NEOSTIGMINE-INDUCED ALTERATIONS AT THE MAMMALIAN NEUROMUSCULAR JUNCTION. II. ULTRASTRUCTURE

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ABSTRACT


Brief and chronic exposure of rats to neostigmine methylsulfate produced marked morphological alterations of the fine structure at the end-plate region of the extensor digitorum longus muscles. These changes were dose and time dependent and were restricted primarily to the subjunctional myofibrillar apparatus and membrane-bound organelles. In addition, significant presynaptic alterations were observed including synaptic vesicle depletion and the appearance of numerous coated vesicles and membrane cisternae, which indicated continuing nerve terminal hyperactivity. With chronic treatment, degeneration and partial recovery of the nerve axon also were observed. The morphological changes of the end-plate region induced by neostigmine did not occur in most fibers after brief denervation and were eliminated entirely by chronic nerve section. Thus, the postsynaptic degenerative changes caused by neostigmine treatment observed in nondenervated animals appear to result primarily from greatly increased synaptic activity and not primarily from a direct neostigmine reaction with the pre- or postsynaptic membranes. Since the myopathic changes observed in this study were produced by neostigmine, a drug which is commonly employed in the routine treatment of human patients with myasthenia gravis, continued use of neostigmine for long-term therapy in noncrisis situations may not be accepted as being free from risk.
The physiological reactions of the rats to neostigmine injections are described by Tiedt et al. (1978).

End-plate morphology of control animals. Above the neuromuscular junctions of control EDL muscles, nerve axons were observed, each with myelin layers closely applied to the neuromuscular junctional folds. Several portions of the transected nerve terminal were present, lying in shallow furrows in the muscle surface.
grooves in the end-plate sarcoplasm (fig. 1). The regular infoldings of the sarcolemma formed junctional folds maintained at a precise distance from the nerve terminal. The cytoplasm in the end-plate region, termed the "soleplate sarcoplasm" by early light microscopists, was distinguished from the extrajunctional peripheral sarcoplasm by its slightly raised contour, which contained several nuclei, numerous mitochondria, and an abundant Golgi-endoplasmic reticulum network. Within the sarcoplasm, the contractile elements were precisely aligned, producing the characteristic striations observed in the light microscope.

Effects of a single injection of 0.1 mg of neostigmine. Thirty minutes after a single injection of neostigmine, the contractile sarcoplasm greater than 20 μm from the neuromuscular junction exhibited relatively normal banding patterns, but within 10 to 20 μm of the neuromuscular junction, the sarcomere banding patterns were progressively disrupted (figs. 2a and 3). Successive sarcomeres exhibited increasingly disorganized Z-discs and misaligned thick and thin filaments. In the immediate subjunctional sarcoplasm, individual sarcomeres were obliterated and Z bands were no longer recognizable, although Z-material was discerned (figs. 2 and 3). This localized supercontraction resulted in partial extrusion of the junctional components from the normal myofiber contour (figs. 2a and 3).

In the immediate subjunctional sarcoplasm, the mitochondria nearest the crest of the junctional folds were grossly swollen or exploded, indicating marked changes in intracellular ionic and osmotic concentrations (fig. 2b). Mitochondrial 0.5 to 10 μm from the junction exhibited progressively fewer alterations, from pronounced intercristal swelling. To normal mitochondrial conformation (fig. 2). Elements of the sarcoplasmic reticulum and T-system appeared as disconnected vesicular fragments (fig. 2b). Separation of inner and outer nuclear membranes, swollen and/or exploded nuclei and distorted Golgi and sarcoplasmic reticulum.

