DISTRIBUTION OF ACETYLCHOLINE RECEPTORS IN THE MYOTOMES OF *XENOPUS LAEVIS* DURING NORMAL DEVELOPMENT

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirement for the degree of Doctor of Philosophy

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January 1980

ABSTRACT

The distribution of acetylcholine receptors in the myotomes of Xenopus laevis was investigated during development by using α-bungarotoxin labelled with tetramethylrhodamine or ^{125}I. The presence of nerve fibres was determined after staining with nitroblue tetrazolium. The results show that acetylcholine receptors are widespread along the muscle cells when the nerve fibres first arrive. Their density increases progressively for about a day and then gradually declines after the embryo hatches. High receptor densities develop at the presumptive synaptic sites about 1-5 hr after the onset of innervation. When segments of developing spinal cord are removed during early stages there is a progressive decrease of receptor patches at the ends of the myotomes, the main innervation site. The loss of these patches is not due to the accompanying muscle inactivity. These findings indicate that during normal development the accumulation of acetylcholine receptors at the synaptic sites is nerve-induced and their maintenance is nerve-dependent.

RESUME

On a étudié la distribution des récepteurs de l'acétylcholine dans les myotomes chez la grenouille (Xenopus laevis) pendant leur développement en utilisant l'α-bungarotoxine accouplée de molécules fluorescentes ou d'iode radioactif. La présence de fibres nerveuses a été constatée en utilisant le nitrobleu tetrazolium. Les récepteurs de l'acétylcholine sont présents le long des cellules musculaires quand les fibres nerveuses font contact avec les muscles. La densité de ces récepteurs extra-synaptiques s'accroît jusqu'à l'éclosion des têtards et ensuite elle diminue graduellement. Certaines régions isolées possédant une haute densité de récepteurs se forment aux jonctions synaptiques d'une à cinq heures après le début de l'innervation. Lorsque des portions de la moelle spinale sont retirées des embryons, les agrégats de récepteurs aux extremités des myotomes (la localité principale d'innervation) disparaîssent progressivement. Ce changement n'est pas causé par l'inactivité musculaire qui accompagne la dénervation. Cette étude a démontré que pendant le développement normal des myotomes l'aggrégation des récepteurs de l'acétylcholine aux jonctions synaptiques est induite par les nerfs, et la présence continuée des fibres nerveuses est nécessaire à la préservation des agrégats de récepteurs.

PREFACE

The present thesis was prepared according to the provisions of guideline number 7 (Manuscript and Authorship) concerning thesis preparation of the Faculty of Graduate Studies and Research of McGill University. This regulation allows the inclusion of original papers suitable for submission to learned journals for publication, with common abstract, introduction of the literature (chapter I) and concluding remarks (chapter IV). Chapters II and III of this thesis are texts to be submitted for publication in the journal "Developmental Biology".

This thesis is entirely original and it was carried out in the laboratory of Dr. M. W. Cohen. The native α-bungarotoxin was prepared by Dr. M. J. Anderson, and different batches of the fluorescent toxin were prepared by Dr. M. J. Anderson and Mr. P. K. Ko.

ACKNOWLEDGEMENTS

I would like to express my appreciation to Dr. M. W. Cohen for his continuous guidance and criticism throughout the period I was under his supervision.

I am thankful to Penny Moody-Corbett for her suggestions and discussions during the preparation of the manuscript, and for her friendship.

My appreciation also goes to Claude Cantin for his expert technical assistance, especially in preparing the photographic material.

I am grateful to Dr. B. M. Kopriwa for her advice and suggestions for radioautography, to Dr. P. A. Lavoie for teaching me the method for iodination of α-bungarotoxin, to Diane Preddie for helping me with the French abstract, and to Robert Thomson for making some of the graphs and preparing the negatives of the photographic plates.

To Sandra James my thanks for typing the thesis.

To Eric Norman, Mary Frances MacIntyre, Mariza Mendes and Drs. D. Runnalls and W. Laskey, my deepest appreciation for their unfailing friendship and encouragement during the several years taken to finalize these studies.

This research was supported by grants to Dr. M. W. Cohen from the Medical Research Council of Canada. Personal support was provided by the MRC of Canada and by an internal award from the Faculty of Medicine of McGill University.

TABLE OF CONTENTS

Chapter I

GENERAL INTRODUCTION

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General Aspects of the Neuromuscular Junction

Reviews by Couteaux (1960, 1972, 1973) and Zacks (1974) report that the first morphological studies on the neuromuscular junction (NMJ) date from the 1830's and were done by Valentin and Emmert. Investigators at that time believed that the nerve fibre terminates in an arc-like ending in the striated muscle and returns to the central nervous system. The idea of a complete termination of the nerve fibre on the surface of the muscle fibre was first proposed by Doyère in 1840 who investigated an invertebrate (Milnesium tardigradum) NMJ. A few years later Wagner also recognized terminal branching of the axon in frog muscle. In the 1860's, NMJs in different vertebrates were described, separately, by Kühne and by Rouget as the region where the nerve fibre penetrates the sarcolemma and branches, forming a complex terminal arborization. Many controversial descriptions followed until the 1940's when Couteaux (1947) made a major contribution to the understanding of the morphology of NMJ. He was the first to recognize the presence of the subneural apparatus as a modification of the innervated muscle fibre (also see Couteaux, 1955, 1963).

Development of the electron microscope and improved histochemical methods have shown that the adult NMJ involves three different cell types: neurones, muscle cells and Schwann cells. The motor nerve terminal arborization (presynaptic structure) is devoid of a myelin sheath and has the non-synaptic side enveloped by a thin process of the Schwann cell. The terminal has neurofilaments, mitochondria and clear synaptic vesicles about 50 nm in diameter, which tend to be clustered against the presynaptic membrane. The axolemma is separated from the sarcolemma by the

primary synaptic cleft of about 30-50 nm which is filled by basal lamina. The sarcolemma (postsynaptic structure) is thickened at the endplate region and is thrown into junctional folds, the secondary clefts, which are about 1 µm deep and occur at regular intervals of about 1 µm (Palade and Palay, 1954; Reger, 1954, 1955, 1958; Robertson, 1954, 1956; Andersson-Cedergren, 1959; Birks et al., 1960a; Zacks and Blumberg, 1961; Ogata et al., 1967; Teräväinen, 1968; Murata and Ogata, 1969; Padykula and Gauthier, 1970; Zacks and Saito, 1970; also see Couteaux, 1972; Zacks, 1974).

Claude Bernard (1856) was the first to demonstrate that the NMJ is a distinct functional entity by using curare which blocked transmission from the nerve to the muscle. Later, around the turn of the century, Heidenhain, Langley, and Edmunds & Roth independently found that nicotine excited the contraction of skeletal muscle and that this effect was prevented by treatment with curare. They concluded that the site of action of nicotine was the muscle and the concept of receptors at the NMJ arose (see Hubbard, 1972; Zacks, 1974). Dale et al. (1936) demonstrated release of acetylcholine (ACh) from the motor nerve ending in response to nerve stimulation. Subsequent electrophysiological recordings of synaptic potentials at the neuromuscular junction proved that ACh was indeed the transmitter and have provided many additional insights regarding the mechanism of ACh release and action (see Kuffler and Nicholls, 1976).

Marnay and Nachmansohn (1938) used biochemical methods and were the first ones to demonstrate a higher concentration of acetylcholinesterase at the region of motor innervation of the frog sartorius. These results were confirmed by Feng and Ting (1938) on toad muscle. The localization of this enzyme at the endplate region was first shown histochemically by

Gomori in 1948. One year later a more specific histochemical reaction was developed by Koëlle and Friedenwald confirming these observations (see Zacks, 1974). Modifications in these histochemical methods allowed other investigators to localize acetylcholinesterase, as well as non-specific cholinesterase, on both the pre- and postsynaptic membranes and in the cleft between them (Birks and Brown, 1960; Couteaux, 1963; Karnovsky, 1964; also see Couteaux, 1972) at the electron microscope level.

From these pioneering studies and with the development of more sophisticated and elaborate technology the basic physiology and morphology of the NMJ has been well established. Briefly, with the arrival of an action potential along the nerve terminal, ACh is released from the pre-synaptic site. It diffuses across the narrow synaptic cleft and binds to acetylcholine receptors, whose binding sites are located on the outer surface of the subsynaptic muscle membrane. This causes a change in the membrane permeability to cations, mainly Na^+ and K^+, which flow down their electrochemical gradient resulting in an excitatory synaptic potential which normally triggers a muscle action potential and contraction. ACh is rapidly hydrolyzed by acetylcholinesterase molecules, which are associated with the basal lamina in the synaptic cleft (Hall and Kelly, 1971; Betz and Sakmann, 1973; McMahan et al., 1978; Sanes and Hall, 1979).

Localization of Acetylcholine Receptors in Skeletal Muscle

The concept of an acetylcholine receptor (AChR) was first introduced by Langley (1907) who applied Elliott's (1905) idea of receptive substance to skeletal muscle. He showed that this substance was specific for curare and nicotine and that it was located beneath the terminations of motor

nerves on skeletal muscle. Buchtal and Lindhard (1937) showed that when ACh was applied to NMJs muscle contractions occurred and these could be blocked by curare. Kuffler (1943) used single frog muscle fibres and demonstrated that application of ACh and nicotine produced depolarization restricted to the synaptic area which was blocked by curare. Nastuk (1951, 1953) confirmed this finding by applying ACh iontophoretically at the NMJ. Del Castillo and Katz (1955) used this technique on isolated frog muscle fibres and found that ACh was effective only when it is applied on the external surface of the muscle fibre and that intracellular application of ACh and other ACh-like substances was ineffective. They concluded, then, that specific AChRs are located on the outer surface of the endplate membrane, while the rest of the muscle membrane is insensitive and impermeable to ACh. Iontophoretic mapping has shown that the cholinergic sensitivity of adult, innervated skeletal muscle is very high at the postsynaptic surface, and it decreases rapidly in adjacent areas (Del Castillo and Katz, 1955; Miledi, 1960a; Peper and McMahan, 1972; Dreyer and Peper, 1974a; Kuffler and Yoshikami, 1975).

An initial attempt for visual proof of the localization of AChRs on the postsynaptic membrane was done by Waser and his colleagues (see Waser, 1967). They used ^{14}C- labelled curarine and toxiferine to demonstrate by radioautographic methods the presence of receptors for these drugs at the NMJs in mouse diaphragm. Unfortunately these radioautographs did not have enough resolution to demonstrate the precise location of curare binding sites.

With the isolation of α-bungarotoxin (α-BuTX) from the venom of the snake Bungarus multicinctus which binds irreversibly and with high specificity to AChRs (Chang and Lee, 1963; Lee and Chang, 1966; Lee and Tseng,

1966) and its conjugation to radioactive isotopes (^{3}H-, ^{125}I-), quantitative and ultrastructural localization studies became possible. It was found that in adult vertebrate skeletal muscle AChRs are found almost exclusively on the postsynaptic membrane, with a density in the order of $10,000/\mu m^2$, while the density is almost one thousand-fold lower away from the NMJ (Barnard et al., 1971; Fambrough and Hartzell, 1972; Hartzell and Fambrough, 1972; Porter et al., 1973; Salpeter and Eldefrawi, 1973; Albuquerque et al., 1974; Burden, 1977a; Orkand et al., 1978). The first EM radioautographic studies suggested that AChRs are located over the entire area of synaptic folds (Porter et al., 1973). Higher resolution EM radioautography showed that most of the label was found at the top one third of the junctional folds, that is, on the juxtaneuronal regions of the postsynaptic membrane, where specialized membrane densities were also seen (Fertuck and Salpeter, 1974; Albuquerque et al., 1974; Porter and Barnard, 1975a). Labelling of α-BuTX with other markers such as horseradish-peroxidase (Lentz et al., 1977), immunoperoxidase (Daniels and Vogel, 1975) or fluorescent dye (Anderson and Cohen, 1974) also demonstrated postsynaptic localization of this toxin. In addition, the use of the freeze-fracture technique has suggested that junctional AChRs may be related to postsynaptic membrane particle aggregates (Heuser et al., 1974; Peper et al., 1974; Rash and Ellisman, 1974; Ellisman et al., 1976; Rash et al., 1978). All these studies demonstrate that in normal adult vertebrate skeletal muscle the junctional AChRs are packed in clusters at the top of junctional folds, facing the motor nerve terminals.

Under special conditions, for example denervation, ACh sensitivity develops over the entire length of the muscle fibres and the receptors have a widespread distribution (Ginetzinsky and Shamarina, 1942; Kuffler,

1943; Axelsson and Thesleff, 1959; Miledi, 1960a, 1962; Katz and Miledi, 1964; Albuquerque and McIsaac, 1970; Miledi and Potter, 1971; Berg et al., 1972; Hartzell and Fambrough, 1972; Dreyer and Peper, 1974b; Porter and Barnard, 1975b; Almon and Appel, 1976a; also see reviews by Guth, 1968; Harris, 1974; Gutmann, 1976; Fambrough, 1979). The presence of extrajunctional receptors has also been shown in skeletal muscle during development in vivo (Diamond and Miledi, 1962; Berg et al., 1972; Burden, 1977a; Bevan and Steinbach, 1977) and in culture (such as Robbins and Yonezawa, 1971; Fambrough and Rash, 1971, 1973; Vogel et al., 1972; Hartzell and Fambrough, 1973; Fischbach and Cohen, 1973; Sytkowski et al., 1973; Anderson et al., 1977).

Both junctional and extrajunctional receptors are large hydrophobic glycoproteins, with an estimated molecular weight between 250,000 - 400,000 daltons and they are integral membrane proteins (Raftery et al., 1976; Dolly and Barnard, 1977; Fambrough et al., 1977; Froehner et al., 1977; Devreotes et al., 1977; also see Rang, 1975; Fambrough, 1979). They both bind to α-BuTX (Brockes and Hall, 1975a) and concanavalin A (Brockes and Hall, 1975b; Brockes et al., 1975; Almon and Appel, 1976b), have the same precipitation curves by rabbit antireceptor serum, the same apparent sedimentation constant and the same position when eluted from agarose columns (Brockes and Hall, 1975b; Brockes et al., 1975). The affinity of the isolated receptors for d-tubocurarine may differ. Brockes and Hall (1975b) found differences between the two types of receptors to this drug whereas other investigators (Alper et al., 1974; Colquhoun and Rang, 1976) did not. However, studies on whole muscle showed a higher affinity of d-tubocurarine for junctional than for extrajunctional receptors (Beránek and Vyskočil, 1967; Chiu et al., 1974; Dolly et al., 1977), similar to

Brockes and Hall's biochemical results (1975b; also see Brockes et al., 1975). Brockes and Hall (1975b) and Brockes et al. (1975) found that junctional AChRs in embryonic and denervated adult rat diaphragms have an isoelectrical point at pH 5.09 - 5.17 whereas the extrajunctional receptors have the point at pH 5.27 - 5.36. The junctional receptors are more stable and remain aggregated at the NMJs for weeks after denervation in frog (Birks et al., 1960b), rat (Hartzell and Fambrough, 1972; Frank et al., 1975) and mouse (Porter and Barnard, 1975b; Ko et al., 1977). Extrajunctional receptors of denervated rat diaphragms have a short life-span (Berg and Hall, 1974; Chang and Huang, 1975; also see below).

Binding of α-BuTX to ACh Receptors

Chang and Lee (1963) used zone electrophoresis on starch to separate the venom of a banded Asian krait, Bungarus multicinctus, into four different fractions: three neurotoxins, namely α-, β- and γ-bungarotoxins, and one cholinesterase rich fraction. More than 80% of the crude venom toxicity was accounted for in these toxins, which exert a neuromuscular blocking action. It was found that α-BuTX (fraction II) is the largest protein fraction of the venom, with about half of the crude venom's toxicity in mice. It inhibited the ACh response of frog rectus abdominis and chick biventer cervicis and rat diaphragm muscles without affecting ACh release from the nerve. It was also found that this blockade of neuromuscular transmission is irreversible, and that α-BuTX binds to AChRs on the postsynaptic membrane of the motor endplate. This postsynaptic action is similar to the one caused by curare although the block by curare is reversible. Additional evidence for a postsynaptic action was given by Lee and Chang (1966) who showed that the toxin acts without affecting the

muscle resting membrane potential or the action potential of the nerve terminal. Protection of the AChRs against α-BuTX by d-tubocurarine has been demonstrated (Lee and Chang, 1966; Miledi and Potter, 1971; Berg et al., 1972; Chang et al., 1973) and suggests that both α-BuTX and d-tubocurarine bind to the same sites on the postsynaptic membrane. Treatment of muscle with d-tubocurarine, ACh, nicotine and carbachol blocks the binding of this neurotoxin, emphasizing the AChRs as the action site of α-BuTX. If the muscle is first treated with α-BuTX and then with these agents, the toxin blocking effect is not reversed, confirming the irreversibility of this reaction.

Mebs et al. (1971) and Lee (1972) studied the primary structure of α-BuTX and found that it consists of 74 residues of 18 amino acids in a single chain cross-linked by five disulfide bridges, forming a small basic polypeptide with molecular weight of about 8,000. This composition is very similar to that of other neurotoxins from different snakes (cobras and sea snakes) and the mode of neuromuscular blocking action is also quite similar for all. A particular group of amino acids common to all these toxins seem to be the "active site". More hydrophobic amino acids are found in α-BuTX and these appear to be responsible for the irreversibility of such binding.

Radioautographic studies have shown that α-BuTX, as well as cobra neurotoxin, binds at the NMJ of skeletal muscle (e.g. Lee and Tseng, 1966; Barnard et al., 1971; Salpeter and Eldefrawi, 1973; Albuquerque et al., 1974; Fertuck and Salpeter, 1974; Orkand et al., 1978; also see Fambrough, 1979), and it also binds to the cholinergic receptors in the electric organs of Electrophorus electricus and Torpedo (e.g. Changeux et al., 1971; Miledi et al., 1971).

Turnover of ACh Receptors

Fambrough and collaborators have studied extensively the problem of turnover of AChRs in cultured chick and rat embryonic myotubes using mainly radioactive α-BuTX. Briefly, this group (Hartzell and Fambrough, 1973; Devreotes and Fambrough, 1975, 1976; Devreotes et al., 1977) and Patrick et al. (1977) found that AChRs in developing myotubes are synthetized about 3 hours before their appearance in the sarcolemma. During this period the newly synthetized receptors appear in the Golgi apparatus and seem to reach the cell membrane by a pathway similar to the one taken by secretory and membrane glycoproteins (Fambrough and Devreotes, 1978). Saponin treatment of chick myotubes (Fambrough and Devreotes, 1978) revealed intracellular AChRs which bound to ^{125}I-αBuTX added into the culture. About 50% of the incorporation of radioactive toxin at the Golgi apparatus decreased after blockade of protein synthesis by puromycin (Fambrough and Devreotes, 1978), while the surface radioactive incorporation continued for several hours after puromycin treatment (Hartzell and Fambrough, 1973; Devreotes and Fambrough, 1975, 1976; Patrick et al., 1977). This intracellular transport process and incorporation into the muscle cell membrane is independent of protein synthesis, but can be blocked by low temperatures and by inhibitors of energy metabolism (Hartzell and Fambrough, 1973; Devreotes and Fambrough, 1976; Devreotes et al., 1977; Fambrough et al., 1977; Fambrough, 1979).

The kinetics of AChR production and incorporation were first studied in embryonic chick and rat myotubes cultured by Hartzell and Fambrough (1973) using iontophoretic application of ACh and quantitative

radioautography after binding the receptors with ^{125}I-αBuTX. They found that the toxin bound to the myotubes with two different rates. A fast component was saturated after 20-min exposure to unlabelled toxin and the slow component increased gradually after similar block. The rate of increase in the number of slow binding sites was limited by the rate of appearance of new receptors into the membrane and it was found to be 90 receptors/μm^2-hr. But the receptor density increased at a rate of 35 receptors/μm^2-hr as the result of simultaneous increase in cell surface. Based on these data they suggested the presence of an intracellular pool of AChRs or a slow activation of non-functional receptors in the membrane for the slow component. The former hypothesis proved to be more accurate and a number of about 10% of the total surface binding sites was found to be contained within this pool of "precursors" (Devreotes and Fambrough, 1975). In addition to the surface receptors and the precursors of the surface receptors a third pool was characterized by these authors as "hidden" receptor pool which contains about 30% of the total surface receptors. These "hidden" receptors only bound to α-BuTX after overnight exposure to the toxin and their relationship to surface and precursor receptors is still unclear.

Degradation of AChRs can be determined indirectly by measuring the release of radioactivity into the culture medium after binding of ^{125}I-αBuTX to the receptors and the radioactivity remaining in the myotubes. Devreotes and Fambrough (1975, 1976) found that the degradation in chick and rat myotubes is a random process with a first order kinetics, and dependent on energy (blocked by ATP-synthesis inhibitors such as 2,4-dinitrophenol, cyanide, azide), temperature (Q_{10} value of about 8), pH (decreases steeply when pH<7 and slowly when pH>7), cell integrity (cell

disruption diminishes degradation), protein synthesis (slowly retarded by puromycin and cycloheximide), and the protein content in the culture medium. It is a proteolytic process. The "half-life" of the radioactive toxin-receptor complex was found to be about 22 hr and the main radioactive component released into the medium was mono-iodotyrosine, a metabolite of αBuTX. Puromycin-treated cultures allowed these authors to demonstrate that the appearance of iodotyrosine in the medium was due to the removal of αBuTX binding site from the membrane, as no new receptors were added after 3 hr of treatment, and that these two processes (incorporation of receptors into the membrane and degradation) are not coupled. These authors suggested internalization of the toxin-receptor complex for degradation by secondary lysosomes (Devreotes and Fambrough, 1976; Fambrough et al., 1977; also see Fambrough, 1979). Merlie et al. (1976) pulse labelled primary foetal calf skeletal muscle with ^{35}S-methionine to label newly-synthetized receptors, and measured the radioactivity remaining in the cells after different time intervals. They obtained a half-life of about 17 hr for the AChRs and this value is close to the one found by Devreotes and Fambrough (see above).

Similarly, turnover of extrajunctional receptors in adult denervated muscles was studied in rat (Chang and Huang, 1975; Brockes and Hall, 1975c; Berg and Hall, 1974, 1975a; Linden and Fambrough, 1979) and in mouse (Grampp et al., 1972). Brockes and Hall (1975c) incubated innervated and denervated rat diaphragm in culture with medium containing ^{35}S-methionine and radioactive receptors were isolated only from denervated preparations, thus indicating that muscle denervation caused synthesis of new receptors. Denervation-induced increase in new protein

synthesis was demonstrated in rat diaphragm muscle (Fambrough, 1970) and in mouse extensor digitorum longus (Grampp et al., 1972). They used different inhibitors of protein synthesis and found that new AChR synthesis after denervation was a result of new synthesis of RNA because actinomycin D reduced the incorporation of uridine into the muscle and delayed the onset of increase in extrajunctional ACh sensitivity. This effect of actinomycin D was only noticeable if the inhibitor was given within the first two days after denervation (Grampp, 1972). Chang and Huang (1975) used ^{3}H-αBuTX to label AChRs in innervated and denervated rat diaphragm and found that the radioactivity of junctional regions remained practically constant for a much longer period (half-life of 9.5 days) than that in the extrajunctional regions (half-life of 19 hr). Actinomycin D was found to depress the generation of AChRs in the denervated diaphragm but not the binding of toxin to existing junctional receptors in the innervated muscle, indicating again synthesis of new receptors as a result of denervation. Linden and Fambrough (1979) denervated extensor digitorum longus and soleus muscles of adult rats, removed the muscles several days after the operation and kept them in culture. They added dense amino acids (^{2}H-, ^{13}C- and ^{15}N-labelled) into the medium and found an incorporation rate of AChRs into the sarcolemma of about 1.4% per hour. The degradation rates for extrajunctional and junctional receptors were found to be about 22 hr and 13 days, respectively. They also found that electrical stimulation decreased the synthesis rate but not the degradation rate. The breakdown of toxin-receptor complex was largely inhibited by 2,4-dinitrophenol and low temperature. Berg and Hall (1975a) also found a big difference between the half-lives of junctional and extrajunctional receptors in innervated and denervated rat diaphragm muscle. They found that the half-life of

junctional receptors in adult normal and denervated muscles was about 6 days, whereas 65% of the extrajunctional receptors in denervated muscle was lost in 24 hr and the half-life of extrajunctional receptors in denervated muscle kept in culture was 8-11 hr.

Differences in half-lives were also found in neonatal rat diaphragms by Berg and Hall (1975a) who injected intrathoracically ^{125}I-αBuTX in the animals on the day of birth and transferred the diaphragms to cultures 2-3 hr later. They found that about 78% of the initial extrajunctional radioactivity was lost in 24 hr as compared to the 49% lost from the junctional regions. Burden (1977a,b) studied the turnover of AChRs labelled with ^{125}I-αBuTX in developing posterior latissimus dorsi of the chick from embryonic day 10 to 18 weeks after hatching. He found that up to one week posthatch the degradation rates of junctional and extrajunctional receptors were identical, a half-life of 30 hr. Two weeks later the junctional receptor degradation rate decreased considerably to a half-life of more than 5 days, while the extrajunctional receptors maintained the original rate. He also showed that muscle activity was an effective regulator of extrajunctional receptor synthesis only after a critical stage. Thus, paralysis by curare of the chick embryo between days 12 and 14 of incubation did not affect the number of extrajunctional receptors whereas paralysis between days 16 and 18 prevented the normal decrease in the number of extrajunctional AChRs.

