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PATTERNS OF MOTOR INNERVATION IN THE PECTORAL MUSCLE OF ADULT *XENOPUS LAEVIS*: EVIDENCE FOR POSSIBLE SYNAPTIC REMODELLING

BY C. HAIMANN*, A. MALLART, J. TOMÁS I FERRÉ† AND N. F. ZILBER-GACHELIN

From the Unité de Physiologie Neuromusculaire, Laboratoire de Neurobiologie Cellulaire, C.N.R.S., 91190 Gif sur Yvette, France

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SUMMARY

1. An anatomical and electrophysiological study was performed on the pectoral muscle of *Xenopus laevis*.

2. Silver-impregnated preparations revealed immature endings, collateral and terminal sprouting and signs of synaptic regression.

3. Twenty percent of the fibres received a dual innervation from two different nerves. The synapses of 25 % of these fibres are formed in close vicinity.

4. Some of the singly innervated and most of the dually innervated end-plates generated only subthreshold electrical activity. Synaptic efficacy in dually innervated muscle fibres with closely spaced or distant endings was, on the average, one third and two thirds, respectively, of that obtained in singly innervated fibres.

5. Fibres with subthreshold electrical activity displayed normal ACh sensitivity.

6. The existence of non-transmitting synapses, of dually innervated end-plate sites and of morphological signs of the sprouting of new endings and the degeneration of old ones suggests that synaptic remodelling may occur in normal adult muscles.

INTRODUCTION

Polyneuronal innervation of single end-plate sites is the rule in developing and reinnervating skeletal muscles of both amphibians (Letinsky, 1974; Bennett & Pettigrew, 1975; Bennett & Raftos, 1977) and mammals (Redfern, 1970; McArdle, 1975). In twitch muscle fibres all but one of the endings are subsequently eliminated. Recent reports have shown that in frog muscle several inputs can be recognized in a single end-plate focus by graded stimulation of the motor nerve (Dennis & Miledi, 1974; Rotshenker & McMahan, 1976; Haimann, Mallart & Zilber-Gachelin, 1976; Vyskočil & Magazanik, 1977). It is not clear whether the polyinnervated end-plate sites in the adult amphibian muscle represent the failure of synapse elimination or the capacity of intact motor nerves to form new synapses in mature muscles.

In the pectoral muscle of *Xenopus laevis* the quantitative analysis of synaptic

* Present address: Laboratorio di Fisologia dei Centri Nervosi, C.N.R., Milano, Italy.
† Present address: Laboratorio de Histología, Universidad de Barcelona and Departamento de Anatomía Patológica, C.S. Principes de España, Hospitalet de Llobregat, Spain.

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efficacy at doubly innervated end-plate sites as compared to singly innervated ones is facilitated by the existence of a double innervation by two independent nerves (Haimann *et al.* 1976; Angaut-Petit & Mallart, 1979). A previous report (Angaut-Petit & Mallart, 1979) has shown that the end-plate potentials (e.p.p.s) in doubly innervated end-plate sites have a relatively small quantal content and are independent of the physical properties of the muscle fibres. In the present study both synaptic activity and certain morphological features of the motor endings suggest a continuous process of synaptic formation in this muscle.

METHODS

All experiments were performed on the pectoral muscle from *Xenopus laevis* (Daudin) aged 5–10 yr. This is a flat superficial muscle of triangular shape which lacks precise rostral and caudal boundaries. The muscle fibres are inserted along the mid line on bony or cartilaginous structures: clavicle, procoracoid and epicoracoid (De Villiers, 1924); from the midline they converge towards the head of the humerus. The rostral edge of the portion of muscle used in this study is formed by those fibres which attach to the more rostral part of the clavicle, whereas the caudal edge is formed by those inserted on the coracoepicoracoid articulation (Fig. 1).

The Bielschowky–Gros silver method was used to stain the motor axons and endings. Several layers of muscle fibres were removed with a freezing microtome and the reaction performed on the entire deep side of the muscle, which was used for the physiological experiments. Vital staining with methylene blue was occasionally used and gave results in agreement with those obtained in silver-impregnated preparations. Karnowsky's (Karnowsky, 1964) and the thiol-acetic acid (Gautron, 1974) methods for revealing cholinesterase were used to detect synaptic contacts.

The standard Ringer solution had the following composition (mM): Na, 118·0; K, 2·5; Ca, 1·8; H_2PO_4, 0·75; HPO_4, 2·25; Cl, 119·1; pH = 7·3. Unless otherwise stated, all the experiments were carried out in a solution in which Na was iso-osmotically replaced by 9–11 mM-Mg to keep end-plate potentials (e.p.p.s) below the spike threshold.

Nerves were stimulated by suction electrodes at a frequency of 0·17 Hz. Intracellular electrodes filled with 3 M-KCl were used for recording. Micropipettes, filled with 2 M-acetylcholine (ACh) of approximately 200 MΩ resistance and connected to a constant current source, were used for ionophoretic application of the drug.

Muscle contraction was measured under isometric conditions with a Grass strain gauge, Model FT03C. A tension of 1 g gave a 10 μm displacement, producing a 10 μV signal.

Synaptic efficacy was evaluated by measuring the mean e.p.p. amplitude of 30–100 responses with the aid of an averaging computer (Didac 800). In all cases where two values had to be compared, their ratio, R, was calculated. In order to know whether the two values were significantly different, we put the different Rs into two classes (>1 and <1) and performed a χ_2 test. If a significant difference was detected, it was always quantified by calculating the geometric mean of the R values, $\bar{R}^g$; i.e. if n values of R were measured, $\bar{R}^g = \sqrt[n]{R_1 \times R_2 \ldots R_n}$. Therefore, $\log \bar{R}^g = (1/n) \Sigma \log R$ in other words, $\bar{R}^g$ is the antilogarithm of the arithmetic mean of $\log R$. Our justification is the following: we wanted a series of ratios, R, whose product was equal to 1 and whose mean value was also 1. This is the property of the geometric mean. If, for instance, $R = a/b$ is equal once to 2/1 and once to 1/2, we would say that there is no difference in the ratios since a and b are interchangeable. Although the arithmetic mean of 2 and 1/2 is 2·5/2 = 1·25, its geometric mean is $\bar{R}^g = 1$; i.e. $\log 2 + \log 1/2 = 0 = \log 1$.

To study the pattern of muscle development at stages 57 to 66 (Niewkoop & Faber, 1967) we injected both a male and a female with 750 i.u. of gonadotrophic hormone, which resulted in egg laying one day later. The tadpoles were fed with powdered nettle and postmetamorphic animals with Tubifex worms.

RESULTS

Innervation of the pectoral muscle

The portion of the pectoral muscle we studied is innervated by two nerves which enter the muscle at its rostral and caudal edges. We will call these nerves anterior (*ant.*) and posterior (*post.*), respectively; they are divisions of a nerve trunk which originates from the second spinal nerve. Stimulating one division and recording on the other has shown that the axons in each division are independent.

