervation (ten animals).

Denervation

Under anaesthesia using 1 ml of a mixture of ketamine hydrochloride (Parke Davis, Pontypool, UK; 80 mg ml^{-1}) and xylazine (Bayer, Leverkusen, Germany; 4 mg ml^{-1}) I.M., the right IAN was exposed by removing a small window of overlying bone in the ascending ramus of the mandible. The nerve was sectioned and the proximal stump ligated and sealed into a length of polyethylene tubing to prevent regeneration. The muscles and skin were closed in layers and a single dose of antibiotic administered (Amfipen, Mycofam, Cambridge, UK; 0·4 ml I.M.). All animals, both immunized and unimmunized, were allowed to recover for 12 weeks.

Immunization

The ferrets were immunized by a subcutaneous injection of 20 μg 7S mouse NGF (Sigma, Poole, UK) in 0·1 ml of a 50:50 emulsion of normal saline and complete Freund's adjuvant (the denervated and control groups were sham-immunized with Freund's adjuvant and saline without the NGF). A booster injection of 50 μg 7S mouse NGF in a similar emulsion was given 4 weeks later, followed by a second boost after a further 6 weeks. One week after the first boost, blood was collected for determination of NGF antibody levels by enzyme-linked immunoabsorbent assay (ELISA) to verify seroconversion, and the denervation was then carried out. After the terminal experiments (17 weeks after the initial immunization), the ELISA was repeated to confirm that immunity to NGF remained throughout the experiment and the activity of the enzyme tyrosine hydroxylase in the superior cervical ganglia was also quantified in order to give an indication of the efficacy of the autoimmunization in producing NGF depletion (Gorin & Johnson, 1980).

ELISA

Plates were coated with 2 μg ml^{-1} 7S mouse NGF in 200 mM sodium carbonate buffer (pH 9·6) overnight at 4 °C. The plates were washed 3 times in PBS–Tween (0·05%) and non-specific staining blocked with bovine serum albumin (BSA; 0·5 mg ml^{-1}) in PBS–Tween for 1 h at 4 °C. The plates were again washed 3 times and the test sera applied at dilutions of 1:100 to 1:25600 in PBS–Tween for 1 h at 37 °C. Washing was repeated and goat anti-ferret immunoglobulin G conjugated with horseradish peroxidase (Kirkegaard & Perry, Dynatech Laboratories, Gaithersburg, MD, USA) added at 1:1000 dilution, 100 μl per well, for 1 h at 37 °C. After washing, 100 μl per well of 0·01% *o*-phenylenediamine in phosphate–citrate buffer (pH 5·0) containing 0·012% H_2O_2 was applied for 5 min and the reaction stopped by adding 50 μl per well of 2·5 N sulphuric acid. Product formation was determined by measuring absorption of light at 492 nm using a Titertek Uniskan 2 scanner.

Serum from all of the immunized animals yielded positive results down to a dilution of at least 1:3200, whereas all animals tested from the other two groups were negative at all dilutions. Further control sera from four animals immunized against vasoactive intestinal polypeptide were also negative for antibodies against NGF.

Tyrosine hydroxylase assay

At the end of each experiment the superior cervical ganglia were dissected free of adherent soft tissue and frozen. Tyrosine hydroxylase activity was determined as described in a previous paper (Doubleday & Robinson, 1992). Individual ganglia were homogenized with 80 μl of 10 mM potassium phosphate buffer (pH 7·4) and 40 μl of the homogenate added to 50 μl of a mixture of 200 mM sodium acetate–acetic acid buffer, 4 mM ferrous ammonium sulphate and 10 μg catalase. After pre-incubation at 37 °C for 5 min, 10 μl of a mixture of L-tyrosine (2 mM) and (6*R*,*S*)-methyl-5,6,7,8-tetrahydropterine (10 mM), a synthetic cofactor (Sigma), in 1 M mercaptoethanol was added and the mixture incubated for a further 10 min at 37 °C. The reaction was terminated by the addition of 100 μl 0·1 M perchloric acid and 0·1 mM disodium EDTA. The mixture was vortexed, placed in an ice bath for 10 min then centrifuged at 12000 *g* for 2 min. The enzyme activity was determined by measurement of L-DOPA (L-β-3,4-dihydroxyphenylalanine) production, using high-performance liquid chromatography (HPLC), in 20 μl aliquots of the reaction mixture. Quantification of L-DOPA was effected by comparison with standard solutions. HPLC employed a 5 μm Spherisorb octadecylsilyl (ODS)-2 column (length, 25 cm; i.d., 4·6 mm) at 40 °C, with 0·1 M phosphate–acetate buffer (pH 3·45) containing 2·5 mM octylsulphonate and 0·05 mM EDTA flowing at 1·0 ml min^{-1}. A BAS amperometric detector was used with a glassy carbon electrode set at 0·6 V *versus* a Ag–AgCl reference electrode.

The mean tyrosine hydroxylase activity in ganglia from the unimmunized animals was 4·25 ± 0·23 nM ganglion^{-1} h^{-1}, (mean ± S.E.M., range 3·2–4·7, $n = 10$). In the immunized animals, the mean tyrosine hydroxylase activity was reduced to 1·66 ± 0·22 nM ganglion^{-1} h^{-1} (mean ± S.E.M., range 0·83–2·4, $n = 11$) i.e. 40% of normal, and this reduction was statistically significant ($P < 0·004$, Mann–Whitney *U* test). Control assays without tyrosine substrate or without homogenate produced no activity.

