

Effects of a Snake α -Neurotoxin on the Development of Innervated Skeletal Muscles in Chick Embryo

(cholinergic receptor/choline acetyltransferase/acetylcholinesterase/
embryonic development of neuromuscular junction)

G. GIACOBINI, G. FILOGAMO, M. WEBER, P. BOQUET, AND J. P. CHANGEUX

Department of Human Anatomy, University of Turin, Turin, Italy; and Department of Molecular Biology, Institut Pasteur, Paris, France

Communicated by François Jacob, March 15, 1973

ABSTRACT The evolution of the cholinergic (nicotinic) receptor in chick muscles is monitored during embryonic development with a tritiated α -neurotoxin from *Naja nigricollis* and compared with the appearance of acetylcholinesterase. The specific activity of these two proteins reaches a maximum around the 12th day of incubation. By contrast, choline acetyltransferase reaches an early maximum of specific activity around the 7th day of development, and later continuously increases until hatching. Injection of α -toxin in the yolk sac at early stages of development causes an atrophy of skeletal and extrinsic ocular-muscles and of their innervation. In 16-day embryos treated by the α -toxin, the endplates revealed by the Koelle reaction are almost completely absent. The total content and specific activities of acetylcholinesterase and choline acetyltransferase in atrophic muscles are markedly reduced.

Snake venom α -neurotoxins bind with a high affinity and a low reversibility to the cholinergic (nicotinic) receptor site (1-3). They have therefore been widely used to assay, characterize, and identify the protein that carries this site (see ref. 4). These neurotoxins can also be used to create a chronic block of neuromuscular transmission at the postsynaptic level and to analyze the consequences of this block in the differentiation, morphogenesis, and stability of a neuromuscular synapse.

In this paper, we report on the cholinergic receptor sites in chick-embryonic muscles and on their evolution during development, in comparison to the appearance of choline acetyltransferase and acetylcholinesterase. Injection of the α -toxin from *Naja nigricollis* at early stages of development causes a marked atrophy of skeletal muscles and of their innervation, accompanied by characteristic changes in their content of choline acetyltransferase and acetylcholinesterase.

MATERIALS AND METHODS

[^3H] α -Toxin. The α_1 -isotoxin was purified from the venom of *Naja nigricollis* by the method of Boquet *et al.* (5) and Karlsson *et al.* (6) and tritiated according to Menez *et al.* (7). The stock solution contained 24 μM α -toxin (10.2 Ci/mmol); 72% of the molecules of α -toxin were active.

Embryos. Chick embryos were obtained from fertilized eggs of a local strain and incubated at 38° at a relative humidity of 70-80%; 3-, 4-, 5-, 6-, 8-, 11-, 12-, 14-, 16-, 18-, and 21-day-old embryos were used. Muscles were dissected under a stereomicroscope and quickly frozen; most samples were lyophilized and stored at -45°. With 3- to 6-day-old embryos, assays were made on posterior limb buds; from the 8th day of incubation and after, the posterior muscles of the leg were used.

The embryos were treated with α -toxin by three successive injections (the 3rd, 8th, and 12th day of incubation) in the yolk sac with a Hamilton syringe, through a small window in the shell. Each time, 20-100 μl of a 1 mg/ml solution in sterile Ringer's solution was injected. Embryos were examined the 16th day of incubation. About 30% of the injected embryos died during development.

Homogenization. The tissue was added to ice-cold Ringer's solution (for [^3H] α -toxin binding), to 0.5% Triton X-100 in 0.2 M Na-phosphate (pH 7.4) (for choline acetyltransferase assay) or to 0.05% Triton X-100 in Ringer's solution (for acetylcholinesterase assay) and homogenized in a PT-10-0D Polytron Homogenizer (Luzern, Switzerland). The concentration of tissue in the homogenates ranged from 1 to 10 mg of dry weight per ml.