![Fig. 1. Normal EDL muscle fiber with several portions of the transected nerve terminal overlying junctional folds. The sarcomere banding pattern shows precise alignment of the contractile elements. A portion of the myelinated motor neuron (N) is present. ×4,400.](image)
Fig. 2. Neuromuscular junction 30 minutes after a single injection of neostigmine (0.1 mg). a. Supercontraction of the subjunctional contractile elements resulted in partial extrusion of the junctional components from the normal myofiber contour. Sarcomere banding patterns at progressively greater distances from the junction are successively less supercontracted. ×2,500. The boxed area is shown at higher magnification in b. Postsynaptically, mitochondria near the junctional fold crests are swollen or exploded (M) indicating marked changes in ionic and osmotic concentrations localized to the junctional area. Mitochondria 0.5 μm from the junctional folds demonstrate intercristal swelling (*). ×16,000. c. Presynaptically, coated vesicles (small arrows), free coat material (large arrow), and membrane cisternae (c) indicate high levels of nerve terminal activity. ×70,000.
profiles provided additional evidence for severe ionic and osmotic disturbances localized to the end-plate region.

In addition to the relatively normal complement of synaptic vesicles in the nerve terminal, there were an unusually large number of coated vesicles, numerous flattened cisternae and slightly distorted mitochondria (fig. 2b), all indicative of greatly increased presynaptic activity. Thus, an abnormal increase in the rates of synaptic vesicle fusion and membrane recycling via coated vesicles was inferred after neostigmine treatment. It should be noted, however, that presynaptic alterations were much less severe than postsynaptic alterations and were similar to alterations observed after several minutes of directly elicited nerve hyperactivity (Rash et al., 1976b).

Eighteen hours after a single injection of neostigmine (0.1 mg) the nerve terminal, junctional fold height and synaptic cleft width appeared normal. However, severe postsynaptic alterations were still observed in most fibers as the subjunctional mitochondria appeared either irreversibly damaged, partially recovered or enlarged with elaborated cristal membranes (fig. 4b). Analysis of the subjunctional contractile apparatus disclosed the following alterations: 1) myofilaments were no longer separated into discrete myofibrils; 2) Z-bands were absent; 3) sarcomere banding patterns were only faintly resolvable; and 4) the sarcoplasmic reticulum was fragmented. The disarray of myofilaments was emphasized by images of cross and longitudinally oriented filaments in close proximity (fig. 4b). Nevertheless, the extent of localized supercontraction was greatly reduced (cf. fig. 2), junctional components were no longer extruded from the fiber contour and partial realignment of contractile elements was evident. Evidence that these changes were confined to the junctional region and were not fixation artifacts was inferred by comparison with the adjacent cell, where the sarcomere banding pattern was normal and mitochondria were of normal size and conformation (fig. 4a).
Fig. 4. Motor end-plate 18 hours after a single injection of neostigmine (0.1 mg). a. Sarcomere banding patterns are no longer discernable subjunctionally, whereas sarcomeres in a nonjunctional region of the adjacent fiber appear normal. Membrane remnant (arrow). ×8,800. b. The nerve terminal, junctional fold height and synaptic cleft width appear relatively normal. Some subjunctional mitochondria appear severely disrupted (M) or enlarged (*) compared to those in the terminal (m). Continuing disarray of myofilaments is emphasized by images of cross (arrow) and longitudinally (large arrow) oriented filaments. ×27,000.
FIG. 5. Neuromuscular junction obtained 12 hours after the last of three injections of neostigmine (0.1 mg) administered at 12-hour intervals. a. Recovery from acute treatment is apparent, although scattered areas of partial disruption of sarcomere banding patterns remain (small arrows). ×4,200. b. A normal synaptic cleft width is present between the junctional folds (JF) and nerve terminal (NT). The nerve terminal exhibits partial depletion of synaptic vesicles, numerous omega-shaped invaginations of the plasmalemma (o) and abundant coated vesicles (cv), coated pits (large arrows), free coat material (small arrows) and membrane cisternae (*). ×96,000.
Thus, the cytostructural alterations induced by neostigmine treatment persisted at least 18 hours after a single (initial) injection.