The involvement of muscle activity in the control of synthesis of extrajunctional receptors after denervation of adult muscle has been demonstrated by different investigators. Direct electrical stimulation of denervated muscle delayed the onset and decreased extrajunctional hypersensitivity (Lömo and Rosenthal, 1972; Drachman and Witzke, 1972; Purves

and Sakmann, 1974; Jones and Vrbová, 1974; Lömo and Westgaard, 1975; Frank et al., 1975; Linden and Fambrough, 1979; Jones, 1979). Inhibition of muscle contraction by blockade of neuromuscular transmission by botulinum toxin (Thesleff, 1960; Tonge, 1974; Bray and Harris, 1975), d-tubocurarine (Berg and Hall, 1975b) and α-BuTX (Chang et al., 1975), and by blockade of nerve activity by application of anaesthetics to nerves (Lömo and Rosenthal, 1972; Pestronk et al., 1976; Cangiano and Fried, 1977; Lavoie et al., 1976, 1977; Gilliatt et al., 1977, 1978) led to high increase in extrajunctional ACh sensitivity. Reduction of muscle activity by spinal cord transection (Johns and Thesleff, 1961) or restraining the limb (Fischbach and Robbins, 1971) caused a moderate increase in extrajunctional ACh sensitivity. Reiness and Hall (1977) pulse-labelled with ^{35}S-methionine denervated rat hemidiaphragms kept in organ culture, and compared the radioactivity incorporated into AChRs of electrically stimulated and unstimulated preparations. They found that stimulation inhibited binding of ^{35}S-methionine into the receptors, and that this inhibition was specific on AChR synthesis because general protein synthesis was not depressed. Linden and Fambrough (1979) also found inhibitory effects of electrical stimulation on the synthesis of AChRs in denervated rat soleus and extensor digitorum longus muscles.

It is known that the number of extrajunctional AChRs which appear during inactivity of innervated muscles is smaller than that caused by denervation (e.g. Pestronk et al., 1976; Lavoie et al., 1976, 1977; Simpson, 1977; Gilliatt et al., 1977, 1978), and that blockade of axonal transport by local application of lidocaine, colchicine and vinblastine induces the development of extrajunctional ACh sensitivity in muscle without producing

muscle inactivity (Albuquerque et al., 1972, 1974; Hofmann and Thesleff, 1972; Anderson and Edström, 1973; Fernandez and Ramirez, 1974). Length of nerve stump has also been shown to affect the supersensitivity developed on the denervated muscle fibre. Thus, when nerve section is done close to the innervation site ACh hypersensitivity develops earlier than the case where the section is more distant (Luco and Eyzaguirre, 1955; Emmelin and Malm, 1965; see Gutmann, 1976; Fambrough et al., 1977; Fambrough, 1979). Decline in extrajunctional hypersensitivity was found prior to onset of neuromuscular transmission during reinnervation of muscle (Miledi, 1960 b; Letinsky, 1975) and to recovery of neruomuscular transmission after blockade by botulinum toxin (Tonge, 1974; Bray and Harris, 1975). Together these studies infer the presence of some "neurotrophic" factors which may play a role on the synthesis of AChRs in the muscle (see Guth, 1968; Hofmann and Thesleff, 1972; Gutmann, 1976, 1977; Fambrough et al., 1977; Fambrough, 1979).

Synaptogenesis in vivo

Morphological studies on the development of the NMJ began last century and until the 1950's histochemical staining methods and the optical microscope were the main tools used to describe the formation of the NMJ. Couteaux (1960, 1973) summarized the then available information and described two main phases during the development of the motor endplate. The first phase involves myotubes and exploring nerve fibres which do not develop lasting contacts with the myotubes, although they may come into close apposition. During the second phase the final nerve-muscle junction is established and the axial nuclei of the developing muscle cells complete their migration to the periphery. The nerve sprouts accompanied by Schwann

cells make contact with the surface of the muscle fibre, close to the peripheral nuclei. Couteaux believed that this new nerve terminal exerts "trophic" action on the muscle, causing the nuclei to divide amitotically and the accumulation of sarcoplasm at the junction site which he called the "primitive eminence".

Ultrastructural studies followed afterwards in various species, including chick (Hirano, 1967; Atsumi, 1971; Sisto Daneo and Filogamo, 1973, 1975; Bennett and Pettigrew, 1974), rat (Kelly, 1966; Teräväinen, 1968; Kelly and Zacks, 1968, 1969; Bennett and Pettigrew, 1974), mouse (de Vos, 1970), human foetus (Fidzianska, 1971; Juntunen and Teräväinen, 1972), amphibian (Kullberg et al., 1977), and in regenerating newt limb (Lentz, 1969). Basically, the formation is similar in all species. Innervation begins with the apposition of the growing motor nerve fibres and the developing muscle membrane. The nerve terminal has very few synaptic vesicles and the sarcolemma is undifferentiated at this stage. Then, the region of the sarcolemma opposite to the nerve terminal thickens and some fuzzy material appears in the cleft. The nerve terminal may be partly enveloped by a Schwann cell and more vesicles accumulate on the inner surface of the presynaptic membrane. These changes occur very rapidly and the postjunctional folds develop subsequently at a slower pace.

Acetylcholinesterase activity detected histochemically was shown to have an initial diffuse distribution in the chick sarcoplasm and after innervation it appears at the NMJ (Mummenthaler and Engle, 1961). Hirano (1967) and Atsumi (1971) also found localized cholinesterase at the chick NMJ after the arrival of the nerve terminals. A similar sequence of events was also found in developing rat skeletal muscle (Kelly, 1966; Teräväinen, 1968; Kelly and Zacks, 1968).

The appearance of ACh sensitivity and the distribution of AChRs during normal development have been studied in rat (Diamond and Miledi, 1962; Berg et al., 1972; Bevan and Steinbach, 1977), chick (Giacobini et al., 1973; Burden, 1977a; Jacob and Lentz, 1979) and frog (Blackshaw and Warner, 1976; Kullberg et al., 1977). The results show a widespread distribution of sensitivity and receptors during early stages of development, before the onset of innervation (Giacobini et al., 1973; Bevan and Steinbach, 1977). Bevan and Steinbach (1977) were able to conclude from their study that high receptor density at the newly-forming NMJ develops after the arrival of nerve fibres to foetal rat sternomastoid muscle. The density of extrajunctional receptors declines gradually and in chick (Burden, 1977a) and rat (Bevan and Steinbach, 1977) muscle reaching undetectable levels about 2 weeks after the onset of synapse formation.

Synaptogenesis in Culture

More insight into the processes involved in synaptogenesis has been obtained in culture where the experimental conditions are simpler and more readily managed (see review by Nelson, 1975). Fambrough and Rash (1971) have shown that mononucleated rat limb bud myoblasts are very rarely sensitive to ACh, whereas all multinucleated myotubes responded to the drug. Other studies on cultures of muscle cells alone from rat and chick embryos have shown that these cells develop uniform ACh sensitivity along the cell in the absence of nerve (Robbins and Yonezawa, 1971; Fischbach and Cohen, 1973). Later in development both chick (Vogel, et al., 1972; Fischbach and Cohen, 1973; Sytkowski et al., 1973) and rat myotubes (Hartzell and Fambrough, 1973) develop sites of higher receptor density in addition to the widespread distribution. This was found by radioautography after

labelling the receptors with ^{125}I-αBuTX (Vogel et al., 1972; Fischbach and Cohen, 1973; Sytkowski et al., 1973; Hartzell and Fambrough, 1973; Frank and Fischbach, 1979), by iontophoretic mapping of ACh sensitivity (Fischbach and Cohen, 1973; Hartzell and Fambrough, 1973; Frank and Fischbach, 1979), or by examination of membrane particles after freeze-fracture (Peng and Nakajima, 1978; Cohen and Pumplin, 1979). Patches of high receptor density have also been shown by binding of fluorescent α-BuTX (Axelrod et al., 1976; Anderson and Cohen, 1977; Anderson et al., 1977; Axelrod et al., 1978; Bloch, 1979; Cohen and Pumplin, 1979). These pure myogenic cell cultures show that muscle cells are capable of synthetizing and packing AChRs into the sarcolemma in the absence of nerve.

When studies were done in co-cultures of muscle and nerve cells, high ACh sensitivity was found at the nerve-muscle contact sites (Kano and Shimada, 1971; Fischbach and Cohen, 1973; Frank and Fischbach, 1979). An increase in ^{125}I-αBuTX binding has also been detected at these sites (Fambrough et al., 1974; Cohen and Fischbach, 1977; Frank and Fischbach, 1979).

It has been recently shown that the ingrowing neurites do not search for already existing receptor patches, instead they induce formation of new receptor patches at the contact site. Anderson and Cohen (1977) pre-labelled AChRs in cultured Xenopus myotomal cells with fluorescent dye conjugated αBuTX and added neural tube cells into the culture. They observed the disruption of pre-existing receptor patches and appearance of new patches at the nerve-muscle contact sites. This effect occurs even if the synaptic activity is blocked with native toxin or curare (Anderson et al., 1977). This nerve-induced receptor localization has been found to be specific for neurites from developing spinal

cord. Sympathetic ganglia and dorsal root ganglia neurites do not induce the development of high receptor density at sites of contact (Cohen et al., 1979). Frank and Fischbach (1979) have also studied this problem in chick myotubes cultured in the presence of spinal cord cells by using iontophoretic mapping combined with distribution of ^{125}I-αBuTX binding sites. They concluded that ingrowing axons do not seek out pre-existing receptor clusters but rather induce the formation of new clusters at the innervation sites within as little as 3 hours of nerve-muscle contact.

Synaptogenesis during Re-innervation of Adult Muscle

Another experimental situation which allows the study of synaptic formation is during re-innervation of adult denervated muscle. The post-synaptic membrane is known to survive for weeks while the axonal termination degenerates within a few days (Birks et al., 1960b). Subsequently, the nerve fibres regenerate and reinnervate the muscle. Re-innervation occurred mainly at the original synaptic sites (Miledi, 1960 b; Bennett et al., 1973; also Jansen and van Essen, 1975; Letinsky et al., 1976).

Letinsky et al., (1976) examined at light and electron microscopy levels the re-innervation of NMJ in the cutaneous pectoris muscle of the frog after crushing the motor nerve for as long as three months. They found that more than 95% of the original synaptic sites were covered by new nerve terminals and little, if any, synapses were made on other portions of the muscle fibre. This precision of re-innervation at the original synaptic site was shown by Marshall et al. (1977) to be independent of the integrity of the muscle cell. These authors denervated and damaged frog cutaneous pectoris muscle and followed the regeneration process with light and electron microscopy. Both damaged nerve and muscle

fibres degenerated and were phagocytized leaving intact the basement membrane which acted as scaffold for regenerating muscle cells. Within two weeks myofibres regenerated and nerve fibres grew back to their surface and functional NMJ were established almost exclusively at original sites. Sanes et al., (1978) extended the study and found that even if the muscle regeneration was prevented the axons still grew back to the original synaptic sites. These original synaptic sites were also shown to be accumulation sites of AChRs after regeneration of damaged muscle cell which were kept denervated throughout the experiment (Burden et al., 1979). Together these results showed that surviving elements in the basal lamina at the original synaptic sites could direct the re-innervation and differentiation of the axonal terminal and the localization of AChRs at these sites even in the absence of one of the synaptic structures. Burden et al. (1979) included the possibility of a Schwann cell influence in the accumulation of AChRs because these cells occupied the previous site of the nerve terminal for a period of time and they are known to release ACh even after denervation (Birks et al., 1960 b).

It is known that ectopic innervation of a muscle fibre by a foreign nerve is only possible after denervation of the host muscle (see Fex and Thesleff, 1967; Bennett and Pettigrew, 1976). Lömo and Slater (1976 a, b; 1978; also Slater, 1978) used this experimental condition to study the subsequent synaptogenesis and ACh sensitivity changes, and their control by muscle activity. They found that fibres which became innervated ectopically lost their extrajunctional ACh sensitivity within two weeks after section of the original motor nerve while the new NMJ formation occurred within the first 2-3 days after denervation. If direct stimulation was started at the time of denervation, no ectopic re-innervation occurred,

whereas if stimulation was started 2-4 days after denervation about one third of the muscle fibres became innervated. Similarly, muscle stimulation started one day after denervation decreased the extrajunctional ACh sensitivity while the stimulation started 2-3 days after denervation did not prevent the increase in extrajunctional hypersensitivity. These authors concluded, then, that muscle activity plays an important role in the maintenance of muscle properties with respect to ACh sensitivity and ability to accept new innervation by controlling the appearance of new receptors in the membrane. They also suggested that the first fibres to receive ectopic innervation were those which first became fully sensitive to ACh. However, they also raised the unlikelihood of the receptor sites themselves playing a direct role in the earliest stages of re-innervation because pharmacological block of the sites does not prevent further innervation (Cohen, 1972; Jansen and van Essen, 1975).

Objective of the Present Study

Although more investigations have since been published (e.g. Blackshaw and Warner, 1976; Burden, 1977a, b; Bevan and Steinbach, 1977; Kullberg *et al.*, 1977; Jacob and Lentz, 1979), at the time the present study was initiated little was known about the distribution of AChRs during synaptogenesis *in vivo*. Electrophysiological (Diamond and Miledi, 1962) and radioactive α-BuTX uptake (Berg *et al.*, 1972) studies had been done in foetal and neonatal rat diaphragms soon after the onset of innervation. Giacobini *et al.* (1973) measured the uptake of ^{3}H-α toxin by embryonic chick wing muscle which was also innervated at the earliest stages studied. Kullberg, from this same laboratory, had found ACh sensitivity in some embryonic myotomal muscle cells of *Xenopus laevis* when

the animals were less than 21 hr old. He detected synaptic activity electrophysiologically about 1-2 hr later, when a few nerve processes were first seen contacting the developing muscle cells (see Kullberg et al., 1977). At about the same time, Anderson, another member of this group, developed a technique which allows the visualization of patches of high AChR density in cell culture and unsectioned preparations by using α-BuTX conjugated with a fluorescent dye (see Anderson and Cohen, 1974). Subsequently, Anderson, Cohen and Zorychta described the sequence of synaptogenesis in cultures of embryonic myotomal muscle and neural tube cells and determined the neural influence on this process (see Anderson and Cohen, 1977; Anderson et al., 1977). Thus, it seemed important to investigate the distribution of AChRs during the course of normal development and to determine whether in vivo as in culture receptor localization at synaptic sites is nerve-induced.

Experimental Approach

Myotomes from the South African frog Xenopus laevis (Fig. 1) offer several advantages for developmental studies: 1) The development of this animal has been extensively studied and a Normal Table (Nieuwkoop and Faber, 1967) can be used for reference to identify precisely the developing stages. 2) Embryos and tadpoles of these animals are readily available in large number throughout the year, as the parents can be kept in good health under normal laboratory conditions and they can be mated regularly by injection of chorionic gonadotropin. 3) The myotomes develop in a rostro-caudal direction and within each myotome the cells appear to develop synchronously (Nieuwkoop and Faber, 1967; Hamilton, 1969; Muntz, 1975). 4) The myotomal cells are innervated mainly at their ends, which are aligned

(Lewis and Hughes, 1960; Mackey et al., 1960; Kullberg et al., 1977), and this facilitates the observations and comparisons between different animals and stages. 5) The myotomes are the first muscle to differentiate and they are easily accessible throughout development.

The distribution of AChRs in Xenopus myotomal muscle cells during development was studied by using α-BuTX bound to ^{125}I- or fluorescent dye. The use of radioactive α-BuTX allows quantitative analysis of the binding sites, the AChRs. The high sensitivity of this method permits the detection of all the radioactive toxin binding sites within the myotomes. Thus, one can measure the total uptake of toxin per myotome by counting the radioactivity with a gamma-counter. Once the background and non-specific uptake are deducted from the total value, the specific uptake is obtained. Non-specific uptake is measured by pretreating the preparations with native toxin. Knowing the specific activity of the toxin, and taking into consideration the molecular weight of the toxin and Avogadro's number (1 mole of a chemical substance = 6×10^{23} molecules), the number of toxin molecules taken up by the myotomes can be calculated. In addition, the same material used for radioactivity measurement can be prepared for radioautography and, consequently, the localization of toxin molecules taken up by the cells can be determined. With adequate exposure time after covering the preparations with photographic emulsion the grain distribution gives that of the binding sites. By comparing the grain density of preparations from animals at different stages of development the relative changes of AChR density with age can be determined. With this method the extrajunctional AChR distribution was studied throughout the development of Xenopus myotomal muscle cells. Due to the much higher accumulation of receptors at the myotomal end regions, the main innervation site, it was

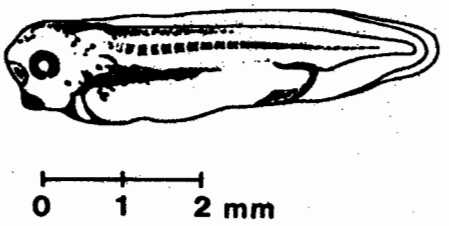
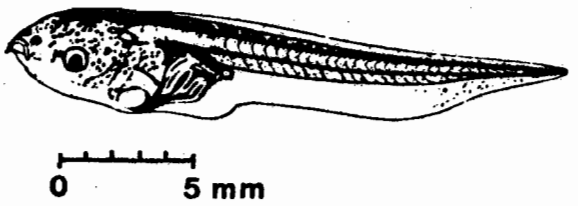
not possible to study simultaneously the grain density at the junctional and extrajunctional areas. This would require different radioautographic exposure times because the adequate time interval for extrajunctional density causes overexposure of junctional density. Another drawback of this procedure is the difficulty to quantify high density sites throughout the myotomes. Thus, for the study of developmental changes of junctional receptor accumulation fluorescent dye-conjugated α-BuTX was used.

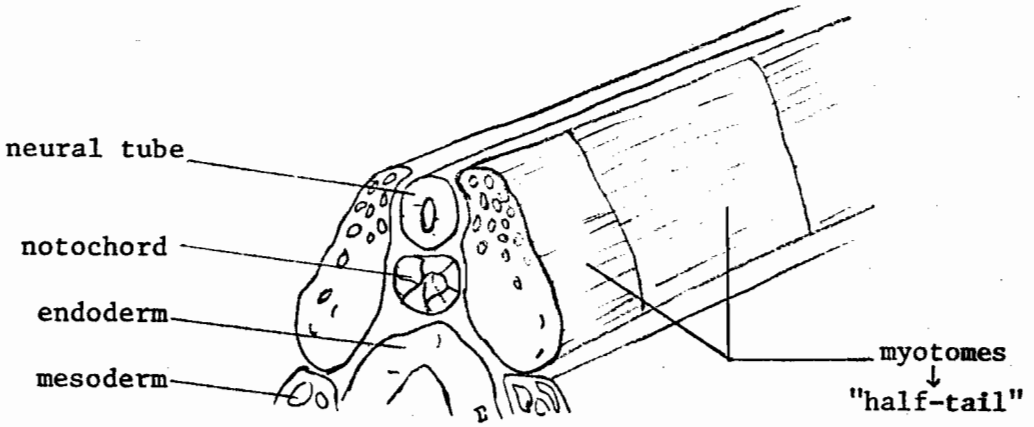
Anderson and Cohen (1974) developed this technique which permits sites of high receptor density to be viewed throughout whole mounts of myotomes. By applying this method the changes in the distribution of high receptor density patches during the course of development were easily observed. The main limitations of this method are its lower sensitivity and difficulty of relating the staining intensity to receptor density. On the other hand it offers high spatial resolution and proved to be advantageous for studying the localization of high receptor density patches.

The presence of nerve fibres was detected by a technique recently developed by Letinsky and DeCino (1978, personal communication). It employs nitroblue tetrazolium, which when reduced to its diformasan state colours the nerve fibres blue. The above authors demonstrated that even the very fine unmyelinated, developing and regenerating nerve fibres were stained by this method. Changes of pH of the staining solution, of concentrations and types of fixative used may result in failure of the staining. Some modifications to the original method were necessary in order to stain the developing nerve fibres in the myotomal preparations, and to avoid excessive staining of the muscle cells and connective tissue.

Immobilization experiments were carried out to examine the effects of muscle inactivity on the distribution of AChRs during development. This was achieved by keeping the embryos and tadpoles in an anaesthetic solution, tricaine methanesulphonate (MS-222, Sandoz). This drug is an efficient anaesthetic for fish and amphibians and it is widely used in the fishing industry for artificial fertilization and transportation purposes (Maeno, 1966; Ohr, 1976; Smit and Hattingh, 1979). The anaesthetic solution was neutralized because MS-222 in solution is very acid (pH<5) and it is known that anaesthetic effect in a neutralized solution is more long-lasting (Ohr, 1976).

Fig. 1 From Nieuwkoop and Faber (1967). Description of some stages studied and their respective characteristics. Dorsal view of stages 17, 19 and 21. Lateral view of all the other stages. Insert at bottom shows portion of a "tail preparation" from which "half-tail preparations" were isolated. See Methods in Chapter II for further details.

Stage	Age	Comments	
17	18 3/4hr	late neural fold stage; myotomes begin to form	
19	20 3/4hr	4-6 myotomes; neural folds touching	
21	22 1/2hr	8-9 myotomes; neural tube completely formed	
24	26 1/4hr	15 myotomes; initial motor responses	
26	29 1/2hr	17 myotomes; beginning of spontaneous movements	
35/36	2d 2hr	embryo hatches	
50	15d	both fore- and hindlimb buds distinct; hindlimb bud constricted at base	



Chapter II

DISTRIBUTION OF ACETYLCHOLINE RECEPTORS IN THE MYOTOMES OF *XENOPUS LAEVIS* DURING NORMAL DEVELOPMENT

ABSTRACT

The distribution of acetylcholine receptors in the myotomes of Xenopus laevis during the course of normal development was investigated by using α-bungarotoxin conjugated with fluorescent dye or ^{125}I. The distribution of nerve fibres was determined after staining with nitroblue tetrazolium. Nerve fibres were first seen, very rarely, at the ends of the myotomes at stage 19 (20.75 hr). With age, the number of myotomes with nerve fibres increased in a rostro-caudal direction. By stage 24 (26.25 hr) they were present in virtually all preparations examined. Occasionally they were also seen over more central regions of the myotomes. Acetylcholine receptors appeared as early as stage 19 and were distributed along the entire length of the myotomes. The density of extrajunctional receptors increased up to stage 36 (50 hr), the time of hatching, and declined gradually afterwards. Patches of high receptor density, revealed by fluorescent α-bungarotoxin, were first found at the presumptive sites of innervation at stage 21 (22.5 hr). They generally developed in a rostro-caudal direction and by stage 26 (29.5 hr) were present in virtually all preparations examined. Some receptor patches were also seen along the myotomes and they were aligned in patterns which resembled the course of nerve fibres. The results indicate that high receptor densities develop at presumptive synaptic sites about 1-5 hr after the arrival of the nerve fibres and are consistent with the notion that receptor localization at these sites is nerve-induced.

INTRODUCTION

In adult vertebrate skeletal muscle acetylcholine receptors (AChRs) are found almost exclusively in the postsynaptic membrane, with a density in the order of $10{,}000/\mu m^2$, while the density is almost a thousand-fold lower away from the neuromuscular junction area (Barnard et al., 1971; Fambrough and Hartzell, 1972; Hartzell and Fambrough, 1972; Porter et al., 1973; Salpeter and Eldefrawi, 1973; Albuquerque et al., 1974; Burden, 1977a; Orkand et al., 1978). How this distribution is achieved during normal development is still not fully understood.

Diamond and Miledi (1962) first studied this problem by using iontophoresis of acetylcholine (ACh) and intracellular recording in the diaphragm of foetal and newborn rats. They found that in foetuses as young as 17 days ACh sensitivity was present along the whole length of the muscle fibres; but even at this stage it was greatest at the site of innervation. Subsequently the widespread ACh sensitivity receded and the mature pattern was attained about 2 weeks after birth. These results were confirmed by Berg et al. (1972) who used ^{125}I-α-bungarotoxin (^{125}I-αBuTX) and measured radioactivity in neonatal rat diaphragm muscle fibres which were divided into regions with and without end-plate. More recently Bevan and Steinbach (1977) studied the distribution of radioactive α-BuTX binding sites on embryonic and neonatal rat sternomastoid and diaphragm muscles with radioautography. They found a uniform distribution of grains along the muscle fibres of 15-day old embryos and one day later most of the fibres had a single spot of higher grain density in the mid-region of the fibre. Cholinesterase (ChE) stain was also detected, indicating these were sites of developing neuromuscular junction. The extrajunctional grain density decreased with

development and was below detectable levels one week after birth. They suggested that AChR accumulation occurs after arrival of the growing nerve fibres.

