Influence of Neostigmine Treatment on Embryonic Development of Acetylcholine Receptors and Neuromuscular Junctions

G. S. SOHAL and W. R. BOYDSTON

Department of Anatomy, Medical College of Georgia, Augusta, Georgia 30912

ABSTRACT   The postulated role of the acetylcholine receptor in the formation of neuromuscular synapses during the course of embryonic development was investigated in the superior oblique muscle of white Peking duck embryos. The possibility that the number of receptors could be experimentally lowered by chronic injections of the anticholinesterase agent, neostigmine methylsulfate, was determined using ^{125}I-α-bungarotoxin. The total number of acetylcholine receptors on incubation day 12, 2 d subsequent to the onset of treatment, was reduced 45% as compared to saline-treated controls. A similar reduction in total receptor content (49%) was also observed on day 19. Radioautographic preparations showed that clusters of acetylcholine receptors were rare and that the grain density of extrajunctional receptors was also reduced. Hence, chronic treatment with neostigimine during development was observed to exert an effect on both the number and distribution of receptors in the developing superior oblique muscle. These changes occurred in the absence of any apparent effect on muscle differentiation in general. Myoblasts and myotubes were present on day 14 and further differentiated into myofibers by day 18 in both neostigmine and saline-treated muscles. The cytology of the developing muscle cells also appeared normal. This is in contradistinction to the striking morphological changes that take place in adult mammalian and avian muscle after anticholinesterase treatment. More significantly, the decreased total receptor content and sparsity of clusters had no apparent effect on the formation of developing neuromuscular junctions at the electron microscopic level. The frequency of neuromuscular junctions in neostigmine-treated muscles was similar to that of the controls. It is concluded that acetylcholine receptor clusters are not required for the events leading to the morphological formation of neuromuscular junctions during in vivo development.

Although initially developing independently, a skeletal muscle and its motor nerve become structurally and functionally dependent on each other during subsequent embryonic development through a complex series of interactions eventually leading to the formation of neuromuscular junctions (NMJs). The mechanisms by which NMJs are established, either during the course of embryonic development or after reinnervation of adult denervated muscle, are little understood. One of the extensively investigated aspects of the NMJ is the presence of acetylcholine receptor (AchR) molecules in the plasma membrane of the muscle fiber. In relatively immature muscle cells the AchRs are distributed uniformly over the entire sarcolemma (7, 17). An integral part of the maturation process of the developing muscle is the change in distribution and number

of AChRs, and in many properties of the receptor such as metabolic turnover times, channel open times, and immunological properties (9, 10, 19, 22, 48, 51, 67). At about the time of the initial innervation, aggregates of AchRs (clusters or hot spots) appear in addition to the relatively low density receptors (7, 8). Although temporally coincidental, the initial appearance of clusters of receptors is not dependent on innervation because the receptor clusters appear on myotubes grown in culture in the absence of neural tissue and in aneural muscle (3, 4, 8, 21, 55, 63). In vitro studies suggest that the growing nerve fibers induce new receptor clusters at the point of nerve-muscle contact (3, 24). Gradually, during the course of embryonic development, the low density receptors disappear and almost all AchRs in the adult muscle fiber are localized to the region

of the NMJ (2, 19, 29, 42). One of the intriguing questions pertaining to synaptogenesis is what role, if any, do the AchR clusters play in formation of the NMJ?

In this paper we report the effects of neostigmine methylsulfate on the embryonic development of AchRs, formation and maintenance of NMJs, and muscle differentiation. Neostigmine methylsulfate and other anticholinesterase drugs have been shown to produce postsynaptic morphological, biochemical, and electrophysiological alterations in adult mammalian skeletal muscle after acute and long-term treatment (1, 18, 20, 23, 30, 34, 37, 38, 49, 52, 64, 66). Initially, the myopathic changes are localized to the junctional region and include disruption of the myofibrillar apparatus, swelling of subjunctional membrane-bound organelles, and a simplification of postsynaptic folds (18, 30, 34, 52). With continued treatment, the degenerative changes spread to regions adjacent to the motor endplate. Furthermore, chronic treatment of adult mammalian muscle with neostigmine reduces the total number of acetylcholine receptors in the range of 42–45% in the endplate region (12, 44). Whether or not neostigmine also decreases the total number of AchRs in the embryonic skeletal muscle and alters muscle morphology during in vivo development is unknown.

MATERIALS AND METHODS

Animals and Drug Regimen

White Peking duck embryos were treated with 1–5 µg neostigmine methylsulfate (prostigmin, Roche Laboratories, Hoffman-LaRoche, Inc., Nutley, NJ) per gram body weight. The drug solution was placed on the vascularized chorioallantoic membrane through an opening in the shell beginning on day 10 and continuing daily until sacrifice. Previous studies have shown that the out-growing trochlear nerve fibers reach their target, the superior oblique muscle, on day 10; neuromuscular transmission begins on day 11, and primitive neuromuscular junctions first become visible on day 13 (56, 58, 59, 62). Control embryos received equal amounts of a sterile, physiological saline solution over the same time period.