**Effects of multiple injections of neostigmine (0.1 mg).** Neuromuscular junctions from animals injected with 0.1 mg of neostigmine three times at 12-hour intervals and fixed 12 hours after the last injection exhibited both pre- and postsynaptic disturbances (fig. 5a). Although the extent of the alterations was variable from fiber to fiber, the reduced damage of postsynaptic constituents was obvious when compared to fibers fixed 30 minutes or 18 hours after a single injection. Sarcomere banding patterns were clearly recognizable in the subjunctional sarcoplasm, although scattered areas of partial disruption still remained (fig. 5a). Continued disruption of the nuclear and cytoplasmic membranes was observed. Further, identifiable mitochondria were of variable size, conformation and distribution. At high magnification (fig. 5b), junctional fold height separation and synaptic width remained unaltered. The nerve terminal, however, exhibited partial depletion of synaptic vesicles, numerous omega-shaped invaginations of the nerve terminal membrane, as well as abundant coated vesicles, coated pits, cisternae, and free coat material, all indicating continuing, abnormally high rates of transmitter release and membrane recycling (Heuser and Reese, 1973; Rash et al., 1976b).

After 7 days of twice daily neostigmine injections, the contractile apparatus, mitochondria, sarcoplasmic reticulum, and T-system remained normal in the non-end-plate regions, whereas in subjunctional areas, continued partial disruption of myofibrils (fig. 6) was observed in most fibers. Mitochondria in both pre- and postsynaptic areas were swollen and distorted, but did not exhibit the intercristal swelling characteristic of brief neostigmine exposure. Many nerve terminals were partially depleted of synaptic vesicles and exhibited a significant increase in free coat material, coated vesicles and large membrane cisternae. Further, hypertrophy of the soleplate sarcoplasm and a concomitant increase of smooth

**Fig. 6.** End-plate region after 7 days of neostigmine injections (0.1 mg). Variable recovery of fibers was noted. Compare the disarray of myofibrillar components at 7 days to those at 36 hours (fig. 5a). Pre- and postsynaptic mitochondria show signs of swelling and damage (arrows). The sarcomere banding pattern in the nonjunctional area of the adjacent fiber is normal. ×6,700.
Fig. 7. End-plate region after 23 days of repetitive neostigmine (0.1 mg) injections. a. Mitochondria in the nerve terminal (small arrow) and nerve axon (large arrow) are swollen. ×5,800. Variability of damage within an end-plate region is apparent in the three terminal regions. The regularity of junctional folds in one portion of the motor end-plate (b, ×26,000) should be compared with the severely altered components in an adjacent area in c. In such areas folds show marked degeneration; vesicular debris is present in the widened synaptic cleft; and abundant coat material (arrow) is present in the nerve. ×102,000.
and rough endoplasmic reticulum profiles and of unidentified tubular membranes were observed (fig. 6), not unlike alterations commonly observed in end-plates of human patients with myasthenia gravis (Engel and Santa, 1973; Santa et al., 1972; Albuquerque et al., 1976; Rashci et al., 1976a).

After chronic treatment with neostigmine (0.1 mg twice daily) for 14, 23 and 56 days, there was marked variability in end-plate fine structure from fiber to fiber and even from area to area within the same end-plate region (fig. 7). The mitochondria within the nerve terminals were often swollen and distorted (fig. 7a). At higher magnification (fig. 7c), abundant coated vesicles and coat material were seen. Thus, throughout all stages examined, continuing hyperactivity of the nerve terminal was indicated. Postsynaptically, the contractile apparatus in moat cells appeared to be partially recovered by 23 days of treatment. In many areas junctional folds appeared normal (fig. 7, a and b), whereas in other areas of the same end-plate, the primary synaptic clefts were greatly widened, junctional folds were simplified or missing and vesicular debris was present in the synaptic clefts (fig. 7c). By 56 days, some cells continued to exhibit moderate damage to cell organelles (cf. Engel et al., 1973) but most appeared essentially recovered from the effects of chronic neostigmine treatment. Junctional fold height and synaptic cleft width were virtually normal. In areas where the plane of section was tangential to the nerve ending, however, images falsely suggesting junctional fold hypertrophy were produced (fig. 8). Such "plane of section artifacts" should not be confused with similar images of end-plates observed in Lambert-Eaton syndrome and analyzed by morphometry (Engel and Santa, 1973).

