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Ultrastructural Study of Effect of $ACTH_{4-10}$ on Nerve Regeneration; Axons Become Larger in Number and Smaller in Diameter

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Summary. Electron-microscopic analysis of the regenerating sciatic nerve in the rat showed that $ACTH_{4-10}$ treatment stimulated the number of regenerating myelinated and unmyelinated fibers to a similar degree. Fourteen days following a crush lesion of the sciatic nerve, 23% more regenerating fibers were present in the $ACTH_{4-10}$-treated group of animals. The increase in number of regenerating fibers was accompanied by a comparable decrease in axon diameter. The $ACTH_{4-10}$ treatment did not change the number of lamellae in the myelin sheath.

Key words: $ACTH_{4-10}$ treatment – Nerve regeneration – Axons

Introduction

The discovery of NGF gave rise to the notion that the outgrowth of nerve fibers may be manipulatable. Since then the effect of various substances on nerve fiber regeneration has been studied, and a stimulating influence has been reported for a number of these products, like that of triiodothyronin (Cockett and Kiernan 1973), of dibutyryl-cAMP (Gershenbaum and Roisen 1980), and of gangliosides (Ceccarelli et al. 1976). In 1980, ACTH was shown to enhance peripheral nerve regeneration (Strand and Smith 1980; Strand and Kung 1980). Studies in our laboratory confirmed these observations and revealed that this was a direct effect on nervous tissue, as the stimulating influence was likewise exerted by $ACTH_{4-10}$ and the $ACTH_{4-9}$ analogue (Org. 2766). Following a crush lesion of the sciatic nerve, treatment with $ACTH_{4-10}$ enhanced the return of sensorimotor function (Bijlsma et al. 1981 a). Histological studies of myelin-stained sections subsequently showed a marked increase in the numbers of regenerating myelinated fibers in $ACTH_{4-10}$-treated rats following sciatic nerve crush. This stimulation was most pronounced during the first stages of regeneration (+195%), but also 3 months after lesioning, the benificial effect of the peptide treatment was still present (+22%; Bijlsma et al. 1983 a). There was no evidence of a more rapid outgrowth, at least the size of the regenerating myelinated fibers did not differ in peptide-treated and vehicle-treated animals. The present study was undertaken to determine in detail the sizes of the regenerating axons and the relationship between axon diameters and the number of myelin lamellae in $ACTH_{4-10}$-treated and vehicle-treated animals.

Materials and Methods

Animals, Surgery, and Treatments

A detailed description of the surgery and the peptide treatment is given in Bijlsma et al. (1981a, 1983a). Briefly, female albino rats (Wistar, TNO Zeist, 120–140 g b.w.) received a crush lesion in the right sciatic nerve, 6 mm distal from the sciatic notch. An epineurial suture was applied at the distal boundary of the injury with handsilk to facilitate identification during morphological studies.

The animals were injected s.c. every other day either with 10 µg $ACTH_{4-10}$ as a long-acting zinc-phosphate complex, or with the vehicle alone. The peptide was a gift from Dr. J.W.F.M. Van Nispen (Organon Int. BV, Oss, The Netherlands).

Tissue Preparation

At 14 days after surgery, the animals were anesthesized with Hypnorm. The sciatic nerve and its tibial branch were exposed and dissected out. The nerve was stretched on a card and placed in abundant fixative (0.1 M cacodylate, 0.01 M $CaCl_2$, 2% glutaraldehyde, pH 7.3) for 2 h. After fixation, the nerves were rinsed three times, 15 min each, in an isotonic solution, containing 3.6% glucose, 0.1 M cacodylate, 0.01 M $CaCl_2$, adjusted to pH 7.3. The tissue was postfixed with OsO_4 (1% OsO_4, 0.1 M cacodylate, 0.01 M $CaCl_2$, 2.9% glucose, pH 7.3) for 2 h, subsequently the nerves were separated

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in a sciatic and tibial part, dehydrated with graded acetones and embedded in Epon.

Light-microscopic Techniques

From three vehicle- and three peptide-treated animals, semithin transverse sections (1 µm) were cut at 3 and 20 mm distal from the crush lesion, using a microtome LKB-Pyramitome (Bromma, Sweden). The sections were stained for myelin with 1 % para-phenylenediamine. Photographs of the stained sections were made at a final enlargement of × 1,000. In an area of 0.1 mm² (original surface), the numbers of myelinated nerve fibers were counted using a Zeiss Particle Size Analyser (TGZ3, Oberkochen, FRG) as described earlier (Bijlsma et al. 1983 a).