Acetylcholine Receptor Binding and Autoradiography

Acetylcholine receptor quantification was performed by conjugating Na^{125}I (Amersham Corp., Arlington Heights, IL) with α-bungarotoxin (α-BTX, Miami Serpentarium, Miami, FL) using the chloramine-T method (5, 28). Upon sacrifice muscles were quickly dissected and each muscle was incubated at 4°C for 4 h in 1 ml Locke's solution containing 0.3 µg ^{125}I α-BTX (sp act 57 Ci/mM). Subsequently, the muscles were washed overnight in Locke's solution at 4°C to remove unbound toxin. The amount of bound ^{125}I α-BTX (hence receptor content) was determined with a gamma counter. The nonspecific binding in our preparations, as determined by preincubation of muscles with cold toxin, was 7%. Total muscle protein was determined from muscle homogenates by the method of Lowry et al. (40) using bovine serum albumin as the standard.

For autoradiography the superior oblique muscles were labeled and washed as described above with the one exception that an iodinated toxin of higher specific activity was used (200 Ci/mM, Amersham Corp.). Subsequent to washing, the muscles were fixed for 4 h in 4% paraformaldehyde-glutaraldehyde solution. They were then washed in Locke's solution for 6 h (4°C), frozen in liquid nitrogen, and longitudinally sectioned on a cryostat at 20 µm. Sections were coated with NTB-2 liquid emulsion (Kodak) diluted 1:1 with water. After a 6-d exposure at 4°C, the slides were hydrated, developed for 3 min at 18–20°C (2 parts H$_2$O to 1 part D19), and fixed. The slides were then lightly stained with hematoxylin and eosin and cover-slipped. Muscles on embryonic days 11, 16, 19, and 23 were processed for autoradiography. Quantitative autoradiographic studies were performed on embryonic day 23. AchR clusters were counted with the aid of a camera lucida drawing tube attached to a light microscope. Silver grain counts of extrajunctional AchRs were made from photographs at a final magnification of 850 times. The areas were measured with a Zeiss MOP-1.

Light and Electron Microscopy

For electron microscopy, muscles were fixed overnight in 4% paraformalde-

hyde-glutaraldehyde, pH 7.4, postfixed in 1% osmium tetroxide, stained en bloc with 0.5% aqueous uranyl acetate, dehydrated through graded ethanols and embedded in Epon 812. Thin sections of 500–1,000 Å were cut on a Sorvall MT2B ultramicrotome (DuPont Instruments, Newtown, CT). Sections were taken at 250-µm increments of the muscle in both longitudinal and cross-sectional orientations. The sections were stained with lead citrate and viewed on a Philips 400 transmission electron microscope. For quantitative electron microscopy on NMJs, muscles on embryonic day 23 were cut lengthwise into two parts and both parts were embedded to allow visualization of the entire width of muscle in cross section. Thin sections were obtained from three different areas of the muscle at increments of 1.5 mm. A neuromuscular junction was defined as the region of contact between a nerve terminal and muscle cell and showed the following characteristics: local thickening of the postsynaptic membrane, a distinct synaptic cleft, and a presynaptic nerve terminal containing synaptic vesicles.

The uptake of neostigmine into the circulation and its action on the muscle were monitored by staining muscles for acetylcholinesterase. Muscles were dissected, frozen in liquid nitrogen, sectioned at 40 µm and processed for demonstration of acetylcholinesterase according to the method of Toop (65). In addition, embryonic motility was monitored by counting vigorous, jerky limb movements through the shell window for an interval of 5 min on embryonic days 12, 14, 16, and 18.

RESULTS

Effects of Neostigmine on Acetylcholine Receptors

A total of 148 duck embryos was injected daily with neostigmine methylsulfate (3.0 µg neostigmine/g body wt) beginning on day 10 and continuing until sacrifice. At this concentration, the drug elicited a maximum response while not increasing embryonic mortality above normal levels.

Continuous neostigmine application reduced the number of acetylcholine receptors in the superior oblique muscle as also observed by others in adult mammalian muscle (12, 44). Total counts per minute on day 12, 2 d subsequent to the onset of neostigmine treatment, were decreased 45% as compared to saline-treated controls (Fig. 1). This relation remained essentially unchanged through day 19 when a 49% reduction in the receptor content was observed. However, values for total muscle protein in neostigmine-treated muscles were comparable to control muscles on both days (Fig. 1). Hence, the decrease in receptor number in neostigmine-treated embryos cannot be explained by an effect of the drug on muscle weight. When
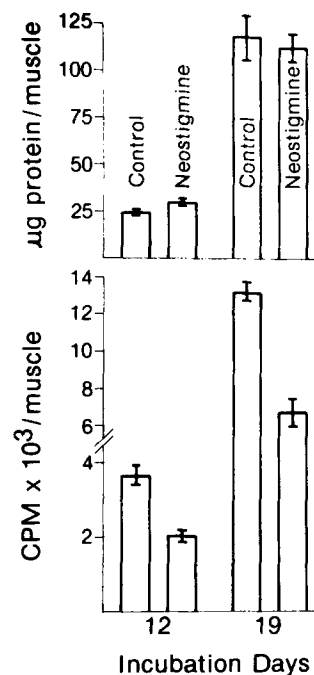