The acquisition and distribution of AChRs has also been investigated in avian muscles. Giacobini et al. (1973) measured the uptake of ^{3}H-α-toxin from Naja in homogenates of chick hind limbs. Toxin binding sites were detected from embryonic day 4 on. They increased rapidly in number up to day 12, and then decreased. Radioautographic studies by Burden (1977a), using ^{125}I-αBuTX, have revealed that individual muscle fibres of the chick posterior latissimus dorsi muscle have a single site of high receptor density by embryonic day 10 and that these sites also contain ChE activity. The anterior latissimus dorsi muscles, whose fibres have several synaptic sites on them, also exhibited several receptor patches along each fibre at these early stages of development. The author concluded that the adult innervation pattern is established in these muscles during embryonic life, and indicated that it is likely that these muscles were already innervated at all stages studied. A decrease in the extrajunctional receptor density was also found throughout development. More recently, Jacob and Lentz (1979) used horseradish-peroxidase conjugated α-BuTX to locate sites of high AChR density on chick embryo anterior and posterior latissimus dorsi muscles at the ultrastructural level. They found the presence of AChRs in patches of plasmalemma unrelated to nerve fibres in myoblast-like cells and myotubes of 10-, 12- and 14-day-old embryos, and surface specializations were consistently seen at these sites. The authors suggested that these sites might be preferential sites of synapse formation although they did not rule out the possibility of these sites ap-

pearing after nerve contact. Indeed Hamburger and Balaban (1963) have detected movements in wings and legs of 6 1/2-day-old chick embryos. Sisto Daneo and Filogamo (1974, 1975) documented the first synaptic structures in these same muscles in embryos between days 9 and 11 of incubation.

Blackshaw and Warner (1976) have found that some myotomal muscle cells of embryos of the South African frog Xenopus laevis become sensitive to ACh when the embryo is less than 21 hr old. Synaptic activity was detected electrophysiologically about 1-2 hours later (Blackshaw and Warner, 1976; Kullberg et al., 1977). Nerve processes were seen leaving the spinal cord and contacting the developing muscle cells at these stages (Kullberg et al., 1977). The distribution of AChRs at these early stages of neuromuscular synaptogenesis in Xenopus myotomes is not known. Eventually the receptors become localized in high density at the sites of innervation (Anderson & Cohen, 1974).

In the present study we have investigated the distribution of AChRs in the myotomal cells of Xenopus laevis during the course of normal development using α-BuTX labelled with ^{125}I or fluorescent dye. We have also investigated the arrival of nerve fibres in the myotomes. The data indicate a very short delay between this latter event and the development of an induced high receptor density at presumptive synaptic sites. Brief accounts of some of this work have been given previously (Chow and Cohen, 1977, 1978).

MATERIALS AND METHODS

The animals used in this study were kept, fed and mated as described previously (Kullberg et al., 1977).

Examination of ACh Receptors Using ^{125}I-α Bungarotoxin

αBuTX was purified as described by Anderson and Cohen (1974), labelled with ^{125}I (carrier-free, New England Nuclear) by the chloramine T (Eastman Kodak) method, and separated from free ^{125}I by chromatography on a column of Sephadex G-25 (see Berg et al., 1972). The iodinated toxin was then diluted with an equal volume of glycerol, and bovine serum albumin (BSA, Sigma) was added to give a final concentration of 5 mg BSA per ml toxin. The toxin concentration varied from 50 to 90 µg/ml. These stock solutions were stored at -16°C. The initial specific activity varied from 10,000 to 33,500 CPM/ng toxin. The radioactive toxin was diluted before each experiment to 1-2 µg/ml with frog Ringer's solution containing 5% dialysed horse serum or calf serum. The composition of the Ringer solution was, in mM: NaCl- 111, KCl- 3, $CaCl_2$- 1.8, tris maleate buffer or HEPES buffer adjusted to pH 7.4 with NaOH- 5-10. All experiments were done at room temperature (21-23°C).

After staging the animals by the external criterion of Nieuwkoop and Faber (1967) their skin, head and belly were removed, leaving "tail" preparations consisting of spinal cord, notochord and their associated myotomes on either side. The length of these "tail" preparations varied with the number of myotomes present at different stages. At early stages of development (19-22) the tail preparations usually had less than 8 myotomes while at later stages (36 and up) as many as 20 myotomes were included. At

all stages the 2-3 most rostral myotomes were excluded. Prior to dissection mobile animals were anaesthetized by immersion in 10^{-4}g/ml MS-222 (tricaine methanesulphonate).

For each stage examined, 6 or more tail preparations were used. The preparations were divided into two groups, one of which was pretreated with native toxin (2 µg/ml) at room temperature for 20 min. Both groups were then treated with ^{125}I-αBuTX (1 µg/ml) for 20 min at room temperature, and washed several times for at least 1 hour at 4°C, first with Ringer solution containing 5% serum and then with Ringer itself. The preparations were fixed in 1.5% glutaraldehyde (0.1M phosphate buffer at pH 7.3) and stored in the refrigerator (4°C) overnight.

The tails were subsequently rinsed with Ringer solution and split longitudinally in order to free each set of myotomes from the spinal cord and notochord. Care was taken to remove damaged myotomes at each end of these "half-tail" preparations. The number of myotomes in each half-tail was then counted and each half-tail was put into a separate plastic vial with Ringer solution for measurement of radioactivity in a gamma-scintillation spectrometer (Nuclear Chicago, model 1185). Background radioactivity was measured in the absence of tissue. This value was deducted and then the uptake per myotome calculated. The molecular weight of the toxin was assumed to be 8,000 (Mebs et al., 1971; Lee, 1972).

Portions of some of the half-tails were dehydrated, embedded in Epon 812, and cut longitudinally in 2µm sections on a Servall Porter-Blum microtome (Sorvall Inc.). After mounting, the slides were coated with nuclear emulsion NTB-2 (Kodak) by the dipping method (Kopriwa and Leblond, 1962), stored at 4°C in light-proof boxes with a package of desiccant Silica-Gel. Exposure times in two experiments were 13 and 21 days. The slides were then

developed with Kodak D-170 developer at 18°C, fixed with 24% solution of sodium hypo-thiosulfate, air-dried, and mounted with Permount and cover-slip. In each of two experiments the same batch of ^{125}I-αBuTX was used for all stages and all sections were processed at the same time. Accordingly the grain efficiency was the same in all sections.

Grains were counted with a Zeiss microscope at a magnification of 1,250 times, within a rectangular eye-piece grid covering a square area of 1,600 μm^2. Five to ten areas were counted in sections from each half-tail. These areas were taken in the central regions of different myotomes, away from the intermyotomal junctions. Background grain counts were measured over areas without tissue and deducted.

Fluorescent Staining of ACh Receptors

αBuTX was conjugated with tetramethylrhodamine isothiocyanate (BBL) as previously described (Anderson and Cohen, 1974; Anderson et al., 1977), and equilibrated with Ringer solution. Stock solutions were diluted to 5-10 µg/ml with Ringer solution containing 5% serum. Tail preparations were obtained as described above, except that the most rostral myotomes were also included. The preparations were stained for 20-30 min. at room temperature. Some embryos were prefixed in 10% neutral formalin at 4°C for 5 min in order to completely arrest development before staining. Such prefixation does not interfere with the staining intensity nor with the toxin specificity (see also Anderson et al., 1977; Ko et al., 1977). For verification of the staining specificity some preparations were also pretreated with unlabelled toxin. The preparations were then washed with several changes of Ringer solution, with and without 5% serum, at 4°C for at least 1 hour and fixed overnight in the refrigerator with 10% neutral formalin, or 4%

paraformaldehyde at pH 7.4, in 0.1M buffer. HEPES buffer was preferred over phosphate buffer because it gave less background autofluorescence. After rinsing again with Ringer the tails were split longitudinally into half-tails, and mounted on microscopic slides with pure glycerol. These preparations were viewed through a Zeiss microscope equipped so that the same field could be seen both with fluorescence and phase contrast optics (Anderson et al., 1977).

Cholinesterase Histochemistry

Embryos at different stages were pre-fixed intact with paraformaldehyde for 10 min. They were then washed in Ringer, skinned and tail preparations dissected out. These were subsequently stained with rhodamine-αBuTX, rinsed and post-fixed in the same fixative (4 hr or overnight) at 4°C. After rinsing with Ringer, the tails were split into half-tails and were then stained for ChE by the method of Karnovsky and Roots (1964). The half-tails were subsequently mounted in pure glycerol and examined for fluorescent staining of AChRs and for ChE.

Staining of Nerve Fibres with Nitroblue Tetrazolium

Letinsky and DeCino (1978, personal communication) have demonstrated that this method stains normal, regenerating and developing unmyelinated nerve fibres in vertebrate skeletal muscle. We have applied it to Xenopus embryos and tadpoles as follows. The animals were staged, anaesthetized if necessary, and skinned in Ringer solution. Following their isolation the tails were prefixed for 5 min in a freshly prepared 0.5% glutaraldehyde solution containing 0.1M phosphate buffer at pH 7.3. After briefly rinsing in 0.1M phosphate buffer the preparations were stained in nitroblue

tetrazolium (NBT) solution for 20 min, rinsed in Ringer and post-fixed in 10% formaldehyde or 4% paraformaldehyde overnight in the refrigerator. Half-tails were subsequently dissected out and mounted with pure glycerol on microscope slides. The staining solution consisted of a mixture of 10mg NBT and 1mg PMS (phenazine methosulfate) in 10ml of 0.1M phosphate buffer, pH 7.3, containing 0.5% glutaraldehyde. The glutaraldehyde was added immediately before use.

An attempt was made to combine this staining technique with fluorescent staining for AChR, but without success. The glutaraldehyde used with NBT caused a very high autofluorescence, hindering the detection of fluorescent receptor patches. Use of formaldehyde or paraformaldehyde, good fixatives for fluorescence, prevented nerve staining.

RESULTS

As a reference point for relating observations in the present study to the state of differentiation of the myotomes it is useful to summarize briefly some of the main developmental stages. Development is initially very rapid. Myotomes begin to form at stage 17 when the embryo is about 19 hr old and by stage 24 (26.25 hr old) all embryos exhibit motor responses which involve neuromuscular transmission (Nieuwkoop and Faber, 1967). Spontaneous synaptic potentials are detected in some embryos as early as stage 21 (22.5 hr), and are consistently observed by stage 24 (Blackshaw and Warner, 1976; Kullberg et al., 1977). At stage 36 (50 hr) the embryos hatch and become free-swimming tadpoles (Nieuwkoop and Faber, 1967). By this stage many of the synaptic contacts exhibit fairly well-developed ultrastructural specializations and the synaptic potentials, which were initially long-lasting, have become considerably briefer (Kullberg et al., 1977). Development subsequently proceeds more slowly and by stage 49 (12 days old) the synaptic potentials have attained their ultimate brief duration and the synapses have a relatively mature ultrastructural appearance (Kullberg et al., 1977).

Developmental Changes in the Number of Toxin Binding Sites

Fig. 1 illustrates how the uptake of toxin varied with time when stage 49 myotomal muscle was incubated with 1μg/ml ^{125}I-αBuTX. The uptake per myotome increased progressively for the first 15 min and then levelled off, suggesting saturation of toxin binding sites. This is in line with previous findings (Anderson and Cohen, 1974) that 1μg/ml toxin completely blocks carbachol (10^{-4}g/ml) induced contraction in 17 min. On the basis of these

results, in all subsequent experiments the myotomal muscle was treated with 1μg/ml ^{125}I-αBuTX for 20 min.

As expected the uptake proved to be highly specific. In three experiments on stage 47 and 49 myotomes the presence of 10^{-4}g/ml carbachol inhibited the myotomal uptake of radioactive toxin by an average of 90% (82%, 93%, 94%). Likewise, pretreatment of myotomes at these same stages with 2μg/ml native α-BuTX for 20 min blocked the uptake of radioactive toxin by 95 ± 0.5% (12 experiments).

The uptake of radioactive toxin by myotomes pretreated and not pretreated with native toxin was measured at different stages of development. At stage 19, the earliest stage studied, there was a small but significant ($P<0.05$) difference between pretreated and non-pretreated myotomes, thereby indicating the presence of AChRs even at this stage (Table 1). The presence of AChRs by stage 19 has also been demonstrated in studies in which ACh sensitivity was assessed electrophysiologically (Blackshaw and Warner, 1976). Beyond stage 19 the specific uptake per myotome increased progressively at first very rapidly and then more slowly (Table 1, Fig. 2). For example, in the 12-hour period between stages 19-28 the uptake approximately doubled every 2 hr of development. This rate subsequently declined such that between stages 49-53 the uptake per myotome doubled over a period of 7-8 days.

In order to assess whether the developmental increase of toxin uptake per myotome was due to an increase in the number of cells per myotome or to an increase in the total number of binding sites per cell, the size of the myotomal cell population was determined at different stages of development. The number of cells per myotome was counted in 5μm thick cross sections (see Fig. 1 in Kullberg et al., 1977). The values for stages 20, 22, 28,

36 and 49 were 169 ± 6, 176 ± 16, 203 ± 6, 254 ± 8 and 277 ± 13, respectively (based on counts of 4 to 7 myotomes in one or two tails per stage). For these measurements no attempt was made to select myotomes having the same rostro-caudal position along the tail so that some of the differences may reflect positional variability rather than developmental variability. In any event it appears that the number of muscle cells per myotome is fairly constant throughout the developmental stages studied and does not change by more than about 65% from stage 20 to stage 49. Accordingly the developmental increase in toxin binding sites per myotome (Fig. 2) must be due to an approximately similar relative increase at the single cell level.

Myotomal muscle cell growth is relatively slight from stage 20 to stage 36 and more extensive afterwards. The myotomal lengths at stages 20, 36 and 49 were 97 ± 3 μm, 162 ± 2 μm, and 268 ± 4 μm, respectively (based on measurements of a total of 50-120 myotomes from 4-8 half-tails at each stage). In transverse sections the cells appear polygonal rather than circular (see Fig. 1 in Kullberg et al., 1977). No marked change in size was apparent between stages 20 and 36. Measurements of "diameters" yielded values of 14.4 ± 0.2 μm for stage 20 (100 cells), 15.1 ± 0.4 μm for stage 36 (60 cells) and 25.0 ± 0.5 μm for stage 49 (150 cells). From these observations it is apparent that at least between stages 20 and 36 the rate of AChR acquisition proceeds much more quickly than the rate of myotomal cell growth.

Distribution of Toxin Binding Sites: Radioautography

It has been well established that amphibian myotomal cells are innervated at their ends (Mackay et al., 1960; Lewis and Hughes, 1960; Filogamo and Gabella, 1967; Kullberg et al., 1977) and that in the tadpoles

these are the sites where ACh receptors occur in high density (Anderson and Cohen, 1974). In order to investigate the developmental changes in receptor distribution leading up to this preferential localization at the ends of the myotomal cells radioautography was carried out on longitudinal sections of some of the half-tails used for measuring receptor numbers (see Methods).

Preferential localization at cell ends

Figures 3-6 show representative radioautographs at different stages of development. A widespread distribution of grains was seen along the myotomes at all stages examined. At stages 20 and 22 the grain distribution appeared uniform along the entire length of the myotomes and only rarely, at stage 22, was there any hint of more grains at the ends of the myotomes (Fig. 3). At stage 24 an extra accumulation of grains at the ends of myotomes was commonly observed and this differential distribution became more marked with development (Figs. 4-6). As expected, pretreatment with native toxin eliminated virtually all grains at all stages (Fig. 7), indicating that the grain distribution in unblocked preparations reflects the distribution of AChRs. These results indicate that receptor localization at the ends of the myotomes begins to occur at about stage 22. More detailed evidence in this regard was obtained in experiments using fluorescent α-BuTX (see below).

Developmental changes along the myotomes

As seen in Figs. 3-6 the grain density along the myotomes varied with the developmental stage. In order to obtain an estimate of these changes, grain densities were measured along the myotomes, away from the cell ends (see Methods). The results are summarized in Table 2 and Fig. 8. It can

be seen that the grain density increased progressively between stages 20 and 36 by an overall factor of about 25 and then declined between stages 36 and 49 by a factor of about 3-5.

Since muscle cell "diameters" are approximately the same up to stage 36, the average amount of sarcolemma in a section should also be the same; therefore up to stage 36 the changes in grain density should be a good reflection of the changes in extrajunctional receptor density. Beyond stage 36 the cell "diameters" increase so that the amount of sarcolemma in a longitudinal section will be less. Accordingly the decline in grain density beyond stage 36 must be an overestimate of the decline in extrajunctional receptor density. Despite this obvious quantitative uncertainty it seems clear that the extrajunctional receptor density increases up to stage 36, when the embryo hatches, and subsequently declines. However the decline is slow and even by stage 49 there is still a substantial density of extrajunctional receptors.

Sites of High Receptor Density: Fluorescent Staining

Fluorescent staining of AChRs permitsvisualization of sites of high receptor density at high resolution in unsectioned tissue (Anderson and Cohen, 1974). Accordingly such staining was carried out in order to obtain more detailed information on the development and distribution of such sites at different stages of development.

Development of sites of high receptor density at the ends of the myotomes

Figures 9-12 show micrographs of preparations stained with fluorescent toxin at different stages of development. Fluorescent patches, indicating sites of high receptor density, were seen at the ends of myotomes as early as stage 21 (Fig. 9), but their occurrence at this stage was rare and

was restricted to only small portions of the ends of very few myotomes. By stage 24 fluorescent stain was observed at the ends of many more myotomes. It consisted of small patches as well as a less intense glow along the intermyotomal junction and extended over a large portion of the myotomal end region (Fig. 10). With age the patches increased in size and ultimately extended over the entire length of the myotomal end regions. At early stages of development the ends of the rostral myotomes were more brightly stained (Fig. 11) than the caudal ones (Fig. 12); and the most caudal myotomes did not exhibit any stain. These rostro-caudal differences were consistently found up to stage 30 and presumably reflect the fact that the caudal myotomes form last and are therefore the least mature (Nieuwkoop and Faber, 1967; Hamilton, 1969). At later stages the ends of all myotomes examined were brightly stained (as in Fig. 11, also see Plate 3 in Anderson and Cohen, 1974).

The frequency of occurrence of fluorescent stain at the ends of the myotomes is summarized in Fig. 13. Stain was never seen at stage 19 and was extremely rare at stage 21. For example a total of 37 half-tails were examined at stage 21 and stain was seen in only one of these, and only at the ends of the first two myotomes. At stage 22, 15-20% of the half-tails exhibited stain at the ends of the first five myotomes. By stage 24 this value had increased to about 60% and stain was also seen at the ends of the more caudal myotomes, but at a progressively lower frequency. By stage 26 almost all, and by stage 28 all, half-tails had stain at the ends of the first nine myotomes. In addition to demonstrating a rostro-caudal gradient during development these results reinforce our radioautographic observations indicating that the development of sites of high receptor density at the

ends of the myotomes begins at about stages 21-22. The values in Fig. 13 are based on the occurrence of fluorescent patches and/or fluorescent glow at the ends of the myotomes. If only fluorescent patches are considered the peak values become: 3% for stage 21, 4% for stage 22, 53% for stage 24, 94% for stage 26, and 100% for stage 28. Presumably the glow reflects an early stage in the development of the patches of high receptor density.

Occurrence of sites of high receptor density along the myotomes

Fluorescent patches were also seen along the myotomes, away from the cell end. These patches were found from stage 24 on, and sometimes occurred on myotomes which had no patches at their ends (Fig. 12). In many cases, the staining along the myotomes consisted of narrow, interrupted bands of stain running either longitudinally or transversely across several cells (Figs. 14-16). Occasionally some isolated patches were also observed. As was the case for the staining at the ends of the myotomes the individual patches of stain along the myotomes were smaller at the earlier stages. The patterns of stain along the myotomes are reminiscent of the patterns seen on nerve-contacted myotomal muscle cells in culture (Anderson et al., 1977; Anderson and Cohen, 1977; Cohen et al., 1979).

Two other lines of evidence also suggest that many of the receptor patches along the myotomes were located at sites of synaptic contact. Combined staining of receptors and of ChE revealed that many of these receptor patches are also sites of cholinesterase activity (Fig. 16). Between stages 41 and 49, about 60% of the receptor patches along the myotomes exhibited ChE staining (Table 3). At stages 32 and 36, the correspondence was found to be about 25%. In addition, at all stages where receptor patches were seen along the myotomes, staining with nitroblue tetrazolium

(NBT) also revealed the presence of nerve fibres, not only at the ends of the myotomes but also coursing along or across the muscle cells away from their ends (see below).

Myotomal Nerve Fibres

In previous studies synaptic potentials were recorded in Xenopus myotomes as early as stage 21 (Blackshaw and Warner, 1976; Kullberg et al., 1977). Electron microscopy also revealed an occasional nerve fibre in the myotomes at this stage (Kullberg et al., 1977). In the present study we have examined in more detail the arrival of the nerve fibres in the myotomes in order to determine the temporal sequence between this event and the development of sites of high receptor density. For this purpose we employed the technique of Letinsky and DeCino (1978, personal communication) who showed that nitroblue tetrazolium stains unmyelinated nerve fibres and motor nerve terminals in developing and adult muscle.

Fig. 17 shows a low magnification micrograph of a stage 24 half-tail stained with NBT. The ends of the first nine myotomes are stained darkly and appear as narrow bands whereas the ends of the two most caudal myotomes do not exhibit such staining. In addition dark blotches of stain are seen along the two most caudal myotomes and other regions exhibit some fainter, more diffuse staining particularly near the ends of the myotomes. The dark blotches reflect staining of damaged cells. This form of intense staining was seen along the entire length of individual myotomal muscle cells which had been inadvertently damaged, at all stages of development. On the other hand the more diffuse, fainter staining was seen only up to stage 26 and was associated at least in part with the thin dermatomal layer of cells which remains fairly tightly attached to the lateral surface of the myotomes

up to this stage. It was usually possible to tease away much of this dermatomal layer but some of the diffuse staining remained, particularly at the ends of the myotomes. The significance of this remaining diffuse stain is not clear. It may be that some of the myotomal muscle cells become stained in this region or that some stained product diffuses away from the immature nerve fibres. In any event, at higher magnification fine lines of stain could be resolved amidst the diffuse staining at the ends of the myotomes (Figs. 18 and 19) and these resembled the nerve fibres seen at the ends of myotomal muscle cells at later stages where there was no interference from diffuse staining (Figs. 20 and 21).

In addition to their presence at the ends of the myotomes, nerve fibres were also seen coursing along the myotomes, from stage 24 on (Figs. 21 and 22). Some nerve fibres ran longitudinally along one or more muscle cells and others coursed transversely across the myotome. The similarity between the patterns of AChR patches (Fig. 15), ChE activity (Fig. 16) and nerve fibre distribution along the myotome (Fig. 21) emphasizes the likelihood that such staining patterns are in fact associated with sites of nerve-muscle contact.

At early stages of development the nerve fibres appeared uniform along their length. However from stage 30 on some of the nerve fibres also exhibited varicosities (Figs. 20-22). As described in previous studies (Lewis and Hughes, 1960; Mackay et al., 1960; Filogamo and Gabella, 1967) the nerve fibres at later stages of development contact the ends of the muscle cells either by encapsulating them with a few short branches or by simply coursing over the cell end without branching (Fig. 20).

The percentage of half-tails exhibiting some nerve fibre staining at

the ends of myotomes at different stages of development is summarized in Fig. 23. Stain was observed as early as stage 19 but only at the ends of the first three myotomes in one out of sixteen half-tails. At stage 21 over 20% of the half-tails had stain up to the fifth intermyotomal junction, and less more caudally. The values were higher at stage 22, and by stage 24 virtually all half-tails had stain along some of their intermyotomal junctions. These values are probably underestimates because some of the half-tails were stained relatively lightly and it appeared that in a few cases the staining method may have failed. Nevertheless comparison with Fig. 13 reveals that nerve fibre staining was detected earlier than AChR staining for every myotome examined along the rostro-caudal axis. From the data in Figs. 13 and 23 one can readily estimate, for each myotome, the embryonic age when nerve fibre stain and receptor stain were seen in 50% of the embryos. These estimates are listed in Table 4 and reveal that on the average nerve fibres appeared at the ends of the myotomes about three hours before the high receptor density. This interval was generally shorter for the four most rostral myotomes, and longer for the more caudal myotomes.

