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EDITORIAL

Highlights from 40 Years' Research on the Neuromuscular Junction*

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My scientific career started when I took part in The International Congress of Physiology and Pharmacology held in Copenhagen 1950. At that time I was a young instructor in the Department of Pharmacology at the Caroline Institute in Stockholm. The professor was Göran Liljestrand, a brilliant and in many ways remarkable person. He was a superb speaker with a sharp tongue which made him feared both in the Faculty and in the Nobel Committee whose secretary he was for several years. His main scientific interests were circulation and respiration but as a teacher he gave his students remarkable freedom to follow their own ideas and their own scientific pursuits.

In Copenhagen I listened to a lecture given by Daniel Bovét on curarizing substances. He told us about succinylcholine and its short-lasting neuromuscular blocking activity. Unfortunately, it also had muscarinic activity. Succinylcholine consists of two molecules of acetylcholine put together and that fascinated two young chemists, Lars-Erik Tammelin and Hans Löw who proposed to me that they would synthesize the compound if I was willing to study its pharmacological effects. I eagerly accepted their offer and started with animal experiments. That was quite in line with previous traditions at the Department of Pharmacology in Stockholm because Liljestrand's predecessor was C. G. Santesson who was one of the pioneers in elucidating the mode of action of various arrow poisons including that of curare from South America.

I confirmed the short-lasting neuromuscular blocking activity of succinylcholine which is rapidly hydrolyzed by cholinesterases. However, the compound I obtained apparently was more pure than that used by Bovét because it lacked muscarinic activity. Hence, I could, together with an anaesthesiologist, try the compound clinically as a relaxant when a short-lasting effect was required

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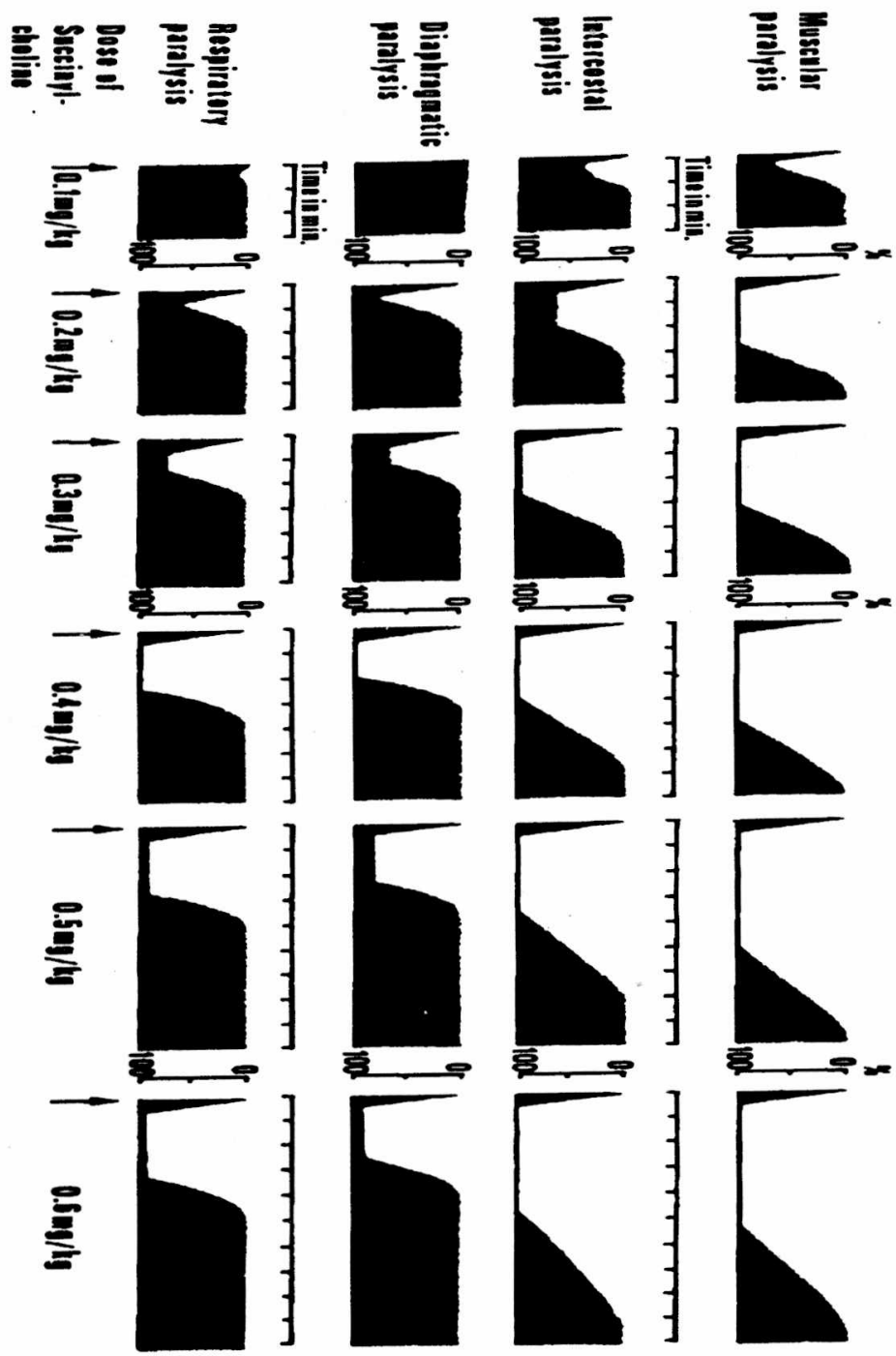