FIGURE 1 Total I^{125}α-bungarotoxin binding per muscle (bottom) and total muscle protein in the control and neo-stigmine-treated superior oblique muscle. Data are expressed as mean ± SEM. N = 9–12 muscles.

normal muscles were preincubated with neostigmine for 1 h, no significant differences in the total number of receptors between the treated and nontreated muscles were observed suggesting that neostigmine does not inhibit α-BTX binding to the receptor. Injections of neostigmine during the course of embryonic development therefore appeared to have an identical effect on the total number of acetylcholine receptors as

reported in adult muscle (12, 44).

In contrast to adult innervated muscle, embryonic muscle is characterized by populations of both junctional and extrajunctional receptors. Thus, the question arises as to how the neostigmine-induced reduction in receptor number is affecting receptor distribution. Fig. 2a shows the typical pattern of receptor distribution on day 23 in saline-treated controls. In
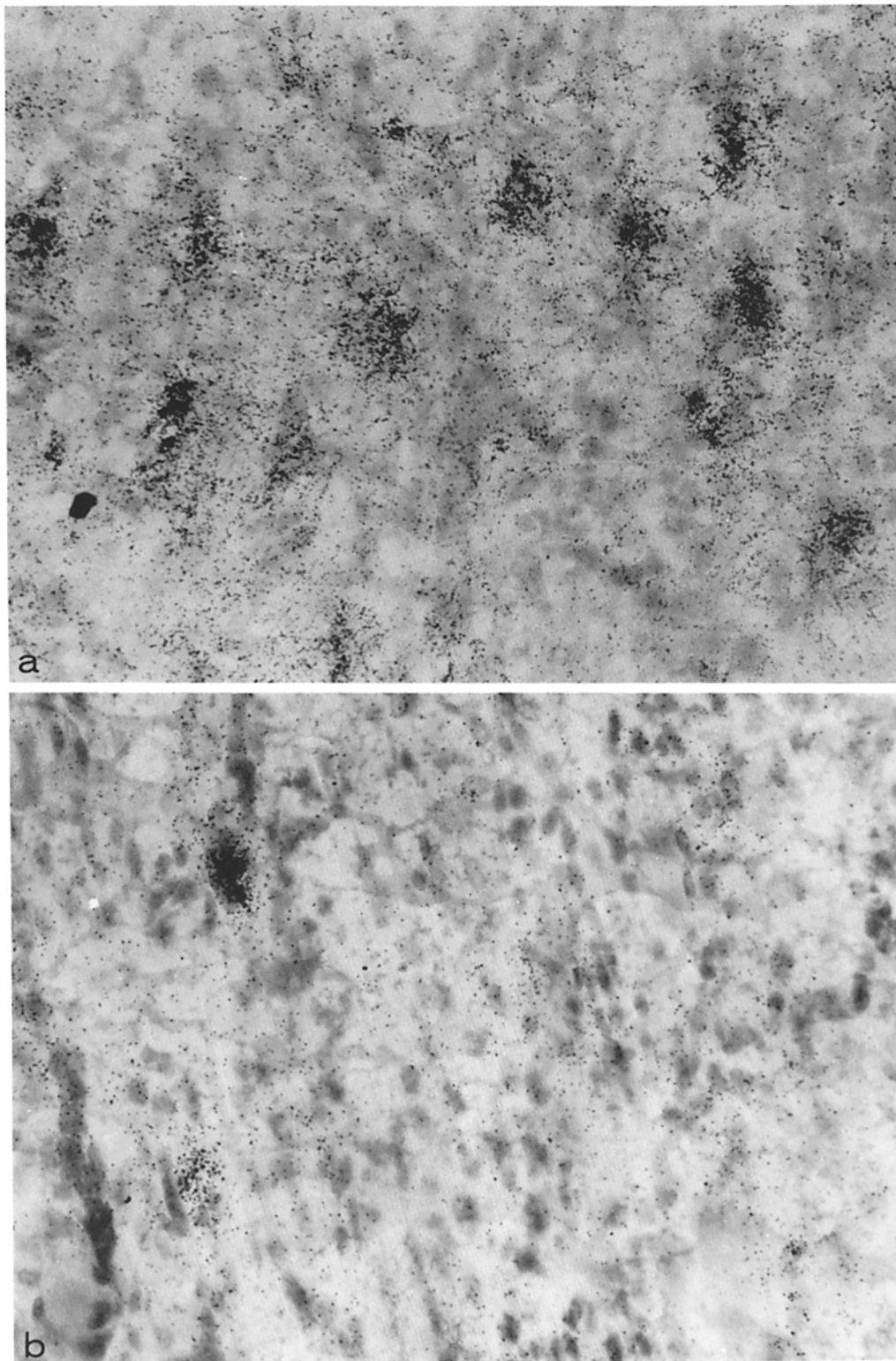

FIGURE 2   Autoradiographic demonstration of acetylcholine receptor distribution in superior oblique muscle on embryonic day 23. a, control; b, neostigmine-treated. Note the sparsity of both clusters of receptors as well as extrajunctional receptors in b. × 525.

addition to the extrajunctional receptors, obvious receptor clusters were seen. However, such clusters were sparse in neostigmine-treated muscles (Fig. 2b). Quantitative study of receptor clusters indicated that ~85% of the clusters were absent in the neostigmine-treated embryos (Table I). When present, the area of the remaining clusters was considerably smaller (Table II). Counts of silver grains indicated that the extrajunctional receptors were also fewer in number in neostigmine-treated embryos (Table II). Thus, reduction in total number of receptors after neostigmine treatment is due to loss of both clusters of receptors and extrajunctional receptors.