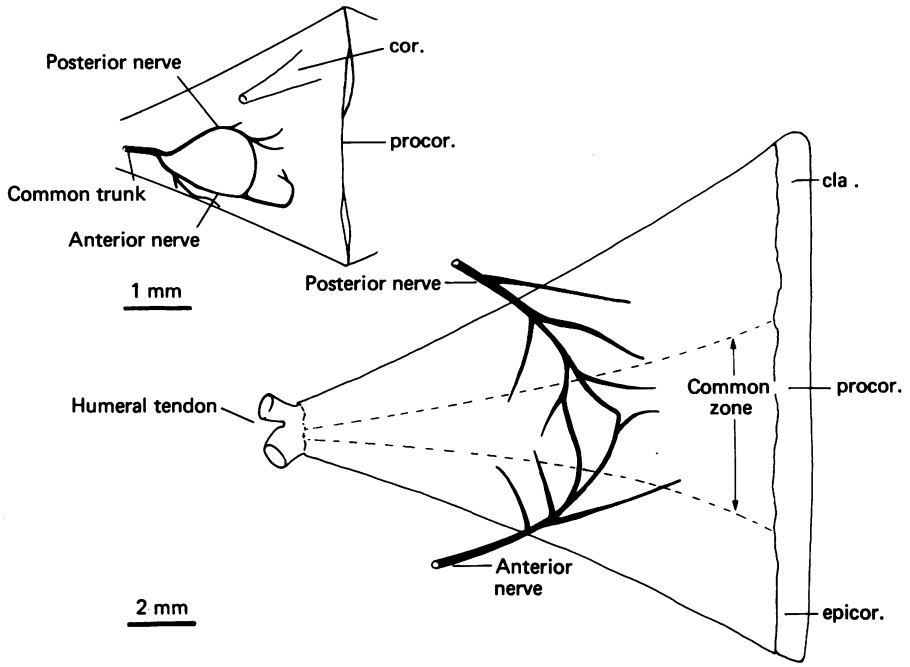

Fig. 1. Schematic diagram of the deep surface of the pectoral muscle showing the distribution of the main nerve branches. Inset: the pectoral muscle at the end of metamorphosis. cor., coracoid; procor., procoracoid; epicor., epicoracoid; cla, clavicle.

By destroying the corresponding spinal ganglion and allowing 2 months for the sensory fibres to degenerate, we could estimate from fibre counts in semithin sections the number of motor axons reaching each edge of the muscle. In two experiments the *post.* and the *ant.* nerves contained, on the average, seventy and thirty-eight axons, respectively, half of which are motor. Since the mean number of muscle fibres estimated from transverse sections is approximately 900, each axon innervates an average of seventeen muscle fibres.

Both nerves first follow a path more or less at right angles to the muscle fibres but soon give abundant branches on the deep surface of the muscle. The distribution of these branches is fairly constant from one muscle to another (Fig. 1). In osmic acid-stained preparations of whole muscles both nerves can be seen to innervate a common territory where axons from each nerve run together inside the same perineural sheath.

This particular pattern of innervation can be traced back to the period of meta-

morphosis (stages 57 to 66 of Nieuwkoop & Faber, 1967). The forelimbs break through at stage 58 (44th day) and, from this stage on, the pectoral muscle can be dissected out. Until stage 64 (53rd day), when the paired sternal anlage fuses, the muscles are attached to the humerus but do not reach the mid line. The nerve trunk common to *ant.* and *post.* reaches the humeral tendon and from there the two nerves diverge towards the corresponding edges of the muscle, where they make a recurrent loop and meet in the middle of the muscle. The typical adult pattern of innervation is obtained by the end of metamorphosis (Fig. 1, inset).

The territory innervated by each nerve was established by systematic intracellular recording of the electrical responses from superficial muscle fibres to stimulation of *ant.* or *post.* Three zones were identified (Fig. 1): (*a*) an anterior zone innervated only by *ant.*, (*b*) a posterior zone, innervated only by *post.*, and (*c*) a medial zone, or 'common zone', in which fibres innervated by *ant.*, by *post.* and by both nerves are found, apparently at random. Two muscles (out of thirty-five) lacked a common zone of innervation.

To estimate the amount of innervation of each nerve and the degree of overlap between them, we measured the isometric tension of the muscle when each nerve separately or both nerves together were stimulated by a train of 400 msec at 100 Hz. A train of stimuli was used because it allows more reliable measurements of the tension overlap (see Brown & Matthews, 1960). A short tetanus was sometimes required to drive muscle fibres above the spike threshold (see below). In sixteen experiments the tension developed upon stimulation of the anterior and the posterior nerves separately was $T_{ant.} = 41\,\%$ (± 10 S.D.), and $T_{post.} = 80\,\%$ (± 9 S.D.) of the total tension obtained either by simultaneous stimulation of both nerves or by direct stimulation of the muscle. These figures agree well with the number of motor units supplied by each nerve obtained from fibre counts. The tension overlap, defined as the difference between the sum of the tensions from separate nerve stimulation and the tension from simultaneous stimulation of the nerves, expressed as a percentage of the latter, was 21 % (± 9 S.D., $n = 16$ muscles). This means that about one fifth of the muscle fibres receive a functional innervation from both nerves (see Fig. 7*A* of the accompanying paper).

Histological observations

Elongated terminal branches running parallel to the main axis of the muscle fibre originated from medullated axons that lost their myelin sheath close to the surface of the fibre (Pl. 1*A*, *D*). Sometimes the parent axon divided into two or three medullated branches, each supplying a distinct motor apparatus on closely spaced areas of the muscle fibre (Pl. 1 *B*, *C* and *D*). Motor endings varied considerably in the number and length of their unmyelinated terminal branches; from one to thirty or more branches per end-bush were encountered in a given muscle. To visualize the areas of synaptic contact, we used the cholinesterase reaction. Plate 1 *D* shows an example of the results obtained by using the thiolacetic method together with a complementary staining by osmic acid for myelin; the deposit of the reaction products reproduced a pattern of arborization which closely resembles the one obtained in silver-impregnated preparations. By contrast, the picture obtained by using Karnow-

sky's method (Pl. 1*E*) outlined a broad area which extends outside the limits of the individual terminal branches, probably by means of a diffusion of the reaction products (McMahan, Spitzer & Peper, 1972).

Four types of motor endings in *Xenopus* muscles have been described (Lännergren & Smith, 1966) using the thiolacetic method for revealing cholinesterase. Three of them, differing in the distribution and length of the arborization, correspond to muscle fibres which have been identified as 'fast' according to their ultrastructural, histochemical and contractile properties (Smith & Lännergren, 1968; Smith & Ovalle, 1973). These three end-plate types were found in our preparations. 'En grappe' endings, typical of tonic muscle fibres, have never been observed in the pectoral muscle; this agrees with the absence of fibres with 'slow' membrane properties (Angaut-Petit & Mallart, 1979). Many of the motor axons and endings observed in silver-impregnated whole-mount preparations produced collaterals. Very often a myelinated axon bifurcated near its terminal portion and, retaining its myelin sheath, branched on to the same or different muscle fibres, where these daughter branches formed endings of various sizes. A case of long-range branching is shown in Pl. 2*A*, where the collateral supplies a distant muscle fibre with an extremely simplified motor ending. Plate 2*D* shows a long axon, presumably unmyelinated at least in its distal portion, sprouting from a preterminal node. Sometimes the collaterals sprouted from the motor endings themselves, as seen in Pl. 2*C*. This muscle fibre was innervated both by a terminal branch from a neighbouring motor ending and by simplified endings from an independent axon. Poorly developed endings were sometimes seen sprouting from the last two or three nodes of a myelinated axon (Pl. 2*B*). In Pl. 3*D* two, presumably independent, axons establish contact with the muscle fibre by thin, poorly branched, elongated terminals. One of these axons does not appear to be completely myelinated. It forms a single bifurcation which runs in a synaptic gutter side by side with some of the branches of the more developed terminal. This particular pattern of innervation is typical of regenerating endings (Letinsky, Fischbeck & McMahan, 1976; Rotshenker & McMahan, 1976; see also the accompanying paper). The simplified motor endings described above bear a striking similarity to those observed during muscle reinnervation where developing endings exhibit, in general, longer ultimate branches and fewer branching points than is usually found in normal adult muscles (see the accompanying paper).