Enzymatic Assays. Choline acetyltransferase was measured by the radiometric method of McCaman and Hunt (8), as modified by Fonnum (9) (microassay). Acetylcholinesterase was estimated (2) by the method of Ellman *et al.* (10), with acetylthiocholine as the substrate. Proteins were measured by the method of Lowry *et al.* (11), using bovine-serum albumin as standard.

Binding of [^3H] α -Toxin. To 0-3 mg (dry weight) of homogenized tissue was added 40 μl of 48 nM [^3H] α -toxin; the final volume was adjusted to 540 μl with Ringer's solution. The mixture was incubated 4-6 hr at room temperature and filtered on a Millipore filter to remove the unbound [^3H] α -toxin. The filter was then washed with 15 ml of Ringer's solution, dried, added to 10 ml of Toluene-POPOP, and counted in an Intertechnique liquid scintillation spectrometer (12).

Histochemistry and Electron Microscopy. Acetylcholinesterase was revealed by the histochemical method of Koelle and Friedenwald (13). For ultrastructural studies, muscles were fixed in 3.5% glutaraldehyde and postfixed in 2% osmic hydroxide; specimens were embedded in Araldite (Ciba) and observed with a Siemens 1 A electron microscope.

RESULTS

Binding of (^3H) α -toxin to embryonic muscles during development

It has been reported that α -toxins from snake venoms bind to vertebrate skeletal muscle (14-18), including chicken muscles (19). Fig. 1 confirms that the tritiated α -toxin from *N.*

nigricollis binds to membrane fragments in homogenates of chick-embryonic muscles. Under our experimental conditions 10 mM decamethonium, a known cholinergic agonist, reduces by 97% the total amount of [3 H] α -toxin bound.* In the presence of 1 mM *d*-tubocurarine, a typical nicotinic antagonist, a similar decrease occurs. These observations were repeated with homogenates of 5-, 12-, 18-, and 21-day embryos. As already extensively discussed (see refs. 1-4, 7, 14-18), the protective effect of cholinergic ligands against α -toxin binding constitutes an excellent criterion for the selective binding of the α -toxin to the cholinergic receptor site. Therefore, the binding of [3 H] α -toxin is assumed to reveal the presence of cholinergic receptor sites at all these stages.

Titration curves of the type shown in Fig. 1 give estimates of the number of α -toxin binding sites, or of cholinergic receptor sites, present per mass of tissue. In 12-day embryos, there are 0.49 nmol of binding sites per g of muscle protein, a value close to that reported with other muscle preparations (14-18), but smaller than that found with membrane fragments purified from *Electrophorus* electric organ (5-15 nmol/g of protein) (7, 12).

In Fig. 2 are presented the changes during development of the total number of toxin-binding sites in the posterior muscles of the leg and of the specific activity of these sites in the same muscles (expressed as nmol/g of protein). In 4-day embryos, the amount of [3 H] α -toxin eventually bound to posterior limb buds remains below the limits of resolution of the method. After the 4th day, sites are detected and their total number increases monotonically but much faster from the 4th to the 12th day than from the 12th day to the 18th. In contrast, the specific activity rises rapidly until the 12th day, where it reaches a maximum and subsequently decreases until the 18th day of incubation.

In Fig. 2 are also compared the changes in the levels of acetylcholinesterase and choline acetyltransferase with those of the cholinergic receptor sites in the same group of muscles. It should be recalled that in embryonic-chick muscle, the cholinesterase activity arises primarily from acetylcholinesterase, rather than from pseudocholinesterase (20, 21). The time-course of the variation in the level of acetylcholinesterase parallels that observed with toxin-binding sites, except, perhaps, that the initial, fast, increase lasts until the 16th instead of the 12th day of incubation. The specific activity of both proteins reaches a peak on the 12th day. With choline acetyltransferase the picture is different. The total transferase activity increases continuously until hatching without a plateau. The specific activity rises rapidly until the 6th day, falls from the 7th to the 11th day, but then increases again after the 11th day of incubation until hatching. During muscle differentiation, then, the content of the protein responsible for acetylcholine synthesis evolves in a manner different from that of the proteins involved in acetylcholine recognition and degradation.