Effects of Neostigmine on Neuromuscular Junction Formation and Muscle Differentiation

To verify the anticholinesterase nature of neostigmine in a developing muscle system, an acetylcholinesterase stain was performed. By staining cholinesterase deposits at the junctional regions, this procedure allows for the demonstration of motor endplates at the light microscopic level. In Fig. 3a, distinct acetylcholinesterase deposits are seen in the control muscle on day 23. This staining was absent when tissues were preincubated with neostigmine for 30 min. Normally, the first appearance of endplates detectable in the superior oblique muscle with this stain is on incubation day 18. On the other hand, an absence of distinct acetylcholinesterase staining of motor endplates was noticed in neostigmine-treated embryos despite

TABLE I

Number of Acetylcholine Receptor Clusters and Neuromuscular Junctions in Control and Neostigmine-treated Superior Oblique Muscles on Embryonic Day 23

Series	AchR Clusters	NMJs
Controls		
C-3	84.33	32.66
C-4	100.93	44.33
C-5	64.15	41.0
Neostigmine-treated		
NST-42	13.16	38.66
NST-43	15.82	33.66
NST-44	9.62	38.09
Mean ± SEM		
Control	83.13 ± 10.63	39.33 ± 3.47
Neostigmine-treated	12.86 ± 1.79*	36.80 ± 1.58

Data are expressed as number of AchR clusters and NMJs per section. In each case one muscle was processed for I^{125} α-BTX autoradiography and the opposite muscle of the same embryo was used for electron microscopy. Counts of AchR clusters were made from every third section. NMJs were counted from thin transverse sections at X 10,000 from three different levels of the muscle (at increments of 1.5 mm).
* $P < 0.001$ (Student's t test).

TABLE II

Acetylcholine Receptor Cluster Area and Extrajunctional Receptor Silver-Grain Density in the Superior Oblique Muscle on Embryonic Day 23

Group	AchR cluster area	Silver grains/100 μm^2
	μm^2	
Control	144.58 ± 9.01 (79)	20.96 ± 1.25 (25)
Neostigmine-treated	90.21 ± 5.69* (94)	12.09 ± 0.57* (47)

Data are expressed as Mean ± SEM. The number of clusters measured and the number of different sites for extrajunctional grain density counts are given in parentheses. The autoradiographic pictures used for the above measurements were those used for the study in Table I.
* $P < 0.001$ (Student's t test).

repeated tissue washing of up to 2 h (Fig. 3b (possible explanations for lack of cholinesterase staining in neostigmine-treated muscles and difficulties with this experiment are considered in the last paragraph of discussion). Upon electron microscopic examination of treated muscles on days 14, 16, 19, and 23, neuromuscular junctions appeared normal morphologically and their temporal appearance coincided with those observed in saline-treated control muscle (Fig. 4a). Hence, no differences in the time of appearance or structure of developing neuromuscular junctions occurred. In view of the loss of a vast majority of clusters it was of considerable interest to determine whether or not the number of neuromuscular junctions in neostigmine-treated embryos was comparable to that of the control. An ideal approach would be to count the number of motor endplates from muscles stained with a combined acetylcholinesterase and silver impregnation method at the light microscope level. Unfortunately, lack of acetylcholinesterase staining in neostigmine-treated muscles precluded this type of analysis. However, some indication of the frequency of neuromuscular junctions was obtained using quantitative electron microscopy. Table I indicates that the frequency of finding neuromuscular junctions in the treated embryos was similar to that of the controls.

A cursory look at the neostigmine-treated embryos at the time of injections gave the impression of normal quantitative motility. However, totally different results were obtained when the number of limb movements were quantitated in a systematic fashion. Table III indicates that embryonic movements were significantly reduced in the treated embryos. Furthermore, this reduced level of activity remained virtually unchanged in the neostigmine-treated embryos whereas a progressive increase in the number of limb movements occurred in the control embryos.

As striking changes in the organization of contractile elements and degeneration of postsynaptic folds occur in adult muscle after long-term anticholinesterase treatment, the effect of neostigmine on muscle differentiation was examined. The normal stages in the development of the superior oblique have been well documented and were not seen to vary in neostigmine-treated embryos (59). Myoblasts and myotubes were present on day 14 and further differentiated into myofibers by day 18. The cytology of the developing muscle fibers was also normal in the sense that the appropriate complement of organelles was seen and myofilaments became well organized and assembled into myofibrils (Fig. 4b). Numerous intramuscular nerve bundles of normal ultrastructure were encountered.