Effects of a single injection of 0.01 mg of neostigmine. Thirty minutes after a single injection of a much lower dose of neostigmine, a
alterations to end-plate fine structure were detected (fig. 9) but were not as severe as those seen 30 minutes after a single injection of 0.1 mg of neostigmine. Postsynaptically, sarcomere banding patterns were essentially unaltered. However, mitochondria in the subjunctional region appeared swollen (fig. 9a, small arrow), whereas those at greater distances appeared less affected (not shown). Occasionally, presynaptic mitochondria appeared swollen (large arrow), warranting closer inspection of nerve terminals at increased magnification. At higher magnification (fig. 9b), the presence of coated vesicles, free coat material (small arrow), membrane cisternae (large arrow) and numerous infoldings of the nerve terminal membrane (asterisk) reflected continued hyperactivity of the nerve terminal. Further, significantly increased postsynaptic activity was inferred from the presence of a large number of "calcium specific granules" in the mitochondria (fig. 9c). Calcium-specific granules are thought to be formed in the mitochondria in the presence of excessive amounts of intracellular calcium. The increase in intracellular calcium concentration may result from excessive release from sacroplasmic reticulum stores. Thus, even this very low dose of neostigmine induced detectable alterations of pre- and postsynaptic fine structure.

Effects of a single injection of 0.1 mg of neostigmine after denervation. To ascertain whether the localized supercontraction of the end-plate region resulted from excess transmitter release and the resulting prolonged postsynaptic depolarization or from direct effects of neostigmine on the postsynaptic membrane constituents, rats were denervated either for 1/2 hour or for 4 days and were subsequently treated for 1/2 hour with neostigmine. After brief denervation and subsequent injection of neostigmine (fig. 10a), end-plates of most fibers appeared virtually normal and exhibited only those morphological changes characteristic of short-term denervation in untreated animals (Ellisman and Rash, 1977). However, end-plate regions in a few fibers (2 end-plates in 10 examined) possessed all of the alterations typical of end-plates from animals given a single injection of neostigmine (0.1 mg) with no prior nerve section (fig. 10b). Presynaptic changes indicative of nerve hyperactivity were observed despite denervation: synaptic vesicle depletion and the presence of abundant coated vesicles, free coat material, membrane cisternae and numerous omega-shaped invaginations of the plasmalemma. Postsynaptically, localized subjunctional supercontraction of the myofibrillar apparatus as well as interstitial swelling of mitochondria near the junctional folds evidenced extreme depolarization and concomitant ionic and osmotic changes of the end-plate region. Thus, in the presence of neostigmine, the remaining activity within some motor units apparently was sufficient to produce alterations in end-plate fine structure. The alternative possibility, that the presence of neostigmine in these briefly denervated motor end-plates may directly activate sufficient transmembrane ion channels to produce supercontraction and damage to postsynaptic membrane organelles, was examined in denervated preparations in which the nerve terminals were degenerating or absent.

After 4 days of denervation, muscle fibers become sensitive to ACh over their entire surfaces, presumably reflecting the synthesis and insertion of additional ACh receptors at extrajunctional sites (Albuquerque and McLsaac, 1970). After 4 days of denervation, nerve terminals were atrophic or absent and end-plates were invaded by Schwann cells. Postsynaptic alterations directly attributable to denervation included minimal alterations of junctional folds and partial misalignment of myofibrils (cf. Ellisman and Rash, 1977). However, 4 days of denervation followed by a 1/2 hour exposure to neostigmine appeared to result in no additional alterations to postsynaptic elements (fig. 10c). Thus, even in denervated muscle fibers which were supersensitive to ACh (Albuquerque and McLsaac, 1970), neostigmine treatment did not affect membrane permeability of junctional or extrajunctional membranes sufficiently to produce muscle supercontraction or damage to membrane organelles.

Alterations in nerve axons during chronic neostigmine. Close examination of the nerve tract of chronically treated fibers (fig. 7a) indicated that the mitochondrial distortions observed in the nerve terminal may extend many microns into the nerve axon. Thus, since there was abundant evidence for prolonged nerve hyperactivity (figs. 2–7), it was appropriate to consider possible alterations of the nerve axon at each of the stages described above.