Effect of α -toxin on neuromuscular development

A 3-day-old hatched chick given 10 μ g of α -toxin intramuscularly dies in 20-30 min from respiration block. Administration of the total of 60-300 μ g of α -toxin in three injections does not kill the embryo, however, which survives until hatching, but it causes a reduction in its size and weight

*A similar reduction occurs in the presence of 1 μ M decamethonium and 100 μ M carbamylcholine, flaxedil, or *d*-tubocurarine.

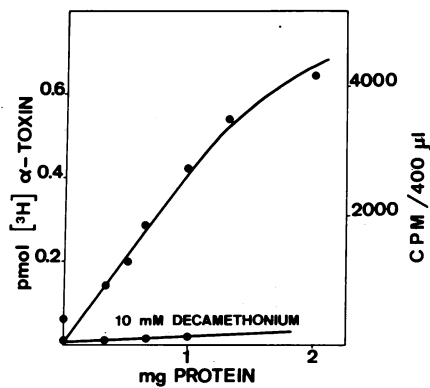


FIG. 1. Binding of [3 H] α -toxin to a muscle homogenate from 12-day embryos. The homogenate was incubated in 3.5 nM α -toxin with or without prior incubation in 10 mM decamethonium.

without evident malformation. The magnitude of the atrophy varies from one injected embryo to the other, but a significant reduction in size was already noticed in 12-day embryos. In 16-day embryos, the dry weight of leg muscles in treated embryos was about half that of untreated embryos (Table 1). At that age the atrophy was evident in all skeletal muscles and also in extrinsic ocular muscles. Interestingly, the innervation of all these muscles was affected as well. Fig. 3 shows the reduction of size of the sciatic nerve, of its spinal roots, and of corresponding sensory ganglia caused by toxin injections. Preliminary observations indicate that the motility of these embryos is considerably reduced.

Histologically (Fig. 4), atrophic muscles from 16-day treated embryos markedly differ from those of normal embryos. At that age, normal muscles contain typical adult fibers, with nuclei at the periphery and regularly organized cross-striated myofibrils. Some mononucleated cells are closely applied to fibers; myotubes are rare (Fig. 4a and b). By contrast, in toxin-treated embryos, the contractile elements are mostly myotubes (4c); in their cytoplasm myofilaments are disorganized (4f) and they no longer make parallel bundles except near the nuclei. A few myoblasts are still present and the connective tissue is more abundant. Few, if any, muscle fibers are present; at both ends these fibers show nuclei arranged in columns (4d). Similar figures commonly seen in muscle cultures or after denervation are signs of dedifferentiation. Phagocytic histiocytes, with dense bodies, lysosomes, vacuoles, and masses of granular or filamentous material are frequently observed (Fig. 4g and h). Preliminary observations at the 12th day show that at this stage atrophic and degenerative changes are much less marked than at the 16th day. The same observations were made by Drachman (22) with botulinum toxin.

The distribution of acetylcholinesterase on muscle fibers was revealed by the method of Koelle. In 16-day normal embryos only the endplate region reacts (4i). In toxin-treated ones, the Koelle reaction is almost completely negative (4l). The few positive spots seen correspond to endplates established with the rare mature muscle fibers present in the atrophic muscle (4m).

Finally, the content of choline acetyltransferase and acetylcholinesterase was estimated in leg muscles and sciatic nerve of toxin-treated and control embryos (Table 1). The total activities of transferase and esterase in leg muscles are, respectively, 5.9- and 8.4-times lower after toxin treatment. The

decrease in the total activities is not simply due to the reduction of muscle size and weight, since the specific activities of the two enzymes drop, respectively, 2.4 and 3.5 times. In sciatic nerve, the decrease of choline acetyltransferase specific activity parallels that seen with muscle. Therefore, toxin treatment affects both the development of the muscle and that of its innervation.