DISCUSSION

Arrival of Motor Nerve Fibres in the Myotomes

The stage at which developing muscle cells are first contacted by growing motor nerve fibres is an essential reference point for the study of neuromuscular synaptogenesis. In the present study NBT staining indicated that nerve fibres first reach the myotomes at stages 19-21. It is, however, necessary to consider the possibility that nerve fibres invade the myotomes at earlier stages but cannot be detected by NBT staining. For example in a study employing radioactive thymidine it was found that the neuroblasts which give rise to some of the spinal cord neurones, the Rohon-Beard cells, undergo their last bout of DNA synthesis at stages 11-12 (Spitzer and Spitzer, 1975). These primary sensory neurones can be recognized in the developing spinal cord by stage 18 and become electrically excitable at stage 20 (Spitzer, 1979). Motor neurone cell bodies have also been recognized at stages 20-22 (Muntz, 1975). However there is no evidence that these or other spinal neurones send processes to the myotomes before stage 19. For example electron microscopic examination has revealed very few processes in the developing spinal cord of stage 19 embryos and a progressive increase in their number thereafter (Hayes and Roberts, 1973). Likewise areas of cell membrane specialization suggestive of synaptic contacts were not seen in the developing spinal cord at stage 19 but were observed at stage 20 (Hayes and Roberts, 1973). Light microscopy, after staining with a modification of Bodian's technique, has revealed Rohon-Beard nerve fibres leaving the spinal cord at stages 20-22 (Muntz, 1975). In view of all of these findings it seems likely that nerve fibres do not

leave the spinal cord before stage 19 and that the NBT staining technique does detect the first nerve fibres to reach the myotomes.

Previous studies have indicated that both primary sensory fibres (Rohon-Beard nerve fibres) and motor nerve fibres run along intermyotomal junctions in Xenopus embryos and tadpoles (Hughes, 1957; Nieuwkoop and Faber, 1967; Muntz, 1975). The question therefore arises whether the NBT stained fibres that we observed at early stages were sensory rather than motor. That some could have been sensory is suggested by the work of Muntz (1975) who found Rohon-Beard nerve processes leaving the spinal cord and reaching the myotomes by stages 20-22. It is not known, however, whether these early Rohon-Beard nerve fibres enter into the type of close apposition with the ends of the myotomal cells that we have observed (see Fig. 18). Furthermore, electrophysiological studies have revealed that synaptic activity begins in the myotomal muscle cells of some embryos as early as stage 21 (Blackshaw and Warner, 1976; Kullberg et al., 1977) thereby indicating that motor nerve fibres in some embryos have reached the myotomes by this stage. Taken together these observations suggest that the earliest sensory and motor fibres probably invade the myotomes at about the same time and it seems reasonable to conclude that the data we have obtained with NBT staining do provide an accurate indication of the arrival of motor nerve fibres in the myotomes.

Acquisition of Acetylcholine Receptors

The present study has shown that the myotomal cells of embryos of Xenopus laevis acquire AChRs as early as stage 19 (about 21 hr), and that the receptors are initially distributed along the entire length of the myotomes. ACh sensitivity was also detected as early as stage 19

(Blackshaw and Warner, 1976). At this stage nerve fibres were seen only rarely thereby suggesting that the receptors may appear on the myotomal muscle cells slightly before the onset of innervation. A similar temporal relationship between the acquisition of AChRs and the arrival of nerve fibres has also been suggested in studies on developing muscles in rat foetuses (Bevan and Steinbach, 1977) and in chick embryos (Giacobini et al., 1973). That developing muscle cells can acquire receptors in the absence of innervation has been extensively documented in cell culture studies (for review, see Fambrough, 1979).

Changes in Extrajunctional Receptor Density

Our findings have indicated that the density of extrajunctional receptors increases rapidly for about one day after the onset of receptor acquisition and then declines slowly after the embryo hatches. An initial rapid increase in extrajunctional receptor density also occurs in other developing muscles. Thus, in the rat sternomastoid muscle extrajunctional receptor density reaches a peak by foetal day 15, less than a day after the onset of receptor acquisition (Bevan and Steinbach, 1977). In the posterior latissimus dorsi (PLD) muscle of chick receptor acquisition is apparent by embryonic day 8 and extrajunctional receptor density has reached a peak by embryonic day 10 (Burden, 1977a). On the other hand the subsequent decline in extrajunctional receptor density appears to proceed much more quickly in rat and chick muscles than in Xenopus myotomal muscle. In rat sternomastoid the density decreases more than 10-fold between foetal days 16 and 20, and is negligible 9 days later (Bevan and Steinbach, 1977). In chick PLD the extrajunctional density remains constant between embryonic days 10 and 14, and then declines by a factor of 25 over the next 5 days (Burden,

1977a). By contrast the extrajunctional receptor density in Xenopus myotomes decreased less than 5-fold during the 10-day interval between stages 36 and 49 (see Fig. 8). One contributing factor may be the lifespan of receptors in the surface membrane. For example in chick PLD the receptors have a half-life of about 30 hr (Burden, 1977a). A similar value has also been obtained for developing rat muscle (Berg and Hall, 1975b). The half-life of receptors in myotomal muscle of Xenopus embryos and tadpoles may be greater since the animals are poikilothermic and are kept at room temperature. A slower decline in extrajunctional receptor density would also be expected if there were continued synthesis and incorporation of new receptors in Xenopus. Such questions concerning receptor metabolism in Xenopus myotomes should be amenable to experimental testing.

Development of Sites of High Receptor Density

The present study has shown that patches of high receptor density first appear at stage 21 and that their number increases with development. That many of these receptor patches were located at sites of innervation is suggested by the following evidence. It is well established that Xenopus myotomal muscle cells are innervated mainly at their ends (Lewis and Hughes, 1960; Mackay et al., 1960). This appears to be the case from the very onset of innervation as judged by electron microscopy and electrophysiology (Kullberg et al., 1977) as well as by the distribution of nerve fibres seen in the present study. Sites of high receptor density were also located mainly at the ends of the myotomal muscle cells from the very beginning of their formation. Furthermore, receptor patches which were situated along the myotomes, away from the cell ends, often formed aligned

patterns which resembled the course of nerve fibres running over these regions of the myotome. Indeed these patterns were similar to those seen at nerve-muscle contacts in cell cultures of Xenopus spinal cord and myotomes (Anderson et al., 1977; Anderson and Cohen, 1977; Cohen et al., 1979). Moreover, many of the receptor patches along the myotomes also proved to be sites of cholinesterase activity. It therefore seems likely that from the onset of their formation many of the receptor patches were located at developing synaptic sites.

The development of a high receptor density at presumptive synaptic sites after the arrival of nerve fibres has also been suggested from studies on the developing sternomastoid and diaphragm muscles in rat foetuses (Bevan and Steinbach, 1977). Such a temporal sequence is consistent with the demonstration in culture that nerve fibres can induce the development of a high receptor density at sites where they contact muscle cells (Anderson and Cohen, 1977; Anderson et al., 1977; Frank and Fischbach, 1979). In culture it has been possible to follow the development of the high receptor density at individual sites of nerve-muscle contact and such experiments have suggested that the final high density can be achieved very shortly after contact is made: the delay is less than 3 hr in chick cultures (Frank and Fischbach, 1979) and less than 6 hr in Xenopus cultures (Cohen et al., 1979). Our results suggest that in vivo the delay between nerve-muscle contact and the development of a high receptor density at that site may be as little as 1-5 hr (Table 4).

It is also possible that some of the receptor patches develop independently of nerve-muscle contact. Indeed many studies of developing muscle cells in culture have revealed the formation of receptor patches in

the complete absence of nerve (Vogel et al., 1972; Fischbach and Cohen, 1973; Sytkowski et al., 1973; Hartzell and Fambrough, 1973; Axelrod et al., 1976; Bekoff and Betz, 1976; Prives et al., 1976; Betz and Osborne, 1977; Land et al., 1977), and this is also the case for cultures of Xenopus myotomal muscle cells (Anderson et al., 1977). Recently Jacob and Lentz (1979) found small receptor patches on developing muscle cells in the anterior and posterior latissimus dorsi muscles of chick embryos 10-14 days old that were not associated with nerve-muscle contacts. Such patches may have developed in the absence of nerve contact. Alternatively, since nerve fibres are already present in these muscles by embryonic day 10 (Sisto Daneo and Filogamo, 1975) it is also possible that the patches developed at sites of nerve-muscle contact and that the nerve subsequently withdrew. Studies in culture have demonstrated that sites of high receptor density at nerve-muscle contacts do persist for a short time after spontaneous withdrawal of the nerve (Cohen et al., 1979; Anderson and Klier, 1979).

Whether receptor patches arise independently of innervation during normal development might well depend upon the state of muscle cell differentiation prior to the arrival of the nerve fibres. Thus it could be that in vivo, as in culture, developing muscle cells eventually form sites of high receptor density even in the absence of contact by nerve. If on the other hand nerve fibres arrive before this stage of muscle cell differentiation, the contacts they form with the muscle cells may act as loci for the development of a high receptor density and reduce the chances of receptor patches developing elsewhere. In either case studies on cultures of Xenopus myotomal muscle (Anderson and Cohen, 1977; Anderson et al., 1977), on cultures of chick muscle (Frank and Fischbach, 1979) and on developing chick

muscle *in vivo* (Jacob and Lentz, 1979) have indicated that once nerve-muscle contact is established the receptor patches which are not associated with these contacts become short-lived and disappear.

Table 1

Uptake of radioactive toxin by Xenopus myotomes at different stages of development. Mean ± standard error. Numbers in parentheses indicate the number of half-tails counted.

		Uptake / myotome (x 10^6 molecules)			
Stage	Age *	Without pretreatment		Pretreated with α-toxin +	
19	20.75hr	7.8 ± 1.4	(5)	2.4 ± 0.3	(5)
20	21.75hr	13.3 ± 0.7	(6)	3.7 ± 1.0	(6)
22	24hr	26.2 ± 2.4	(7)	6.5 ± 1.0	(7)
24	1d2.25hr	44.5 ± 1.7	(6)	8.5 ± 1.0	(6)
28	1d8.5hr	131.2 ± 7.8	(6)	14.6 ± 1.4	(5)
36	2d2hr	231.4 ± 25.8	(11)	20.1 ± 5.6	(7)
41	3d4hr	236.2 ± 27.0	(6)	15.1 ± 7.3	(7)
45	4d2hr	401.0, 428.8, 460.5		71.5 ± 11.9	(7)
47	5d12hr	474.7 ± 25.8	(37)	31.1 ± 6.6	(38)
49	12d	1241.9 ± 78.2	(40)	60.5 ± 9.6	(40)
52	21d	3011.7 ± 543.2	(4)	544.3 ± 94.1	(4)
53	24d	3784.7 ± 38.9	(4)	371.9 ± 6.9	(4)

* from Nieuwkoop & Faber, 1967
\+ 2 µg/ml α-bungarotoxin for 20 min

Table 2

Developmental changes in grain density in the mid-regions of myotomes. Mean ± standard error. Numbers in parentheses indicate the number of areas counted. The specific activity of the radioactive toxin and the radioautographic exposure time in experiment A were different from those in experiment B.

		Grain density (grains / 1600 μm^2)			
Expt.	Stage	Without pretreatment		Pretreated with α-Toxin	
A	20	29.6 ± 5.0	(8)	12.7 ± 0.8	(8)
A	22	55.0 ± 7.6	(9)	24.6 ± 2.2	(9)
A	24	114.4 ± 10.1	(8)	17.0 ± 2.8	(5)
A	36	416.5 ± 33.9	(5)	5.6 ± 2.8	(5)
B	36	117.1 ± 9.7	(10)	3.7 ± 0.9	(10)
B	41	115.9 ± 8.6	(10)	0.9 ± 0.3	(10)
B	45	72.8 ± 5.3	(10)	2.4 ± 1.0	(10)
B	47	59.8 ± 7.1	(10)	1.4 ± 0.6	(10)
B	49	43.8 ± 5.2	(10)	1.7 ± 0.8	(10)
A	49	86.9 ± 6.3	(5)	5.9 ± 0.9	(5)

Table 3

Occurrence of patches of ACh receptor stain with cholinesterase activity along the myotomes at different stages of development.

Stages	Number of ACh receptor patches examined	Percentage of receptor patches with cholinesterase
32	106	25.5
36	236	23.7
41	252	66.7
43	204	90.2
45	37	46.0
46	173	67.1
47	204	65.4
49	6	100.0

Table 4

Comparison of embryonic age at which 50% of half-tails exhibit nerve fibres and ACh receptor patches at the ends of their myotomes.

Rostro-caudal position of myotome	Nerve fibres (hr)	ACh receptor patches (hr)	Age difference (hr)
1	25.2	26.1	0.9
2	25.8	25.6	-0.2
3	24.2	25.6	1.4
4	24.2	26.1	1.9
5	24.3	27.7	3.4
6	24.6	28.8	4.2
7	24.5	29.1	4.6
8	24.8	29.1	4.3
9	25.0	29.3	4.3
10	25.0	30.5	5.5

Fig. 1 Uptake of ^{125}I-α bungarotoxin by stage 49 myotomes after different times of incubation with 1 µg/ml of toxin. Each value is a mean based on 4 half-tails. Bars indicate standard errors.

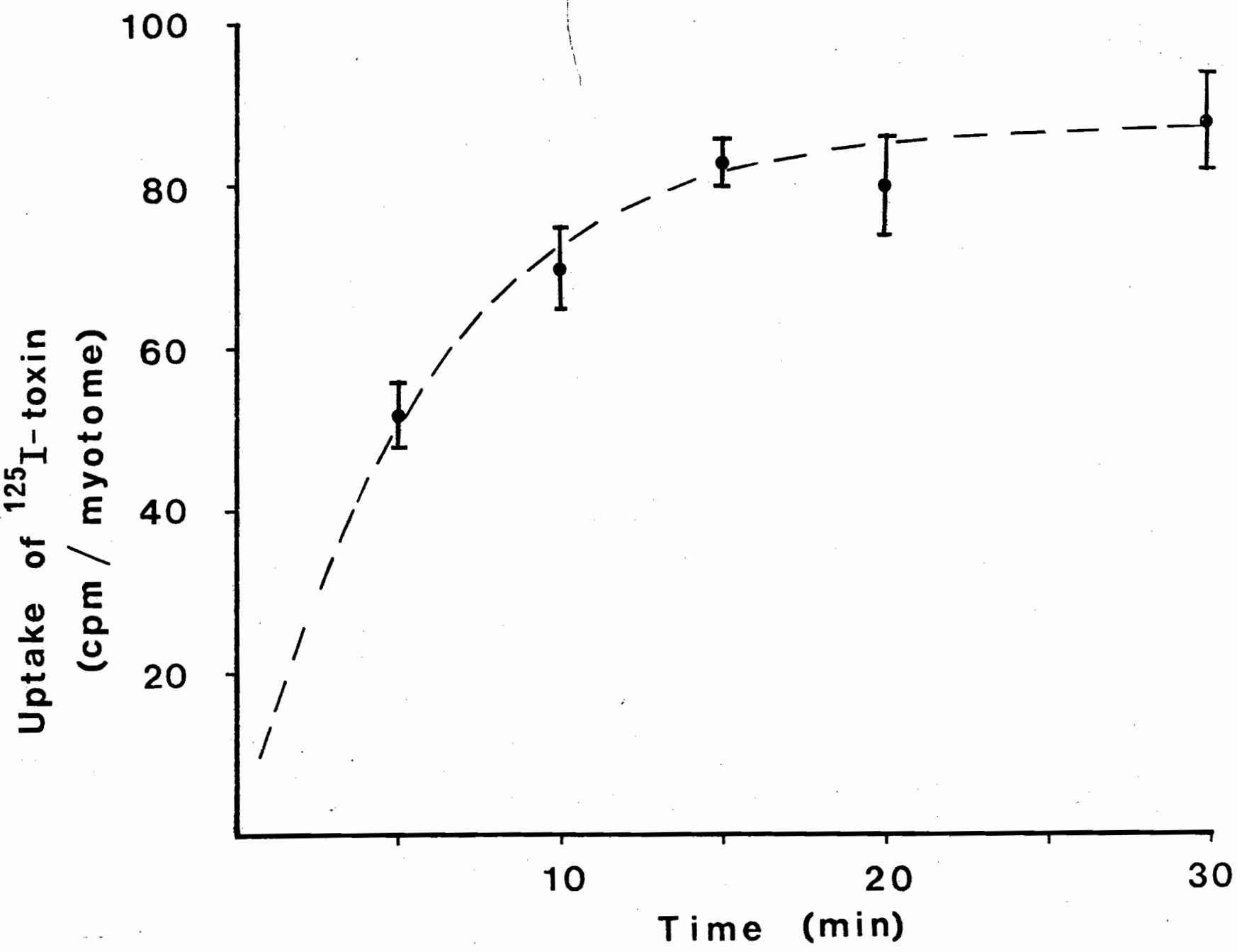
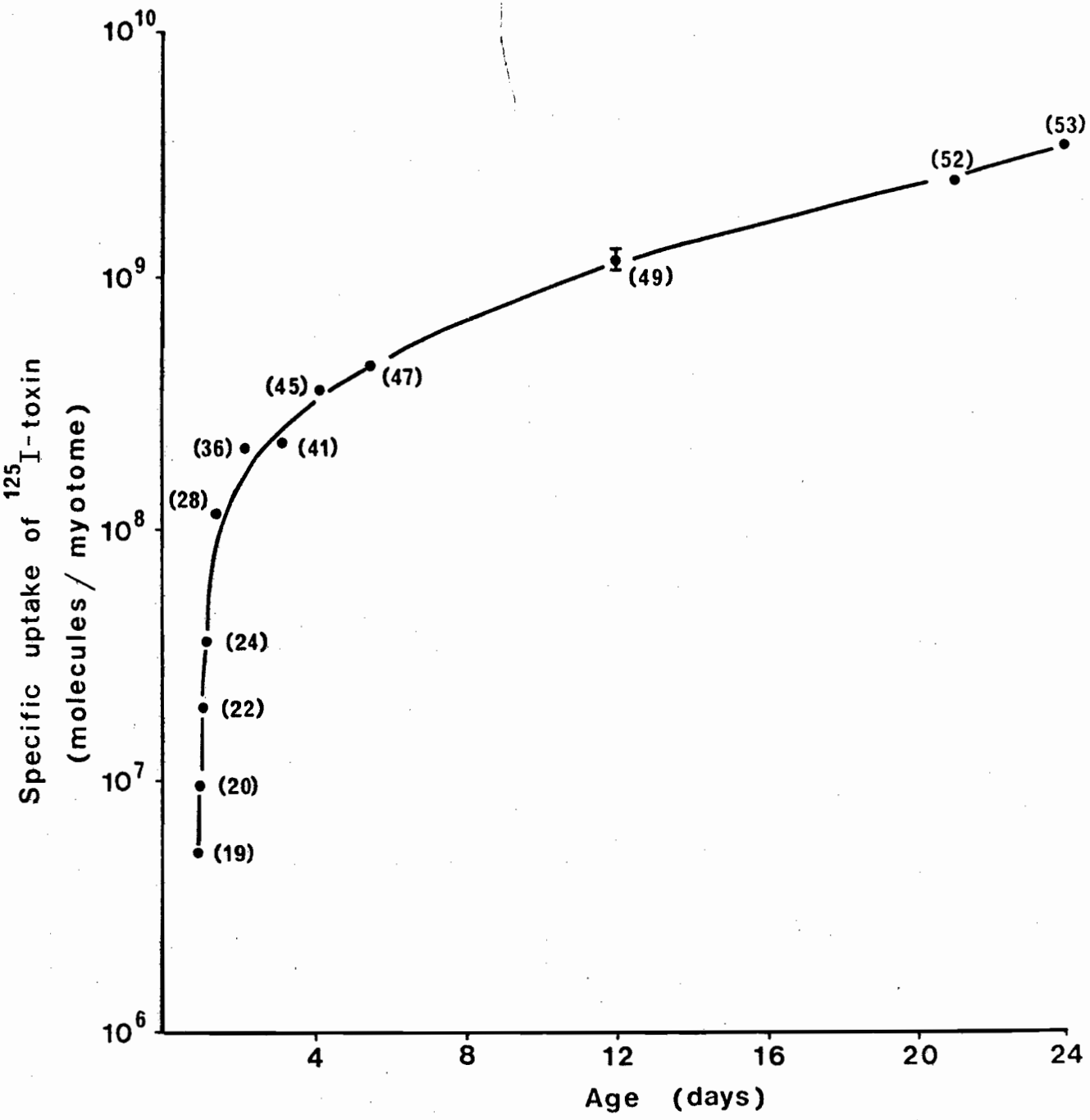

100
80
60
40
20
Uptake of ^{125}I-toxin
(cpm / myotome)
10
20
30
Time (min)

Fig. 2 Specific uptake of ^{125}I-α bungarotoxin per myotome at different stages of development. Based on values in Table 1. Numbers in parentheses indicate the corresponding stages.

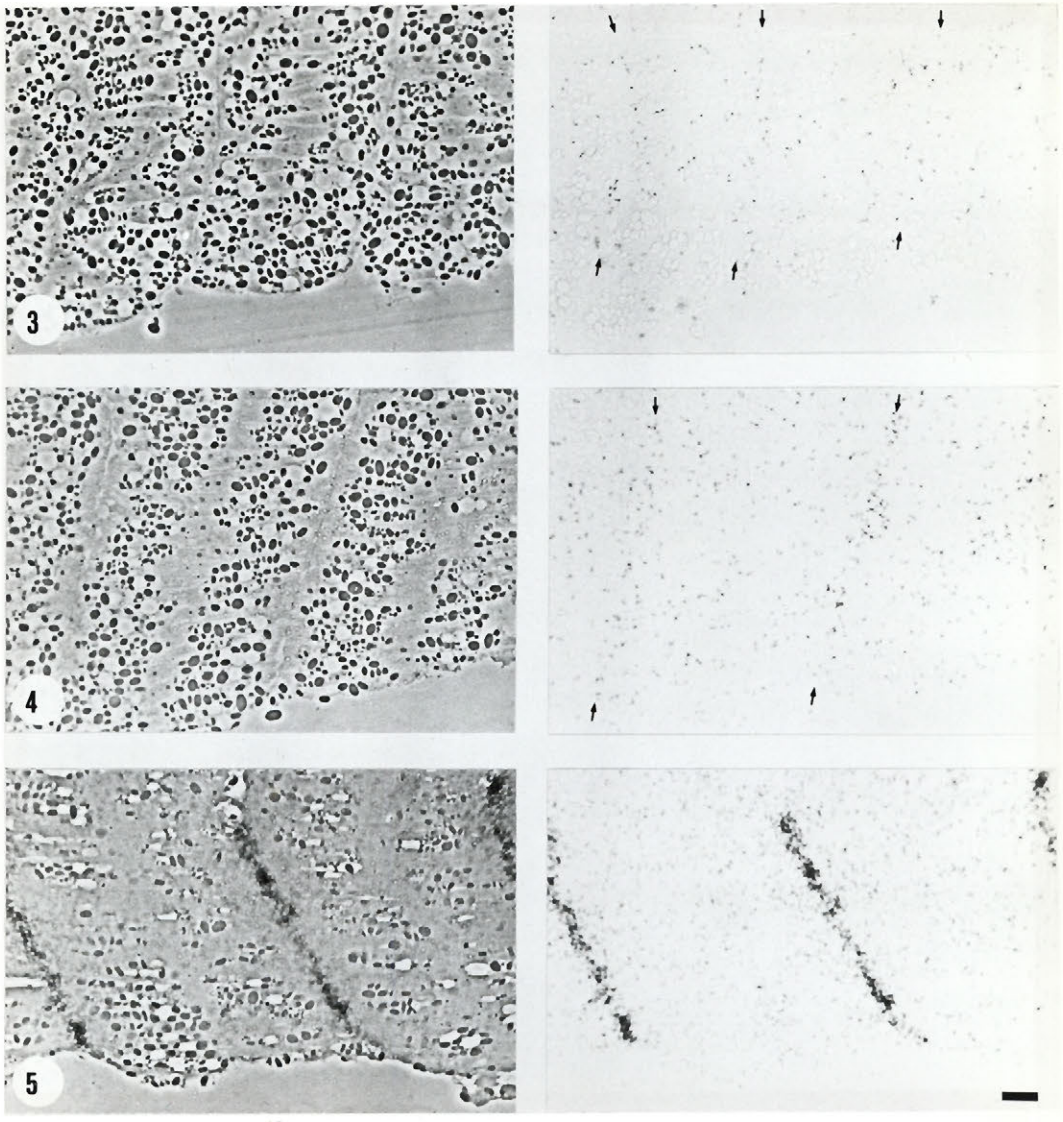

10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
Specific uptake of ^{125}I-toxin
(molecules / myotome)
(53)
(52)
(49)
(47)
(45)
(41)
(36)
(28)
(24)
(22)
(20)
(19)
4
8
12
16
20
24
Age (days)

Figs. 3-5 Radioautographs (right) and corresponding phase contrast micrographs (left) of longitudinal sections of myotomes at different stages of development. All sections were 2 μm thick and the emulsion was exposed for 13 days. The specific activity of the radioactive toxin was also the same for all radioautographs. Arrows indicate intermyotomal junctions. Oval and round phase dark structures are yolk granules, which are mostly consumed by stage 41. Calibration bar is 20 μm.

Fig. 3 Stage 22. Note the widespread distribution of grains along the myotomes and some grains at the myotomal end regions.

Fig. 4 Stage 24. The density of grains along the myotomes is greater than at stage 22 and the presence of more grains at the ends of the myotomes is apparent.

Fig. 5 Stage 36. Note the marked accumulation of grains at the ends of the myotomes as well as the relatively high grain density along the myotomes.