Electron-microscopic Techniques

The EM study was restricted to the level 3 mm distal from the crush lesion. Intrafascicular areas were trimmed out using glass knives from the same material as used for the light-microscopic analysis. Ultrathin sections were cut with diamond knives, stained with uranyl-acetate (5 %, 20 min) followed by lead-citrate (4 min; Reynolds 1963) and examined in an electron microscope (Zeiss, EM109). Twenty-five photographs (18 × 18 cm) were made throughout this selected part of the sciatic nerve at a final magnification of × 8,045. This area represented an area of 0.01 mm² and contained about 100 myelinated fibers. The numbers of myelinated and unmyelinated fibers were quantified and the diameters were measured and calculated using an electronic digitizer connected to a desk-top calculator (Hewlett-Packard 9864A and 9820). The regenerating fibers were divided into the following four classes:

I: unmyelinated axons, one axon per Schwann cell (axons that probably will become myelinated);

II: myelinated axons with less than three loops of myelin (early stage of myelination);

III: myelinated axons with three or more loops of myelin;

IV: unmyelinated axons, more axons per Schwann cell (axons that probably will stay unmyelinated).

Approximately 50 myelinated axons were photographed at a final magnification of × 22,670, and the number of myelin lamellae surrounding these axons were counted, using a binocular dissection microscope. Also the axon circumference and area were determined with the HP digitizer equipment.

Results

Light-microscopic Observations in the Sciatic and Tibial Nerve

Effect of ACTH on Regenerating Myelinated Nerve Fibers. The numbers of myelinated nerve fibers were counted in the selected regions in the three $ACTH_{4-10}$- and the three vehicle-treated animals in the sciatic and tibial nerve, 14 days following crush lesioning. The results are presented in Table 1. The $ACTH_{4-10}$ treatment resulted in a stimulation of the numbers of myelinated fibers by 23 % and 164 % in the sciatic and tibial nerve, respectively.

Table 1. Numbers of regenerating myelinated nerve fibers in the sciatic (3 mm from the crush lesion) and tibial nerve (20 mm from the crush lesion), 14 days following sciatic nerve crush. The fibers were counted in 0.1 mm² of the original area, after photographic enlargement of × 1,000

	Vehicle	$ACTH_{4-10}$	% Stimulation
Sciatic nerve	959 ± 79	1,180 ± 44	23*
Tibial nerve	96 ± 44	254 ± 47	164*

Means ± SEM are shown
* $P < 0.05$ (two-tailed) Student's t-test ($n = 3$)

Electron-microscopic Observations in the Sciatic Nerve

A. Effect of $ACTH_{4-10}$ on Classes of Regenerating Fibers. To count the number of myelinated and unmyelinated fibers, and to determine the effect of a chronic $ACTH_{4-10}$ treatment on the number of regenerating fibers, enlargements were made, representing 0.01 mm² of the sciatic nerve (Fig. 1). As shown in Fig. 2 and Table 2, the percentage of the fibers in the four different classes (see Materials and Methods) was not influenced by the $ACTH_{4-10}$ treatment. However, the total numbers of regenerating fibers in two of the four classes representing more than 90 % of the total fibers, are stimulated by $ACTH_{4-10}$ (Table 2). The increase of the number of regenerating myelinated fibers (III, + 22 %) was accompanied by a comparable increase in the number of regenerating unmyelinated fibers (IV, + 24 %). The number of fibers in classes I and II were few and differences were not significant.

Median diameters of the fibers are presented in Table 2. In all classes the median diameters in the $ACTH_{4-10}$-treated animals are slightly smaller than in the vehicle-treated animals, but as will be shown in the following, with the exception of the class of the myelinated fibers the differences did not reach significance.

Frequency histograms of the diameters of the regenerating myelinated and unmyelinated fibers are shown in Figs. 3 and 4, respectively. From both histograms it appears that after $ACTH_{4-10}$ treatment, diameters of the regenerating fibers are smaller. Statistical analysis was performed on two groups of axon diameters by lumping together all axons with a diameter ≤ 2 µm, and all axons with diameter > 2 µm (Table 3). Indeed, in $ACTH_{4-10}$-treated animals the percentage of small myelinated axons was higher than in the vehicle-treated animals, whereas the percentage of larger myelinated axons was lower. Statistical analysis of the diameters (≤ 0.5 and > 0.5 µm) of the unmyelinated fibers (Table 3) did not reveal significant differences as a result of the $ACTH_{4-10}$ treatment.