DISCUSSION

The development of motor innervation in chick embryo comprises three major steps (for review, see refs. 23, and 24): (i) from the 4th to the 6th day of incubation, root motor fibers

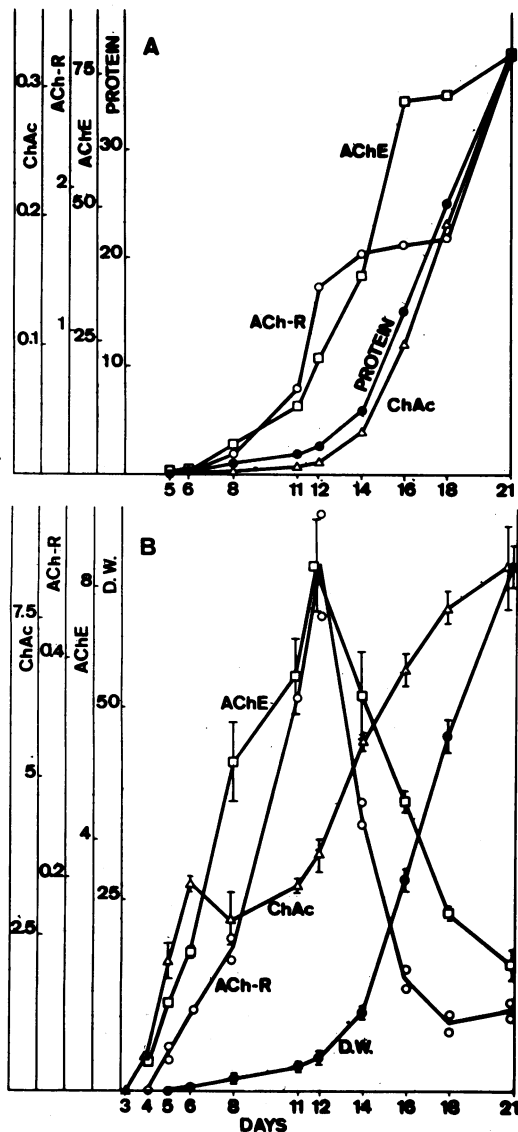


FIG. 2. Developmental changes of choline acetyltransferase (ChAc), acetylcholinesterase (AChE), cholinergic receptor (ACh-R), dry weight (D.W.), and protein in embryonic leg muscles. (A) Total activities and protein (ChAc and AChE: μM per muscle group and hour; ACh-R: pM per muscle group; protein: mg). (B) Specific activities and dry weight (ChAc: μM per g of protein per hour; AChE: μM per mg of protein per hour; ACh-R: nM per g of protein; D.W.: mg). Bars represent SEM; ACh-R determinations were made in most cases in duplicate (at each stage, single measures are indicated).

TABLE 1. Consequences of an early α -toxin injection on acetylcholinesterase and choline acetyltransferase content of innervated leg muscles in 16-day chick embryos

	Dry weight (mg)	Choline acetyltransferase		Acetylcholinesterase	
		Specific activity	Total activity	Specific activity	Total activity
<i>Muscle</i>					
Control (2)*	27.5	7.58	0.11	3.13	47
Treated (3)	11.7	2.98	0.02	0.87	5.7
Ratio Control					
Treated		<u>2.35</u>	<u>2.5</u>	<u>3.6</u>	<u>8.4</u>
<i>Nerve</i>					
Control (2)		57.2			
Treated (3)		26.7			
Ratio Control					
Treated		<u>2.1</u>			

* Same units as for Fig. 2. The embryos received 300 μg of α -toxin in three injections at the 3rd, 8th, and 12th days of incubation, and were killed the 16th day when leg muscles were dissected out. In the controls the same opening in the shell was made as in the experimental animals. A slight increase in choline acetyltransferase results from this operation and explains the difference seen between the numbers given here and in Fig. 2.

gradually invade limb-bud muscles; the first myotubes appear with a diffuse localization of acetylcholinesterase; embryonic movements of neurogenic origin (25–27) are present; (ii) from the 6th to the 12th day, the contractile elements grow and differentiate; a massive formation of myotubes occurs; acetylcholinesterase is gradually restricted to the myo-tendon junctions (23); (iii) from the 12th day until hatching, muscle fibers replace the myotubes, and the nerve endings differentiate into motor endplates with acetylcholinesterase localized at the subneural apparatus (23). The small irregular potentials of the electromyogram evolve in typical action potentials (28) and acetylcholine sensitivity, diffuse in the early stages, becomes restricted to the endplate region.