Terminal experiments

At this stage the operator was blind to the experimental group of the animal. The animals were anaesthetized using sodium pentobarbitone (Sagatal, May & Baker, Dagenham, Essex, UK; 36 mg kg^{-1} I.P. for induction and 3–6 mg I.V. as required for maintenance). Body temperature was maintained with a heated blanket thermostatically controlled by a rectal thermistor. The right cephalic vein and trachea were cannulated and the ECG and exhaled CO_2 were monitored throughout the procedure. The animal's head was stabilized by a head pole attached by screws into the frontal sinus and clamped to a metal table. The mandible was also stabilized by

a stainless-steel rod attached to the upper and lower posterior teeth, sealed in place with acrylic cement. The mid-line of the skin overlying the chin and neck was marked with an indelible felt-tip pen.

The left IAN was exposed via a skin incision over the left angle of mandible. The masseter muscle was excised and the overlying bone, ascending ramus of the mandible, condyle and coronoid processes were removed. Skin flaps were sutured to a brass ring to form a pool which was filled with liquid paraffin at 37 °C. The exposed nerve was sectioned proximally and placed on platinum wire electrodes.

Tooth recordings

Two cavities were cut into each of the lower canine teeth with inverted cone burs and into the second and third incisors using hand-rotated 0·3 mm twist drills. The first incisors were too small to be used. Silver wire electrodes (0·125 mm diameter) were placed into the cavities, which were then packed with a mixture of Ag and AgCl and supported with a layer of acid-etch bonded composite resin (Silar Concise, 3M, St Paul, MN, USA). The left IAN was stimulated electrically (10 V, 0·1 ms, 10 Hz) and an average of 25 responses was recorded from each of the teeth, using an SPI2 interface and software (Grafitek, UK). In most recordings the resulting compound action potential could be clearly distinguished from stimulus artifact and this could be clarified by alterations to the level of stimulation, in the region of threshold intensity. Conduction velocities were calculated using the latency of the fastest component in the compound action potential and the peak-to-peak amplitudes were measured. The number of contralateral teeth in which transmedian innervation could be demonstrated was calculated for each experimental group and the results compared using χ^2 tests, with Yates correction for small numbers.

Skin recordings

The epineurium and perineurium of the exposed left IAN was divided and fine filaments containing single cutaneous mechanoreceptive fibres were dissected out and placed on the platinum wire recording electrodes. Units with receptive fields in the hairy skin overlying the lower lip and chin, within an area 12 mm each side of the mid-line, were characterized and classified using a key modified from Horch, Tuckett & Burgess (1977; Table 1). Von Frey hairs were used to map the receptive fields and evaluate the response to movement of the hairs. The response to skin stretch was determined by gently stretching the skin across the receptive field with the tips of two pairs of fine forceps placed outside the area. This was repeated in two directions, at 90 deg to each other. Steady deformation of the skin was achieved using the tips of bipolar stainless-steel electrodes (2 mm inter-electrode distance) held in a micromanipulator and the same electrodes were used for electrical stimulation (0·1 ms, up to 10 mA) to permit calculation of the conduction velocity. Approximately thirty units were characterized in each animal and filaments from the whole of the nerve were screened in an attempt to avoid missing any transmedian units.

Data were pooled for each group of animals and statistical comparisons made between groups using the Kruskal–Wallace test (analysis of variance for non-parametric data), the Mann–Whitney U test or the χ^2 test.

Table 1. Key used for classification of cutaneous mechanoreceptors (after Horch *et al.* 1977)

Conduction velocity ($m\ s^{-1}$)	Mechanical stimulation of the skin		Mechanoreceptor type
< 2		Responds to slow or lingering stimuli; may have an 'after discharge' when stimulus ceases.	C
2–25		Very sensitive to movement of skin (e.g. by hand tremor or respiration), down or guard hair.	Aδ
> 25	Response to steady deformation of skin persists for more than 30 s.	Has resting discharge or responds to skin stretch; regular discharge pattern, 15–30 impulses s^{-1}; single punctate focus.	Ruffini
		Little or no resting activity; does not respond well to skin stretch; evoked activity may be irregular; has 1–5 punctate foci.	Haarscheiben
	Response to steady deformation lasts less than 30 s.	Responds to movement of single guard hairs, but not very sensitive to down hair movement.	Guard hair
		Does not respond to movement of single guard hairs; may respond to clumps of guard hairs, but most sensitive to stimulation of skin between the hair.	Field

Mechanoreceptive units were classified according to their axonal conduction velocity as either Aβ, Aδ or C. The Aβ group was subdivided into Ruffini, Haarscheiben, guard hair or field receptors by their differing responses to mechanical stimulation.