Effect of $ACTH_{4-10}$ on the Axon/Myelin Relationship. Circumferences and diameters of the regenerating axons were determined and the numbers of myelin

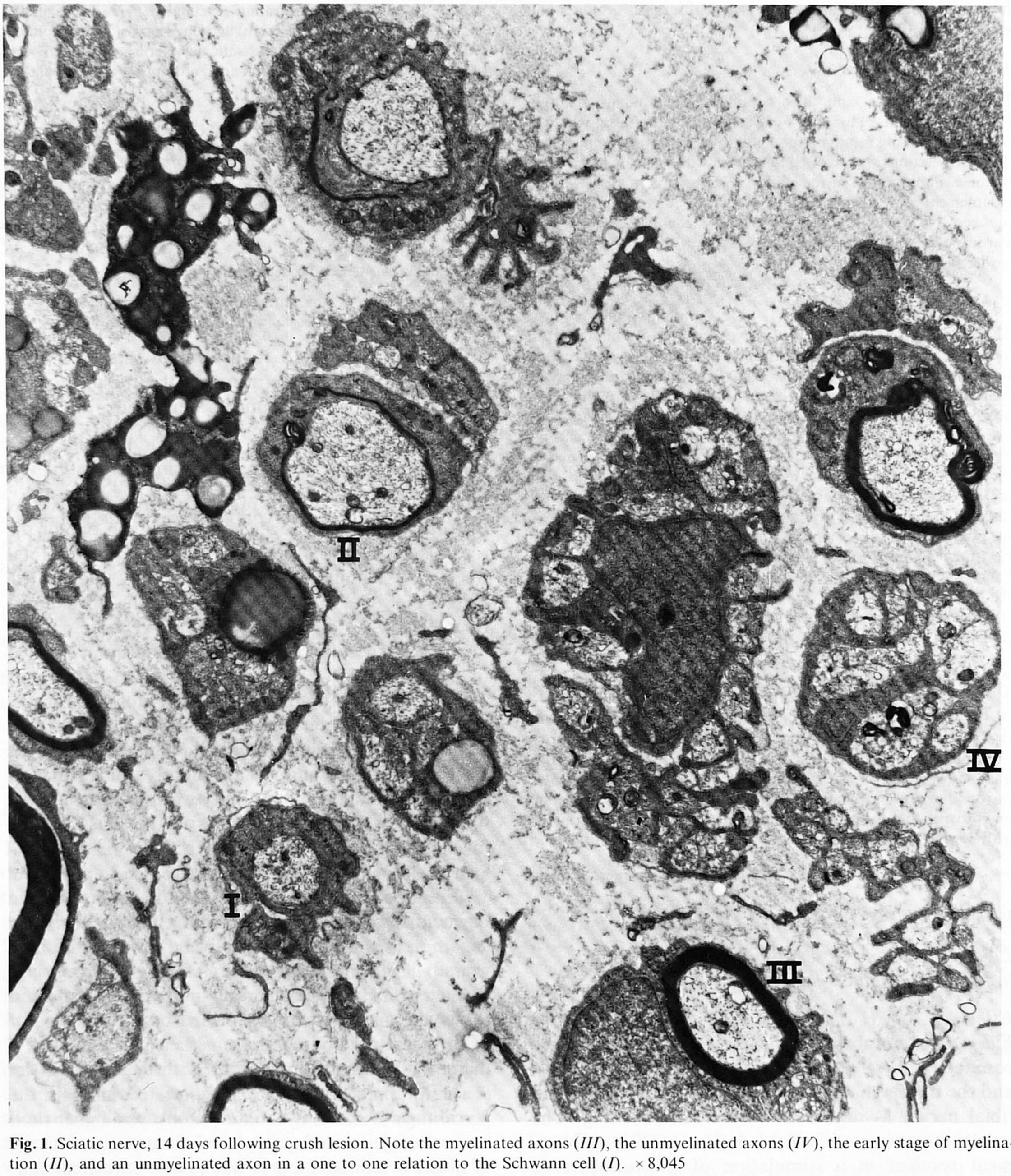

Fig. 1. Sciatic nerve, 14 days following crush lesion. Note the myelinated axons (*III*), the unmyelinated axons (*IV*), the early stage of myelination (*II*), and an unmyelinated axon in a one to one relation to the Schwann cell (*I*). × 8,045

lamellae were counted in approximately 50 myelinated nerve fibers from each animal. It appeared that the axon circumference was smaller, though not significantly, in the $ACTH_{4-10}$- than in vehicle-treated animals (Table 4).