The present data indicate that toxin-binding sites, and therefore cholinergic receptor sites, as well as acetylcholinesterase, are already present in embryonic muscles at step (i). The total and specific activities of these two proteins increase markedly during step (ii), which chronologically corresponds to the fusion of myoblasts into myotubes (see ref. 18). The decrease of specific activities observed during step (iii) results from the slower increase of the content of cholinergic receptor and acetylcholinesterase, and from the faster increase in muscle weight due largely to an enrichment in myofibrillar proteins (29, 30). During this last period, a redistribution of the sensitivity to acetylcholine and of acetylcholinesterase on muscle surface takes place.

The evolution of the choline acetyltransferase content during embryonic development follows a different program. First of all, it is clear from Hebb's and Israël's work (31, 32) that this enzyme is almost exclusively present in the neural moiety of the innervated muscle and, therefore, characterizes nerve terminals. The observed time course is similar to that already described by Giacobini (33) for axial and thigh muscles. The fast increase in specific activity during the first step of development likely corresponds to the arrival of motor fibers;

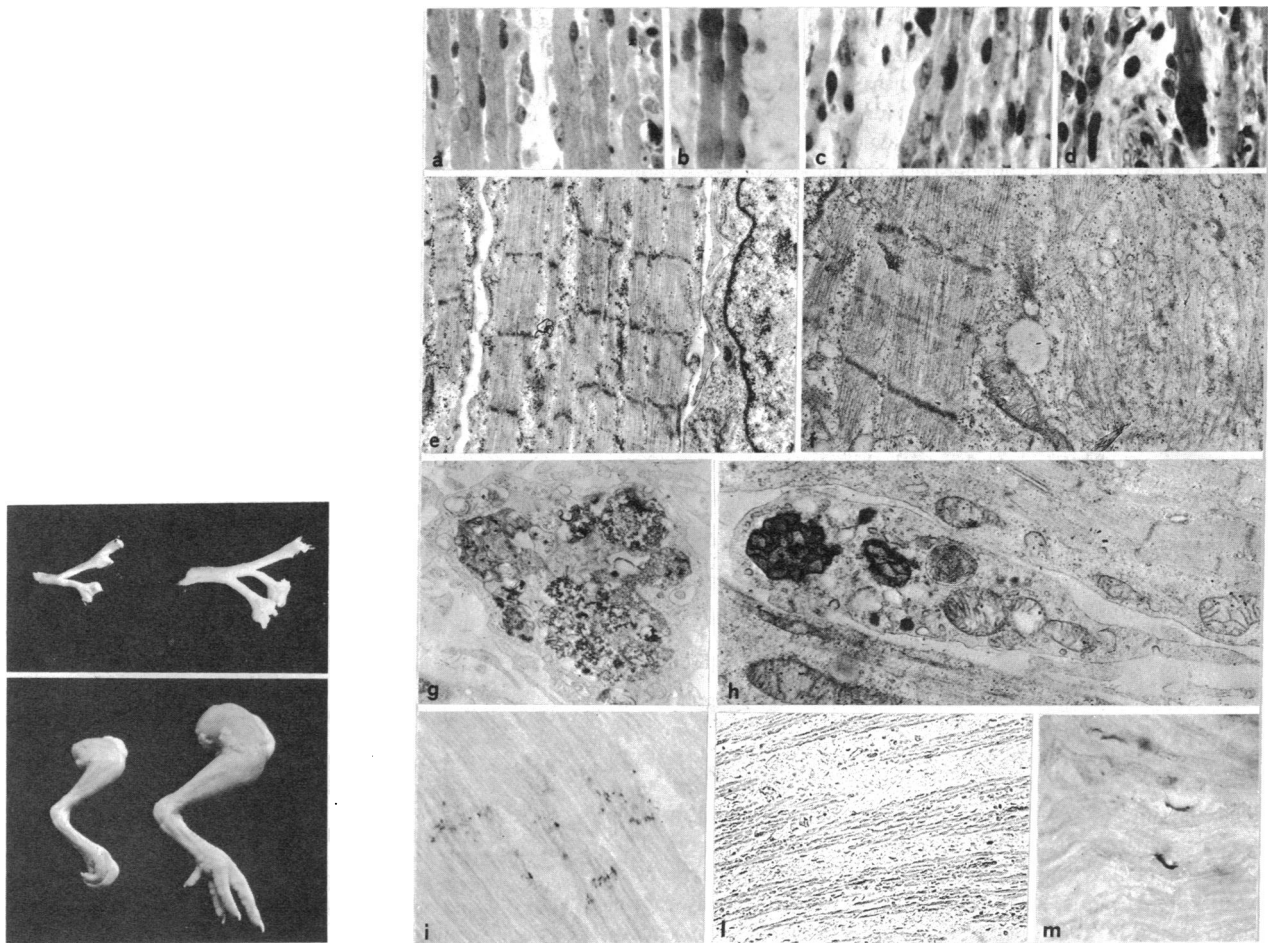


FIG. 3 (left). Legs from 19-day-old control (right) and α -toxin-treated (left) embryos. Top: the corresponding sciatic nerves (with their plexuses) are represented.

FIG. 4 (right). Consequences of early α -toxin injection on the anatomy of innervated leg muscles in 16-day-old embryos. (a) and (b) control embryo: muscle fibers with adhering mononucleated cells ($\times 110$); (c) and (d) α -toxin-treated embryos: myotubes and dedifferentiation processes ($\times 110$); (e) control embryo: regularly organized myofibrils ($\times 22,000$); (d) α -toxin-treated embryo: disorganized myofilaments ($\times 36,000$) (g) and (h) α -toxin-treated embryo: phagocytic histiocytes ($\times 25,000$ and $30,000$); (i) control embryo: Koelle-positive reaction at motor endplates ($\times 110$); (l) α -toxin-treated embryo: Koelle-negative reaction after prolong incubation ($\times 110$); (m) α -toxin-treated embryo: exceptional motor endplates ($\times 540$).