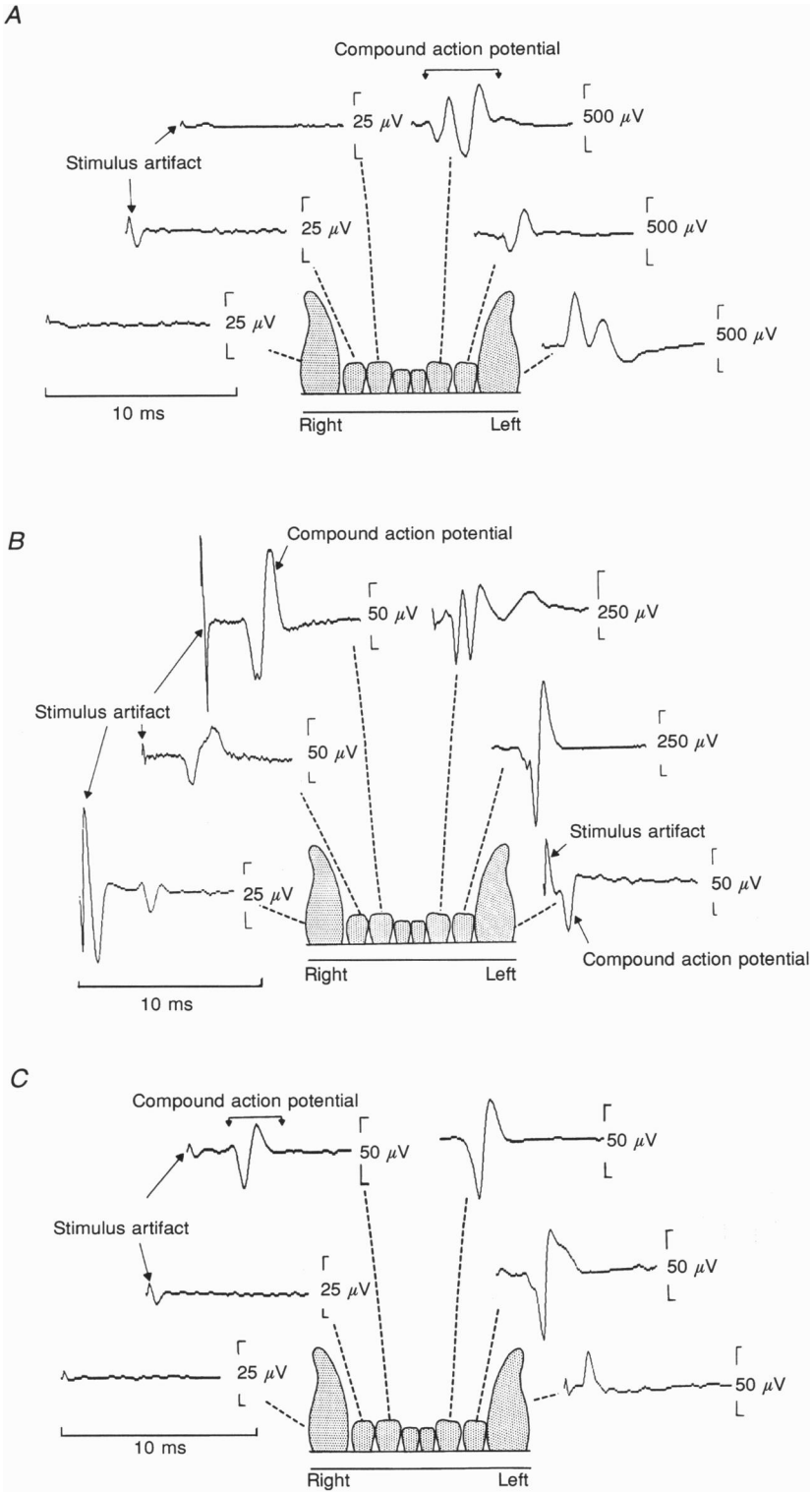

Figure 1. Examples of compound action potentials recorded from the anterior teeth whilst electrically stimulating the left IAN

A, the control group; *B*, the denervated group, in which the right IAN had been cut and regeneration prevented 12 weeks earlier; *C*, the immunized and denervated group, which had been previously immunized against NGF and then denervated as in *B*. The left IAN was stimulated at 10 V, 0·1 ms. All the records represent the average of 25 successive responses.

Table 2. Percentage of different mechanoreceptor types in the ipsilateral (left) skin, in each of the three experimental groups

Receptor type	Control (%)	Denervated (%)	Immunized and denervated (%)	Statistical significance
Ruffini	31	37	37	$P > 0.15$
Field	42	41	40	$P > 0.86$
Guard	10	8	10	$P > 0.85$
Haarscheiben	4	4	3	$P > 0.93$
Aδ	13	10	10	$P > 0.21$

The proportion of each receptor type was compared between experimental groups by multiple χ^2 tests. Percentages are given to the nearest integer.

RESULTS

None of the animals appeared to suffer any adverse effects as a result of the autoimmunization or denervation.

All recordings were made whilst stimulating, or recording from, the intact left IAN and the teeth and skin on this side are therefore referred to as *ipsilateral*. The transmedian teeth and skin, on the denervated right side, will be referred to as *contralateral*.

Innervation of tooth pulps

Data for all ipsilateral teeth in each experimental group were pooled, as were data for the contralateral teeth in each experimental group. There was a wide range of peak-to-peak amplitudes recorded from different teeth and different animals, as would be expected due to differences in electrode positioning and dentine thickness (both affecting impedance between the electrodes), and integrals of rectified compound action potentials were no more consistent. The data does, however, provide a crude measure of the number of fibres in the tooth pulp and statistical comparisons were therefore made.

Ipsilateral teeth

There was no significant difference between the three experimental groups in either the median peak-to-peak amplitude of the compound action potentials or their median fastest conduction velocities (control group: 149 μV (interquartile range 91–306) and 24·5 m s^{-1} (range 22–28); denervated group: 167 μV (range 90–253), and 25 m s^{-1} (range 23–28); immunized and denervated group: 155 μV (range 75–218) and 27 m s^{-1} (range 22–29). Both parameters $P > 0.7$, Kruskal–Wallace test).

Contralateral teeth (transmedian innervation)

In the control group, no responses could be recorded in any of the animals, between the right (contralateral) lower anterior teeth and the left IAN. After denervation, however, a response could be recorded from the right second incisor in nine out of ten animals (in the tenth animal a response was recorded from the adjacent third incisor), from the third incisor in eight out of ten animals and from the right lower canine in four animals. Examples of the responses obtained from each experimental group are shown in Fig. 1*A–C*. Following immunization and denervation there was a significant reduction in the number of contralateral teeth from which responses could be recorded ($P < 0.03$, χ^2 test). Responses were recorded from the right second incisor in six out of eleven animals; the third incisor in one and from none of the right canine teeth.