The total number of myelin lamellae of all examined myelinated nerve fibers was quite similar in both groups of animals, being 20 ± 2 and 20 ± 1; despite the fact that the $ACTH_{4-10}$-treated animals had more smaller axons ($\leq 2\,\mu m$). Also when the regenerating myelinated

Table 2. Effect of $ACTH_{4-10}$ on regenerating axons in rat sciatic nerve, 14 days following crush lesioning. Axons are divided over four classes: I, unmyelinated, one axon per Schwann cell; II, myelinated, with less than three loops of myelin; III, myelinated, with more than three loops of myelin; IV, unmyelinated, more axons per Schwann cell. Median diameters of the axons are shown as well as the numbers of the axons and their distribution over the different classes. Furthermore, the mean area of the axons and the total area of axoplasm are presented. From these data the weighed mean area per axon could be calculated. It appeared that the value obtained from the $ACTH_{4-10}$-treated animals was 23% lower compared to vehicle-treated animals

Vehicle fiber class	Median ± variance	*n*	% of total	% Stimulation vs vehicle	Area (μm^2) surface/axon	Total area (μm^2)	Weighed mean area per axon
I	1.04 ± 2.70	27 ± 4	3.1 ± 0.7		1.356 ± 0.199	36.612 ± 5.373	1.069 ± 0.073
II	2.11 ± 1.72	16 ± 4	1.9 ± 0.5		3.484 ± 0.623	55.744 ± 9.968	
III	2.44 ± 2.97	113 ± 6	13.1 ± 1.7		5.137 ± 0.495	580.481 ± 55.935	
IV	0.60 ± 2.40	708 ± 86	81.9 ± 3.6		0.355 ± 0.055	251.340 ± 38.940	
		864 ± 60	100			924.177 ± 63.768	
ACTH							
I	1.02 ± 2.62	34 ± 5	3.2 ± 0.4	+ 26	1.081 ± 0.076	36.754 ± 2.584	0.829 ± 0.046*
II	1.84 ± 2.20	13 ± 2	1.2 ± 0.2	− 19	3.035 ± 0.449	39.455 ± 5.837	
III	2.08 ± 2.54	138 ± 2**	13.1 ± 0.9	+ 22**	4.144 ± 0.433	571.872 ± 59.754	
IV	0.53 ± 2.40	878 ± 55*	82.5 ± 0.9	+ 24*	0.266 ± 0.018	233.548 ± 15.804	
		1,063 ± 58	100	23**		881.629 ± 48.490	

* $P < 0.05$, Student's *t*-test;
** $P < 0.01$, Student's *t*-test, peptide vs. vehicle