In several instances we observed abnormal endings like those shown in Pl. 4; here, the terminal branches end in pyriform or bulbous structures. Sometimes the whole terminal arborization was retracted and only the stumps of the main branches could be seen. These remnants appeared thickened and presented swellings in which the hypertrophied 'neurofibriles' could be distinguished. The structural abnormalities we observed in our preparations of normal muscles correspond to those observed by Cipollone (1897) in frogs and Tello (1907) in rabbits in cases of post-traumatic degeneration of motor endings. End-bulbs have also been observed by Riley (1977) and O'Brien, Östberg & Vrbová (1978) in motor axons during spontaneous neonatal elimination of motor endings. Plate 4 illustrates a case in which normal and degenerated endings are close together on the same muscle fibre; both originate from the same Ranvier node in the nerve trunk. This probably exemplifies a case of motor-

ending replacement (see Barker & Ip, 1965). In other instances isolated degenerated endings were observed, indicating that perhaps the muscle fibre is temporarily denervated.

Motor endings originating from either collateral or terminal sprouting may innervate muscle fibres already provided with a motor innervation. In a number of cases both endings occurred at the same point on the muscle fibre. Here, both endings

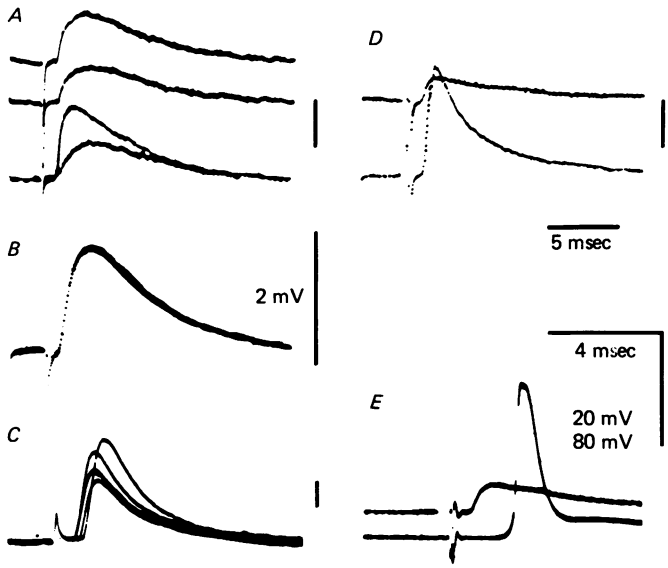

Fig. 2. Examples of e.p.p.s recorded from pectoral muscle fibres. *A*, e.p.p.s of variable rise time obtained from a single synaptic focus upon stimulation of one of the nerves at constant stimulus strength. Since the different components could not be separated by changing the stimulus intensity, we can conclude that each component arises from different but closely spaced terminals supplied by a single axon whose branching points do not have the same security factor for spike propagation. In *B*, thirty e.p.p.s from the same end-plate as in *A* have been averaged with the aid of a computer. In *C*, the latency jumps of the e.p.p. are probably due to variable delays in propagation across a preterminal branching point. In *D*, the stimulation of one of the nerves evoked e.p.p.s at two synaptic foci on the same fibre separated by 2·5 mm. The recordings were obtained by placing one electrode at each synaptic spot. *E*, the muscle was bathed in standard Ringer solution and the microelectrode located at a point on the fibre where the *post.* nerve gave only subthreshold potentials; propagated action potentials from an end-plate supplied by the *ant.* nerve were recorded from the same electrode position; note different voltage calibrations for the e.p.p. and for the action potential. Same vertical and horizontal calibrations for *A*, *B*, *C* and *D*.

lie side by side a few μm apart (Pls. 2*C* and 3*A*, *C*), or have some of their terminal branches crossing over one another (Pl. 3*B*). They can occupy the same synaptic gutter (Pl. 3*D*). This third disposition, a relatively common feature of reinnervating muscle (see the accompanying paper and Letinsky *et al.* 1976), was observed only occasionally in the normal adult. The existence of doubly innervated end-plate sites in adult muscles has already been reported by Ramón y Cajal (1881) and Dogiel (1890) in frogs and by Tello (1917) in the tongue of the rabbit. This last author explains his observations by the wealth of motor input to this organ.

Electrophysiology of the neuromuscular junctions

The impalement of a muscle fibre close to a nerve terminal partially blocked by Mg^{2+} allowed us to record e.p.p.s which usually had a rise time of 1–1·5 msec. However, the rise time sometimes fluctuated around a longer mean value, which did not change appreciably when the electrode was moved several hundred μm away. This probably occurs when multiple endings are supplied by a single axon (e.g. Pl. 1 *B*, *C*).

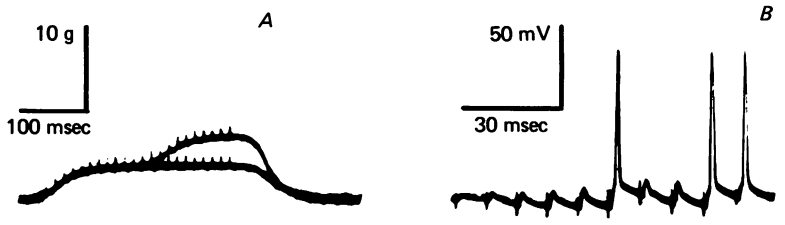

Fig. 3. Evidence for subthreshold synaptic activity in pectoral muscle. In *A*, tetanic tension elicited by two identical trains at different intensities of stimulation. The increase in intensity recruits a motor unit which would be activated only by the facilitation of subthreshold potentials. *B*, intracellular recording; subthreshold e.p.p.s generated action potentials by facilitation during a train of stimuli. Both experiments were performed in standard Ringer solution.

Examples of e.p.p.s of variable rise time and latency are shown in Fig. 2*A*, *B* and *C*. An intermittent failure or delay of the invasion by the presynaptic action potential of one or more of the main terminal branches could cause these variations (Hatt & Smith, 1976). A similar effect has been obtained by applying tetrodotoxin to one of the terminal branches with the aid of a micropipette (Katz & Miledi, 1968).

Large variations in e.p.p. amplitude were observed not only from one fibre to another but also between different junctions from the same muscle fibre (Fig. 2*E*), in contrast to the frog sartorius, where all junctions from the same muscle fibre have a similar efficacy (Weakly, 1978). In twelve muscles the ratio of the largest to the smallest e.p.p. amplitude from different fibres in each muscle varied from 7·4 to 129. The geometric mean of the distribution was 21·8, similar to that observed in frog muscles under similar experimental conditions (Weakly, 1978).

In standard Ringer solution neuromuscular transmission at some end-plates was achieved only through facilitation of e.p.p.s by a train of stimuli. Fig. 3*A* shows the isometric contraction of the muscle in response to threshold stimulation. By increasing the stimulus intensity, we activated another motor unit which also remained subthreshold until a critical level of facilitation was obtained. This is shown in Fig. 3*B*.

Polyneuronal innervation of muscle fibres

About 20% of the muscle fibres of the pectoral muscle are innervated by more than one neurone. Poly-innervated fibres received two, sometimes three, nerve terminals supplied either by the same or by different nerves. Although poly-innervated muscle fibres with synapses supplied by the same nerve can be found throughout the muscle, our study was concerned essentially with fibres located in the common zone whose endings were supplied by different nerves.