Our failure to observe massive morphological alterations in neostigmine-treated embryonic muscles suggested that either the embryonic muscle is not as susceptible to anticholinesterase myopathy as the adult muscle or that for some reason the anticholinesterase myopathy does not occur in avian muscles. Since all of the reported anticholinesterase myopathy studies were done on mammals we examined the effects of neostigmine treatment on the adult duck muscle. Adult ducks were given intraperitoneal injections (0.05 mg/kg body weight in 0.1 ml solution of neostigmine methylsulfate) twice daily for 7 d. Shivering and salivation was observed shortly after the injections. Ducks injected with 0.25 mg/kg body weight of neostigmine of 0.5 ml solution died within 10 min of injection. After sacrifice, superior oblique muscles were examined with electron microscopy. Myopathic changes similar to those reported for the adult mammalian muscle were observed and a limited evidence of this is provided in Fig. 5.
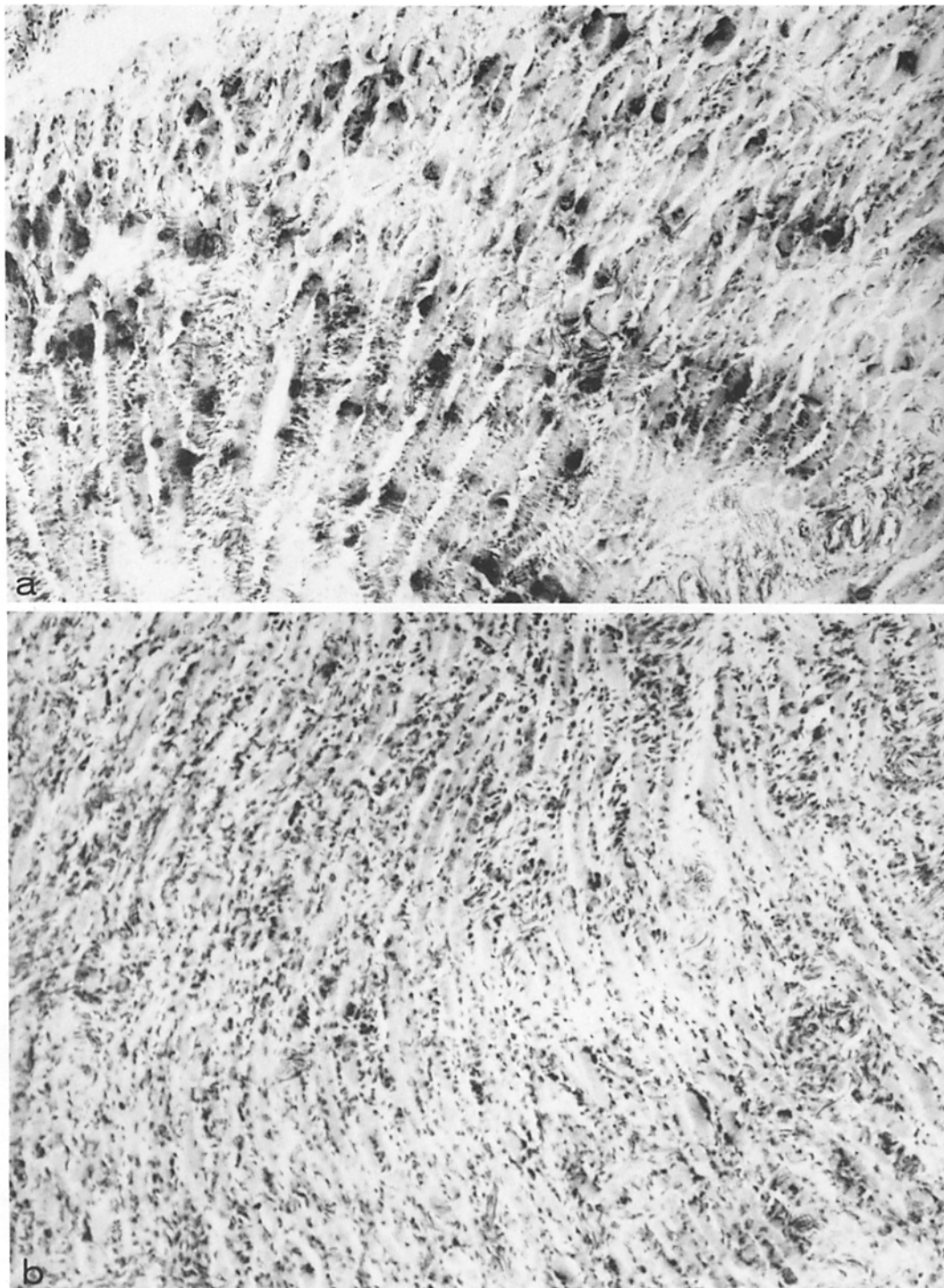
FIGURE 3   Acetylcholinesterase staining of superior oblique muscle on embryonic day 23. *a*, control; *b*, neostigmine-treated. Distinct cholinesterase deposits are absent in the neostigmine-treated muscle. × 200.