the decrease, which lasts from the 6th to the 8th day of incubation, would be caused by the relatively faster growth of muscle tissue. The final increase of specific activity would be related to increasing number and complexity of the endplates. The development of choline acetyltransferase activity, therefore, does not parallel that of receptor and that of acetylcholinesterase [as in spinal and sympathetic ganglia (34, 35)]. The specific activity of choline acetyltransferase increases earlier than that of the two postsynaptic proteins, suggesting a causal relationship in this sequence of events.

Chick embryo provides a convenient preparation to study the effect of the α -toxin on the development of innervated muscle, since the embryo survives high doses of α -toxin. Embryos injected with 60–300 μg of α -toxin become atrophic, with a marked reduction in the development of skeletal muscles. A similar atrophy was observed by Drachman (22) after injection of botulinum toxin or *d*-tubocurarine. Minor differences are noticed, however: a less-marked replacement of muscle by fat and a reduction of length of the bone leg in the α -toxin-treated embryo. These differences may be related to

the fact that Drachman's injections were made later than ours.

The atrophic muscle presents signs of delayed differentiation, since contractile elements are mostly represented by myotubes after 16 days of incubation. However, increased connective tissue, the enlarged blood vessels, the regressive forms of muscle fibers, and the phagocytic histiocytes suggest that dedifferentiation might occur as well. Most of these effects resemble those seen upon surgical denervation. The effects of the α -toxin are not restricted to muscle. In the 16-day toxin-treated embryo, motor endplates are almost completely absent, the size of the sciatic nerve is reduced, the choline acetyltransferase content of muscle and sciatic nerve decreased, etc. Moreover, spinal roots and sensory ganglia show a marked reduction in size.