Doubly innervated end-plate sites. E.p.p.s elicited by stimulation of *ant.* and *post.* nerves displayed a minimum rise-time at the same spot on the muscle fibre, suggesting that axons of different origin form synaptic contacts in close vicinity. We confirmed this by systematically recording the e.p.p.s every 250 μm along the muscle fibre and plotting the rise-time of the response to stimulation of each nerve as a function of the distance. The localization of the synaptic site could thus be determined precisely and appeared to be the same for *ant.* and *post.* The same result was

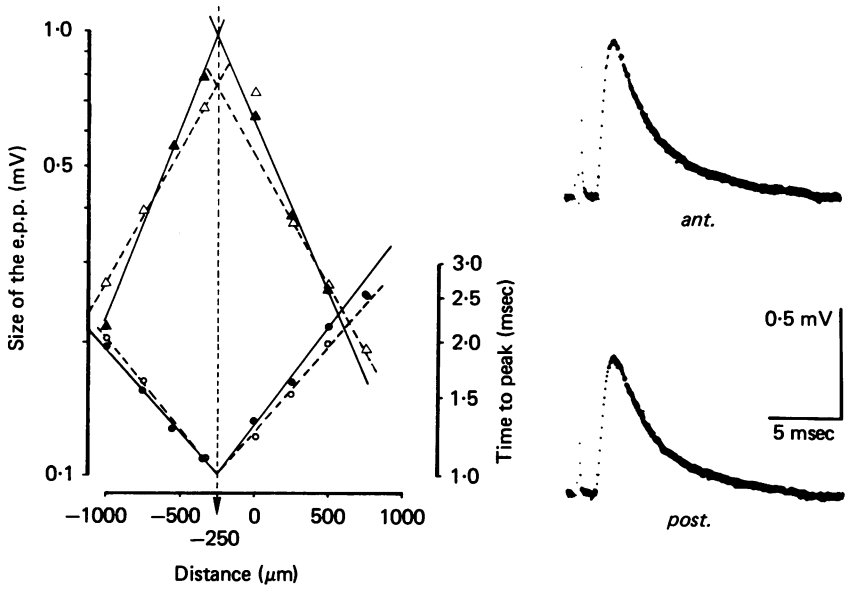

Fig. 4. On the right, e.p.p.s from a synaptic site after *ant.* and *post.* stimulation; each trace represents the average of ten sweeps. The graph represents the exponential variation of e.p.p. amplitude (triangles) and rise time (circles) along the muscle fibre. Filled symbols: *ant.*; open symbols: *post.* Both nerves evoke an e.p.p. at the same synaptic focus.

obtained with greater accuracy, by plotting the amplitude of the responses to stimulation of *ant.* and *post.* against the distance (Fig. 4). Doubly innervated end-plate sites were found in about 25 % of the fibres with a dual innervation from *ant.* and *post.* End-plates innervated by more than two axons were not observed in this series of experiments.

A quantitative analysis of e.p.p. amplitude was performed in twenty-two doubly innervated and in more than 100 singly innervated end-plate sites. E.p.p.s from doubly innervated end-plates were divided into four classes: *A*, the whole population of e.p.p.s; *B*, the smaller element; *C*, the larger element; and *D*, the sum of both elements. Table 1 shows that the larger amplitudes predominate at singly, and the smaller at doubly innervated end-plates. A χ^2 test indicated that each of the *A*, *B*, *C* and *D* distributions was significantly different from the e.p.p. distribution in singly innervated muscle fibres. If we compare the individual amplitudes of the e.p.p.s from dual end-plates in each muscle with the average e.p.p. size of singly innervated end-plates from the same muscle, we can express the relative size of the former to

the latter as a series of ratios whose geometrical mean, $\bar{R}^g = 0{\cdot}31$ (S.D. log $R = 0{\cdot}63$, $n = 30$), is significantly different from 1 (t test, $P < 0{\cdot}001$). Thus, the mean size of the e.p.p.s from dual end-plates is approximately $\frac{1}{3}$ of that obtained at singly innervated sites. Twenty-three of the ratios were < 1; 7 were > 1, due to the presence of a

TABLE 1. Distribution of e.p.p. amplitudes at doubly and at singly innervated end-plate sites

	E.p.p. amplitude (mV)		
	0–1·9	2–3·9	>4
Doubly innervated			
A Both e.p.p.s $n = 44$	36	4	4(*)
B Small e.p.p.	21	1	0(*)
C Large e.p.p. $n = 22$	15	3	4(*)
D Sum of both e.p.p.s $n = 22$	11	6	5(**)
Singly innervated $n = 131$	36	40	55

Each of the *A*, *B*, *C* and *D* e.p.p. amplitude distributions is significantly different from that found at singly innervated end-plates (* $P < 0{\cdot}001$, ** $P < 0{\cdot}05$).

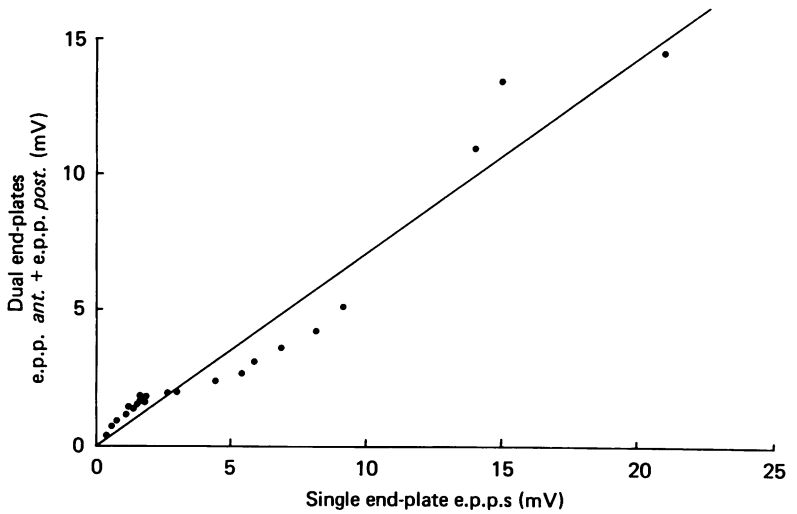

Fig. 5. Values obtained by adding both e.p.p.s from a sample of twenty-two doubly innervated end-plate sites were plotted against e.p.p. amplitudes from an equal sample of singly innervated end-plate sites. Both sets of values were ranked according to increasing size and each point corresponds to the same rank in both series. The straight line was obtained by linear regression from the experimental points; its slope is 0·71 and the correlation coefficient is 0·97.

large e.p.p. in some of the doubly innervated end-plates. To investigate this point further, we compared the sum of the amplitudes of both e.p.p.s from the whole population of doubly innervated end-plates with an equal number of randomly selected e.p.p.s from singly innervated muscle fibres. Both samples were ranked according to increasing size. In Fig. 5 each point corresponds to a given rank in each

population. A linear relationship was obtained with a correlation coefficient of 0·97 and a slope of 0·71, significantly different from 1 (t test $P < 0·001$), indicating that the sum of both inputs at dual end-plates is, on the average, 30 % *less* than that obtained at singly innervated ones. When comparing both elements from dual end-plates, the amplitude ratios were, in most cases, in the range of 1–5, although in some instances they could be 100 times greater. Cases in which both e.p.p.s had a large amplitude were never observed.