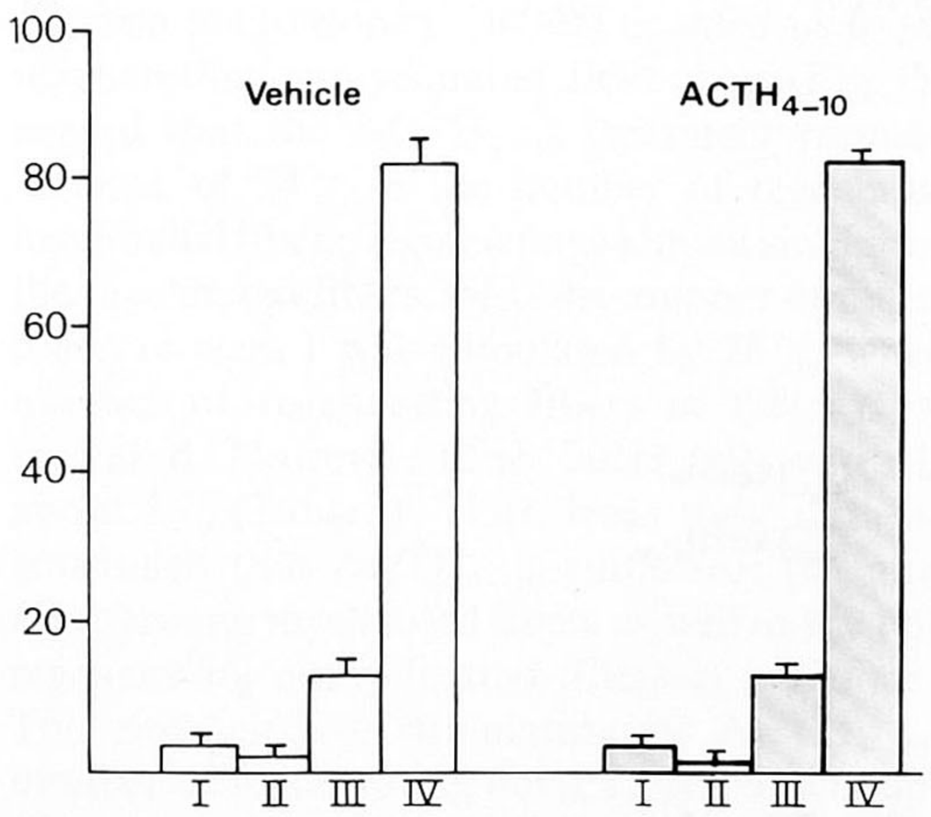

Fig. 2. Distribution pattern of the regenerating myelinated and unmyelinated fibers in the sciatic nerve, 14 days following crush lesion. *I*: nonmyelinated axons in a one to one relation with the Schwann cell; *II*: myelinated axons, with less than three loops of myelin; *III*: myelinated axons, with more than three loops of myelin; *IV*: unmyelinated axons with more axons per Schwann cell. Every other day the animals were injected with 10 µg $ACTH_{4-10}$ or with the vehicle alone (s.c.). Means are shown + SEM ($n = 3$)

axons were divided in two classes, i.e., $\leq 2\,\mu m$ and $> 2\,\mu m$, there were no differences in number of myelin lamellae between the two different groups (Table 4). The ratios (mean circumference of the axon/number of myelin lamellae) turned out to be not significantly

Table 3. Effects of $ACTH_{4-10}$ on diameters of regenerating axons. Percentage of regenerating axons in the sciatic nerve, 14 days following crush lesion with or without ACTH treatment. The myelinated axons are divided in two classes: $\leq 2\,\mu m$ and $> 2\,\mu m$ and the unmyelinated axons in two classes: $\leq 0.5\,\mu m$ and $> 0.5\,\mu m$

		Vehicle	$ACTH_{4-10}$
Myelinated axons (III)	$\leq 2\,\mu m$	17 ± 2%	37 ± 4%[a]
	$> 2\,\mu m$	83 ± 2%	63 ± 2%[a]
Unmyelinated axons (IV)	$\leq 0.5\,\mu m$	54 ± 8%	56 ± 7%
	$> 0.5\,\mu m$	46 ± 8%	44 ± 7%

[a] Two-factor analysis of variance, $P < 0.001$ (two-tailed), means ± SEM are shown ($n = 3$)

Table 4. Effects of $ACTH_{4-10}$ on myelination. Numbers of myelin lamellae were counted in the regenerating myelinated fibers in vehicle- and $ACTH_{4-10}$-treated animals. Also the ratio (axon circumference/number of myelin lamellae) was calculated

	Vehicle	$ACTH_{4-10}$
Number of myelin lamellae		
around axons ≤ 2 µm	19 ± 1	19 ± 1
around axons > 2 µm	20 ± 2	22 ± 2
mean number	20 ± 2	20 ± 1
Median axon circumference	7.99 ± 10.08 µm	7.29 ± 10.23 µm
Mean axon circumference	8.30 ± 0.37 µm	7.55 ± 0.35 µm
Ratio (mean axon circumference/ myelin lamellae	0.521 ± 0.053	0.440 ± 0.032