An important question is raised by the mechanism through which the α -toxin causes these important alterations in the development of skeletal muscles and of their innervation. First, we emphasize that the α -toxin at the doses used is not teratogenic: its effects seem limited to systems involving

typical nicotinic receptors. Second, most present evidence indicates that α -toxin affects neither central nervous activity nor peripheral propagation of impulses (1). Although this last alternative cannot be completely ruled out, the most likely interpretation is that the observed effects arise from blockade by the α -toxin of the cholinergic receptor site of embryonic muscle. All the postsynaptic effects of delayed differentiation and dedifferentiation would then be simply caused by the lack of postsynaptic activation and, therefore, of muscle contraction.

The existence of presynaptic effects raises a question. How does a postsynaptic blockade influence the motor nerve terminal? A first possibility is that the cholinergic receptor surface contributes to the recognition between motor nerve fibers and muscle surface in the early stage of development: the α -toxin would then interfere with this recognition step (36). However, as early as the 4th day of incubation, when the first injection of α -toxin is made, Alconero (26) and Ripley and Provine (27) have observed neurogenic movements; at this early stage functional contacts are already made between the free nerve endings and the contractile elements. Moreover, at day 16, in injected embryos, some abortive endplates are seen. These two observations suggest that the α -toxin impairs the *development* of the endplate rather than the *establishment* of the neuromuscular contact.

Another possibility is that, during development, the muscle cell informs the nerve terminal of its state of activity. One channel that might transmit this information could be the sensory pathway from the muscle to the motoneuron. The extrinsic ocular muscles offer a situation particularly relevant to this point, since they receive essentially only motor innervation (37). In these muscles as well, the α -toxin causes a marked reduction in size, both of the muscle and of the corresponding motor nerves. Here, at least the sensory pathway could not be involved. We are, therefore, led to postulate that a *retrograde signal* passes *directly* from the postsynaptic to the presynaptic cell in a direction opposite to that of the transmission of impulses. The chemical nature of this signal is completely unknown.

Whatever the mechanism involved, it is clear that development of neuromuscular synapses depends upon an essential correlation between center and periphery (38–40). In addition, as in other systems (41–43), their differentiation and stability appears highly coupled with their functioning. The eventual implication of this property in learning processes has been discussed recently (44, 45).

We are indebted to Dr. M. G. Robecchi-Giacobini and to Miss M. Penna for help with electron microscopy and to Drs. Menez and Fromageot for the tritiation of the α -toxin. This work was supported by the Italian National Research Council Grant 720075404 (G. G.) and 710019304 (G. F.), the National Institutes of Health, and by funds from the Centre National de la Recherche Scientifique, the Délégation Générale à la Recherche Scientifique et Technique, the Collège de France, and the Commissariat à l'Énergie Atomique.