Doubly innervated fibres with separated end-plate sites. Muscle fibres with a double innervation at separated end-plate sites were more frequent than those with a double innervation at a single end-plate site. In fibres with distant end-plate sites each synaptic focus was identified either by exploring the whole length of the fibre with an intracellular electrode or by using the cholinesterase reaction as a marker and then measuring the end-plate distance with the aid of a calibrated eyepiece. Mean end-plate separation was found to be 2·5 mm (S.D. = 0·9 mm; n = 19; range, 1·4–3·5). Fig. 2 *D*, *E* shows examples of responses obtained in fibres innervated by distant synapses; in *D*, both foci are supplied by the posterior nerve and in *E* by different nerves.

To obtain a relative estimate of the magnitude of the synaptic input to doubly innervated muscle fibres, we compared e.p.p. amplitude to the mean e.p.p. amplitude obtained at singly innervated fibres from the same muscle. The geometrical mean of the ratios of e.p.p. amplitude from doubly innervated fibres to the average e.p.p. size obtained at singly innervated ones was $\bar{R}^g$ = 0·49 (S.D. log R = 0·47, n = 17), significantly smaller than 1 (t test $P < 0·05$). When comparing the *sum* of both inputs to the same fibre to the average e.p.p. size from singly innervated end-plates, we found no significant difference. We can therefore conclude that e.p.p. amplitude in fibres with two distant synapses is about half the average amplitude obtained in fibres with a single input.

Eight fibres with two separated end-plate sites – one singly and the other doubly innervated – were found, but no significant difference in e.p.p. amplitude was observed between the singly innervated site in these fibres and the singly innervated site in fibres with no separated end-plates. E.p.p.s from the doubly innervated site were significantly smaller than those from doubly innervated sites in fibres with no separated end-plates. It thus seems that the existence of an additional junction at some distance on the same fibre reduces the synaptic efficacy at doubly innervated end-plate sites.

In the estimation of synaptic efficacy, a possible source of error could result from e.p.p. amplitude dependence on the membrane input impedance, which might not necessarily be the same in dually and in singly innervated muscle fibres. However, this seems unlikely since Angaut-Petit & Mallart (1979) have shown that there are no differences in passive membrane properties between both populations of muscle fibres.

Acetylcholine sensitivity of fibres with subthreshold potentials

It is now well documented that procedures leading to the suppression of the muscle action potential induce the extension of the extrajunctional ACh receptors (for review, see Lømo & Westgaard, 1976). Since some of the e.p.p.s found in the present

series of experiments were unable to trigger propagated electrical activity in response to a single stimulus even in a standard Ringer solution, we investigated ACh sensitivity in these fibres. The results of a typical experiment are shown in Fig. 6. A singly innervated fibre was found in which the mean e.p.p. amplitude was only 0·32 mV in 10 mM-Mg^{2+} Ringer solution and 5·8 mV in standard saline solution, far from the

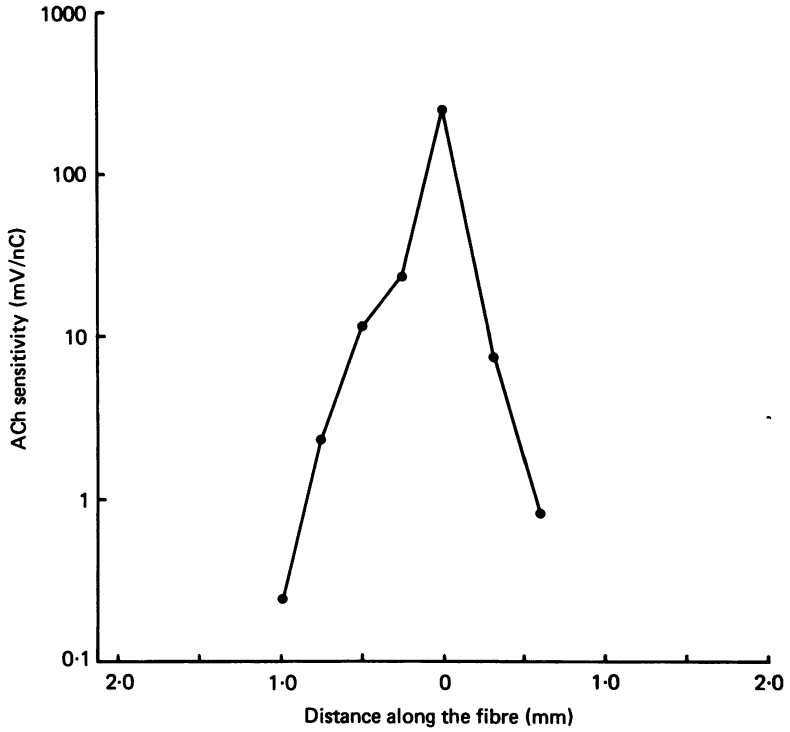

Fig. 6. Decay of ACh sensitivity as a function of distance from an end-plate which responded to nerve stimulation by subthreshold e.p.p.s. Sensitivity is expressed in millivolts of depolarization produced per nanocoulomb of ionophoretic current.

40 mV depolarization required to trigger an action potential. ACh sensitivity falls abruptly outside the end-plate area and becomes undetectable at about 1 mm on either side. This distribution of ACh sensitivity was found regularly at all end-plates, irrespective of their ability to produce suprathreshold e.p.p.s.

DISCUSSION

Two main findings emerge from this study: first, a substantial number of motor terminals are poorly developed, bearing morphological signs of immaturity or of regression; secondly, when two different axons innervate the same muscle fibre, the mean amount of transmitter released by both axons together is, on the average, smaller than the amount released at singly innervated sites.

Motor endings of normal muscles of adult *Xenopus* present considerable variations in size. Endings in the smaller size range are frequently formed by a few fine, elon-

gated terminal branches which resemble those observed during muscle reinnervation (see the accompanying paper and Tomás i Ferré, 1977), or during *de novo* synaptogenesis in regenerating muscles (Tomás i Ferré, 1977). Immature looking endings sprouting from motor axons of normal adult muscles have already been observed in frogs (Dogiel, 1890, Fig. 3), in rabbits (Tello, 1917); Ramón y Cajal, (1925) and, more recently, by Barker & Ip (1965) and Tuffery (1971) in the cat. The similarity between the immature looking endings and the terminals formed by regenerating axons could indicate cases of synapse formation in normal mature muscles. These findings, together with the observation of morphological signs of regression in some motor endings, support the view of Barker & Ip (1965), Tuffery (1971) and Ip (1974), that adult muscles are subjected to a continuous process of synaptic degeneration and regeneration (see also Cardasis & Padykula, 1979). In agreement with this interpretation, clear electron microscopic evidence of synaptic degeneration in normal adult animals has been obtained in the central nervous system of the rat (Sotelo & Palay, 1971) and in the ciliary muscle of the monkey (Townes-Anderson & Raviola, 1978).

Some of the e.p.p.s recorded from fibres in the *Xenopus* pectoral muscle are not large enough to trigger a muscle action potential except when facilitated by a train of high frequency stimuli. Subthreshold e.p.p.s have also been recorded in singly innervated twitch fibres of frog iliofibularis muscle (Orkand, 1963). These subthreshold e.p.p.s could be generated by poorly developed terminals. This is based mainly on the direct correlation between end-plate area and quantal content found by Kuno, Turkanis & Weakly (1971) and between the size of the terminals and the size of the e.p.p.s reported in the accompanying paper.

Small amplitude e.p.p.s were more frequently observed in doubly innervated end-plate sites. Doubly innervated end-plates appear to be, on the average, three times less efficient than singly innervated end-plates. Double end-plates may be formed by two immature endings in the course of reinnervation of an unoccupied end-plate site, although new synaptic formation on a virgin area of the membrane cannot be excluded. Where there is a large disparity in the sizes of both responses, we can imagine that one of the axons occupied the synaptic space before the other, or that one axon had reinnervated a synaptic area left partially vacant by the retraction of some of the terminal branches of an old motor ending. Since some of the small-sized endings which innervate double end-plates do not look immature, we can speculate that the double innervation of end-plate sites is relatively stable.