3
4
5

Figs. 6, 7 Radioautographs (right) and corresponding phase contrast micrographs (left) of stage 49 myotomes. No more yolk granules are found. Same experimental conditions as Figs. 3-5.

Fig. 6 Note that the grain density is highest at the ends of the myotomes but it is also substantial along the myotomes.

Fig. 7 Myotomes pretreated with native toxin. Uptake of radioactive toxin is prevented both at the ends and along the myotomes. Note the very low level of non-specific uptake. Arrows indicate the intermyotomal junction.

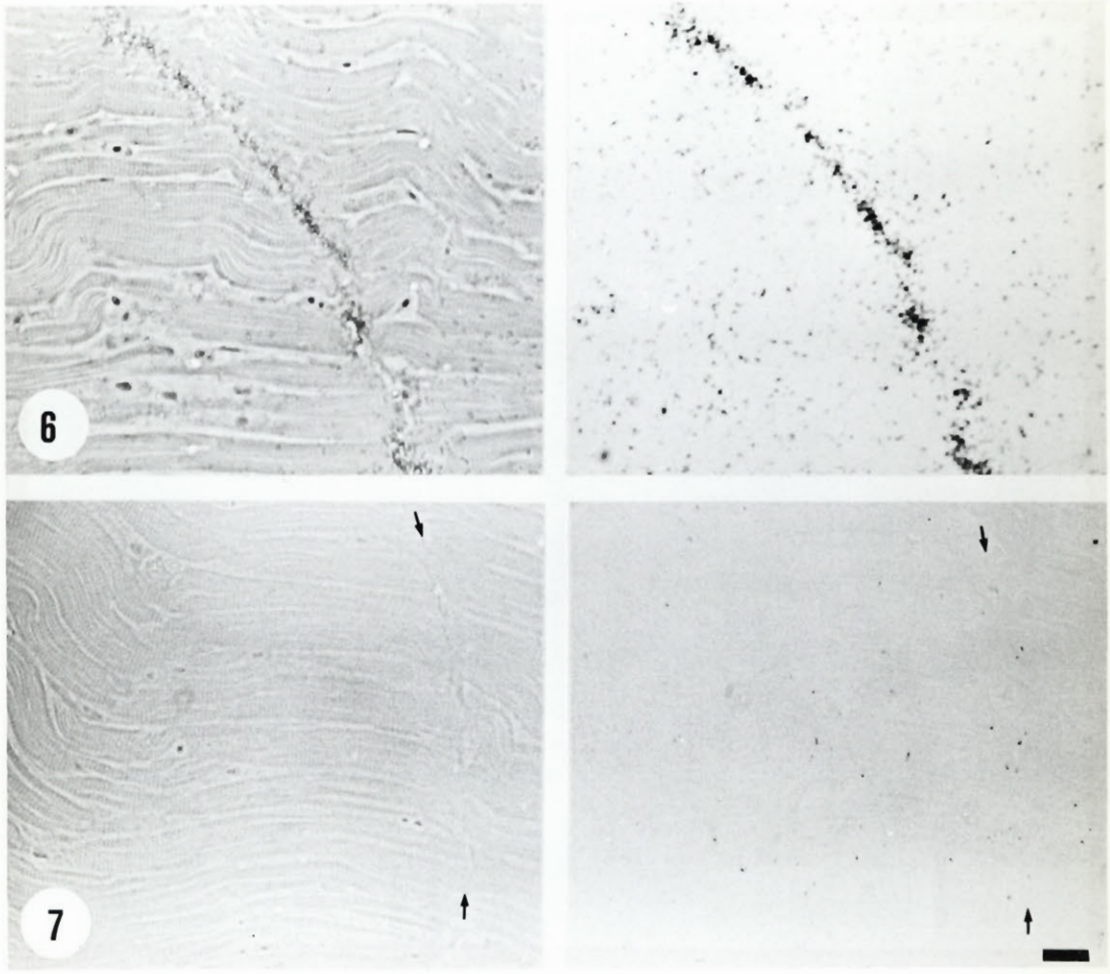

6
7

Fig. 8 Specific grain density along the myotomes at different stages of development. Values relative to density at stage 49. Based on data from experiments A (●) and B (X) in Table 2. Numbers in parentheses indicate corresponding stages. Note the rapid increase between stages 20 and 36, and the subsequent slower decline.

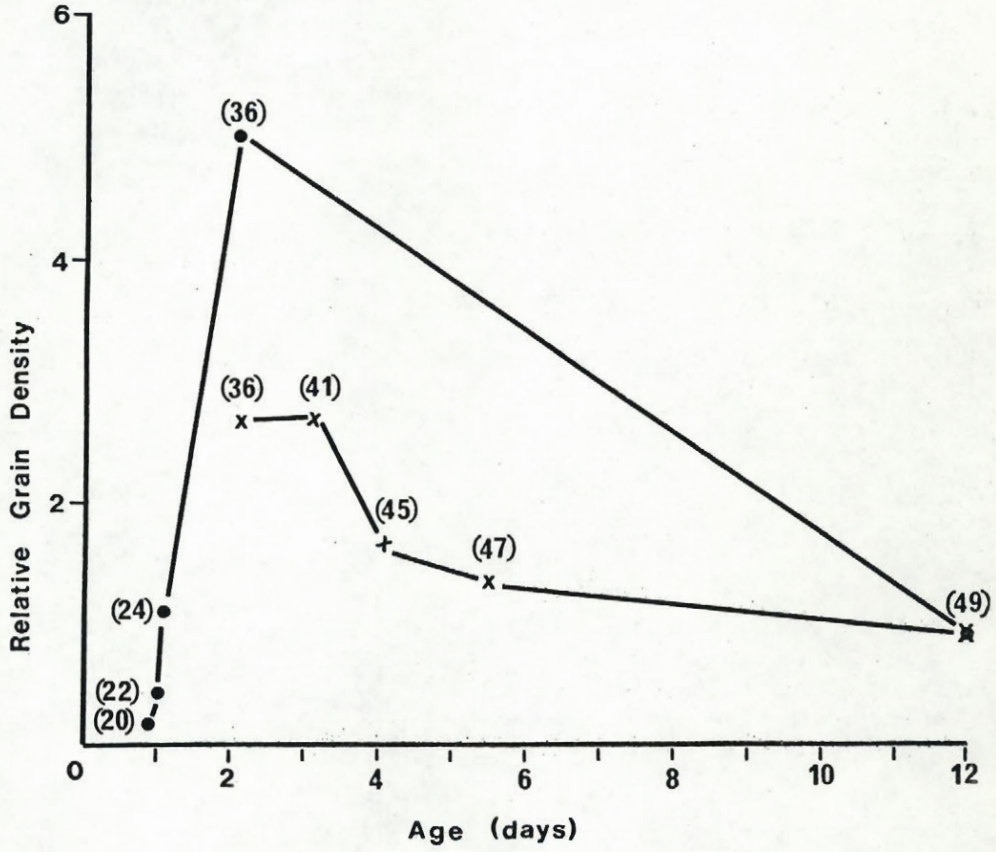

6
4
2
0
Relative Grain Density
(36)
(36)
(41)
(45)
(47)
(24)
(49)
(22)
(20)
2
4
6
8
10
12
Age (days)

Fig. 9 Phase contrast (left) and fluorescent (right) micrographs of part of a teased myotomal muscle cell from stage 21 embryo. Small patches of fluorescent stain are seen at the cell end (arrows). The large bright globules on the right of the fluorescent micrograph are yolk granules and do not represent specific staining. Calibration bar is 10 μm.

Fig. 10 Intermyotomal junction from a stage 24 half-tail showing fluorescent glow and some small patches of brighter fluorescence. Calibration bar is 10 μm.

Fig. 11 Rostral portion of half-tail of a stage 28 embryo, showing bright fluorescent patches at the ends of the myotomes. Note that the patches extend over much of the myotomal end regions. Calibration bar is 20 μm.

Fig. 12 Caudal portion of the same half-tail shown in Fig. 11. Note the much lower incidence of fluorescent patches at the ends of the most caudal myotomes (on the right of the micrograph). Note also the aligned group of patches extending into the mid-region of one of the myotomes. Calibration bar is 20 μm.

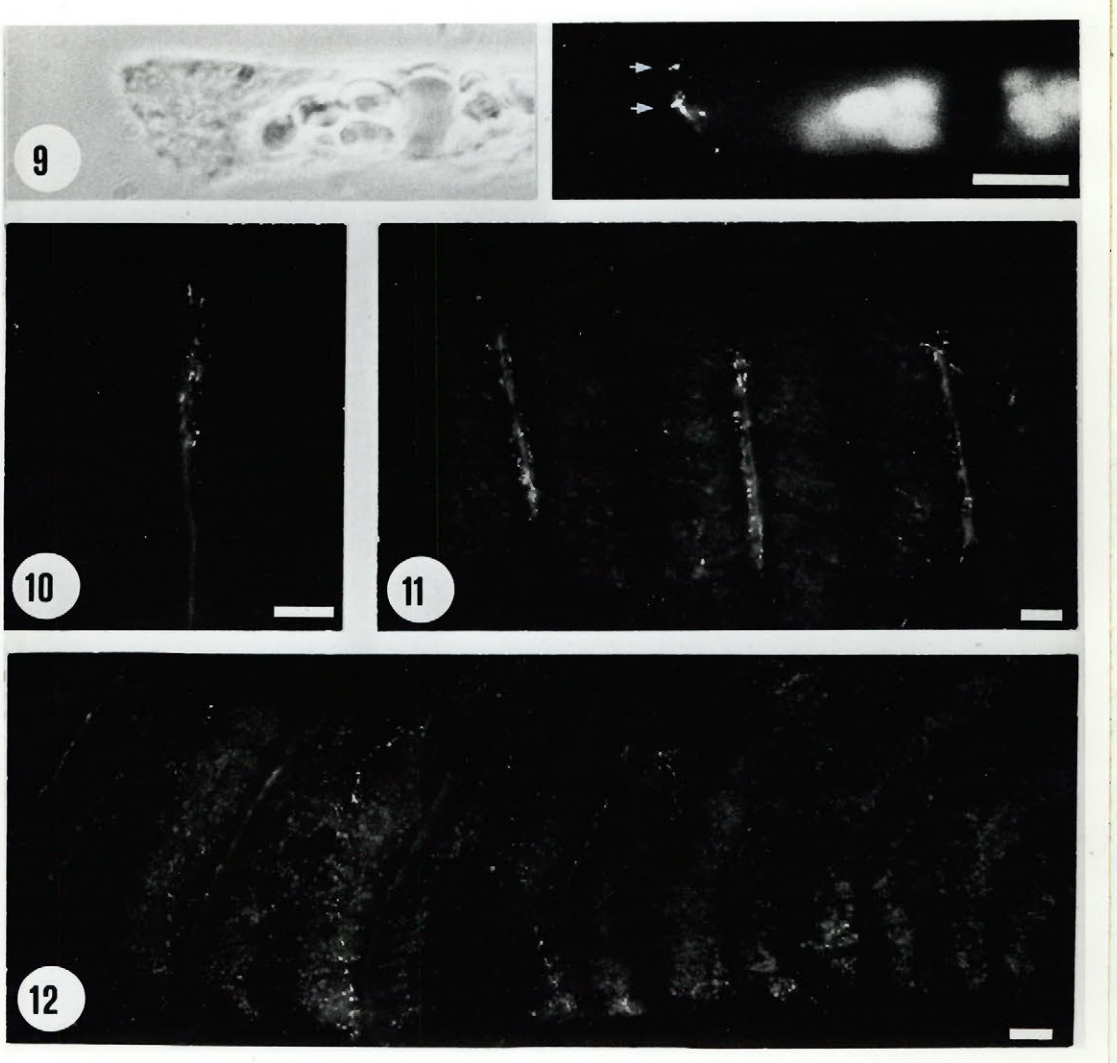

9
10
11
12

Fig. 13 Occurrence of ACh receptor stain at the ends of myotomes at different stages of development. Numbers in parentheses indicate the stages. Note the progressive increase with age in the percentage of myotomes with stain as well as the rostro-caudal differences. Numbers of half-tails examined were: 24 at stage 19, 37 at stage 21, 47 at stage 22, 45 at stage 24, 35 at stage 26, 15 at stage 28.

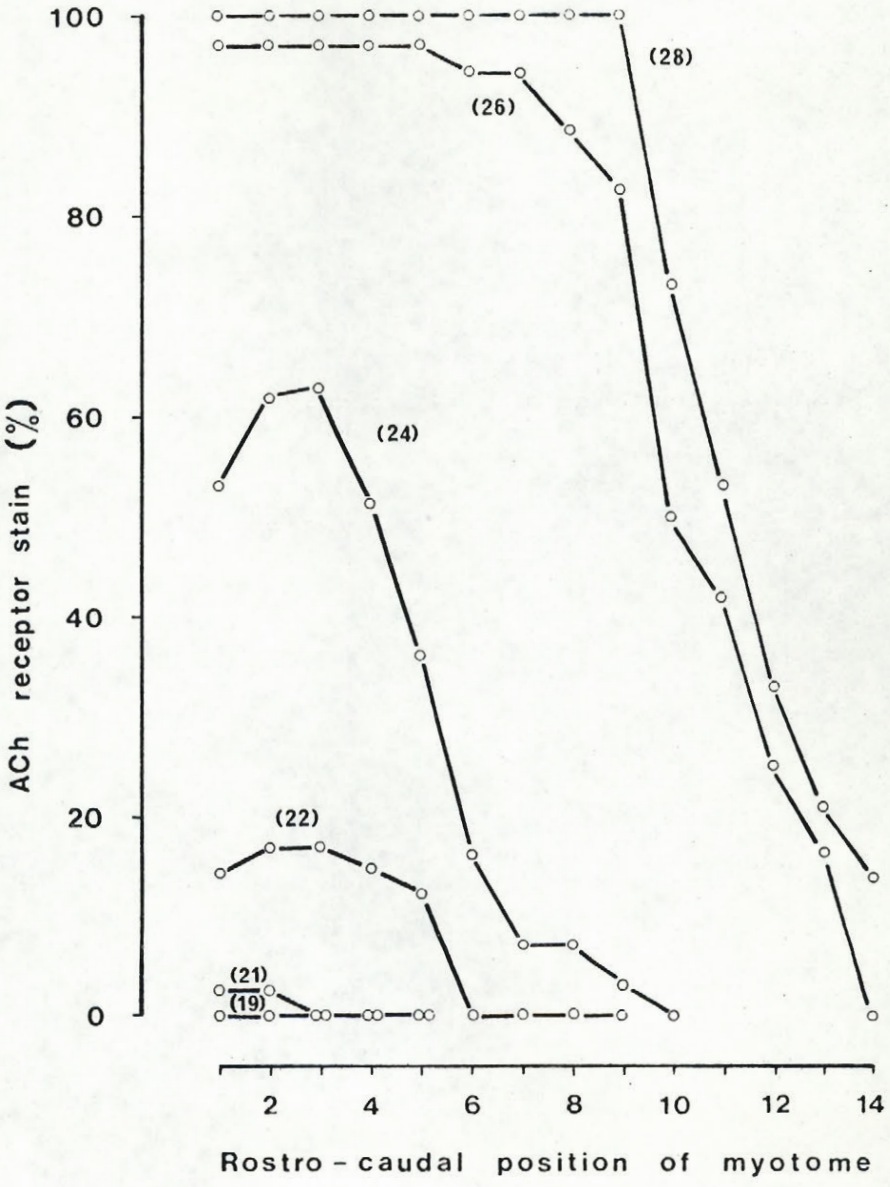
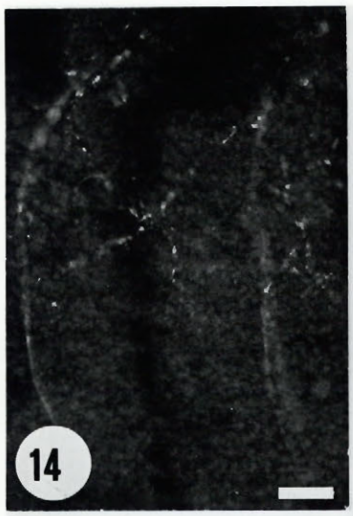
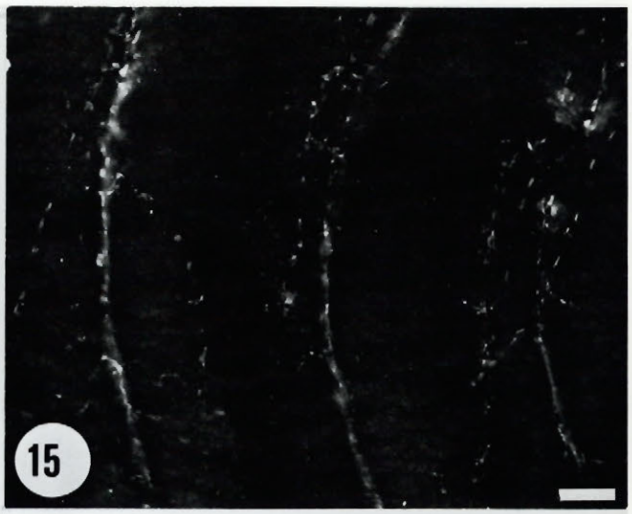

100
80
60
40
20
0
ACh receptor stain (%)
(28)
(26)
(24)
(22)
(21)
(19)
2
4
6
8
10
12
14
Rostro - caudal position of myotome

Fig. 14 Fluorescent patches forming path-like patterns across myotome of a stage 24 half-tail. Fluorescent glow and patches are also apparent at the ends of the myotomes. Calibration bar is 20 µm.

Fig. 15 Fluorescent patches forming path-like patterns acorss the myotomes of a stage 31 half-tail. Calibration bar is 20 µm.

Fig. 16 Patches of fluorescent stain (right) along the mid-region of a number of myotomal muscle cells of a stage 47 half-tail. Corresponding phase contrast micrograph (left) shows ChE activity at the same sites. Calibration bar is 10 µm.


14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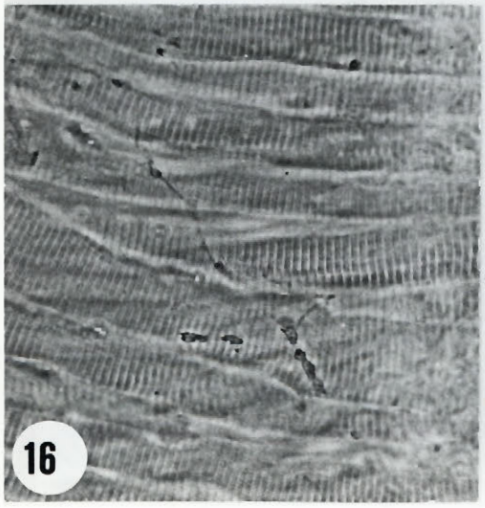

16

Fig. 17 Stage 24 half-tail stained with NBT. The ends of the first nine myotomes are darkly stained whereas the ends of the two most caudal myotomes (on the right hand side of the micrograph) are not stained. See text for further description. Calibration bar is 50 µm.

Fig. 18 Presence of fine nerve fibres (arrows) at the ends of stage 21 myotomal cells. Calibration bar is 20 µm.

Fig. 19 Stage 24 myotomes showing presence of fine nerve fibres (arrows) along with some diffuse staining at ends of myotomes. Calibration bar is 20 µm.

Fig. 20 Nerve fibres with varicosities at the ends of stage 49 muscle cells. Calibration bar is 10 µm.

Fig. 21 Segment of a stage 31 half-tail showing nerve fibres with varicosities at the intermyotomal junctions as well as along the myotomes. Calibration bar is 20 µm.

Fig. 22 Stage 41 half-tail showing a nerve fibre with varicosities coursing across the myotome. Calibration bar is 20 µm.

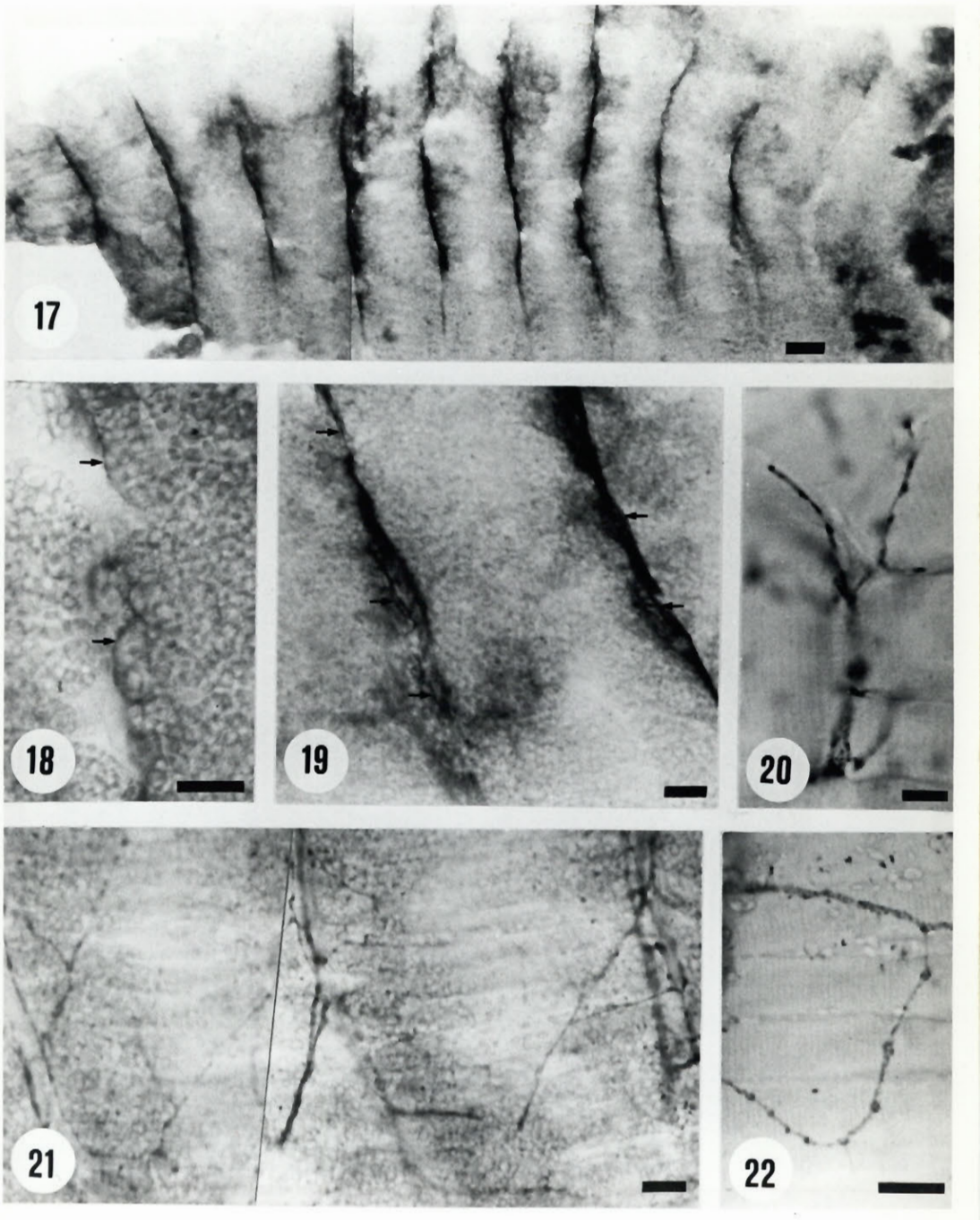
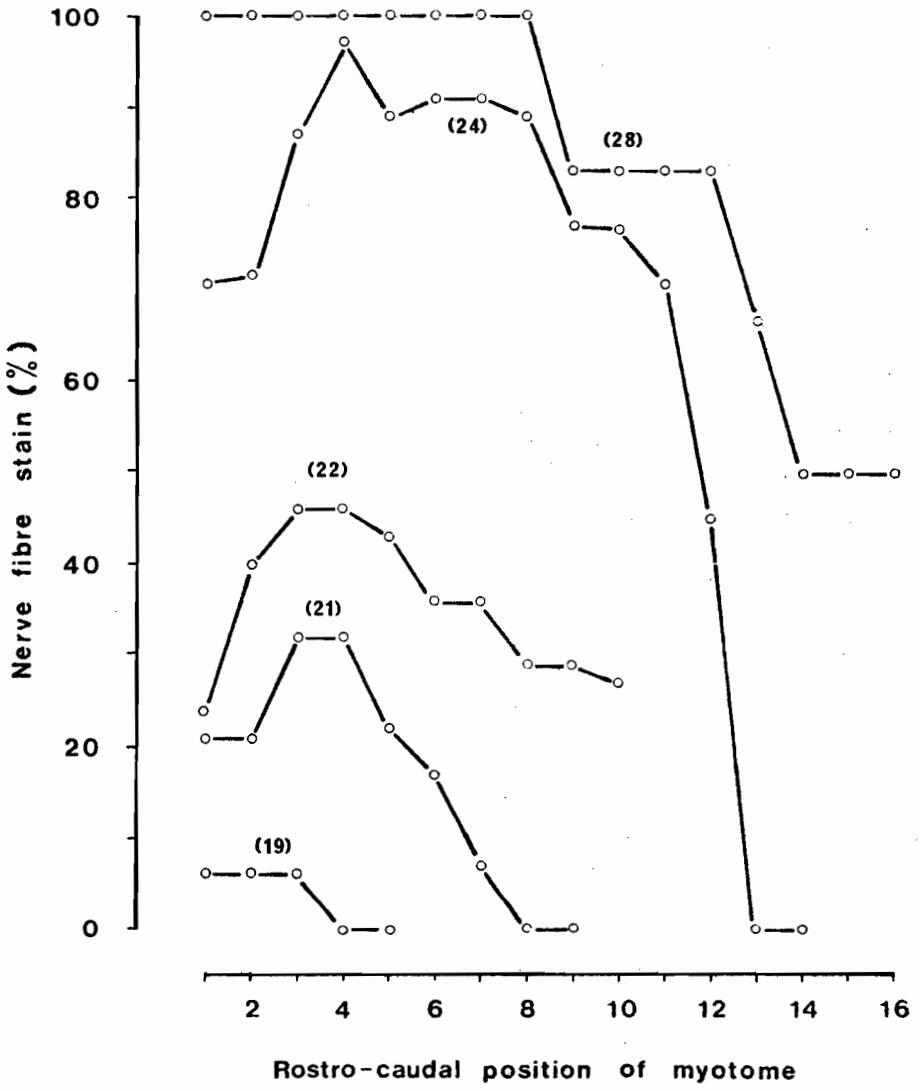

17
18
19
20
21
22

Fig. 23 Occurrence of nerve fibre stain at the ends of myotomes at different stages of development. Numbers in parentheses indicate respective stages. Note the progressive increase in the percentage of myotomes with nerve fibres as well as the rostro-caudal differences. Numbers of half-tails examined were: 16 at stage 19, 19 at stage 21, 28 at stage 22, 35 at stage 24, 7 at stage 28.