DISCUSSION

The results of the present study indicate that continued treatment of the embryo with neostigmine before, during, and after the period of synaptogenesis in the superior oblique muscle significantly reduces the total number of AchRs. Despite such a reduction in AchRs and the absence of clusters of receptors, neuromuscular junctions of normal ultrastructure were observed. Further, the progressive differentiation of the myogenic cells to the myofiber stage was unaffected as a result of neo-

stigmine treatment. These findings suggest that the total receptor content and the ability of receptors to cluster may not be essential elements either for the formation and maintenance of the morphological aspects of the neuromuscular junctions or for the growth and maturation of the muscle. Whether the neuromuscular junctions were functionally normal in the treated embryos could not be ascertained with the present approach. This could only be determined by electrophysiological recordings. Reduced levels of embryonic motility can be interpreted to mean fewer physiologically normal junctions.
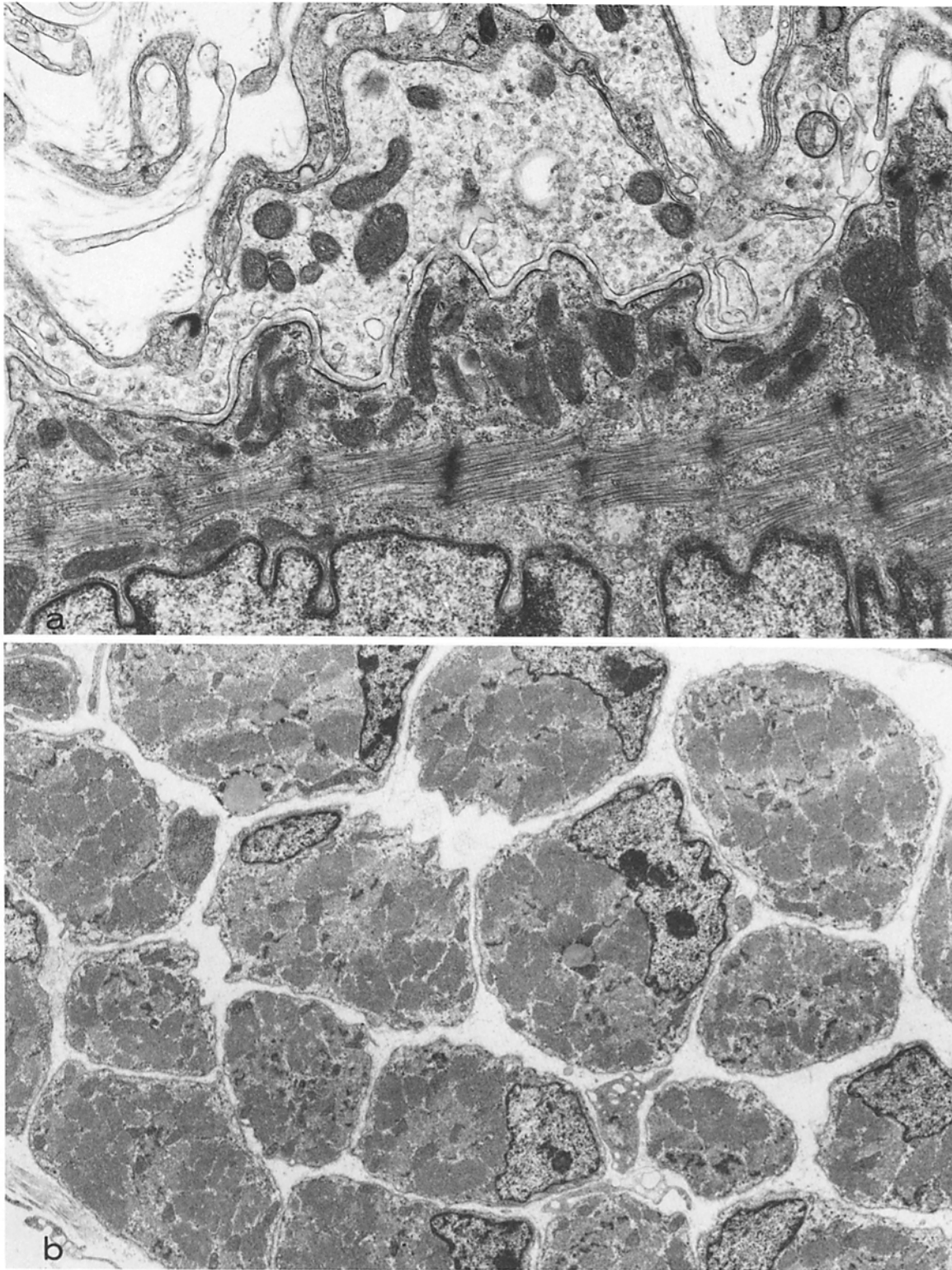
FIGURE 4   a, neuromuscular junction in the neostigmine-treated superior oblique muscle on embryonic day 23. × 15,3000. b, low magnification cross-sectional view of neostigmine-treated superior oblique muscle on embryonic day 23. × 5,775.