There was a significant difference between the median conduction velocity of the responses recorded from the contralateral teeth and the ipsilateral teeth in both the denervated (contralateral 15 m s^{-1} (interquartile range 10·5–20·5); ipsilateral 25 m s^{-1} (range 23–28); $P < 0.0001$, Mann–Whitney U test) and immunized and denervated groups (contralateral 16 m s^{-1} (interquartile range 14–20);

Table 3. Conduction velocities of fibres supplying different mechanoreceptor types in the ipsilateral (left) skin, in each of the three experimental groups

Receptor type	Control (m s^{-1})	Denervated (m s^{-1})	Immunized and denervated (m s^{-1})	Statistical significance
Ruffini	35 (32–42)	37 (34–40·25)	36 (33–40)	$P > 0.15$
Field	37 (32–43)	38 (34–44·75)	37 (33–43)	$P > 0.09$
Guard	34 (31–37·5)	36 (33–41)	35 (32–37·5)	$P > 0.57$
Haarscheiben	35·5 (31–41)	33·5 (32·75–36·25)	37 (31·5–39·5)	$P > 0.75$
Aδ	19 (12·5–22·5)	20 (16–24)	20 (14–23)	$P > 0.42$

Median values, together with the interquartile range in brackets, are shown. Variation between experimental groups was assessed using Kruskal–Wallace tests (analysis of variance for non-parametric data).

Table 4. Receptive field areas of the different mechanoreceptor types in the ipsilateral (left) skin, in each of the three experimental groups

Receptor type	Control (mm^2)	Denervated (mm^2)	Immunized and denervated (mm^2)	Statistical significance
Ruffini	4 (2–4)	4 (3–6)	4 (2·25–6)	$P > 0·2$
Field	4 (4–9)	6 (4–9)	4 (4–6·25)	$P > 0·06$
Haarscheiben	1·5 (1–2·5)	3 (1–5·25)	1 (1–3)	$P > 0·3$
Aδ	8 (4–12)	4 (4–8)	6 (4–7·5)	$P > 0·2$

Median values, together with the interquartile range in brackets, are shown. Variation between experimental groups was assessed using Kruskal–Wallace tests (analysis of variance for non-parametric data).

ipsilateral 27 m s^{-1} (range 22–29); $P < 0·0001$), but there was no significant difference between the denervated group and the immunized and denervated group in the median conduction velocities of the responses recorded from the contralateral teeth ($P > 0·4$). The median peak-to-peak amplitude of the compound action potentials recorded from the contralateral teeth was smaller than that of the corresponding ipsilateral teeth, for both the denervated (contralateral 23 μV (interquartile range 8–64); ipsilateral 167 μV (range 90–253); $P < 0·0001$, Mann–Whitney U test) and immunized and denervated groups (contralateral 42 μV (interquartile range 12–47); ipsilateral 155 μV (range 75–218); $P < 0·0001$). However, there was no significant difference between the denervated group and the immunized and denervated group in the median peak-to-peak amplitude of the compound action potentials recorded from the contralateral teeth ($P > 0·7$).

Cutaneous mechanoreceptors

A total of 270 units were sampled in the control animals, 274 after denervation and 305 after immunization and denervation. No C fibre units were recorded.

Ipsilateral skin

There was no significant difference between the three experimental groups in the proportions of the different mechanoreceptive types, their median conduction velocities or median receptive field areas (Tables 2–4).

Transmedian skin

In the control group, four units (out of a total of 270 sampled) were located which had receptive fields extending across the mid-line (one Ruffini and three field receptors) by a maximum of 1 mm. After denervation the number had significantly increased ($P < 0·0001$, χ^2 test) to forty-two units, out of a total of 274 sampled, with transmedian receptive fields (eighteen Ruffini, twenty-one field and three Aδ), extending up to 4 mm across the mid-line. After immunization and denervation, eleven units, from a total sample of 305, had transmedian mechanoreceptive fields (four Ruffini, three field and four Aδ) and these extended a maximum of 1 mm across the mid-line. This proportion was not significantly different from the controls ($P > 0·2$, χ^2 test) but was significantly smaller than after denervation alone ($P < 0·0001$; Fig. 2).

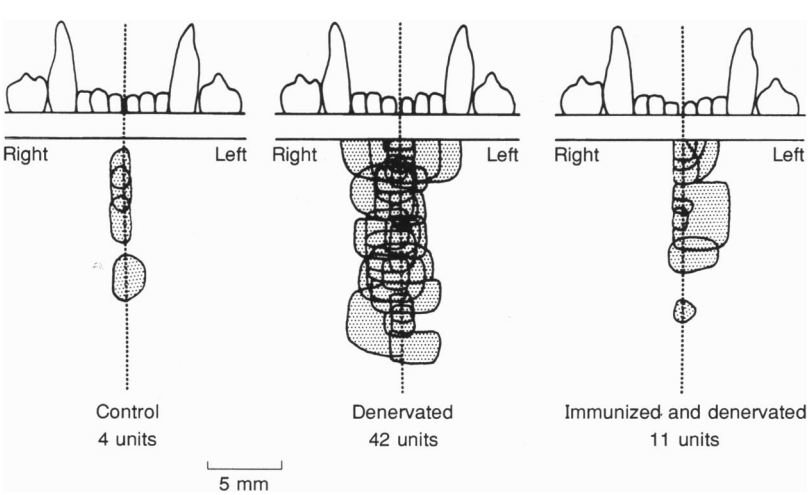

Figure 2
Composite diagram of all the cutaneous mechanoreceptive fields with a transmedian component in each of the three experimental groups.