100
80
60
40
20
0
Nerve fibre stain (%)
(28)
(24)
(22)
(21)
(19)
2
4
6
8
10
12
14
16
Rostro-caudal position of myotome

Chapter III

EFFECTS OF DENERVATION ON SITES OF HIGH ACETYLCHOLINE RECEPTOR DENSITY IN DEVELOPING MYOTOMES OF *XENOPUS LAEVIS*

ABSTRACT

The relationship between innervation and the development of acetylcholine receptor patches at presumptive sites of synaptic contact was studied in developing Xenopus myotomes. When segments of developing spinal cord were removed from embryos at stages 19-20, re-innervation as judged by nerve fibre staining with nitroblue tetrazolium occurred within a day after the operation, and receptor patches were observed at the ends of the myotomes, the main site of innervation. Myotomes from embryos similarly denervated between stages 22-24 exhibited a marked reduction in nerve fibre staining one day later but immature nerve fibres were seen at the ends of some myotomes by the second day. Likewise the myotomes exhibited fewer receptor patches at their ends one day after spinal cord resection. Two and five days later the frequency of occurrence of these receptor patches increased but was still less than at the ends of control myotomes. Myotomes from which spinal cord was removed at stage 36 exhibited a progressive loss of nerve fibres and receptor patches at their ends one, two and four days later. Scattered receptor patches were observed along the myotomes. Unoperated animals kept anaesthetized for up to four days did not show any decrease in receptor patches at the ends of the myotomes. These data indicate that denervation at early stages of development disrupts the maintenance of acetylcholine receptor patches at presumptive synaptic sites and that such an effect is not due to the accompanying muscle inactivity.

INTRODUCTION

Studies on cell cultures of embryonic spinal cord and muscle have demonstrated that nerve fibres can induce the development of a high density of acetylcholine receptors (AChRs) at sites where they contact the muscle cells (Anderson et al., 1977; Anderson and Cohen, 1977; Frank and Fischbach, 1979). That such a process may also occur during the development of the neuromuscular junction in vivo is suggested by the findings that patches of high receptor density develop at presumptive sites of innervation after the arrival of the nerve fibres in developing muscle of rat foetuses (Bevan and Steinbach, 1977) and Xenopus embryos (Chow and Cohen, 1980). A nerve-induced maintenance of AChRs at sites of innervation has also been proposed for the normal development of chick neuromuscular junction (Jacob and Lentz, 1979).

If the development of a high receptor density at the neuromuscular junction is nerve-induced, then prevention of innervation might be expected to prevent the development of the usual sites of high receptor density. Furthermore, studies on cell cultures have indicated that the maintenance of the high receptor density at nerve-muscle contacts is dependent on the continued presence of the nerve (Cohen et al., 1979; Anderson and Klier, 1979). Accordingly one might also expect that denervation during early stages of neuromuscular synaptogenesis in vivo would lead to a corresponding loss of the high receptor density at the original synaptic sites. In the present study we have attempted to prevent the innervation of Xenopus myotomal muscle cells, and to denervate the cells at early stages of neuromuscular synaptogenesis, by removing appropriate portions of the spinal cord from embryos and young tadpoles.

Our results indicate that removal of spinal cord does lead to a marked decrease in the number of receptor patches at presumptive synaptic sites and that this effect is not due to the accompanying lack of muscle activity.

MATERIALS AND METHODS

Spinal Cord Resection

Embryos were removed from the mating tank less than a day after fertilization, and put in a solution consisting of 1/20 Ringer (see Chow and Cohen, 1980), 50 µg/ml Gentamycin (Schering), and 50 UI/ml Mycostatin (Gibco), until the time of operation. The latter two agents eliminate the fungal and bacterial contamination usually associated with the eggs (Laskey, 1970).

After staging according to Nieuwkoop & Faber (1967), the selected embryos were handled under sterile conditions. One embryo at a time was put in a Petri dish containing a layer of Sylgard 184 (Dow Corning) on its bottom. The embryo was positioned dorsal side up in a narrow tapering trough in the centre of the dish. The dish was filled with a solution consisting of 60% L-15 (Gibco), 0.25 µg/ml Gentamycin, and 0.25 UI/ml Mycostatin. The dorsal skin was slit open with a fine pin, exposing the spinal cord (SC) beneath it. The developing SC was separated from the adjacent tissues with the pin and transected just below the developing brain stem. It was then gently lifted away from a number of myotomes and removed. The operated animals were then transferred into another Petri dish with fresh solution, without Gentamycin. After collecting 4-6 of these operated animals they were put in another sterile Petri dish and maintained in 7 ml of L-15-Mycostatin solution until the skin grew back (usually 1-2 days). Subsequently the animals were transferred into 40 ml beakers containing 1/20 Ringer which was continuously aerated. Sham operations were also performed by slitting open the dorsal skin and

keeping the animals in the same solutions as the denervated ones. Control animals were kept in 1/20 Ringer solution throughout their development.

The extent of SC resection varied with the stage of the animal. At the earliest stages (19-24) only short lengths of SC could be removed and there was considerable variability in this regard. For example at stages 19-20 the resected portion usually extended for less than four myotomes but sometimes included more caudal regions where myotomes had not yet formed. On the other hand at stage 36 considerably longer lengths were consistently removed, usually for 8-12 myotomes.

The SC-resected animals were prodded daily with a fine pin on the skin over the myotomes from which the SC had been removed. If the animal responded with reflex contractions it was assumed that the denervation had been incomplete or that re-innervation had occurred, and the animal was discarded. In cases where there was no response, subsequent staining with nitroblue tetrazolium (NBT) sometimes revealed the presence of nerve fibres in the myotomes (see Results) thereby suggesting some interruption elsewhere along the reflex pathway. Sham animals developed normally and always responded to prodding by quickly swimming away.

Operated and control animals were staged and skinned, and "tail" preparations were isolated. Some of these preparations were stained for AChRs with tetramethylrhodamine labelled α-bungarotoxin (α-BuTX) (Anderson and Cohen, 1974; Anderson et al., 1977); in some cases they were subsequently stained for cholinesterase as well (Karnovsky and Roots, 1964). Other tail preparations were stained for nerve fibres with NBT (Letinsky and DeCino, 1978, and personal communication). A detailed description of these staining procedures can be found in the previous paper (Chow and Cohen, 1980). SC-free half-tails and controls which were

pretreated with unlabelled α-BuTX (2 μg/ml for 20 min) before exposure to fluorescent α-BuTX did not exhibit any staining along their entire length.

After fixation, the myotomes from which SC had been removed were identified in the dissecting microscope in each tail preparation so that "denervated" and innervated regions could subsequently be compared. In most experiments involving staining with fluorescent α-BuTX the SC-free portion of the half-tail was assessed as a whole. In some experiments the staining was also assessed for each of the myotomes within the SC-free portion.

The staining patterns in sham-operated animals were similar to those in normal animals and therefore no distinction is made between these two groups in the Results.

Immobilization

Embryos were freed from their jelly coat and vitelline sac at stages 17-20, at stage 24, and at stage 36. The animals were then transferred into beakers containing a solution of MS-222 (tricaine methanesulphonate, Sandoz; 10^{-4}g/ml) in 1/20 Ringer which had been previously neutralized with 0.1 M NaOH solution, using phenol red as a pH indicator. This anaesthetic solution was continuously aerated and was changed every 8-12 hours. The animals were kept in it for 1-4 days and were then stained for AChRs, cholinesterase activity and nerve fibres as described above. Three to six animals were used for each stage and for each period of exposure to the anaesthetic solution. Control animals were kept in 1/20 Ringer.

The animals in MS-222 remained immobile even when prodded daily with a fine pin. Just before staining they were briefly returned to

anaesthetic-free 1/20 Ringer in order to assess development of functional nerve-muscle contacts. In all cases the animals regained motor behaviour in 5-20 min.

RESULTS

The initial aim of these experiments was to determine whether high receptor densities develop at presumptive sites of innervation when innervation is prevented. In order to prevent innervation segments of SC were removed from embryos at stages 19-20, when nerve fibres are just beginning to invade the myotomes (see Chow and Cohen, 1980). However it became apparent that we could not consistently achieve aneural myotomes at these stages for even as little as one day. The experiments were repeated on stage 22-24 embryos, with more success, but even in these cases myotomes did not consistently remain without nerve fibres for more than one day. We therefore repeated the experiments on stage 36 animals in which larger segments of SC could be removed. The results are presented below according to the stage at which SC was removed. Some general observations on the effects of SC resection are described first.

Spinal Cord Resection

General observations

The tails of animals with SC resected continued to grow after the operation and were sometimes even longer than controls. Judged by other external criteria (Nieuwkoop and Faber, 1967) the animals appeared to develop at a normal rate for up to two days but by the third and fourth days lagged behind sham and normal animals by as much as two stages (up to 1 day). However all SC-resected animals exhibited grossly abnormal swimming behaviour and were unable to propel themselves for more than a few millimetres even when prodded in the head or distal tail region. In fact, in most cases the portion of the tail beyond the region of the

operation was curved dorsally and even when it was prodded the resulting reflex contractions were ineffective in causing any consistent motion in one direction. Despite these severe motor deficits most animals survived for three days, and some survived for more than five days. Some of the surviving animals also exhibited additional abnormalities by the fourth day such as a puffed head and abdomen, and these animals were discarded.

SC-resected animals were followed for up to five days. They were then staged and skinned, and tail preparations were isolated from them. The skin in the region of SC resection often lacked melanophores and proved to be more adherent to the myotomes thereby making the skinning procedure more difficult and increasing the chances of damage to the surface myotomal muscle cells. The rate of consumption of yolk granules was also reduced in the myotomes of denervated animals and when viewed with fluorescence optics these myotomes usually exhibited more background fluorescence.

Resection at stages 19-20

Developing SC was removed from a total of thirty-three embryos. When tested one day later nine of these animals were responsive to prodding of the skin overlying the SC-free myotomes and were discarded (see Methods). By two days only one of thirteen animals was responsive. In those cases where the animals did not respond the myotomes were then examined for AChR patches or for nerve fibres.

Despite the lack of reflex behaviour nerve fibres were observed at the ends of most of the SC-free myotomes as well as along the lengths of the myotomes. Four SC-free half-tails were examined one day after SC resection and all had a few nerve fibres at the ends of their myotomes.

By two days the two SC-free half-tails examined exhibited significant numbers of nerve fibres. On both days it appeared that some of the SC-free myotomes had fewer nerve fibres at their ends than controls. In such cases there was often additional diffuse staining at the ends of the myotomes and the nerve fibres lacked prominent varicosities (Fig. 1). These latter nerve staining features are also seen at earlier stages during normal development (Chow and Cohen, 1980) and suggest that the nerve fibres were less mature than in controls. It is therefore apparent from these results that spinal cord resection at stages 19-20 resulted in no more than a short-lived and incomplete denervation. Since the developing SC is quite soft at these stages small fragments of the resected SC may have remained in the animals thereby contributing to incomplete denervation of the apposed myotomes. In addition there may have been invasion by nerve fibres from neighbouring regions with intact SC.

The pattern of receptor localization was examined in SC-free and control myotomes after staining with fluorescent α-BuTX. A total of 14 half-tails were examined one day after spinal cord resection and 20 half-tails were examined on the second day. In all cases the SC-free myotomes exhibited similar patterns of receptor staining as controls. As shown in Fig. 2 patches of fluorescent stain were seen at the ends of the SC-free myotomes and also occurred as interrupted narrow bands along the myotomes. Such patterns are similar to those seen during normal development (Chow and Cohen, 1980). Although quantitative differences are difficult to exclude, no marked reduction in the number of receptor patches at the ends of the SC-free myotomes was apparent. However, in view of the lack of reliability in eliminating nerve fibres from the myotomes (see above) the present results do not permit any definite conclusions regarding the

dependence of the receptor patches on innervation.

Resection at stages 22-24

Two of seventy-eight animals responded to prodding in the SC-free region one day after the operation, and they were discarded. By two days eleven of forty-nine animals which had been allowed to survive were discarded for the same reason. Ten animals which survived for five days did not respond to prodding of the skin over the SC-free myotomes. The animals which did not respond to prodding were then staged, skinned and stained for ACh receptor patches, cholinesterase activity and nerve fibre.

One day after SC resection two SC-free half-tails were examined after staining with NBT and in each case very few nerve fibres were seen. Examples are illustrated in Figs. 3 and 4, and stand in contrast to the much more mature pattern of nerve fibres seen in control myotomes (Fig. 5). Myotomes located near the ends of the resected region tended to have more nerve fibres (arrowheads) than those in the central region (arrows) (Fig. 3). On the second day after SC resection all SC-free myotomes from two half-tails examined exhibited some nerve fibres at their ends (Figs. 6 and 7) but not as many as at the ends of control myotomes (Fig. 8). In addition, the fibres in the SC-free myotomes lacked varicosities and there was also diffuse staining at the ends of SC-free myotomes (Figs. 6 and 7). Since these features are seen at earlier stages during normal development (Chow and Cohen, 1980) and since there was a virtual absence of nerve fibres on the first day after SC resection it seems likely that re-innervation of the myotomes had begun by the second day.

A marked reduction was also observed in the number of AChR patches at the ends of SC-free myotomes one and two days after SC resection. Examples of a control half-tail (Fig. 9) and of SC-free (Figs. 10 and 12)

and innervated (Fig. 11) regions of half-tails two days after resection are shown. They reveal that in some cases there was a virtual absence of receptor patches at the ends of the SC-free myotomes (Fig. 12). In other cases the ends of the SC-free myotomes had a scattering of small receptor patches (Figs. 10 and 13). One day after SC resection in 14 half-tails in which each myotome was assessed separately it was found that 77% of the SC-free myotomes (116) exhibited a marked reduction in the number of receptor patches at their ends. These myotomes were located in the centre of the SC-free regions and presumably were the ones where denervation was most complete. Altogether a total of 50 half-tails were examined one day after cord resection and each one contained SC-free myotomes which exhibited these marked reductions in receptor staining at their ends. By two days after cord resection 80% of the half-tails examined (42) still exhibited reduction in receptor patches, but not as pronounced as on day one. Seven of these half-tails were examined separately and 72% of the SC-free myotomes (71) showed reduction in receptor patches at their end regions. In addition 20 half-tails were examined from animals which survived for 5 days after SC resection. Fifty percent of these exhibited no apparent reduction in the occurrence of receptor patches at the ends of the SC-free myotomes and in the other half-tails, although some reduction was apparent there were no regions with an absence of receptor patches as seen after one and two days. It thus appears that with the return of the nerve fibres there was also a new development of receptor patches at the ends of the myotomes which had originally been denervated.

Differences between SC-free and control myotomes were also observed with respect to the patterns of receptor staining along the lengths of the myotomes, especially one and two days after cord resection. As shown

in Fig. 14 some of the SC-free myotomes exhibited large numbers of receptor patches along their length but these sites appeared to be scattered in a random fashion and never took the form of narrow interrupted bands that typically occur in control myotomes. These scattered receptor patches were seen more frequently on the second day after SC resection and may be analogous to those which develop on non-innervated myotomal muscle cells in culture (Anderson et al., 1977). Interestingly about 35% of these receptor patches were also sites of cholinesterase activity (Table 1). Many of the receptor patches on non-innervated muscle cells in culture are also sites of cholinesterase activity (Moody-Corbett and Cohen, 1979).

Resection at stage 36

None of the seventy-two tadpoles operated at stage 36 responded to prodding of the skin over the SC-resected region throughout the experiment, indicating a more effective cord removal than at earlier stages. The effectiveness of the SC resection was confirmed after staining with NBT. On the first day 8 operated half-tails were examined and 94% of their SC-free myotomes (75) exhibited a noticeable reduction in the number of nerve fibres at their ends. Similar changes were seen in 83% of the SC-free myotomes (62) examined in 6 half-tails two days after SC resection. By four days nerve fibres were virtually absent in all the SC-free myotomes (40) from 3 half-tails. Figs. 15 to 17 show a progression of this reduction in nerve fibre staining from the day of operation to four days later. Unlike nerve fibres in stage 36 myotomes (Fig. 15) those in SC-free myotomes one day after SC resection (Fig. 16) generally lacked varicosities and did not extend into the central regions of the myotomes. By four days nerve fibres were rarely seen and consisted of

only very short segments at the ends of a few myotomes (Fig. 17).

Similar progressive changes were also seen after staining with fluorescent α-BuTX. Two of the four half-tails examined one day after SC resection showed some reduction in receptor patches at the ends of SC-free myotomes. Fourteen half-tails were examined on the second day after SC resection and 70% of these exhibited a marked reduction in the number of receptor patches at the ends of the SC-free myotomes. When examined separately 67% of the SC-free myotomes (18) from 2 operated half-tails showed much fewer fluorescent patches at their ends than the controls. In some cases there was almost a complete absence of receptor patches whereas in other cases a few scattered patches were observed at the ends of the myotomes (Figs. 18 and 19). In addition non-aligned receptor patches were also observed along the length of the SC-free myotomes (Figs. 18 and 19). Similar marked changes were seen in each of 10 half-tails four days after SC resection, at the ends of the SC-free myotomes as well as along their length (Figs. 20-23). Many of these receptor patches along the SC-free myotomes were also found to be associated with cholinesterase activity (Fig. 24, Table 1).

Immobilization

Animals maintained in MS-222 remained immobilized and appeared to develop normally except that after 4 days some lagged behind control animals by up to one stage. All immobilized animals regained their mobility within 5-20 min when removed from anaesthetic. They were then skinned and stained for AChRs or nerve fibres. In the immobilized animals the skin was more adherent to the myotomal cells, making its removal more difficult, especially in animals which were kept in anaes-

thetic for 3 or 4 days. The preparation of half-tails after fixation was also more difficult than in the normal animals because the notochord was also more adherent to the myotomes. More yolk granules were also found in the immobile animal preparations.

Although treatment with MS-222 began at different stages of development (see Methods), the effects were basically similar. In view of this the changes observed for the different stages at various time intervals will be described together.

Nerve fibres were found at all intermyotomal junctions one day after the beginning of immobilization, and the staining pattern was similar to the one seen in the control animals. However, less stain was seen in animals immobile for two days and by three days no more stain was found in any anaesthetized animal. As all animals recuperated from anaesthesia within 5-20 min when put in solution without MS-222, it is clear that the myotomes were in fact innervated. However we have no explanation why long-term anaesthesia interferred with staining of nerve fibres by NBT.

Brightly stained receptor patches were found at the ends of all myotomes in animals kept immobile for one, two and four days without apparent reduction in their number (Figs. 25 and 26). The frequency of aligned fluorescent patches along the myotomes was also at least as great as in control animals. It is therefore apparent from these findings that the development and maintenance of high receptor densities at presumptive synaptic sites does not require muscle activity. This is in agreement with previous studies on cultured myotomal muscle cells (Anderson and Cohen, 1977; Anderson et al, 1977).

DISCUSSION

Survival of Receptor Patches at Presumptive Synaptic Sites After Denervation

The present study has shown a progressive loss of nerve fibres after SC resection in stage 36 tadpoles and a concomitant decrease in AChR patches at the original sites of synaptic contact. This suggests that the maintenance of receptor patches at the innervation sites was discontinued shortly after the onset of denervation. Similar observations have also been reported in cultures where patches of AChRs at sites of nerve-muscle contact disappeared within a day after spontaneous withdrawal or section of the neurite (Anderson and Cohen, 1977; Cohen *et al.*, 1979; Anderson and Klier, 1979). These results suggest that both *in vivo* and in culture the maintenance of high receptor density at sites of nerve-muscle contact is dependent on the continued presence of the nerve fibre, at least during the first day or so after contact is established.

Whether the disappearance of these synaptic aggregates of AChRs was a result of muscle inactivity was tested by immobilizing the animals with an anaesthetic solution (MS-222) for up to four days, and no disruption of receptor patches at the myotomal end regions was observed. Thus, in spite of the lack of muscle activity the continued presence of intact nerve fibres was sufficient to maintain the receptor patches at the innervation sites. Synapse formation and accumulation of AChRs at the contact sites likewise occur in cultures of *Xenopus* myotomal muscle and SC cells in the presence of curare or α-BuTX (Cohen, 1972; Anderson and Cohen, 1977; Anderson *et al.*, 1977; Cohen *et al.*, 1979). All these results suggest

that muscle activity is not essential for the accumulation of AChRs at the synaptic sites.

A second possibility for the disruption of receptor patches is the formation of toxic substances as a result of nerve fibre degeneration after SC resection. It has been suggested that the higher ACh sensitivity detected after a small piece of silk thread (Jones and Vrbová, 1974) or a segment of degenerating nerve (Jones and Vyskočil, 1975; Lömo, 1976; Lömo and Westgaard, 1976) is placed on the adult rat soleus muscle is a result of substances released by active phagocytes which invade that region. As no Schwann cell is found in association with the motor nerve fibres in Xenopus myotomes until after stage 36 (Kullberg et al., 1977), phagocytosis of the degenerating nerve fibres in the present experiments may have been delayed and some neural product of the degenerative process may have contributed to the disappearance of the junctional receptor patches.

An alternative explanation is that denervation results in the removal of some neural factor which is essential for the continued maintenance of the patches. Many authors have described experiments where the action of neurotrophic substance(s) on skeletal muscle properties is suggested (see reviews by Guth, 1968; Harris, 1974; Gutmann, 1976, 1977; Fambrough, 1976, 1979). However, most of these studies dealt with extrajunctional ACh receptors and sensitivity, and with other properties of the muscle fibres. The neurotrophic influences on the localization of junctional ACh sensitivity or receptor patches has been investigated only in a few culture studies. Cohen & Fischbach (1977) have shown that muscle fibres cultured near cholinergic nerve fibres have higher ACh sensitivity than more distant muscle cells. They suggested that a diffusible substance from the nerve explant induces the higher ACh sensitivity.

Jessell, Siegel & Fischbach (1979) found that extracts of brain and ciliary ganglia increase the number of AChRs and receptor clusters on cultured chick myotubes. Recently, Cohen et al. (1979) have shown in studies on Xenopus embryonic myotomal muscle cells cultured with embryonic SC cells, or with explants of sympathetic ganglia (SG) of juvenile animals or dorsal root ganglia (DRG) of tadpoles that only SC neurites were capable of inducing receptor accumulation on the muscle cells. They suggested that SC neurites contain a specific property which is lacking in DRG and SG neurites and which is important for inducing and maintaining receptor localization at sites of nerve-muscle contact. The present findings are therefore most simply explained by assuming that SC resection caused the nerve fibres to degenerate terminating the action of a neural factor for the maintenance of receptor patches at presumptive synaptic sites.