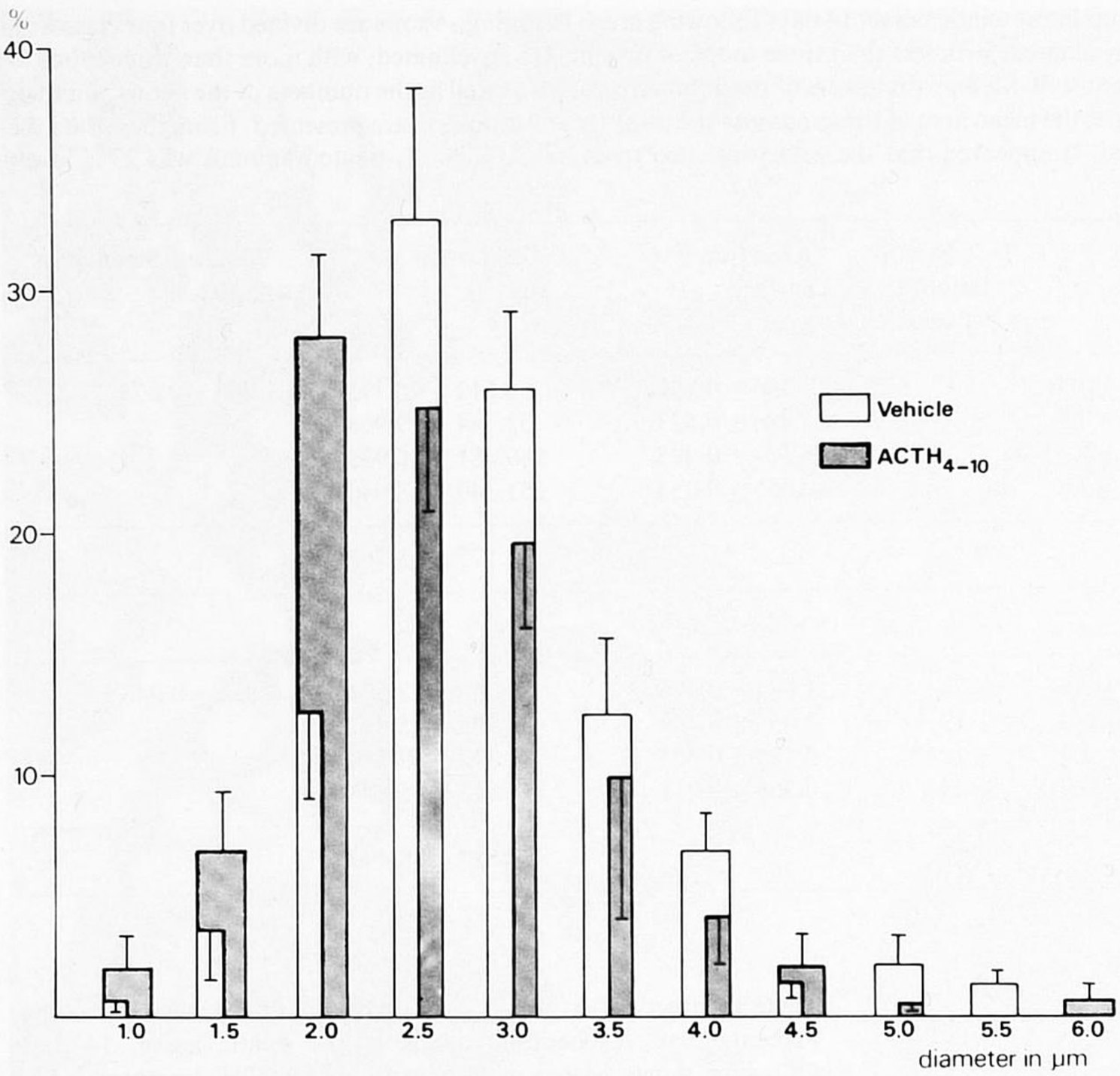

Fig. 3. Frequency histograms of the regenerating myelinated axons in the sciatic nerve, 14 days following crush lesion. Every other day the animals were injected s.c. with 10 µg $ACTH_{4-10}$ or with the vehicle alone. Means are shown $\pm$ SEM ($n = 3$)