Table 5. Conduction velocities of fibres supplying different mechanoreceptor types with transmedian receptive fields, in each of the three experimental groups

Receptor type	Control ($m\ s^{-1}$)	n	Denervated ($m\ s^{-1}$)	n	Immunized and denervated ($m\ s^{-1}$)	n	Statistical significance
Ruffini	34	1	38·5 (36–40·25)	18	38·5 (32·75–52·5)	4	$P > 0{\cdot}4$ †
Field	40 (30–58)	3	43 (38·5–48)	21	46 (43–47)	3	$P > 0{\cdot}7$ †
Aδ	—	—	18 (16–19)	3	15 (13·5–18·75)	4	$P > 0{\cdot}3$ *

Median values, together with the interquartile range and the numbers in each group (n), are shown. Variation between experimental groups was assessed using either Kruskal–Wallace tests (analysis of variance for non-parametric data, †) or the Mann–Whitney U test (*).

There was no significant difference between the three experimental groups in the median conduction velocities of each low-threshold mechanoreceptor type with a transmedian receptive field (Table 5). However, following denervation the median area of each receptive field type was significantly larger in those fields with transmedian components than in those confined entirely to the ipsilateral side (Ruffini receptors $P < 0{\cdot}003$, field receptors $P < 0{\cdot}007$ and Aδ receptors $P < 0{\cdot}01$, Mann–Whitney U tests). This difference was not seen in either the control group or the immunized and denervated group (values shown in Tables 4 and 6).

DISCUSSION

The results of this study revealed that sectioning and blocking regeneration of the inferior alveolar nerve is followed by an increase in transmedian innervation from the contralateral nerve, by axons supplying tooth pulps and by cutaneous low-threshold mechanoreceptive fibres. Collateral reinnervation of tooth pulps has previously been demonstrated both electrophysiologically and histologically (Robinson, 1981; Holland & Robinson, 1985), and it is generally accepted that these axons are principally nociceptive (Anderson, Hannam & Matthews, 1970). Prior to this study, however, none of the electrophysiological investigations in adult mammals have revealed collateral sprouting of the cutaneous low-threshold Aβ fibres. Jackson & Diamond (1984) suggested that collateral sprouting of low-threshold mechanosensory fibres only occurred in rats aged between 15 and 20 days and did not develop in the adult. Horch (1981), examining the cat hindleg, reported that adjacent intact mechanosensitive axons did not reinnervate denervated Haarscheiben receptors (type 1, slowly adapting cutaneous mechanoreceptors) and Devor *et al.* (1979), studying the rat foot, also stated that only high-threshold fibres underwent collateral sprouting. These results seem to be at variance with several clinical reports, in which return of light touch sensation to denervated skin appears to be due to ingrowth by neighbouring nerves (Robinson, 1988). Inbal *et al.* (1987) reported on five patients in whom cutaneous reinnervation occurred by what appeared to be collateral sprouting; totally anaesthetic areas at a distance from the site of nerve injury recovered sensitivity long before regenerating fibres could have arrived. The only animal studies to have revealed collateral sprouting of large fibres are the histological investigations of Kinnman (1987). Using anterograde tracing with wheat germ agglutinin–horseradish peroxidase in adult rats, he showed that coarse as well as fine cutaneous sensory nerve axons can extend collateral sprouts outside their normal segmental territory. The failure of previous electrophysiological studies to reveal sprouting of low-threshold fibres could have resulted from sampling or resolution difficulties. The experiments of Jackson & Diamond (1984) and Diamond *et al.* (1992) used whole-nerve recordings rather than recordings from single units and it is not clear how many units were sampled in the study by Devor *et al.* (1979). From a large sample of single units we found relatively few fibres which had a transmedian receptive field; the maximum was nine in one animal (which had undergone contralateral denervation) and in most animals there were three or less. If the total number of myelinated fibres in the IAN is considered (an average of 5111 has been reported in the cat; Holland & Robinson, 1990), it would

Table 6. Receptive field areas of the different mechanoreceptor types with transmedian components, in each of the three experimental groups

Receptor type	Control (mm^2)	n	Denervated (mm^2)	n	Immunized and denervated (mm^2)	n
Ruffini	4	1	6 (5·5–10·5)	18	4 (2·5–4)	4
Field	6 (3–8)	3	8 (6–14)	21	6 (6–8)	3
Aδ	—	—	16 (12–18)	3	7 (4·5–20)	4

Median values, together with the interquartile range in brackets and the numbers in each group (n), are shown.

not be surprising if whole-nerve recording did not allow resolution of responses from the few transmedian fibres which extended by a maximum of only 4 mm. We are unable to compare our results with those of Horch (1981) as we did not locate any Haarscheiben with transmedian receptive fields. This does not necessarily indicate that these are incapable of forming collateral sprouts as we identified relatively few Haarscheiben in the ipsilateral skin (approximately 4% of the total number of units characterized). The proportions of each mechanoreceptor type with transmedian receptive fields appeared to be similar to the proportions in the ipsilateral skin.

The percentage of different receptor types appears to differ widely in different investigations; Brown & Iggo (1967), examining the hindlimb of the cat and rabbit, found 22·6% of the units in the cat and 28·4% in the rabbit to be slowly adapting type I (Haarscheiben), 8·6 and 3·3% to be slowly adapting type II (Ruffini) and 41 and 16·6% to be guard hair (rapidly adapting). In the hairy skin of the rat hindlimb, Lynn & Carpenter (1982) reported that 66% of A fibre units were rapidly adapting hair follicle afferents and Lewin & McMahon (1991) found that 87% of fibres were in this category. On the dorsum of the human hand, however, figures of 28% for rapidly adapting type 1 (field), 32% for slowly adapting type I (Haarscheiben) and 32% for slowly adapting type II (Ruffini) have been quoted (Edin & Abbs, 1991). These varying percentages may be due to inter-animal variation or to variation at different sites. We found a low proportion of guard hair afferents (8%), which did not seem surprising as there were few guard hairs, mainly down hairs, in the experimental area, and roughly equal proportions of Ruffini (type II slowly adapting, 31–37%) and field (rapidly adapting, 40–42%) receptors. There was no significant change in these proportions in the ipsilateral skin after contralateral denervation or immunization with NGF. Our sampling method therefore appears to be consistent and unaffected by the experimental conditions.