To explain the reduced size of the endings at doubly innervated end-plate sites, we may imagine some kind of competition occurring. However, our observations do not fit into the simple model of competition between axons for a limited synaptic space. If this were the case, then the sum of both responses from double end-plates would be approximately equal to the average e.p.p. amplitude at singly innervated synapses, not *less*. A plausible explanation would be that an inhibitory influence is exerted between closely spaced nerve endings and leads to an impaired development of the terminals (see Diamond, Cooper, Turner & Macintyre, 1976). Since most e.p.p.s from distant end-plates on the same fibre are smaller than those from singly innervated fibres, we could also suppose that a restraining influence could be exerted, although to a lesser degree, between end-plates separated by 2–3 mm. Recent data

from muscles with experimentally induced ectopic end-plates indicate that supernumerary synapses appear depressed and are eventually eliminated (Grinnell, Letinsky & Rheuben, 1979; Bennett & Raftos, 1977; Kuffler, Thompson & Jansen, 1977; Tonge, 1977; Dennis & Yip, 1978).

The control of ACh sensitivity by muscle activity is now firmly established (Lømo & Westgaard, 1976). However, factors other than propagated electrical activity may be operant since multiply innervated muscles in frog (Nasledov & Thesleff, 1974) and in fish (Frank & Jansen, 1976) do not generate action potentials, yet they do not display widespread ACh sensitivity. Also, in transected muscles in which the distal part became hypersensitive (Vyskočil & Gutmann, 1976), high ACh sensitivity persisted for a long time after the return of normal electrical activity. Conversely, muscle fibres in the course of development (Letinsky, 1975) or recovering from botulinum poisoning (Bray & Harris, 1975) lose their extrajunctional sensitivity although they displayed only subthreshold transmission.

Before we can conclude that ACh sensitivity is regulated by subthreshold activity, we must be sure that the threshold for the spike cannot be readily reached by the facilitation of small e.p.p.s when the motor nerve discharges repetitively. Although we cannot rule out this possibility, it seems improbable, since trains of stimuli hundreds of milliseconds long at 100 Hz are often required to enable small e.p.p.s to reach the threshold for the spike (Angaut-Petit & Mallart, 1979). This high stimulation frequency corresponds to the maximal rate of firing of frog motoneurons when driven by electrical stimulation (Magherini, Precht & Schwindt, 1976). We may wonder, however, how often this situation occurs in the living animal.

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Note added in proof. While this paper was in press, a report on terminal sprouting and degeneration at frog motor endings has been published by A. Wernig, M. Pécot-Dechavassine & H. Stöver (*J. Neurocytol.* **9**, 277–303).

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EXPLANATION OF PLATES

Photomicrographs of nerve endings from whole mount preparations of the pectoral muscle of *Xenopus laevis*. Bielschowsky's silver technique was used throughout unless otherwise specified. The calibration bars represent 50 μm.

PLATE 1

Examples of neuromuscular junctions of different degrees of complexity.

A, simple end-bush; all the main (primary) terminal branches arise from the last Ranvier node of the parent axon (arrow). The primary branches are thick and varicose; they bifurcate into thin, straight secondary branches of various lengths that first form an approximately right angle to each other and then curve to adopt a path parallel to the longitudinal axis of the muscle fibre. Some of the secondary branches bifurcate into fine tertiary branches, and so on.

B, double end-bush consisting of a large arborization supplied by the main axon and of a smaller one supplied by a collateral arising from a preterminal node (double arrow). The large arborization contains thick primary branches originating from the last two Ranvier nodes (arrows). Since the degree of branching relative to the total length of the terminal branches is less important in the end-bush supplied by the collateral, we could infer that this ending is ontogenetically more recent than the other one.

C, triple end-bush formed by the terminal arborization of the main axon and by two arborizations supplied by collateral branching at the last two Ranvier nodes (arrows). Note that the more proximal collateral gives the more developed arborization.

D, thiolacetic acid method for revealing cholinesterase combined with the osmic acid method for staining myelin (in lighter shades of grey). The main axon bifurcates into myelinated branches; the unmyelinated portions of the end-bush are found at the sites marked by the cholinesterase reaction. This disposition is similar to that shown in *A* by Bielschowsky's method.

E, Karnowsky's reaction for cholinesterase showing the contour of the terminal apparatus. Some of the myelinated portions of the axon are probably also outlined by the reaction (arrow).

PLATE 2

Examples of neuromuscular junctions presumably in course of development.

A, a collateral arises from the antepenultimate node of a terminal axon (white arrow) and travels over four muscle fibres to form a simplified motor ending, probably representing a new synapse formation. Another collateral arises from the penultimate node (arrow) and forms a small end-bush (barely visible in the photograph) on the same muscle fibre as the main ending.

B, a single axon gives three nerve endings: one at its tip and two at the last two Ranvier nodes. All three show a very poor development. This disposition is essentially the same as in Pl. 1*C*, but here the endings are more immature.

C, a muscle fibre is innervated by two poorly developed arborizations (arrowheads) supplied by two primary branches of a terminal axon. It also receives a branch from an end-bush innervating a neighbouring muscle fibre (arrow). This branch is thick and coarse throughout most of its length but becomes thin and tapered at the tip, where the synaptic contacts are presumably made.

D, a long and delicate collateral originating from a preterminal Ranvier node (empty arrow). It gives off a small bifurcated branch (arrow) to the same muscle fibre that receives the main end-bush. At an additional distance of 200 μm from the small bifurcation it supplies the neighbouring muscle fibre with a poorly developed ending (double arrow).

PLATE 3

Examples of dually innervated end-plate sites. Same magnification for *A* and *B*.

A, methylene blue staining. A muscle fibre is innervated by two separate axons (arrows) that establish synaptic contacts in close vicinity. Note the different degree of development of both arborizations.

B, a synaptic site is innervated by two independent axons (white and black arrows); the axon indicated by the white arrow starts bifurcating at a short distance from the muscle fibre. The terminal arborization of both axons partly overlap each other (empty arrow).