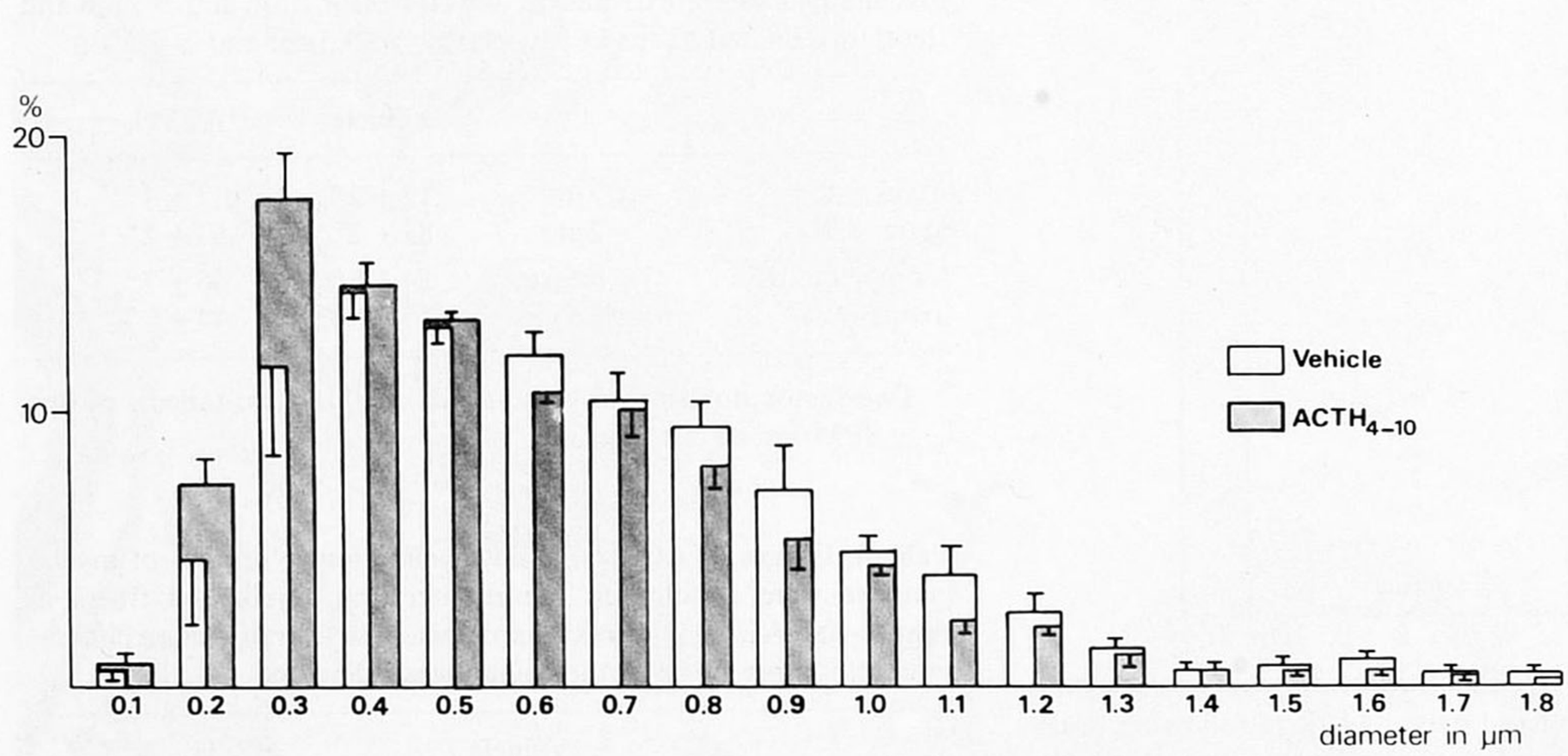

Fig. 4. Frequency histograms of the regenerating unmyelinated axons in the sciatic nerve, 14 days, following crush lesion. Every other day the animals were injected s.c. with 10 µg $ACTH_{4-10}$ or with the vehicle alone. Means are shown $\pm$ SEM ($n = 3$)

lower in the $ACTH_{4-10}$-treated animals as compared to vehicle-treated animals (0.440 ± 0.032 vs. 0.521 ± 0.053, respectively).

Discussion

In a previous light-microscopic study (Bijlsma et al. 1983a), we demonstrated that $ACTH_{4-10}$ stimulated the number of outgrowing myelinated fibers. The present study was performed to study as accurately as possible the sizes of the axons in treated and untreated axons, in particular those of the unmyelinated fibers. Also we were interested in the relationship between axon diameters and number of myelin lamellae. We decided to limit this investigation to one time point during the process of regeneration. Day 14 was chosen

since at this stage: (1) the ACTH effect was markedly expressed, and (2) myelination had proceeded to a degree that axons with markedly different numbers of myelin lamellae were present.