It appears that collateral reinnervation by the low-threshold mechanoreceptive cutaneous fibres occurs by a spread in the area innervated by the units adjacent to the denervated area, as the units with transmedian mechanoreceptive fields were larger than the ipsilateral units. A similar increase in receptive field size was reported by Devor *et al.* (1979) for Aδ units that had undergone collateral spread.

Using single unit recordings in cats, Cadden, Lisney & Matthews (1983) found that fibres supplying the pulps of the lower teeth had conduction velocities in the Aβ, Aδ and C fibre ranges, with a maximum recorded velocity of 57·7 m s^{-1} for the extradental component. They noted that slower conduction velocities were recorded for the intradental component, presumably because the nerve fibres had smaller diameters within the pulp. The recordings in our study were made from coronal electrodes and thus included both intradental and extradental components. The maximum conduction velocity recorded was 30 m s^{-1} for the ipsilateral teeth and 23 m s^{-1} for the contralateral teeth. As noted previously it is difficult to record C fibre activity using tooth electrodes and none was detected in the present experiments. In view of these limitations we are unable to comment on whether or not differential sprouting of Aβ, Aδ or C fibres occurs in the dental pulp.

A possible alternative explanation for the apparent expansion of receptive fields following contralateral denervation is that, rather than ingrowth from adjacent fibres, pre-existing non-functional afferent collaterals are 'uncovered' or 'disinhibited'. If these non-conducting collateral fibres do occur, then they are also unable to transport radiolabelled amino acids injected into the trigeminal ganglion; Byers & Matthews (1981) found no evidence of transmedian innervation of normal mandibular teeth using this method in the cat. In addition, Robinson (1983) has previously demonstrated collateral reinnervation of transplanted and reimplanted extracted teeth, in which all of the pre-existing nerve supply must have been disrupted.

The second major observation in the present study was that collateral reinnervation by cutaneous Aβ and Aδ fibres, and Aδ fibres supplying the tooth pulps, was reduced by immunization against NGF. Thus we propose that NGF (or possibly a closely related neurotrophin, see below) plays a major role in the development of collateral reinnervation by these fibre groups. This interpretation of our results presumes that adult ferrets immunized with mouse NGF have developed antibodies which combine with their own endogenous NGF, resulting in chronic NGF deprivation. As a source of ferret NGF is not available, direct assay cannot be performed. However, we and other workers (Gorin & Johnson, 1980; Doubleday & Robinson, 1992) have shown that when adult rats are immunized with mouse NGF they develop marked biochemical and morphological changes in the sympathetic nervous system which are similar to those which develop in adult mice injected with specific antiserum to mouse NGF. Using the same immunization protocol as the present study we have demonstrated, in adult rats, a 40–60% fall in superior cervical ganglion tyrosine hydroxylase levels (an enzyme required for noradrenaline synthesis), together with a 20% reduction in target organ noradrenaline levels and consequent upregulation of cardiac β-adrenoceptor responses (Doubleday, Reynolds & Chess-Williams, 1992). In the present study all the immunized ferrets developed specific antibodies to mouse NGF and a systemic effect was revealed by a marked decrease in the activity of tyrosine hydroxylase in the superior cervical ganglia.

Although mature sensory neurones are not dependent upon NGF for survival, they do continue to express NGF receptors (Richardson & Riopelle, 1984) and appear to require NGF for normal homeostasis and function (Johnson, Rich & Yip, 1986). NGF also appears to be

important in the response of a peripheral nerve to injury. Sensory denervation of the iris either in culture or *in vivo* leads to the appearance of NGF activity (Ebendal, Olson, Seiger & Hedlund, 1980) and the biochemical and histological changes which usually occur central to a peripheral nerve injury may be reduced by application of NGF to the proximal nerve stump (Fitzgerald, Wall, Goedert & Emson, 1985). NGF may also provide trophic support and guidance for regenerating axons. After nerve section there is an increase in NGF-like activity in the distal segment of the nerve (Richardson & Ebendal, 1982) and Schwann cells show an increase in both mRNA for NGF (Bandtlow, Heumann, Schwab & Thoenen, 1987) and the low-affinity NGF receptors (Taniuchi, Clark & Johnson, 1986). Administration of NGF to an injury site enhances the regeneration of myelinated axons (Rich, Alexander, Pryor & Hollowell, 1989) and NGF has been shown to increase the expression of a cell adhesion molecule on the Schwann cells which may influence the growth of regenerating neurites (Seilheimer & Schachner, 1987).