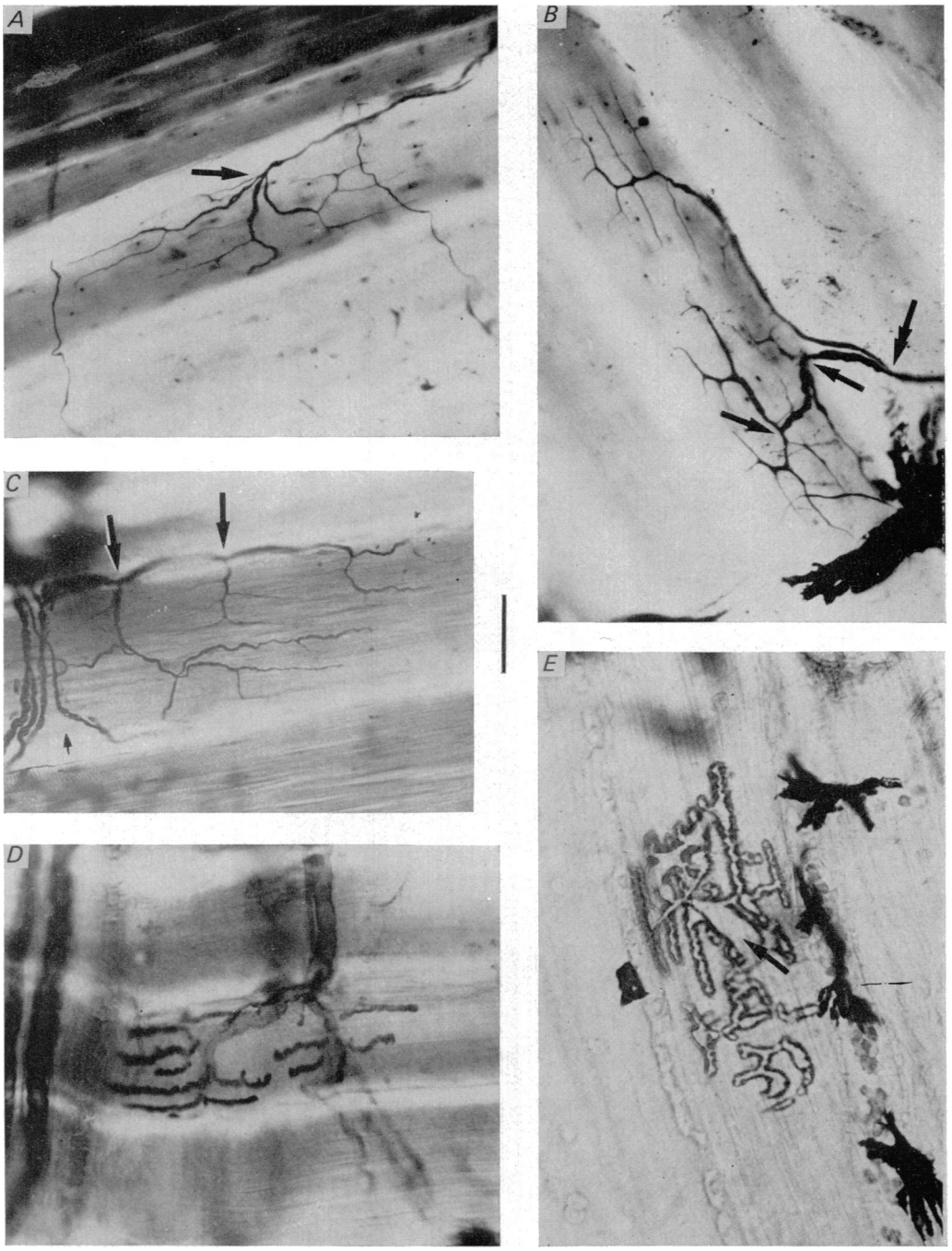
C, two separate axons (arrows) make synaptic contacts in close vicinity; both arborizations show a low degree of development.

D, two axons approach the end-plate site parallel to each other and bifurcate at almost exactly the same point of the muscle fibre surface. Terminal branches of each axon lie side by side, apparently in the same gutter (arrowheads). They are unusually long and poorly branched, typical features of developing neuromuscular junctions.

PLATE 4

Example of synaptic replacement.

A, an axon, originating from a Ranvier node on the main nerve branch, supplies a well-developed terminal arborization (arrow). Two thin terminal branches originate from the same node in the nerve trunk and lie on the top of the muscle fibre, where they end in bulbous structures (double arrow). These are shown at a higher magnification in *B*. These thin branches are probably remnants of a former terminal arborization. A sensory axon (s) is visible in *A*.