In adult skeletal muscle denervation does not cause marked disruption of receptor clusters at the original synaptic sites. For example, postsynaptic ACh sensitivity remained practically normal for many weeks after denervation in frog muscle whereas the nerve terminals degenerated within one week (Birks et al., 1960b; Miledi, 1960). High junctional ACh sensitivity remained stable at the original synaptic regions for sometime after denervation in frog muscle (Dreyer and Peper, 1974b); in rat muscle (Axelsson and Thesleff, 1959) and in mouse muscle (Albuquerque and McIsaac, 1970). Similarly, the number of receptors at the original endplates remained constant for at least two weeks after denervation in rat muscle (Hartzell and Fambrough, 1972; Frank et al., 1975), and so did the junctional receptor patches in mouse muscle (Ko et al., 1977). This persistence of the adult junctional receptor may result in part from its

rather slow turnover rate (Berg and Hall, 1975a; Chang and Huang, 1975; Fambrough et al., 1977; Burden, 1977b). Studies by Burden (1977a,b) on developing chick wing muscles have revealed a fast turnover of junctional receptors for at least 2 weeks after the formation of the neuromuscular junction. During this period the half-life of the receptors is 30 hr whereas by 3 weeks after hatching the half-life is greater than 5 days. It is possible that a fast turnover rate of receptors is also found at the developing synaptic sites in Xenopus myotomal muscle and this may have contributed partly to the fast disappearance of receptor patches seen in the present study.

Another contributing factor is suggested by studies on receptor mobility. Axelrod et al. (1976) have shown that AChRs that aggregate in clusters on cultured rat myotubes do not move freely, and that synaptic AChRs in adult mouse muscle fibres are also relatively immobile. Fambrough and Pagano (1977) have made similar observations at the rat diaphragm neuromuscular junction. It may be that motor nerve fibres act to immobilize the synaptic receptors at early stages of development as well (Anderson and Cohen, 1977) whereas at later stages other factors such as components in the basal lamina play the same role (Burden et al., 1979). Therefore at early stages in the development of the neuromuscular junction the presence of the nerve would be crucial for the continued maintenance of the high receptor density and in its absence the receptors would disperse in a manner analogous to that found for receptor patches on cultured rat myotubes after treatment with metabolic inhibitors (Bloch, 1979).

Additional relevant information is suggested by the denervation experiments of Lömo and Slater (1976a, b). They induced ectopic

innervation in adult rat soleus muscle by cutting the soleus nerve and implanting the "foreign" fibular nerve over the muscle. Two to four days later they cut the foreign nerve and examined ACh sensitivity in the muscle fibres over the next few days. They found spots of higher sensitivity in the region of the degenerating fibular nerve. Acetylcholinesterase spots also developed at these sites if the muscles were stimulated directly during that period. They suggested that these spots correspond to the early synapses made by the foreign nerve before it degenerated and that the high ACh sensitivity of the postsynaptic membrane becomes stable at very early stages of synaptogenesis. Thus, the presumptive synaptic patches in these experiments were much more stable than the ones during the early development of the myotomal neuromuscular junction. Although the junctional receptor patches are newly-formed in both cases, the age of the muscle and nerve is quite different. It is possible that some intrinsic properties develop with age in the muscle cells which enhance the stability of the junctional receptors after nerve contact, regardless of the period during which nerve and muscle remain in contact. Also the age of the respective nerves is quite different, and the same arguments used for muscle can be applied to the innervating fibres. Another possibility is simply the species difference and at the moment there is no other evidence to assess these alternatives.

A more parallel comparison can be made with Slater's recent report (1978) on resection of the phrenic nerve in mouse within 24 hours of birth. He found that it was still possible to stain the previous endplates with fluorescent α-BuTX 5 weeks later, although the staining intensity was weaker than seen in normal 5-week-old mice and at birth. The author suggested that the normal pattern of staining apparently depends

on the continued presence of the nerve. Thus, it seems that the maintenance of patches of high receptor density at the innervation sites of developing Xenopus myotomal and mouse diaphragm muscle cells depends on the continued presence of their motor neurones. The much more rapid disappearance of the receptor patches in Xenopus may be attributable to the fact that the neuromuscular junctions were denervated when they were 1-day-old, whereas in the mouse they were probably 5-6 days old (see Bevan and Steinbach, 1977).

Formation of Receptor Patches at Presumptive Sites of Re-innervation and Innervation

At stages 19-20 only short segments of SC could be removed and the absence of nerve fibres was at most short-lived. An immature type of nerve fibre staining was already found one day after the cord resection, and by the following day the number of nerve fibres along the intermyotomal junctions had increased. Similarly, bright fluorescent stain was found at the intermyotomal junctions one and two days after SC resection. At stages 19-20 there are no receptor patches and nerve fibres are rare (Chow and Cohen, 1980). The fluorescent patches found along the intermyotomal junctions one day after cord resection cannot be due to previous innervation. Presumably they developed as a result of new innervation. Alternatively it could be argued that the process of receptor patch formation continues at the ends of the myotomes independently of the presence of nerve fibres, and that the absence of receptor patches there when cord resection is performed at later stages is due to their disruption by the degenerating nerve fibres. However such an explanation would still not account for the formation of the aligned receptor patches which

were seen along myotomes during normal development as well as in cases where nerve fibres had grown into the myotomes after cord resection.

All the embryos which had their developing SC resected at stages 22-24 had few nerve fibres along the corresponding myotomes one day later and also exhibited a marked reduction in AChR patches at the ends of the myotomes. On the second day most of the SC-free myotomes showed a few immature-type nerve fibres, similar to the pattern seen at intermyotomal junctions of normal embryos around stages 24-26. Only fine fibres, with no varicosities were seen at these regions. This suggests the arrival of new nerve fibres from adjacent innervated segments or from remaining fragments of SC. By the second day there was still a marked reduction in the number of receptor patches at presumptive synaptic sites whereas by the fifth day 50% of the half-tails appeared to have normal staining patterns. These results suggest that the arrival of nerve fibres caused receptor patches to form again at the ends of the myotomes.

As shown in the previous paper, myotomes at stages 22-24 are not fully innervated. Presumably the cord resection at these stages not only denervated already-innervated myotomes but also prevented further arrival of nerve fibers for a period of 1-2 days. During normal development patches of high receptor density begin to form at the ends of the myotomes at stage 21 and only by stage 28 are most of the intermyotomal junctions stained with fluorescent α-BuTX. Therefore at stages 22-24 receptor patch formation is still incomplete. This suggests that many of the fluorescent patches seen on the second day and probably all of the patches seen on the fifth day along intermyotomal junctions were formed under the influence of the newly-arrived nerve fibres.

Nerve-induced receptor localization at developing synaptic sites has previously been reported during ectopic re-innervation of adult skeletal muscle in rat (Lömo and Slater, 1976a,b) and has also been shown in cultures of embryonic muscle and nerve from chick (Frank and Fischbach, 1979) and from Xenopus (Anderson et al., 1977; Anderson and Cohen, 1977). The studies on Xenopus cultures further revealed that muscle impulse or contractile activity is not essential for this process. The present observations on embryos which were kept anaesthetized from an age prior to the onset of motor behaviour (stage 24) confirm that receptor localization at synaptic sites in vivo also occurs in the absence of muscle activity.

Development of Extrajunctional Receptor Patches

It is well known that denervation causes hypersensitivity of the adult skeletal muscle fibres to ACh which is related to the appearance of new receptors throughout the extrajunctional regions of the fibres (see reviews by Guth, 1968; Harris, 1974; Gutmann, 1976; Fambrough, 1979). Patches of higher extrajunctional ACh sensitivity (Katz and Miledi, 1964a; Albuquerque and McIsaac, 1970, Dreyer and Peper, 1974b) and of higher extrajunctional ACh receptor density (Ko et al., 1977) have also been observed.

In the present study denervation also appeared to result in the development of extrajunctional receptor patches. These patches appeared to be randomly distributed and occurred under conditions where SC resection was effective in producing a maintained denervation. Thus, it seems that as in adult denervated muscle, the developing myotomal cells also acquire receptor patches after denervation. Interestingly many of these patches

were also associated with cholinesterase activity. A similar association was also found on Xenopus myotomal cells cultured in the absence of nerve (Moody-Corbett and Cohen, 1979). It thus appears that Xenopus myotomal muscle cells are capable of synthetizing and localizing AChRs and cholinesterase in the absence of nerve fibres. Once the motor nerve arrives it appears to specify where these sites of localization develop.

Table 1

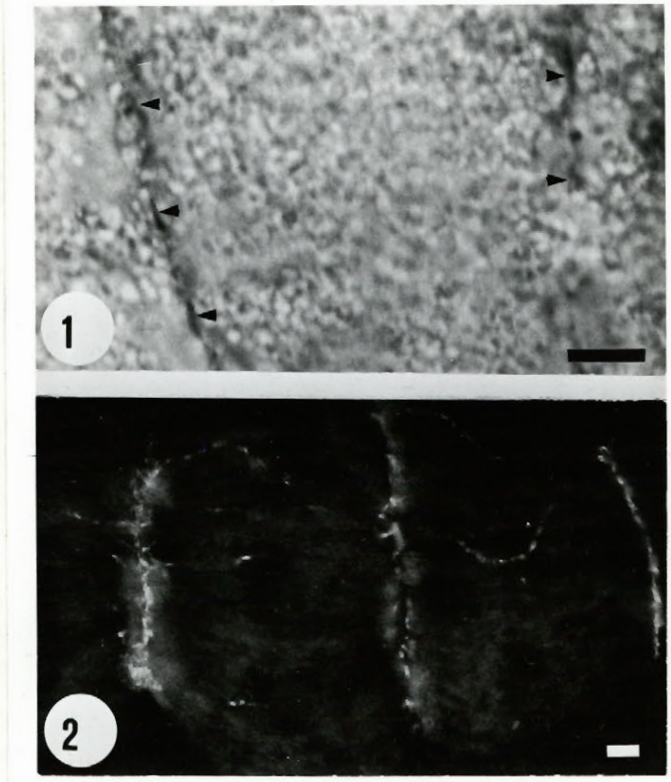
Occurrence of AChR patches associated with cholinesterase activity along the SC-free myotomes from animals operated at stages 24 and 36.

Stage* at time of SC resection	Duration of SC resection (days)	Corresponding stage	No. of AChR patches examined	% of patches with cholinesterase
24	1	36	37	35
24	2	42	200	36
36	2	42	1048	46
36	4	47	1598	75

* Nieuwkoop and Faber, 1967

Fig. 1 Myotomes of a stage 36 half-tail stained with NBT, one day after SC resection at stage 19. Note the short segments of nerve fibres at the ends of the myotomes (arrowheads). Many yolk granules are found in the myotomes. Calibration bar is 20 μm.

Fig. 2 Myotomes of a stage 36 half-tail, stained with fluorescent α-BuTX, one day after SC resection at stage 19. Note the bright fluorescent stain at the intermyotomal junctions as well as along the myotomes forming path-like patterns. A few isolated fluorescent patches are also seen. Calibration bar is 20 μm.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Figs. 3-8 Segments of half-tails stained with NBT, one (Figs. 3-5) and two (Figs. 6-8) days after SC resection at stage 24. Calibration bar for Figs. 3 and 6 is 50 µm and is 20 µm for Figs. 4, 5, 7 and 8.

Fig. 3 SC-free segment of a half-tail (stage 34) one day after SC resection. Note the presence of a few fine fibres (arrowheads) and the virtual absence of nerve fibres along the three central intermyotomal junctions (arrows).

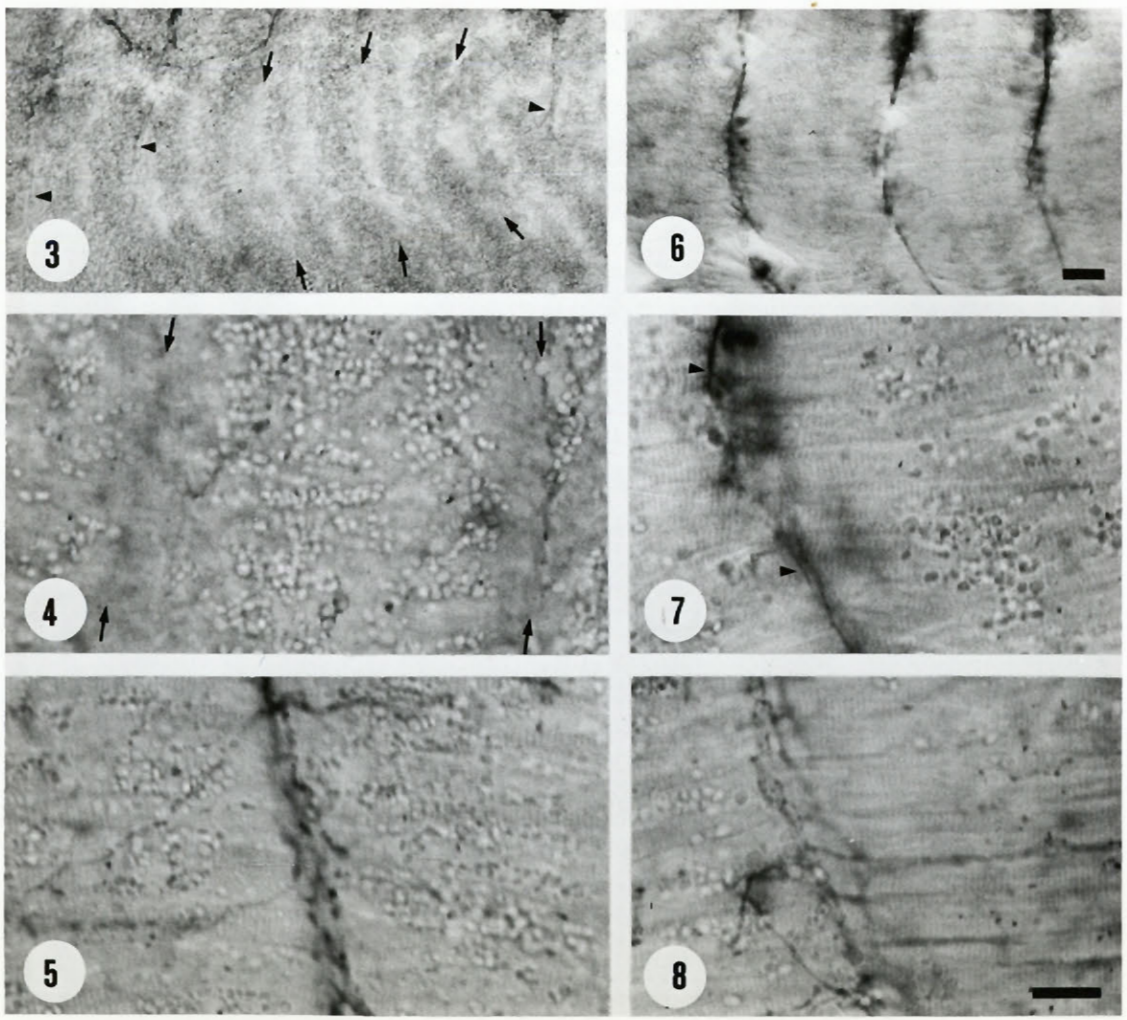
Fig. 4 Higher magnification of an one-day SC-free myotome showing the lack of nerve fibres at the intermyotomal junctions (arrows).

Fig. 5 Control myotome (stage 34) showing nerve fibres with varicosities at the intermyotomal junctions, and branches coursing over several myotomal cells.

Fig. 6 SC-free segment of a half-tail (stage 39) two days after SC resection. Note the immature type staining of nerve fibres along the intermyotomal junctions, associated with some diffuse staining.

Fig. 7 Higher magnification of a two day SC-free myotome showing the fine nerve fibres (arrowheads) at the intermyotomal junction. This intermyotomal junction corresponds to the one on the right in Fig. 6. Some diffuse stain is also present.

Fig. 8 Control innervated myotome (stage 39) showing the nerve fibres with varicosities and branches.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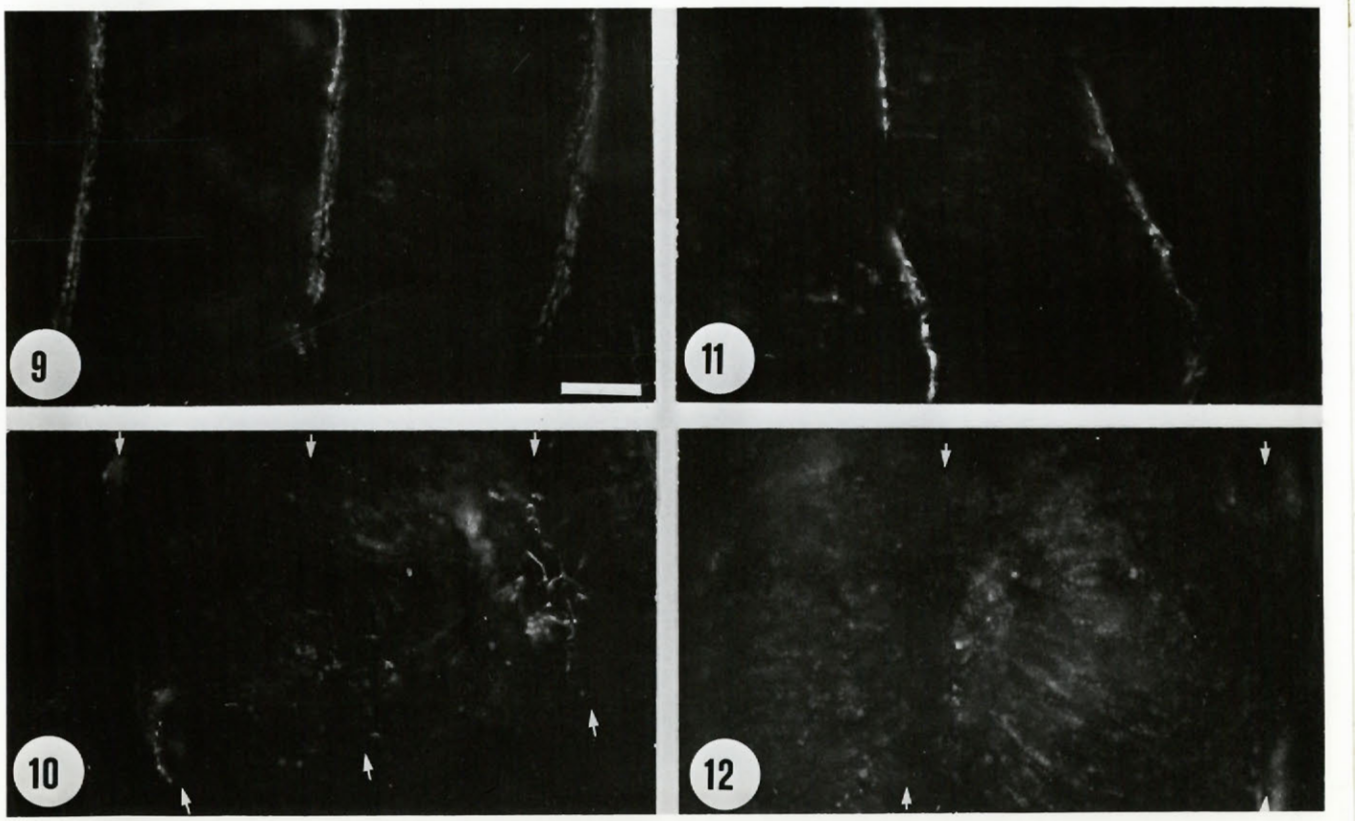
Figs. 9-12 Segments of stage 40 half-tails showing patches of high AChR density, after staining with fluorescent α-BuTX. Calibration bar is 50 μm.

Fig. 9 Segment of a control preparation showing bright fluorescence along the whole length of the intermyotomal junctions.

Fig. 10 SC-free segment of a half-tail two days after SC resection in a stage 24 embryo. Note the sparsity of staining at the intermyotomal junctions (arrows).

Fig. 11 Innervated segment of a half-tail two days after SC resection in a stage 24 embryo. The staining pattern is normal. The gap along one of the intermyotomal junctions is due to separation of myotomal cells during preparation of the slide.

Fig. 12 SC-free segment of the same half-tail of Fig. 11 showing the lack of fluorescent staining along the intermyotomal junctions (arrows).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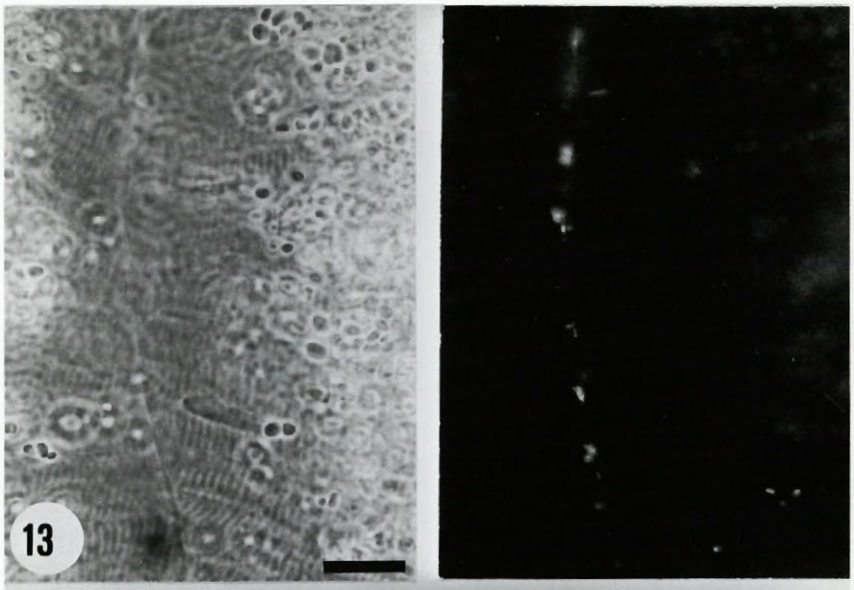
Figs. 13, 14 Phase contrast (left) and corresponding fluorescence (right) micrographs of stage 40 myotomes 2 days after SC resection at stage 24. Calibration bar is 20 μm.

Fig. 13 SC-free intermyotomal junction showing small fluorescent patches along the junction. Note the discontinuities in the staining along the junction.

Fig. 14 SC-free myotome showing isolated fluorescent patches scattered along the length of the myotome.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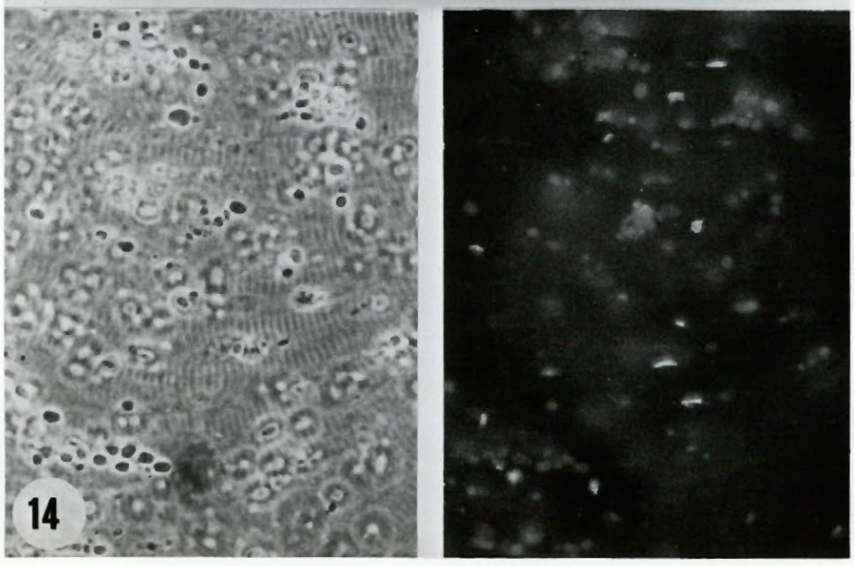
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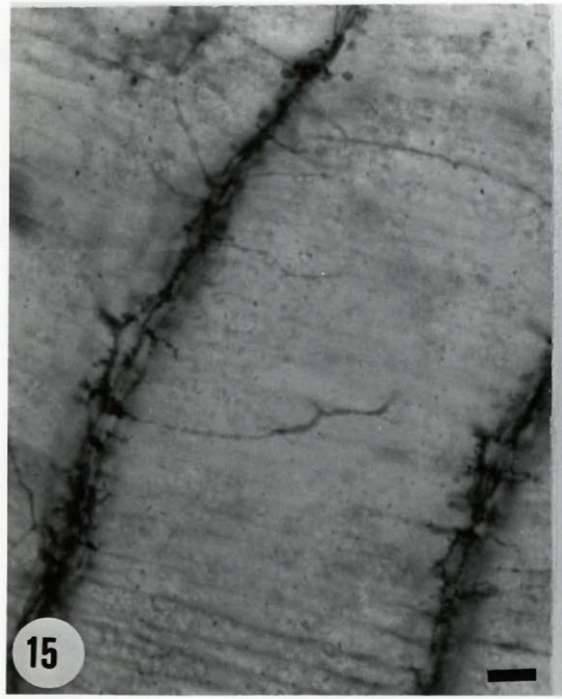
Figs. 15-17 Segments of NBT-stained half-tails. Calibration bar is 20 μm.

Fig. 15 Normal stage 36 half-tail, showing mature nerve fibres with varicosities and branchings.

Fig. 16 One day after SC resection at stage 36. Note the fine fibres without varicosities and few short branches (arrowheads).