Fig. 1
Schematic presentation of the duration and the degree of muscle paralysis in the anaesthetized man by different doses of succinylcholine (from Thesleff 1952).

(von Dardel and Thesleff 1952). Fig. 1 is from my Ph.D. thesis (Thesleff 1952). Soon the drug gained clinical popularity and I am glad to say that succinylcholine even now, almost 40 years later, is in world-wide use as a muscle relaxant.

The next step in my research was to try to learn the exact mode of action of succinylcholine at the neuromuscular junction. At that time, Paton and Zaimis (1949) had shown that decamethonium, a structurally related substance, depolarized the postsynaptic endplate and blocked transmission by what is called a depolarization block. To perform similar experiments with succinylcholine required electrophysiological techniques and the obvious place to learn such was at Ragnar Granit and his coworker Anders Lundberg in Stockholm (Lundberg and Thesleff 1953, Granit *et al.* 1953). At that time electrophysiology was much more difficult and cumbersome than the present generation of scientists can imagine. DC-recordings required dozens of serially connected batteries which had to be fully charged to prevent detrimental potential drifts in the amplifier. To improve the rate of successful experiments we shared a bottle of wine whenever everything worked technically. Once I mastered these techniques I continued these studies in Lund where I had been promoted to associate and later on to professor of pharmacology.

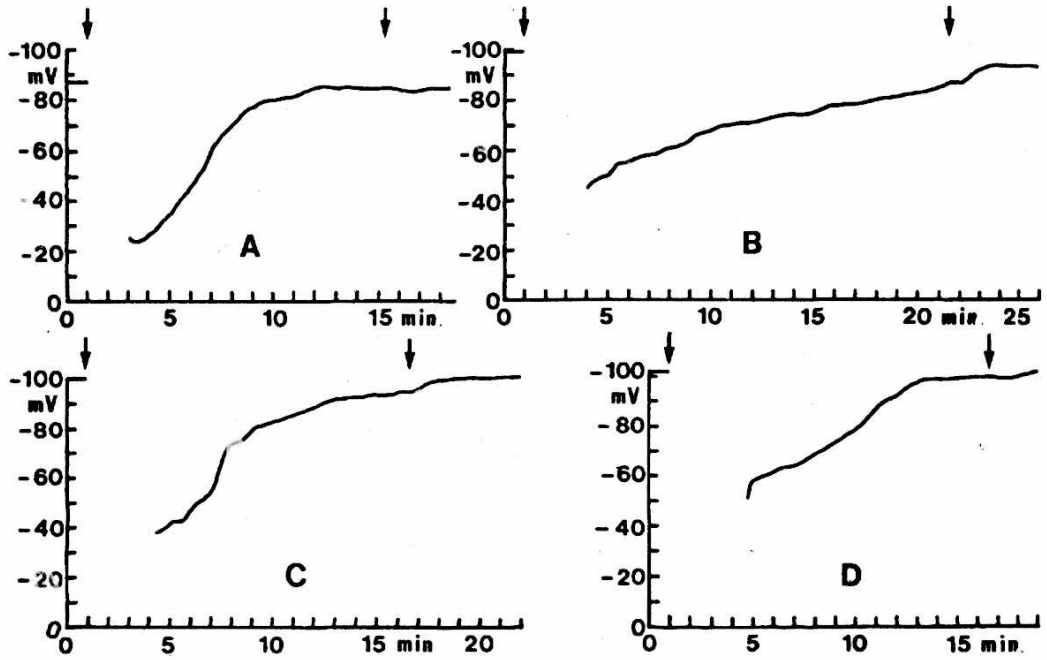

Fig. 2
The resting membrane potential at the endplate region of single frog muscle fibres. At the arrows the following agonists were added to the organ bath. A: acetylcholine 10^{-5}; B: Nicotine 1.5 x 10^{-5}; C: decamethonium iodide 4 x 10^{-5}; D: succinylcholine iodide 2 x 10^{-5} g/ml (from Thesleff 1955b).