However, the drastic reduction in the total number of receptors and the fact that most junctions are without AchR clusters could also account for reduction in activity.

Our observations are consistent with findings in other systems regarding the role of AchRs in synapse formation. A study by Anderson et al. (3, 4) has suggested that the clusters of receptors may be formed along the course of neurite-muscle contacts in cultured *Xenopus* myotubes. A detailed mapping study of AchR clusters on chick myotubes grown in culture was recently carried out by Frank & Fischbach (24). By mapping AchR clusters before and after synapse formation, they observed that the ingrowing nerve fibers induced new clusters of receptors rather than seeking out the preexisting ones. The uninnervated AchR clusters gradually disappeared
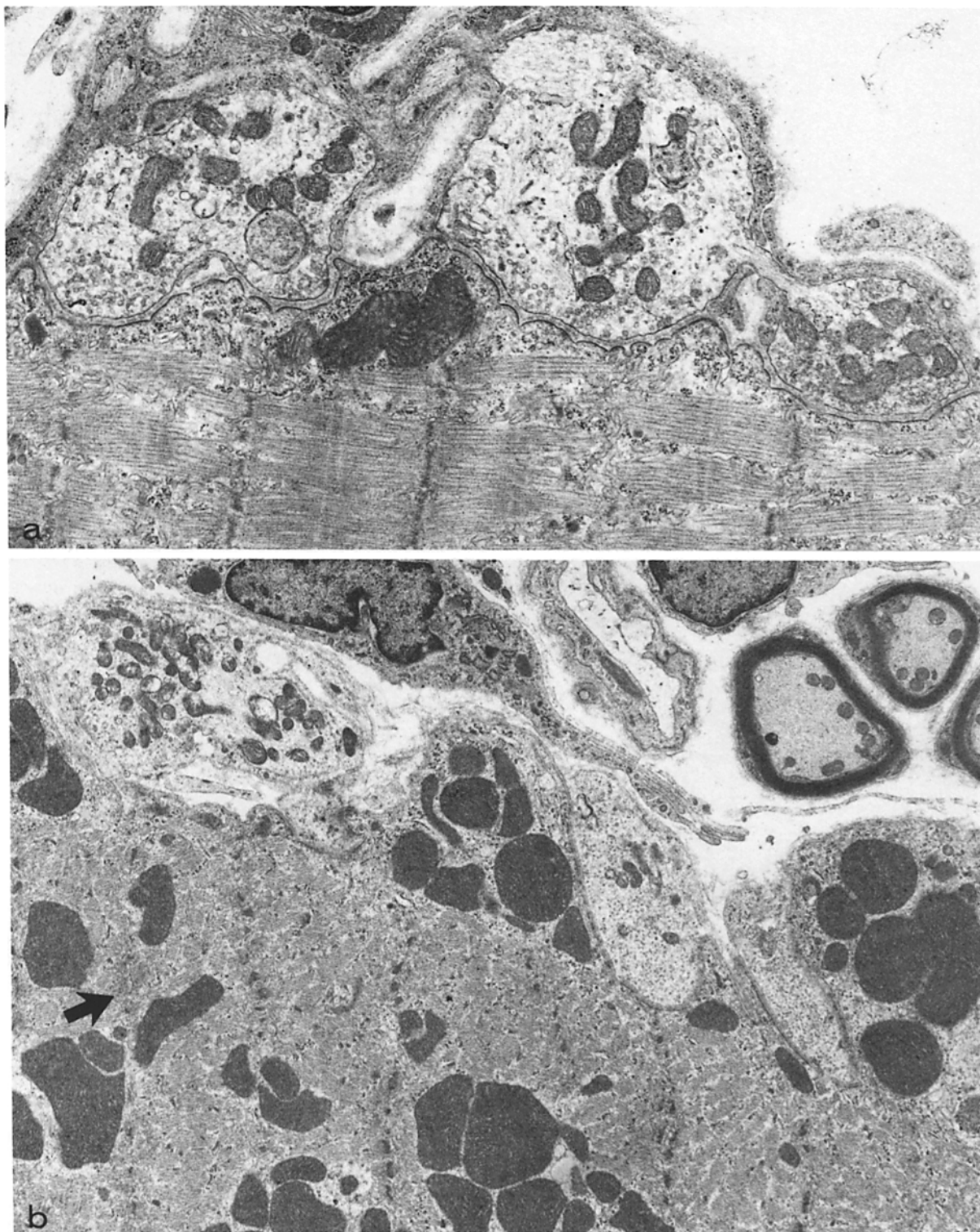
FIGURE 5   a, neuromuscular junction in superior oblique muscle of control adult duck. × 18,480. b, superior oblique muscle of neostigmine-treated adult duck. Note dissolution of Z-discs (arrow), large mitochondria and general disorganization of muscle. Damaged mitochondria were observed in the presynaptic nerve terminal but not in intramuscular nerve fibers. Indistinct pre- and postsynaptic membranes and displacement of nerve terminals were frequently observed. Considerable damage to subsynaptic organelles although not present in this picture also occurred. × 8,250.