Fig. 17 Four days after SC resection at stage 36. Note the lack of nerve fibres along the intermyotomal junction on the left (arrows) and the very short segments of nerve fibres (arrowheads) at the intermyotomal junction on the right.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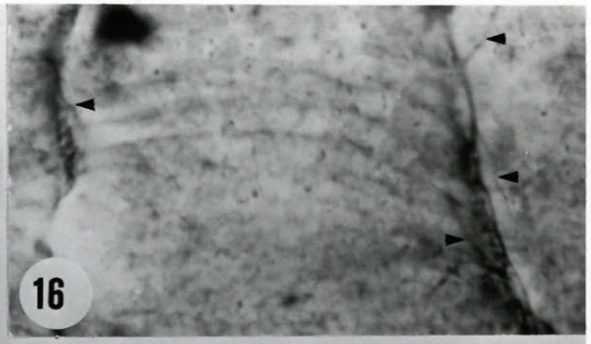
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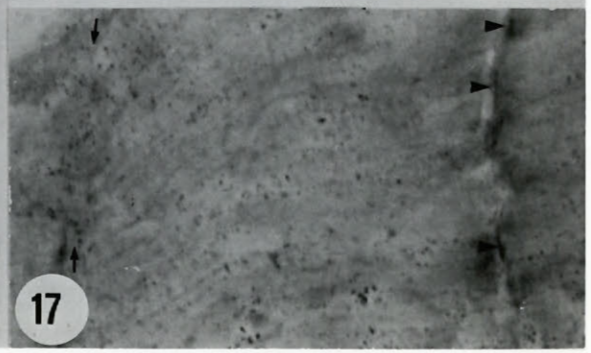
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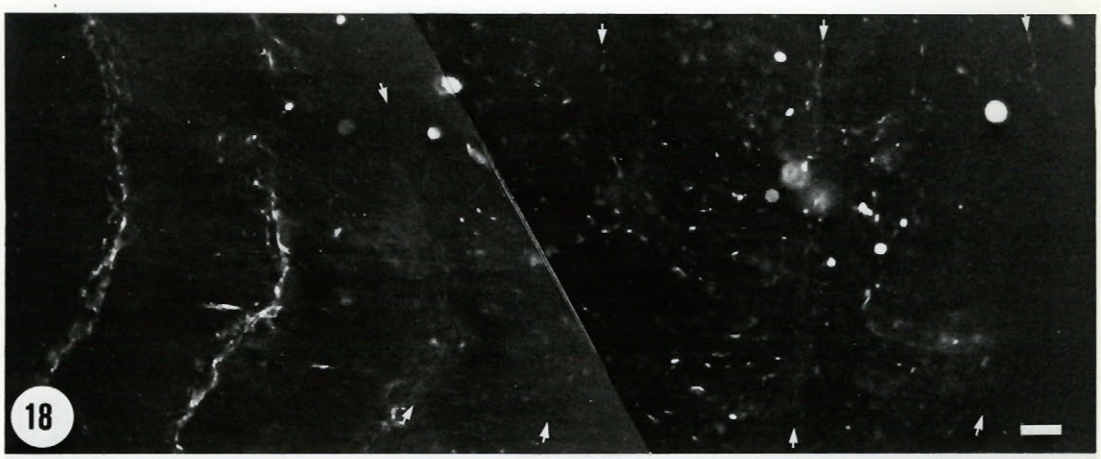
Fig. 18 A segment of a half-tail 2 days after SC resection at stage 36, showing 4 denervated intermyotomal junctions on the right and 2 innervated intermyotomal junctions on the left. The SC-free junctions (arrows) have some short segments of fluorescent stain or no stain at all. Fluorescent stain is seen along the entire innervated junctions. The large white round spots are autofluorescent debris. Calibration bar is 20 µm.

Fig. 19 SC-free myotomes of a half-tail 2 days after SC resection at stage 36. The right intermyotomal junction (arrows) is devoid of receptor patches and the left junction (arrows) has a few small patches along its upper portion. Also note the many receptor patches scattered along the myotome, away from the end regions. Calibration bar is 20 µm.

Fig. 20 SC-free portion of a half-tail 4 days after SC resection at stage 36, showing lack of receptor stain at the intermyotomal junctions (arrows) and patches scattered along the myotomes. Calibration bar is 20 µm.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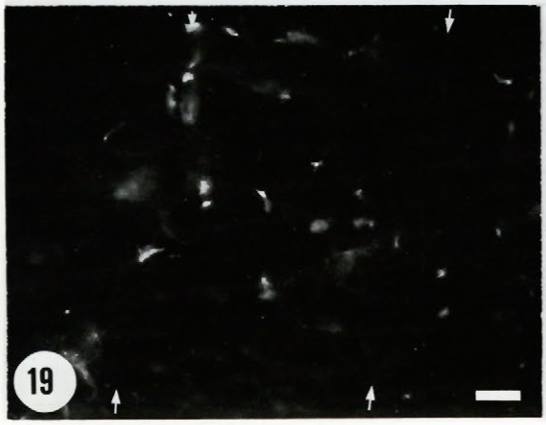
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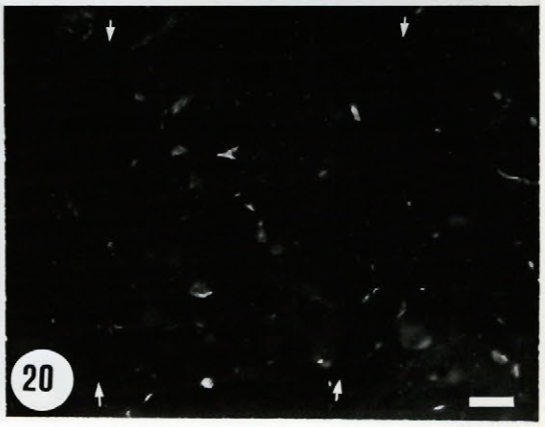
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Figs. 21-23 Phase contrast (left) and fluorescence (right) micrographs of intermyotomal junctions from control and SC-free animals 4 days after spinal cord resection at stage 36. Calibration bar is 20 μm.

Fig. 21 Control preparation of stage 45 tadpole showing patches of AChRs at the ends of the myotomal muscle cells.

Fig. 22 SC-free intermyotomal junction at stage 43 showing a few patches of AChRs. Note also the scattered patches away from the cell end regions.

Fig. 23 SC-free intermyotomal junction at stage 43 with very few receptor patches.

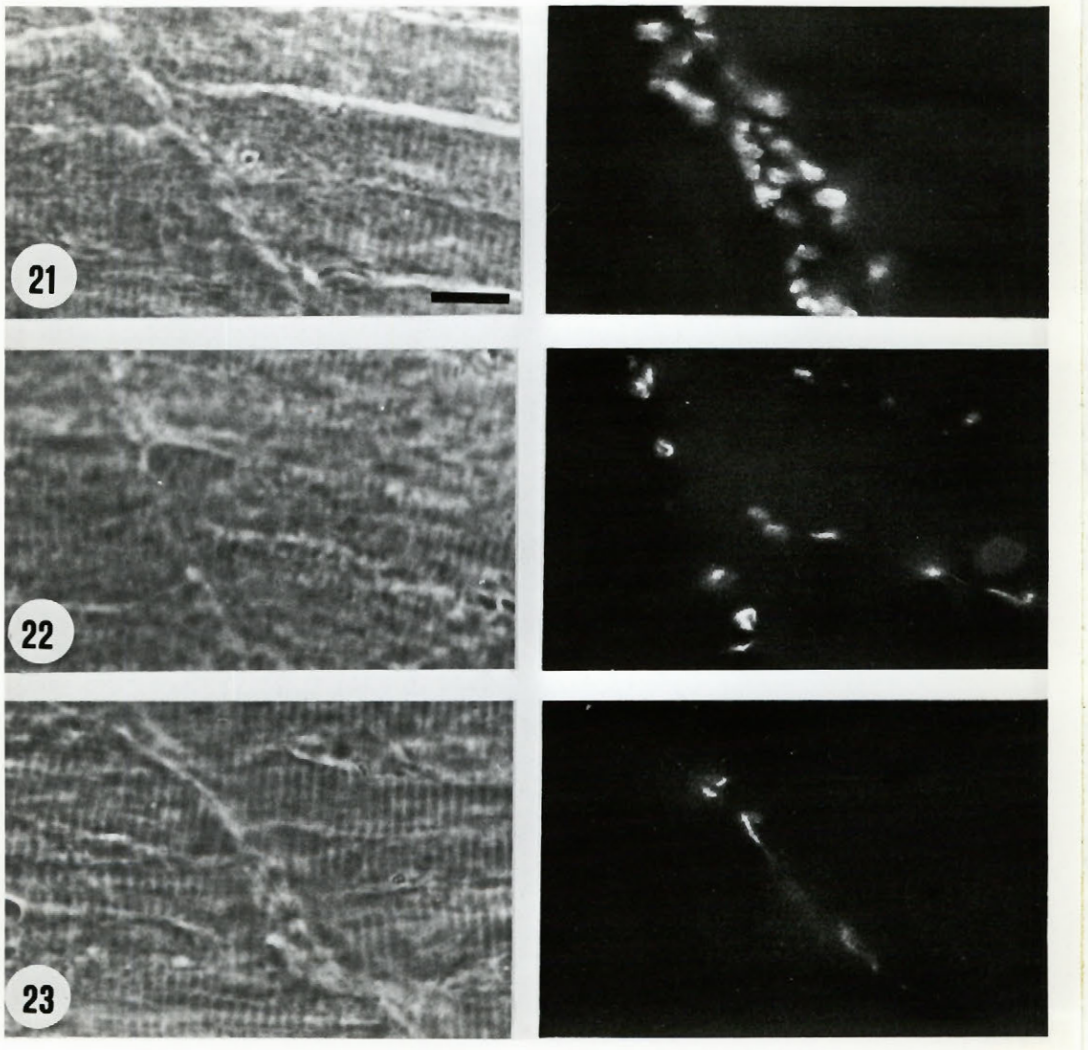
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Fig. 24 Part of a myotome 4 days after SC resection at stage 36. Above: scattered AChR patches along the myotome. Below: cholinesterase patches. Arrowheads indicate sites which exhibited both receptor patches and cholinesterase patches. Calibration bar is 20 µm.

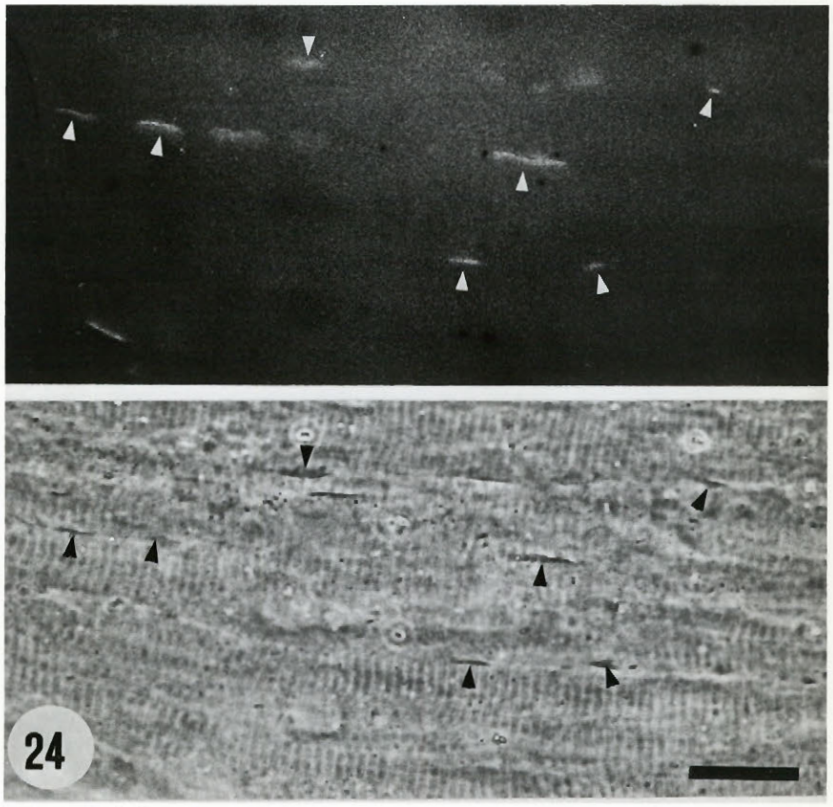
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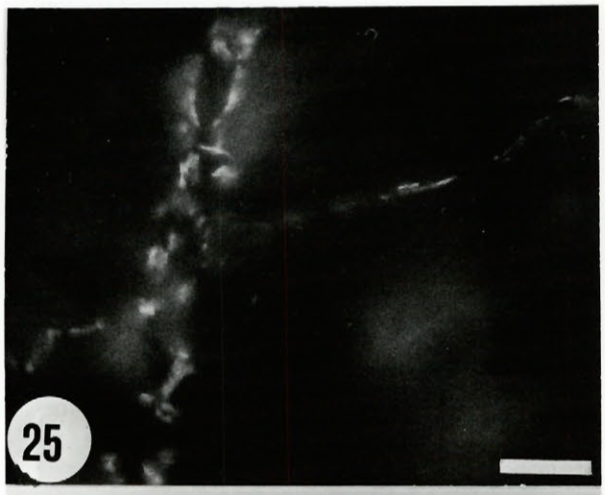
Fig. 25 Fluorescence micrograph of myotomes from an animal which was kept in MS-222 for 2 days, from stage 36 to stage 46. Note the bright stain at the intermyotomal junction and the path-like stain along the myotome. Calibration bar is 20 µm.

Fig. 26 Portion of a stage 45 half-tail from an animal anaesthetized for 4 days (stage 20-stage 45). Note the bright stain at the ends of the myotomal muscle cells. Calibration bar is 20 µm.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Chapter IV

CONCLUSIONS

CONCLUSIONS

The present results have indicated that the embryonic muscle cells already have AChRs when the nerve fibres arrive and that with innervation a high receptor density develops at the nerve-muscle contact site. If removal of the spinal cord is done after the nerves contact the myotomes, the patches of junctional AChRs disappear with time, probably as a consequence of nerve degeneration. However, if the spinal cord resection is done earlier, either before or about the time of arrival of the nerve fibres, the "denervation" effects are short-lasting because re-innervation occurs rapidly at these early stages. Thus, with the arrival of new nerve fibres patches of receptors are formed at the contact sites. These results suggest that the nerve fibres can induce accumulation of AChRs at the synaptic site and that the continuous presence of these nerve fibres is required for the maintenance of a high receptor density in the developing subsynaptic membrane. This is achieved even in the absence of muscle activity as demonstrated in the experiments where the animals are kept immobile for a period of time.

During normal development receptor patches are also found along the length of the myotome, and their aligned patterns suggest that they are associated with nerve fibres. Thus, while the ends of the myotomal cells are the main sites of innervation for these muscles other synaptic sites form during the course of development. Patches of high receptor density also develop in the absence of innervation but these are found scattered along the myotomal muscle cells in an apparently random fashion. The developing myotomal muscle cells seem to have the capacity to form patches

of high receptor density whether or not nerve is present. What the nerve does is to determine where these receptor patches form.

In view of these considerations two important questions arise. What is the nature of neural influence on the localization of ACh receptors during development? How is the stability of such receptor accumulations achieved?

Studies on embryonic muscle cell cultures of chick (Vogel et al., 1972; Fischbach and Cohen, 1973; Sytkowski et al., 1973; Bekoff and Betz, 1976; Prives et al., 1976; Betz and Osborne, 1977; Frank and Fischbach, 1979; Cohen and Pumplin, 1979; also see Fambrough, 1979), mouse (Powell and Friedman, 1977), rat (Axelrod et al., 1976; Land et al., 1977) and frog (Anderson et al., 1977) have shown that these cells are capable of forming clusters of AChRs in the absence of nerve fibres. If spinal cord cells are added to muscle cell culture, higher sensitivity to ACh and higher receptor density patches are found at the nerve-muscle contact sites in chick (Fischbach and Cohen, 1973; Cohen and Fischbach, 1977; Betz and Osborne, 1977; Frank and Fischbach, 1979) and in frog muscle cultures (Anderson et al., 1977; Anderson and Cohen, 1977). These studies suggest strongly that the presence of nerve fibre on the muscle cell induces the accumulation of AChRs at the site of contact. This increased aggregation of receptors may be caused by some diffusible substance from the nerve explant (Cohen and Fischbach, 1977; Jessell et al., 1978). Cohen and Fischbach (1977) showed that after incubating co-cultures with ^{125}I-αBuTX the chick embryo muscle fibres adjacent to spinal cord explants had higher grain density than those fibres located farther away. The fibres closer to the SC explants also had more receptor patches. Jessell et al. (1978) reported an increase in the total number of binding sites

and number of receptor clusters in chick myotube cultures grown in medium with membrane-free extracts from embryonic chick spinal cord, brain, retina or cerebellum, or grown in the presence of chick ciliary ganglionic nerves. It may be therefore that an inducing factor is liberated from motor nerve fibres during the development of the synapse and that this factor is responsible for the localization of AChRs at the synapse.

Alternatively, non-diffusible factors associated with the outside of the axonal membrane or substances present in the basal lamina at the synaptic region may play an important role in the accumulation of AChRs. To date no evidence for the former alternative has been found, and some indication for the latter choice has been reported. Studies by Burden et al. (1979) have shown that even in the absence of the motor nerve the regenerating pectoris muscle fibres in adult frog accumulated AChRs at the original synaptic sites. They suggested that this process may be directed by some elements which remained in the basal lamina at the original nerve-muscle contact sites. That the synaptic portion of the basal lamina contains elements not present in the extrasynaptic regions has been demonstrated by Sanes and Hall (1979). Together, these studies suggest the presence of some non-diffusible substance in the synaptic region of the basal lamina which is not present in the extrajunctional portions and which determines the localization of AChRs in regenerating myofibres. However, neither the origin nor the nature of such a factor is suggested in these studies. Considering that these investigations were done in adult skeletal muscle, and the studies mentioned in the previous paragraph suggested the existence of substance released by the nerve fibres which induced the accumulation of AChRs, it seems reasonable to speculate that the nerve fibres release a factor which becomes incorporated into the developing synaptic

basal lamina and determines the formation of high receptor density patches.

In considering the stability of AChRs in the sarcolemma recent studies have demonstrated the mobility of extrajunctional receptors and the immobility of AChR patches. Axelrod et al. (1976, 1978) described these events in the membrane of cultured rat skeletal muscle cell by pre-labelling them with a fluorescent toxin. Then they bleached fluorescent areas of the membrane to a non-fluorescent state and measured the subsequent fluorescence recovery. They were able to calculate the diffusion rate of the receptors and found that during its lifetime the receptor probably moves no more than a few tens of micrometers in the plane of the muscle cell membrane by Brownian movement. They also found that receptors that accumulate in clusters are no longer freely mobile within the myotube membrane. Similar immobility was also found in adult mouse diaphragm muscle where junctional receptors were labelled with fluorescent α-BuTX (Axelrod et al., 1976). Axelrod et al (1978) also inhibited the lateral motion of AChRs in developing rat myotubes by adding concanavalin A or anti-α-BuTX-antibody into the culture. They suggested that such effects may be a result of respectively, cross-linking of surrounding concanavalin-A receptors which would "cage in" the AChRs, or via cross-linking of AChRs and the antibodies. This lateral movement is independent of metabolic energy and resists specific conditions which affect extrinsic cell surface proteins (such as detergent and protease treatment, disulfide bond cleavage) and cytoplasmic microfilaments and microtubules (such as colchicine, vinblastine and cytochalasin B treatment). More recently, Bloch (1979) described reversible dispersal of AChR clusters in cultured rat myotubes by treatment with the metabolic inhibitor azide. When the inhibitor is removed the receptors re-aggregate both close to and far away

from the original cluster sites. The author suggested that the metabolic inhibitor probably acts by blocking ATP synthesis and that metabolic energy is used either directly or indirectly to hold the AChR clusters together. This result is different from observations by Axelrod et al. (1978) who failed to see cluster loss in the presence of azide. Bloch attributed this difference to the lower temperature (22°C) used in the experiments by the latter group, while his experiments were conducted at 37°C.

Whether the stabilization of the AChR clusters at the neuromuscular junctions is due to the presence of cytoskeletal elements underneath the subsynaptic membrane or due to the presence of extracellular basal lamina has not been established yet. Orida and Poo (1978, 1979), and Axelrod and colleagues (see above) have found that agents known to disrupt microtubules (colchicine and vinblastine) or microfilaments (cytochalasin B) do not affect the clustering of the receptors. Bloch (1979) also did not find significant effect of colchicine on receptor cluster stability and on dispersal of clusters by azide, but he found an inhibitory effect on the reaggregation of AChRs after removal of this metabolic inhibitor. He suggested that the AChR clusters are stabilized by cytoskeletal elements dependent on the intracellular level of ATP and cations, and that such cytoplasmic elements may bind to and immobilize the receptors which diffuse to the cluster site. Using horseradish-peroxidase-αBuTX and electron microscopy Jacob and Lentz (1979) showed that in chick embryos between 10- and 14-day incubation the developing muscle cells have specializations at the neuro-muscular junctions. Even at these early stages amorphous and fuzzy material is found on the external surface of the high receptor density sites, and dense material is present on the internal side of these

regions. Heuser and Salpeter (1979) described the presence of a distinct web of cytoplasmic filaments beneath the synaptic membrane of the electric organ of Torpedo and a basal lamina above this area. They delineated possible contact points between these structures and the postsynaptic membrane, and suggested interaction between them on the aggregation of AChRs. This is in line with the work by Burden et al. (1979) which also implicated the synaptic basal lamina in the localization of AChRs. They found that new AChRs accumulated at these sites when the damaged muscle was allowed to regenerate in the absence of nerve fibres. Original synaptic basal lamina has also been shown to be the preferred site for re-innervation even after disruption of the muscle cell (Marshall et al., 1977), and for cholinesterase association (McMahan et al., 1978).

The present study has shown that during the course of normal development of Xenopus laevis myotomes the formation of AChR patches at the nerve-muscle contact sites depends on the presence of the innervating nerve fibre and it starts around stage 21. Kullberg et al. (1977) showed at the EM level that no structural specialization is seen at the nerve-muscle contact sites in stages 21-24 Xenopus embryos, and that the narrow clefts have only a small amount of flocculent material. More cytological differentiation is seen between stages 25 and 36; moderately dense, fine material is found in the slightly wider clefts and the first specializations (thicker sarcolemma and elevation of the muscle surface) are seen at stage 27. Together these two studies suggest that in Xenopus embryos the presence of well differentiated synaptic and cytoskeletal structures is not essential for the accumulation of AChRs at the early stages of development. Instead, the existence of a neural factor which acts

directly on the immobilization of receptors during early synaptogenesis seems to be quite possible. Thus, combining all the above considerations one can propose that during early stages of synaptogenesis in the myotomes of Xenopus laevis, when the axonal membrane is in close association with the developing muscle cell, a neural factor is released from the nerve terminal, diffuses through the narrow synaptic cleft and directly induces accumulation of AChRs at the synaptic site by interacting with the receptor molecules to make them immobile. Later on, the appearance of extracellular (e.g. basal lamina) and intracellular (cytoskeletal) elements will contribute to further maintain these high receptor densities, by keeping the factor in the synaptic basal lamina and providing some kind of anchorage to the receptors, respectively.

SUMMARY

SUMMARY

1- The distribution of acetylcholine receptors in the myotomes of Xenopus laevis was investigated during the course of normal development by using α-bungarotoxin conjugated with fluorescent dye or ^{125}I.

2- The distribution of nerve fibres in the myotomes was determined after staining with nitroblue tetrazolium.

3- In confirmation of previous studies it was found that the main innervation site is at the ends of the myotomes and the development proceeds in a rostro-caudal direction.

4*- Nerve fibres were first seen, very rarely, at the ends of the most rostral myotomes at stage 19. With age, the number of myotomes with nerve fibres increased in a rostro-caudal direction,and by stage 24 they were found in almost all preparation examined. Occasionally nerve fibres were seen extending into central regions of the myotomes.

5*- Acetylcholine receptors were detected as early as stage 19 and were found to be distributed along the entire length of the myotomes.

6*- The density of extrajunctional receptors increased up to stage 36 and then declined, but was still substantial at stage 49.

7*- Patches of high receptor density, revealed by fluorescent α-bungarotoxin, were first found at the presumptive sites of innervation at stage 21. They developed in a rostro-caudal direction, and by stage 26 were present in virtually all preparations examined. Patches of high receptor density were also seen along the myotomes and they were aligned in path-like patterns which resembled the course of nerve fibres.

8*- The above results indicate that the high receptor density sites develop at the presumptive synaptic sites about 1-5 hours after the arrival of the nerve fibres, which is consistent with the notion that receptor localization at these sites is nerve-induced.

9*- Removal of the developing spinal cord at stage 36 resulted in a progressive loss of nerve fibres and a progressive loss of high receptor density patches at the synaptic sites over a period of 4 days.

10*- Animals anaesthetized by immersion in 10^{-4}g/ml tricaine methanesulphonate for up to 4 days did not show any disruption of receptor patches at the ends of the myotomes.

11*- It is concluded that at early stages of development the maintenance of acetylcholine receptor patches at presumptive synaptic sites is dependent on the continued presence of the nerve but not on muscle activity.

Claims to originality

Asterisks in the above summary indicate original contributions.

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