Results from previous studies in our laboratory (Owen *et al.* 1989; Doubleday & Robinson, 1992) have shown that immunization against NGF blocks collateral reinnervation by high-threshold fibres in the guinea-pig and rat. These findings were in agreement with those of Diamond and co-workers (1992) who prevented collateral spread from mechano-nociceptive and heat-nociceptive fibres by administering NGF antiserum daily to rats. They also showed that intradermal injections of NGF could increase the rate of nociceptive fibre sprouting and also initiate sprouting *de novo* within normally innervated skin. Thus it would appear that all sensory collateral reinnervation is NGF dependent and presumably results from the increased expression of NGF in the target tissues. Ebendal *et al.* (1980) demonstrated a rise in the level of NGF in irides after either sensory or sympathetic denervation, followed by a fall when the irides were reinnervated. This rise in NGF could either be due to an increase in production of the growth factor as a result of denervation, or an accumulation of NGF which is no longer being depleted by retrograde axonal transport. Bandtlow *et al.* (1987) located NGF mRNA by *in situ* hybridization in epithelial cells, smooth muscle cells and fibroblasts, which suggests that NGF is produced by target cells of sensory and sympathetic neurones; studies of target cells *in vitro* have also revealed the production of NGF (Vos, Stark & Pittman, 1991). However, Rush (1984) examined the sympathetically denervated iris using immuno-histochemistry and only detected NGF in the Schwann cells and not in the smooth muscle target cells. NGF receptor-like immunoreactivity has also been located on various mechanoreceptors (Merkel cells, which form part of the Haarscheibe complex, hair follicle receptors and capsulated receptors) and their associated axon terminals (Ribeiro-da-Silva, Kenigsberg & Cuello, 1991). Various dental cells have been shown to express NGF-like immunoreactivity during development (Mitsiadis, Dicou, Joffre & Magloire, 1992) and NGF mRNA has been demonstrated in adult dental pulp associated with fibroblasts (Byers, Wheeler & Bothwell, 1992). NGF mRNA expression apparently increases following dentine injury and is accompanied by profuse sprouting of pulpal fibres. NGF receptor-like (NGFR) immunoreactivity has also been demonstrated in the dental pulp associated with fibroblasts in the subodontoblast zone (Byers, 1990), axons and Schwann cells in mature teeth and also in blood vessel walls of immature pulps (Fried & Risling, 1991). Byers (1990) reported that NGFR immunoreactivity appears to be related more to the nerve terminals than the axons themselves. She demonstrated occasional labelling of the unmyelinated axons in rat dental and periodontal tissue and no labelling of the myelinated axons, although 60% of the predentine free nerve endings had intense membrane labelling and there was extensive labelling of the Ruffini mechanoreceptor nerve endings in the periodontal ligament. Enhanced expression of NGFR mRNA has been shown to occur in the surviving non-neuronal cells after denervation of the teeth (Byers *et al.* 1992) but, as far as we are aware, no studies as yet have demonstrated an increase in both NGF mRNA and NGF protein in sensory neurone target cells in either the tooth pulp or skin following denervation.

Recently, four other members of the NGF family have been cloned, namely brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5; for review see Vantini, 1992). Other workers have demonstrated that polyclonal antibodies raised against NGF also recognize BDNF (Acheson, Barker, Alderson, Miller & Murphy, 1991) and NT-3 (Negro, Corsa, Skaper & Callegro, 1993), which raises the possibility that immunization against NGF may also suppress these factors. Current knowledge of the physiology of BDNF and NT-3 is limited and does not allow us to differentiate endogenous suppression of these factors.

REFERENCES

Acheson, A., Barker, P. A., Alderson, R. F., Miller, F. D. & Murphy, R. A. (1991). Detection of brain-derived neurotrophic factor-like activity in fibroblasts and Schwann cells: inhibition by antibodies to NGF. *Neuron* **7**, 265–275.

Anderson, D. J., Hannam, A. G. & Matthews, B. (1970). Sensory mechanisms in mammalian teeth and their supporting structures. *Physiological Reviews* **50**, 171–195.

Bandtlow, C. E., Heumann, R., Schwab, M. E. & Thoenen, H. (1987). Cellular localisation of nerve growth factor synthesis by in situ hybridization. *EMBO Journal* **6**, 891–899.

Brown, A. G. & Iggo, A. (1967). A quantitative study of cutaneous receptors and afferent fibres in the cat and rabbit. *Journal of Physiology* **193**, 707–733.

Byers, M. R. (1990). Segregation of NGF receptor in sensory receptors, nerves and local cells of teeth and periodontium demonstrated by EM immunocytochemistry. *Journal of Neurocytology* **19**, 765–775.

Byers, M. R. & Matthews, B. (1981). Autoradiographic demonstration of ipsilateral and contralateral sensory nerve endings in cat dentine, pulp and periodontium. *Anatomical Record* **201**, 249–260.

Byers, M. R., Wheeler, E. F. & Bothwell, M. (1992). Altered expression of NGF and P75 NGF-receptor by fibroblasts of injured teeth precedes sensory nerve sprouting. *Growth Factors* **6**, 41–52.

Cadden, S. W., Lisney, S. J. & Matthews, B. (1983). Thresholds to electrical stimulation of nerves in cat canine tooth-pulp with A beta-, A delta-, and C-fibre conduction velocities. *Brain Research* **261**, 31–41.

Devor, M., Schonfeld, D., Seltzer, Z. & Wall, P. D. (1979). Two modes of cutaneous reinnervation following peripheral nerve injury. *Journal of Comparative Neurology* **185**, 211–220.

Diamond, J., Holmes, M. & Coughlin, M. (1992). Endogenous NGF and nerve impulses regulate the collateral sprouting of sensory axons in the skin of the adult rat. *Journal of Neuroscience* **12**, 1454–1466.

Doubleday, B., Reynolds, G. P. & Chess-Williams, R. (1992). Regulation of cardiac α- and β-adrenoceptors by the sympathetic nervous system. *British Journal of Pharmacology* **107**, 185P.

Doubleday, B. & Robinson, P. P. (1992). The role of nerve growth factor in collateral reinnervation by cutaneous C-fibres in the rat. *Brain Research* **593**, 179–184.

Ebendal, T., Olson, L., Seiger, A. & Hedlund, K.-O. (1980). Nerve growth factor in the rat iris. *Nature* **286**, 25–28.

Edin, B. & Abbs, J. H. (1991). Finger movement responses of cutaneous mechanoreceptors in the dorsal skin of the human hand. *Journal of Neurophysiology* **65**, 657–670.