- Lee, C. Y. & Chang, C. C. (1966) *Mem. Inst. Butantan Simp. Int.* **33**, 555–572.
- Changeux, J. P., Kasai, M. & Lee, C. Y. (1970) *Proc. Nat. Acad. Sci. USA* **67**, 1241–1247.
- Miledi, R., Molinoff, P. & Potter, L. (1971) *Nature* **229**, 554–557.
- Hall, Z. W. (1972) *Annu. Rev. Biochem.* **41**, 925–952.
- Boquet, P., Izard, Y., Jouannet, M. & Meaume, J. (1966) *C.R. Acad. Sci. Ser. D*, **262**, 1134–1137.
- Karlsson, E., Eaker, D. & Porath, J. (1965) *Biochim. Biophys. Acta* **127**, 505–520.
- Menez, A., Morgat, J. L., Fromageot, P., Ronseray, A. M., Boquet, P. & Changeux, J. P. (1971) *FEBS Lett.* **17**, 333–335.
- McCaman, R. E. & Hunt, J. M. (1965) *J. Neurochem.* **12**, 253–258.
- Fonnum, F. (1969) *Biochem. J.* **115**, 465–472.
- Ellman, G. L., Courtney, K. D., Andres, V. & Featherstone, R. (1961) *Biochem. Pharmacol.* **7**, 88–95.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
- Weber, M., Menez, A., Fromageot, P., Boquet, P. & Changeux, J. P. (1972) *C.R. Acad. Sci. Ser. D*, **274**, 1575–1578.
- Koelle, G. B. & Friedenwald, J. S. L. (1949) *Proc. Soc. Exp. Biol. Med.* **70**, 617–622.
- Miledi, R. & Potter, L. (1971) *Nature* **233**, 599–603.
- Barnard, E., Wieckowski, J. & Chiu, T. H. (1971) *Nature* **234**, 207–209.
- Fambrough, D. M. & Hartzell, H. C. (1972) *Science* **176**, 189–191.
- Berg, D., Kelly, R. B., Sargent, P. B., Williamson, P. & Hall, Z. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 147–151.
- Patrick, J., Heinemann, S., Lindström, J., Schubert, D. & Steinbach, J. H. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 2762–2766.
- Vogel, Z., Sytkowski, A. J. & Nirenberg, M. W. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 3180–3184.
- Millo, A. (1961) *Riv. Biol.* **54**, 251–261.
- Goodwin, B. C. & Sizer, I. W. (1965) *Develop. Biol.* **11**, 136–153.
- Drachman, D. B. (1967) *Arch. Neurol. (Chicago)* **17**, 206–218.
- Filogamo, G. & Gabella, G. (1967) *Arch. Biol.* **78**, 9–60.
- Couteaux, R. (1963) *Proc. Roy. Soc. Ser. B* **158**, 457–480.
- Visintini, F. & Levi-Montalcini, R. (1939) *Schweiz. Arch. Neurol. Psychiat.* **4**, 119–150.
- Alconero, B. B. (1965) *J. Embryol. Exp. Morphol.* **13**, 225–266.
- Ripley, K. & Provine, R. (1972) *Brain Res.* **45**, 127–134.
- Boethius, J. (1967) *J. Exp. Zool.* **165**, 419–424.
- Dickerson, G. W. (1960) *Biochem. J.* **95**, 33–40.
- Baril, E. F. & Herrman, H. (1967) *Develop. Biol.* **15**, 318–330.
- Hebb, C. O., Krnjević, K. & Silver, A. (1964) *J. Physiol. Lond.* **171**, 504–513.
- Israël, M. (1970) *Arch. Anat. Microsc. Morphol. Exp.* **59**, 67–98.
- Giacobini, G. (1972) *J. Neurochem.* **19**, 1401–1403.
- Marchisio, P. C. & Consolo, S. (1968) *J. Neurochem.* **15**, 759–764.
- Giacobini, G., Marchisio, P. C., Giacobini, E. & Koslow, S. H. (1970) *J. Neurochem.* **17**, 1177–1185.
- Sytkowski, A. J., Vogel, Z. & Nirenberg, M. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 270–274.
- Cowan, W. M. & Wenger, E. (1967) *J. Exp. Zool.* **164**, 267–280.
- Shorey, M. L. (1909) *J. Exp. Zool.* **7**, 25–64.
- Hamburger, V. (1934) *J. Exp. Zool.* **68**, 449–494.
- Jacobson, M. (1970) in *Developmental Neurobiology*, ed. Holt, (Rinehart and Winston, New York), p. 228.
- Thoenen, H., Mueller, R. A. & Axelrod, J. (1969) *J. Pharmacol. Exp. Ther.* **169**, 249–264.
- Black, I. B., Hendry, I. A. & Iversen, L. L. (1971) *Brain Res.* **34**, 229–240.
- Thoenen, H. (1972) *Pharmacol. Rev.* **24**, 255–267.
- Changeux, J. P. (1972) *Communications* **19**, 37–47.
- Changeux, J. P., Courrège, P. & Danchin, A. (1973) *Proc. Nat. Acad. Sci. USA*, submitted.