A continuum of alterations of the nerve axons was observed after chronic treatment of
FIG. 9. End-plate region 30 minutes after a single injection of a low dose of neostigmine (0.1 mg). a. Sarcomere banding patterns appear normal although some subjunctional mitochondria (small arrow) and occasional presynaptic mitochondria (large arrow) appear swollen. ×1,200. The boxed area is shown at a higher magnification in b. b. Coated vesicles, free coat material (small arrow), membrane cisternae (large arrow) and a convoluted plasmalemma (asterisk) reflect continued hyperactivity of the nerve terminal. ×32,000. c. Postsynaptic hyperactivity is inferred from the large number of "calcium-specific granules" (small arrows) in the mitochondria. ×21,000.
rats with 0.1 mg of neostigmine. Although some pre- and postsynaptic mitochondria in the neuromuscular junction were severely distorted after 1/2 hour treatment with neostigmine, mitochondria within the myelinated nerve axon appeared normal and the myelin remained closely applied to the nerve cell membrane (fig. 11a; see also fig. 3b). In rats exposed to neostigmine for 36 hours, however, the nerve plasma membrane was separated from the myelin in many areas. Further, mitochondria were often slightly swollen and distorted (fig. 11b). By day 7, areas of myelin separation and of artifactual "myelin figures" were observed, presumably representing conformational rearrangement of the disrupted axolemma and/or Schwann cell myelin (fig. 11c). The severity of nerve-myelin disruption appeared maximal by day 14 (fig. 11d) and was partially reversed by day 23 (not shown). The area of myelin disruption also involved Schmidt-Lanterman clefts (C in fig. 11b) and appears to represent ionic or osmotic damage restricted to myelinated (motor) nerve axons.

Discussion

Acute and chronic treatment with neostigmine methylsulfate, a reversible acetylcholinesterase inhibitor, produced structural and functional (Tiedt et al., 1978) alterations localized to the neuromuscular junction. With chronic treatment, damage to some synaptic folds was noted and correlated with a parallel decrease in junctional ACh sensitivity (Tiedt et al., 1978). These postsynaptic alterations may suggest the occurrence of a compensatory response of the end-plate to the increased amount of acetylcholine in the synaptic cleft.

Acute treatment with a low dose of neostigmine (0.01 mg) resulted in minor presynaptic alterations of the neuromuscular junctions in rat EDL muscles, including a significant increase in the number of coated vesicles and membrane cisternae, and the appearance of occasional areas partially depleted of synaptic vesicles. Postsynaptic changes included the presence of a large number of "calcium-specific" granules in the subjunctural mitochondria and a moderate swelling of the mitochondria deeper in the subjunctural cytoplasm. Exposure for 1/2 hour at a much higher dose (0.1 mg) potentiated these pre- and postsynaptic alterations and caused localized subjunctional supercontraction, sarcoplasmic reticulum disruption and mitochondrial damage. Such damage to membrane-bound organelles indicates major alterations in the intracellular ionic and osmotic concentrations. For example, the localized supercontraction may be evidence for increased release and/or decreased sequestration of calcium, presumably from the disrupted or damaged sarcoplasmic reticulum and mitochondria. Since the neostigmine-induced alterations were restricted to the end-plate region and were associated with significantly increased presynaptic activity, the myopathic changes may reflect a greatly increased end-plate permeability produced by the increased amounts of ACh in the synaptic cleft. Notably, such supercontractions restricted to the end-plate regions have long been associated with depolarizing neuromuscular blockade, as originally described by Langley (1907).

After repeated injections of neostigmine, partial recovery of the mitochondria, sarcoplasmic reticulum and myofibrillar apparatus was evident. However, continued partial depletion of synaptic vesicles was observed in some nerve terminals and correlated with a decrease in miniature end-plate potential frequency and quantal content of the end-plate potential (table 2 and fig. 3; Tiedt et al., 1978). In addition, it was evident that disruptive changes occurred which were specifically associated with chronic neostigmine treatment. Variable but marked degeneration of some synaptic folds and the