To my surprise the depolarization of the endplate induced by succinylcholine and other similarly acting drugs rapidly disappeared with time while the neuromuscular block remained (Fig. 2). It was as if the cholinergic receptor had desensitized when stimulated during a prolonged period of exposure to the agonist (Thesleff 1955a,b). After those preliminary studies I had an opportunity to visit

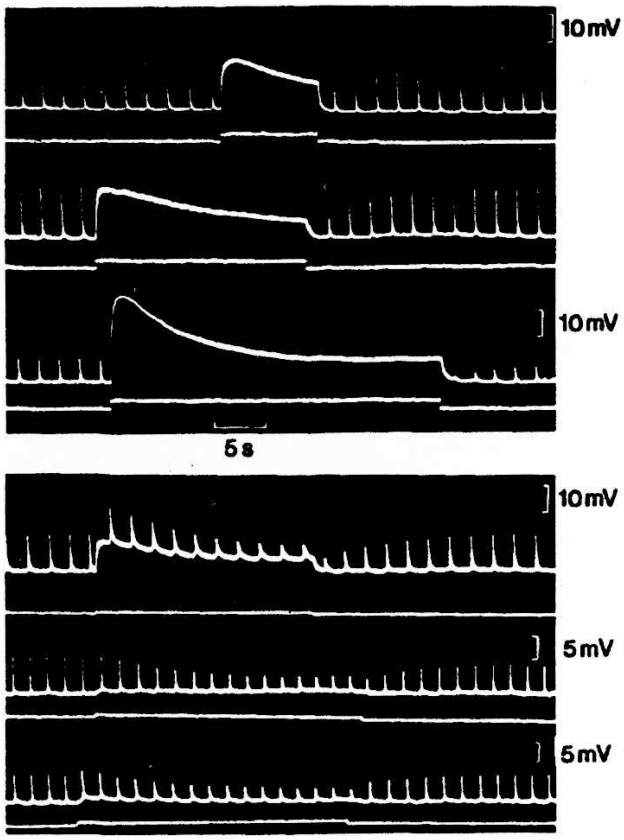

Fig. 3
Desensitization produced by different conditioning doses of acetylcholine at a single endplate spot in the frog sartorius muscle. Double-barrel pipettes were used, test pulses and conditioning doses being delivered through separate pipettes. Monitor calibration (10 mV scale) = 0.2×10^{-8} A (from Katz and Thesleff 1957).

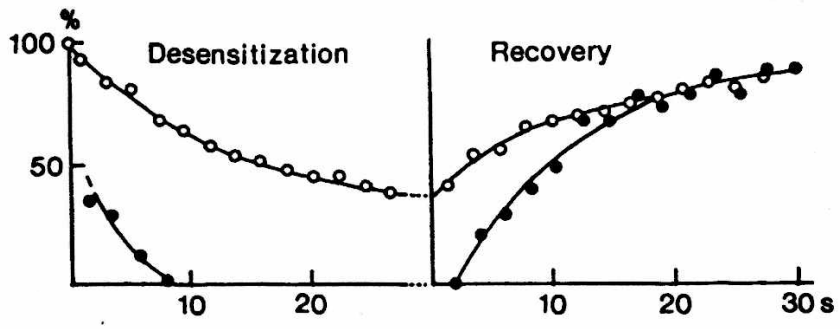

Fig. 4
Amplitude of test responses, during and after application of two desensitizing doses of an agonist. Note that the onset of desensitization could be slower than the recovery process (from Katz and Thesleff 1957).