We counted the myelinated nerve fibers in 0.1 mm^2 area of the sciatic and tibial nerve 14 days following crush lesioning, to determine the degree of stimulation by $ACTH_{4-10}$. As shown previously, restriction of quantitation of this area gives a reliable indication of regeneration in the whole nerve (Bijlsma et al. 1983a). Comparable results were obtained as in the previous study, i.e., at the 3-mm level a stimulation of the number of regenerating myelinated fibers by 23% (previously 22%) and at the 20-mm level a stimulation of even 164% (previously 83%; Table 1). The observation that the stimulation by $ACTH_{4-10}$ in the tibial nerve was more pronounced in the present study might well be explained by the fact that at 14 days after the lesion, the first regenerating myelinated fibers have just passed the 20-mm level. This early stage of regeneration was also more affected by $ACTH_{4-10}$ treatment in the sciatic nerve at the 3-mm level 8 days after crush lesioning, i.e., +195%, as shown in the previous study. It may be concluded that the stimulatory effect of $ACTH_{4-10}$ on the number of the regenerating myelinated fibers was repeated accurately. The use of the electron microscope ($\times$ 8,045) enabled us to study the regenerating unmyelinated fibers too (Fig. 1). It appeared that the $ACTH_{4-10}$ treatment resulted in an increase of 24% in the number of regenerating unmyelinated fibers, a percentage almost similar to that of the myelinated fibers. Also the number of regenerating fibers in class I was stimulated by 26%, whereas the number of regenerating fibers in class II was not increased. However, these fibers only contribute for about 1% (Table 2). Thus, from these data it can be concluded that $ACTH_{4-10}$ stimulates the number of regenerating myelinated fibers as well as the number of regenerating unmyelinated fibers in a similar degree. This non-selective stimulation by $ACTH_{4-10}$ of the number of regenerating nerve fibers, was coupled to a slightly smaller median diameter of the outgrowing axons (Table 2).

In case of the myelinated and unmyelinated fibers, one can see from the frequency histograms (Figs. 3, 4) that the distribution pattern of the fibers in the peptide-treated animals is a little shifted to smaller diameters, but only in the regenerating myelinated axons a statistical difference could be observed (Table 3). Gershenbaum and Roisen (1980) studied the effects of $(But)_2$-cAMP on in vivo nerve regeneration after sciatic nerve crush in rats and found that there were consistently more regenerating fibers in rats treated with the nucleotide than in those treated with saline. The mean fiber diameter in the $(But)_2$-cAMP-treated group was larger and was taken as an indication of an enhanced maturation rate.

As functional recovery of sciatic crush-lesioned rats is stimulated by $ACTH_{4-10}$ treatment (Bijlsma et al. 1981 a) we had expected to find evidence in the present study for an enhanced growth rate of the regenerating nerve fibers. This could have been expressed in a larger diameter of the outgrowing axons. In our peptide-treated group of animals, however, the axon circumferences of the regenerating myelinated nerve fibers were smaller than in the untreated group, whereas the myelin sheaths in the two groups appear to be comprised of a similar number of lamellae. As a consequence the calculated ratios (axon circumferences/number of lamellae) turned out to be lower in the peptide-treated animals than in vehicle-treated animals (0.440 ± 0.032 vs. 0.521 ± 0.053), which is completely explained by the lower axon circumferences in the $ACTH_{4-10}$-treated animals. No effect of $ACTH_{4-10}$ treatment was present on myelination as such. The present data substantiate that $ACTH_{4-10}$ stimulates the number of outgrowing fibers. There remains a possibility that the regenerating fibers, though smaller in size, grow more rapidly in length. Our previous light-microscopical studies, however, do not favor this idea (Bijlsma et al. 1983a).

Although the mechanisms by which ACTH-like peptides influence peripheral nerve regeneration are unknown, the basic thought has been that these neuropeptides stimulate the protein-synthesizing machinery (Schotman et al. 1980; Bijlsma et al. 1981a, b; Strand and Kung 1980). However, the data presented in this paper allow another explanation. In the transverse sections the total areas of the axoplasm in treated and untreated animals are almost similar (Table 2). The mean size of the axons in treated animals is approximately 23% less, but the stimulation in number of regenerating fibers is also 23%. This observation is in agreement with our data on cell-free protein synthesis in the rat lumbar spinal cord (Bijlsma et al. 1983 b). It appeared that $ACTH_{4-10}$ treatment did not change the synthetic activity following sciatic nerve crush. Whether or not a shift in protein synthesis from functional to structural proteins as shown by several authors (Hall et al. 1978; Hall 1982; Benowitz et al. 1981; Giulian et al. 1980; Skene and Willard 1981), occurs in the rat as a consequence of $ACTH_{4-10}$ treatment during regeneration processes, remains to be elucidated.

In conclusion, the present findings support the notion that chronic $ACTH_{4-10}$ treatment stimulates the number of regenerating myelinated fibers and unmyelinated fibers after sciatic nerve crush in the same way. The regenerating fibers are characterized by smaller diameters of the axons.

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