Fig. 10. End-plate regions 30 minutes after a single injection of neostigmine (0.1 mg) following denervation for 30 minutes (a and b) and 4 days (c). a. Most end-plates appear to be unaltered by neostigmine after 30 minutes of denervation. Note that the pre- and postsynaptic mitochondria (arrows) appear normal. x 12,000. b. However, a small proportion of the end-plates exhibit alterations characteristic of a single injection of a high dose of neostigmine without prior nerve section. These changes include intracristal swelling (large arrows) and disappearance of Z-bands (small arrows). x 5,200. The junctional folds in a and b are well delineated after 20 hours of staining for cholinesterase. (The anticholinesterase effects of neostigmine occasionally necessitated prolonged staining in order to identify junctional areas at the light microscope level.) c. In contrast to fibers denervated for 30 minutes (a and b), fibers denervated for 4 days before neostigmine treatment exhibit degenerated nerve terminals (arrows) and Schwann cell (S) invasion of the end-plate region. No additional alterations were caused by neostigmine. x 12,000.
appearance of vesicular debris in the synaptic clefts were observed and are tentatively correlated with decreased miniature end-plate potential amplitude, reduced junctional fold ACh sensitivity (Tiedt et al., 1978), and decreased α-bungarotoxin binding (Chang et al., 1973). These changes, at least superficially, resemble the destructive alterations seen in human patients with myasthenia gravis (Albuquerque et al., 1976; Rash et al., 1976a; Engel and Santa, 1973).

In most fibers, denervation for 1/2 hour followed by a 1/2 hour exposure to neostigmine (0.01 mg) resulted in only those morphological alterations typical of denervation. This is the response observed in the presence of other anticholinesterase agents after prior nerve section (Fenichel et al., 1972, 1974a; Laskowski et al., 1975). It should be emphasized, however, that electron microscopy is not an appropriate sampling technique for the analysis of relatively rare events. For example, only 2 endplates of the 10 examined from the 1/2-hour denervated preparations exhibited changes in end-plate fine structure (identical to those seen with 1/2-hour neostigmine exposure with no prior nerve section). Such uncommon occurrences might easily be overlooked. Nevertheless, it is believed that these infrequent instances of end-plate damage provide additional evidence for the mode of action of neostigmine. Since nerve terminals remain functional after a 1/2-hour denervation, a small proportion of the motor end-plates presumably are activated at a rate sufficient to produce the observed postsynaptic alterations. Conversely, the absence of postsynaptic damage in most fibers after short-term denervation indicates that neostigmine does not directly activate sufficient transmembrane ion channels to produce supercontraction or damage to membrane organelles. Additional evidence for this conclusion is provided by the lack of supercontraction or mitochondrial damage after long-term denervation.

Finally, chronic treatment with neostigmine (0.1 mg twice daily) resulted in previously undescribed alterations of the nerve axon and separation of the myelin from the neurolemma. Speculations concerning the mechanisms producing damage to the nerve axons are not warranted at this time.

In summary, neostigmine methylsulfate at high doses produces severe alterations in end-plate fine structure which are probably related to repeated and prolonged increases in pre- and postsynaptic membrane permeability. These alterations are virtually eliminated by prior nerve section, supporting the proposal that increased acetylcholine in the synaptic cleft underlies the myopathic changes. The deleterious alterations described in this study conducted on rat neuromuscular junctions are similar to certain of the alterations observed in human myasthenia gravis for which neostigmine is a common therapeutic agent. Although this report does not demonstrate that neostigmine treatment accelerates the degenerative course of the disease, it does indicate that long-term therapy with neostigmine may not be considered as being free from risk.

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References

FIG. 11. Nerve axons after acute and chronic neostigmine (0.1 mg) treatment. a. Thirty minutes after a single injection, some pre- and postsynaptic mitochondria are severely distorted (arrows), while those in the myelinated nerve axon appear normal. The myelin remains closely applied to the nerve cell membrane. x 5,400. b. After 36 hours of neostigmine exposure, mitochondria are often swollen (arrow) and the myelin and plasma membrane are separated in several places (large arrow). One area of distortion actually represents the myelin configuration at a Schmidt-Lanterman cleft (C). x 3,200. c. After 7 days of chronic treatment, areas of myelin separation (arrow) and of artificial "myelin figures" (•) are present. Presumably these represent conformational rearrangement of the disrupted axolemma and/or Schwann cell myelin. x 8,500. d. After 14 days of chronic treatment, myelin separation appears maximal (arrow). x 8,200.