The Department of Biophysics at University College, London and to work with Sir Bernard Katz. That was in 1956, shortly following their discoveries of the quantal nature of the transmitter release process and the Department was a veritable Mecca for anyone interested in synaptic transmission. Together with del Castillo, Katz had developed a technique for dual barrel microionophoretic application of drugs to the neuromuscular junction (del Castillo and Katz 1955). This technique seemed eminently suited for studies of the process of receptor desensitization by agonists (Katz and Thesleff 1957). Fig. 3 shows desensitization produced by different conditioning doses of acetylcholine at a single frog endplate. Fig. 4 gives examples of the amplitude of test responses during the onset and recovery from desensitization induced by two conditioning doses of acetylcholine. Note that the onset of desensitization could be slower than recovery. Furthermore, we showed that the affinity of the agonist for the desensitized receptor was higher than for the non-desensitized one. We thought of the following kinetic schemes to explain the desensitization process (Fig. 5). The two upper models required that onset of desensitization always was faster than recovery and that was clearly not the case, so we discarded them. The cyclic model, however, fitted the observations including a higher affinity for the desensitized form of the receptor. This model has subsequently been tested by various laboratories and is still accepted as the one which best describes the phenomenon. It also means that, even in the presence of very low concentrations of agonist drugs, a portion of the receptors is in the desensitized state. Desensitization, as we know it today, is a reversible allosteric change of the receptor molecule which causes its ion channel to close. It is apparently an intrinsic molecular property of all ligand-gated ion channel receptors and therefore of great pharmacological and physiological interest (for a recent review see Ochoa *et al.* 1989). The detailed molecular mechanism underlying desensitization, i.e. the closure of the ion channel, despite the presence of the agonist, is still unknown. Several agents and procedures have been shown to modulate desensitiation. For instance, there a number of neuropeptides, present in the motor nerve, known to speed up the process as are increased intracellular calcium levels and phosphorylation processes. Physiologically, it is considered that desensitization of receptors plays a significant role in the operation of neuronal networks associated with memory and learning processes (Changeux *et al.* 1987, Changeux and Heidmann 1987). Desensitization is a safety device which prevents cells from being overstimulated with cell death as a result. From a pharmacological point of view it, naturally, is of primary importance for the understanding of the mode of action of a number of drugs including that of succinylcholine.

Obviously, the ionophoretic microapplication technique was valuable for studies of receptor properties. When I returned back to Sweden, I therefore started to use that technique to investigate the phenomenon of denervation supersensitivity in mammalian skeletal muscle (Axelsson and Thesleff 1959). This study was prompted by reading the fascinating monograph "*Supersensitivity of Denervated Structures*" by Cannon and Rosenblueth (1949) and was made together with Johann Axelsson who now is professor of physiology in Reykjavik. Fig. 6 shows that in an innervated cat muscle only the endplate region responds to microionophoretic application of acetylcholine while in a 14-days denervated muscle the entire membrane is sensitive to the drug. This was a surprising finding and later we observed that a new type of sodium channels, which were resistant to the blocking

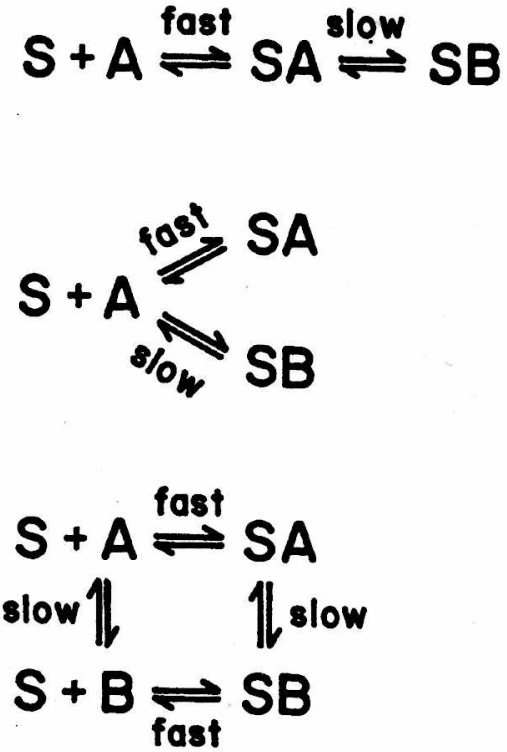

Fig. 5
The two upper schemes represent two successive or simultaneous reactions, a depolarizing reaction which reaches equilibrium very rapidly and a desensitizing reaction which proceeds more slowly. Symbols: S, agonist concentration; A, free receptors; SA, effective drug-receptor compound; SB, desensitized compound. In the two upper schemes the onset of desensitization has to be faster than recovery while in the lowest scheme the recovery process from B to A is slowed by the presence of the agonist as was experimentally observed (from Katz & Thesleff 1957).