suggesting that that the initial AchR clusters may not represent preferred sites for synapse formation. It is now clear that the growing neurities induce clusters of receptors at the region of transmitter release. The mechanism by which neurites induce this clustering is as yet unknown. Several reports indicate that a factor of neural origin may be responsible for clustering of receptors (13, 15, 33, 47). Extracts of spinal cord or brain have been shown to contain a factor that increases the total number of AchRs and the number of receptor clusters on muscle cells grown in culture (33, 47). Media conditioned by neuroblastoma

Group	Movements per minute			
	Embryonic days			
	12	14	16	18
Control	12.2 ± 0.8	23.3 ± 0.7	28.8 ± 1.7	38.5 ± 1.5
Neostigmine-treated	3.9 ± 0.3*	3.7 ± 0.6*	4.2 ± 0.4*	5.9 ± 0.7*

Data are expressed as mean ± SEM. n = 5.
* P < 0.001 (Student's t test).

X glioma hybird cells can also increase the total number of AchR clusters on myotubes in culture (13). The molecular mechanism underlying the action of these factors is completely unknown.

When embryonic muscle cells are cultured with neurons in the presence of curare, synapses are formed (14, 45, 50). Similarly, when neuromuscular transmission is blocked with pre- or postsynaptic blocking agents (such as botulinum toxin, hemicholinum, and bungarotoxin), neuromuscular junctions of normal ultrastructure develop in vivo (25, 26, 56, 59, 60). In vitro studies by Steinbach et al. (61) on the clonal lines of nerve and muscle cells indicate that neither the presence of active AchRs nor the release of acetylcholine are a prerequisite for synapse formation. Kidokoro and Yeh (35) observed that when Xenopus neurons are cultured with rat myotubes, functional synapses are established in the absence of accumulation of AchR molecules at the region of nerve-muscle contact. Rein-nervation of the adult skeletal muscle occurs when AchRs are blocked with α-bungarotoxin (31). Considering the above observations together with the findings of the present study, it appears that none of the known aspects of the cholinergic transmission are essential for the formation of NMJs. It is quite conceivable that something else associated with the region of the nerve-muscle contact (perhaps some component of the basal lamina) along with a factor of neural origin may play an instructive role in synapse formation (6, 11, 32, 41, 54). Regenerating axons have been shown to innervate the intact basement membrane at the original site of the NMJ in otherwise disrupted and phagocytized muscle fibers (11, 41, 54). Recent studies have indicated that the basal lamina at the NMJ is immunologically different from that of the extrasynaptic region of the muscle fibers (53).

The mechanism by which neostigmine produces biochemical and morphological alterations at the vertebrate NMJ has not yet been established. Such changes have been attributed to an excess amount of acetylcholine in the synaptic cleft resulting from acetylcholinesterase inactivation (12, 34, 49, 66). Since myopathic changes require an intact nerve supply and can be prevented by inactivating AchRs, it has been suggested that necrosis may be mediated by an influx of Ca^{++} (1, 52). An alternate hypothesis involving Na^+ flux has also been proposed (37). Recent evidence by Leonard & Salpeter (38) indicates that low external Ca^{++} prevents myopathic changes and on the contrary low Na^+ concentration has little effect. In the present study myopathy was absent in the embryonic muscle whereas necrosis similar to that observed in the adult mammalian muscle occurred in the adult duck. It will be interesting to examine the relative involvement of Ca^{++} in embryonic vs. adult muscle after esterase inactivation. Presently, only hypothetical mechanisms can be proposed by which neostigmine prevents clustering of AchRs during embryogenesis. Neostigmine may prevent the release or activation of chemical factors from neurons which are responsible for clustering of AchRs, or

it may interfere in the mobilization or insertion of receptors in the plasma membrane. It is also possible that neostigmine may alter the synthesis and/or degradation rate of AchRs.

Finally, a comment should be made with respect to our failure to observe positive cholinesterase staining of endplates in neostigmine-treated embryos. Since the appearance of cholinesterase at the embryonic neuromuscular junctions in vitro (36, 50) and after reinnervation of adult muscle in vivo (39) has been shown to be dependent on muscle activity and since activity in our embryos was drastically reduced, it would appear that cholinesterase may be absent in our treated muscles. However, when embryonic neuromuscular transmission is blocked in vivo, motor endplates stain normally with cholinesterase stain (16, 27, 46, 57). Furthermore, cholinesterase staining has also been observed on amphibian muscle cells cultured in the absence of neural tissue (43, 68). A more likely explanation for lack of cholinesterase staining in the present study may be that staining was blocked by neostigmine. This is supported by the fact that when normal muscle was pretreated with neostigmine for 30 min and subsequently thoroughly washed, cholinesterase staining was blocked. Furthermore, despite up to 2 h of extensive washing of muscles from embryos chronically treated with neostigmine, no staining occurred. Thus, our observations suggest that lack of staining in treated embryos may be due to a direct inhibitory effect of neostigmine rather than a repressive effect.

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