A
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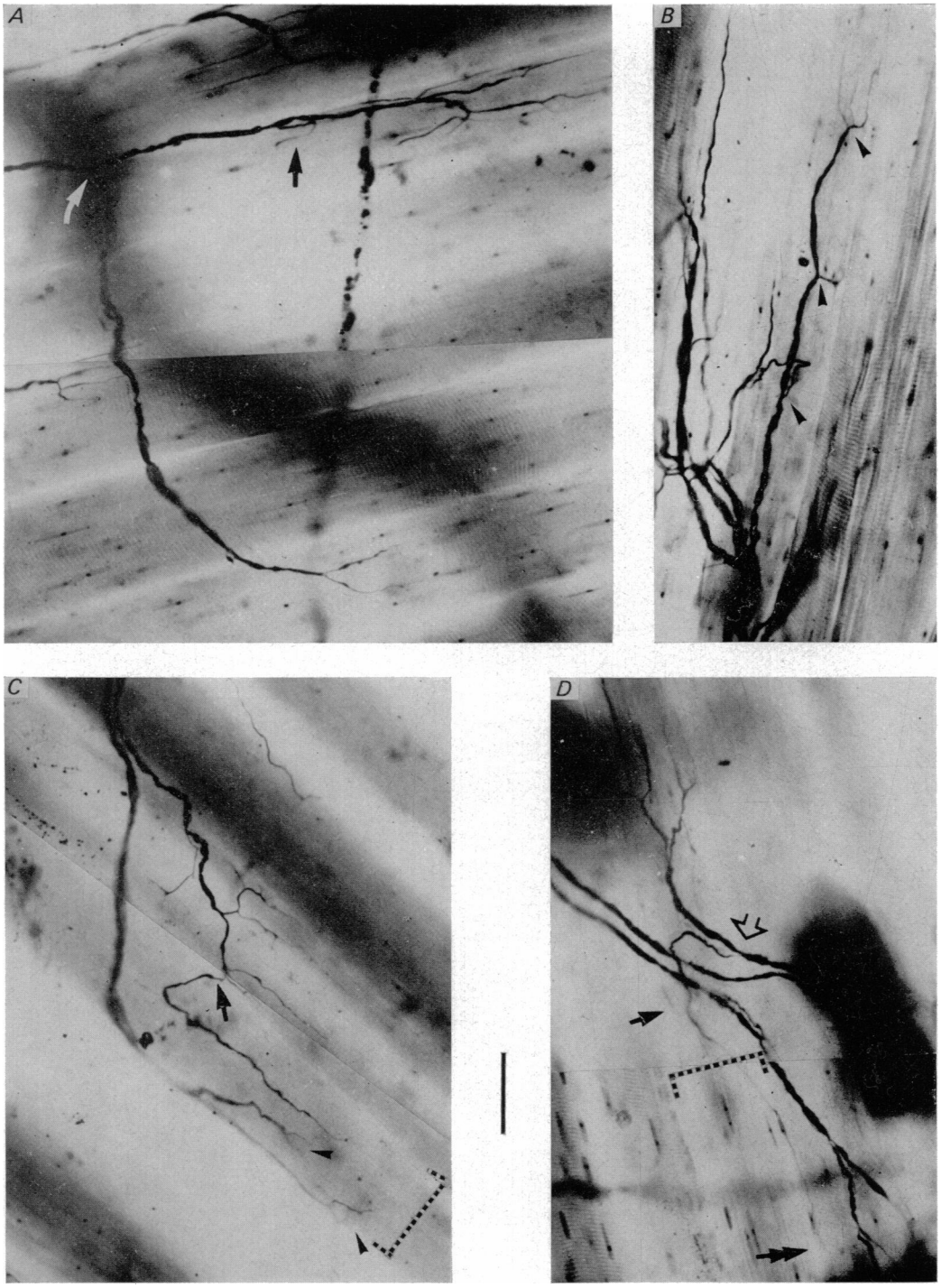
A
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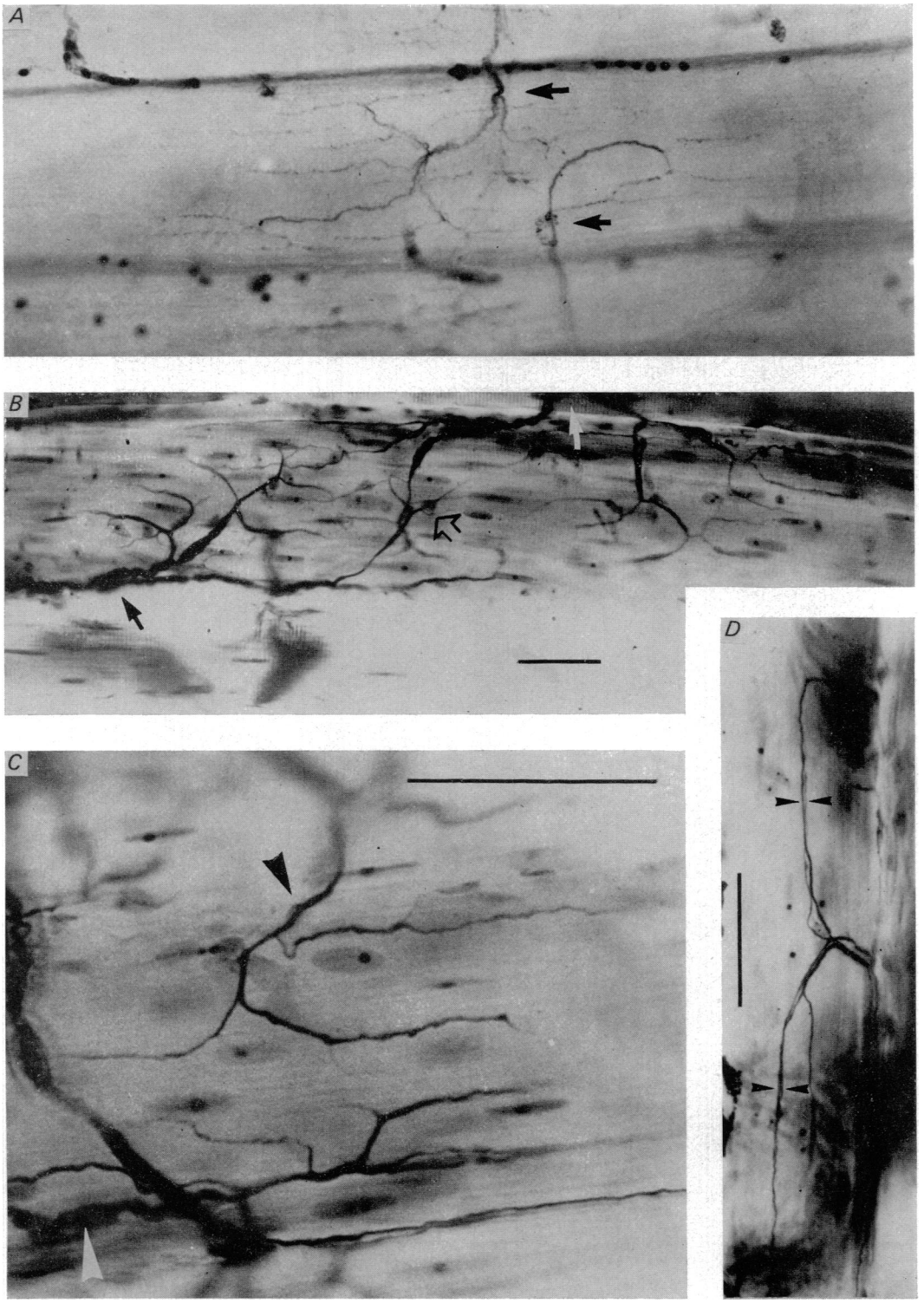
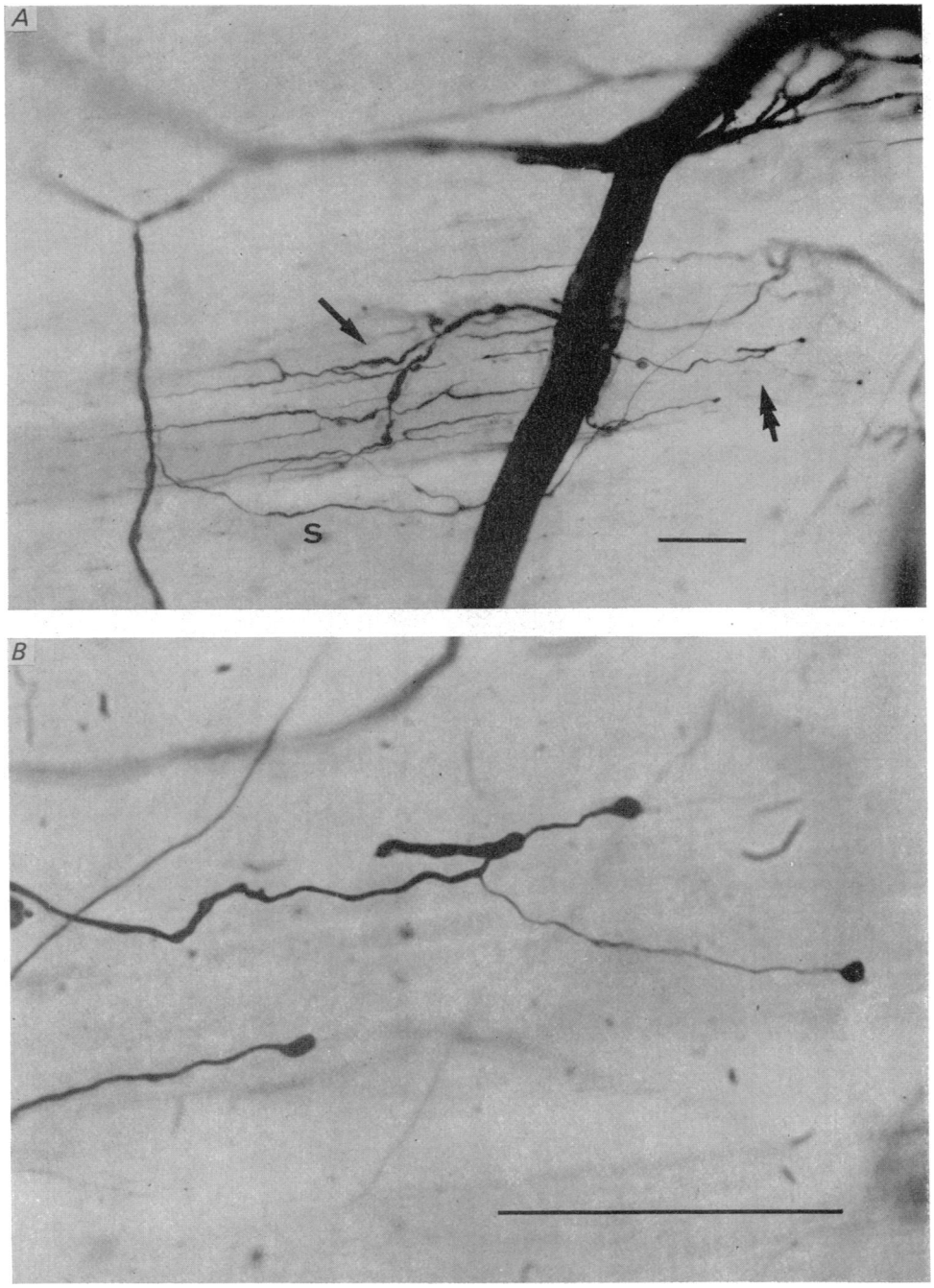
D



C. HAIMANN AND OTHERS



C. HAIMANN AND OTHERS