Fitzgerald, M., Wall, P. D., Goedert, M. & Emson, P. C. (1985). Nerve growth factor counteracts the neurophysiological and neurochemical effects of chronic sciatic nerve section. *Brain Research* **332**, 131–141.

Fried, K. & Risling, M. (1991). Nerve growth factor receptor-like immunoreactivity in primary and permanent canine tooth pulps of the cat. *Cell and Tissue Research* **264**, 321–328.

Gorin, P. D. & Johnson, E. M. (1980). Effects of long term nerve growth factor deprivation on the nervous system of the adult rat: an experimental autoimmune approach. *Brain Research* **198**, 27–42.

Holland, G. R. & Robinson, P. P. (1985). Reinnervation of the canine tooth pulp after section of the inferior alveolar nerve in the cat. *Brain Research* **329**, 300–303.

Holland, G. R. & Robinson, P. P. (1990). The number and size of axons central and peripheral to inferior alveolar nerve injuries in the cat. *Journal of Anatomy* **173**, 129–137.

Horch, K. (1981). Absence of functional collateral sprouting of mechanoreceptor axons into denervated areas of mammalian skin. *Experimental Neurology* **74**, 313–317.

Horch, K. W., Tuckett, R. P. & Burgess, P. R. (1977). A key to the classification of cutaneous mechanoreceptors. *Journal of Investigative Dermatology* **69**, 75–82.

Inbal, R., Rousso, M., Ashur, H., Wall, P. D. & Devor, M. (1987). Collateral sprouting in skin and sensory recovery after nerve injury in man. *Pain* **28**, 141–54.

Jackson, P. C. & Diamond, J. (1984). Temporal and spatial constraints on the collateral sprouting of low-threshold mechanosensory nerves in the skin of rats. *Journal of Comparative Neurology* **226**, 336–345.

Johnson, E. M., Rich, K. M. & Yip, H. K. (1986). The role of NGF in sensory neurons *in vivo*. *Trends in Neurosciences* **9**, 33–37.

Kinnman, E. (1987). Collateral sprouting of sensory axons in the hairy skin of the trunk: a morphological study in adult rats. *Brain Research* **414**, 385–389.

Lewin, G. R. & McMahon, S. B. (1991). Physiological properties of primary sensory neurons appropriately and inappropriately innervating skin in the adult rat. *Journal of Neurophysiology* **66**, 1205–1217.

Lynn, B. & Carpenter, S. E. (1982). Primary afferent units from the hairy skin of the rat hind limb. *Brain Research* **238**, 29–43.

Mitsiadis, T. A., Dicou, E., Joffre, A. & Magloire, H. (1992). Immunohistochemical localization of nerve growth factor (NGF) and NGF receptor (NGF-R) in the developing first molar tooth of the rat. *Differentiation* **49**, 47–61.

Negro, A., Corsa, V., Skaper, S. D. & Callegro, L. (1993). Nerve growth factor antibodies recognize neurotrophin-3. *Neurochemical Research* **18**, 705–709.

Owen, D. J., Logan, A. & Robinson, P. P. (1989). A role for nerve growth factor in collateral reinnervation from sensory nerves in the guinea pig. *Brain Research* **476**, 248–255.

Ribeiro-da-Silva, A., Kenigsberg, R. L. & Cuello, A. C. (1991). Light and electron microscopic distribution of nerve growth factor receptor-like immunoreactivity in the skin of the rat lower lip. *Neuroscience* **43**, 631–646.

Rich, K. M., Alexander, T. D., Pryor, J. C. & Hollowell, J. P. (1989). Nerve growth factor enhances regeneration through silicone chambers. *Experimental Neurology* **105**, 162–170.

Richardson, P. M. & Ebendal, T. (1982). Nerve growth activities in rat peripheral nerve. *Brain Research* **246**, 57–64.

Richardson, P. M. & Riopelle, R. J. (1984). Uptake of nerve growth factor along peripheral and spinal axons of primary sensory neurons. *Journal of Neuroscience* **4**, 1683–1689.

Robinson, P. P. (1981). Reinnervation of teeth, mucous membrane and skin following section of the inferior alveolar nerve in the cat. *Brain Research* **220**, 241–253.

Robinson, P. P. (1983). An electrophysiological study of the reinnervation of reimplanted and autotransplanted teeth in the cat. *Archives of Oral Biology* **28**, 1139–1147.

Robinson, P. P. (1986). A peripheral stimulus initiates the collateral reinnervation of cat teeth. *Brain Research* **366**, 397–400.

Robinson, P. P. (1988). Observations on the recovery of sensation following inferior alveolar nerve injuries. *British Journal of Oral Maxillofacial Surgery* **26**, 177–189.

Rush, R. A. (1984). Immunohistochemical localisation of endogenous nerve growth factor. *Nature* **312**, 538–540.

Seilheimer, B. & Schachner, M. (1987). Regulation of neural cell adhesion molecule expression on cultured mouse Schwann cells by nerve growth factor. *EMBO Journal* **6**, 1611–1616.

Taniuchi, M., Clark, H. B. & Johnson, E. M. (1986). Induction of nerve growth factor receptor in Schwann cells after axotomy. *Proceedings of the National Academy of Sciences of the USA* **83**, 4094–4098.

Vantini, G. (1992). The pharmacological potential of neurotrophins: a perspective. *Psychoneuroendocrinology* **17**, 401–410.

Vos, P., Stark, F. & Pittman, R. (1991). Merkel cells *in vitro*: Production of nerve growth factor and selective interactions with sensory neurons. *Developmental Biology* **114**, 281–300.

Acknowledgements

We would like to thank Dr Ian Douglas and Dr Gavin Reynolds for their help with the ELISA and tyrosine hydroxylase assay, respectively.

Received 21 June 1993; accepted 4 May 1994.