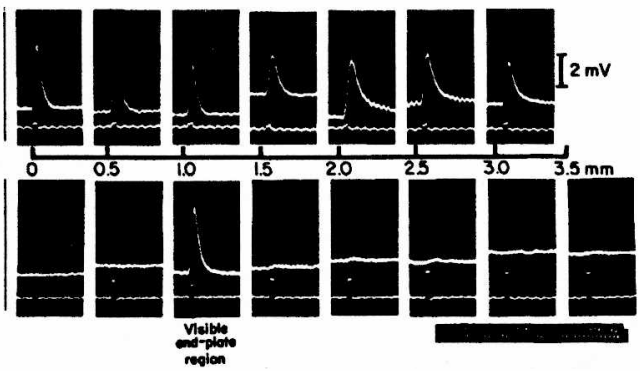

Fig. 6
The sensitivity of a muscle fibre from the cat tenuissimus muscle to microionophoretic application of acetylcholine to points separated by distances of about 0.5 mm. In an innervated fibre (lower records) only the visible endplate region was sensitive while in a 14-days denervated fibre each membrane point responded to acetylcholine (upper records). Time marker 100 Hz (from Axelsson and Thesleff 1959).

effect of tetrodotoxin, were also inserted into the denervated muscle membrane (Redfern *et al.* 1970, Redfern and Thesleff 1971, Harris and Thesleff 1971). Denervation induced the synthesis of a new population of acetylcholine receptors and sodium channels which were inserted into the extrajunctional membrane (Grampp *et al.* 1972). Stimulated by my friend Ernest Gutmann in Prague I came to devote several years to the study of such phenomena. They are responsible for supersensitivity and fibrillation in muscle (Thesleff 1960, 1982) and are induced not only by denervation but also by any other means which cause prolonged paralysis of the muscle. In the nervous system similar mechanisms seem to operate and many of the effects and side reactions of centrally acting drugs could be related to such changes in neuronal receptor numbers and distribution. Today we know, thanks to the pioneering studies of Terje Lömo and coworkers in Oslo (see Lömo and Gundersen 1988), that muscle inactivity and thereby lowering of the intramuscular concentration of free calcium ions triggers the synthesis and insertion of new cholinergic receptors (Birnbaum *et al.* 1980, Forrest *et al.* 1981) and ion channels into the muscle membrane. While, as shown by Changeux and others (Fontaine *et al.* 1986, Fischbach *et al.* 1989), the endplate properties are controlled by trophic substances released from the motor nerve.

I also used the isolated human nerve-intercostal muscle preparation to study neuromuscular transmission in patients with *myasthenia gravis* (Elmqvist *et al.* 1960, Dahlbäck *et al.* 1961). We observed a reduction in the amplitude of spontaneous miniature endplate potentials as compared to potentials from healthy subjects (Elmqvist *et al.* 1964). Unfortunately, we attributed it to a presynaptic defect of either transmitter formation or release in myasthenic patients. This erroneous belief was no doubt due to our previous observation that the drug hemicholinium, which blocks the synthesis of acetylcholine, caused a similar reduction in the size of released transmitter quanta (Elmqvist *et al.* 1963, Elmqvist and Quastel 1965). It is now known that *myasthenia gravis* is a postsynaptic disease in which the number of cholinergic receptors is reduced by an autoimmune process and thereby the size of spontaneous and evoked endplate potentials.

I have tried to analyze denervation phenomena by the use of botulinum neurotoxins. These toxins block the release of acetylcholine from nerve terminals and cause thereby a long-lasting neuromuscular block. Without affecting the morphology of the synapse and the possible transfer of trophic substances between nerve and muscle (see recent reviews by Simpson 1986 and Sellin 1987). When using the toxin, type A, we made a number of observations which I will briefly recall. One observation was that increasing the extracellular calcium level antagonized the neuromuscular block produced by the toxin (Thesleff 1960). This prompted us to try potassium channel blockers as antagonists towards the paralysis caused by the toxin (Lundh *et al.* 1977). Among such blockers are the aminopyridines, either 4-aminopyridine or 3,4-diaminopyridine. By blocking presynaptic potassium channels they prolong the duration of the nerve terminal action potential and thereby greatly increase the amount of calcium influx into the nerve terminal (see a monograph on the subject, Lechat *et al.* 1982). These compounds were potent antagonists to the neuromuscular block produced by botulinum toxin and were able to restore muscle activity in animals fully paralyzed by the toxin. 3,4-diaminopyridine was in that respect more potent than 4-aminopyridine and furthermore it less readily crossed the blood-brain barrier making its effects

restricted mainly to the peripheral nervous system (Molgó *et al.* 1980). We have tried that drug in the treatment of the Lambert-Eaton myasthenic syndrom, a disease with symptoms similar to those in botulism (Lundh *et al.* 1984). 3,4-diaminopyridine is today recommended as the drug of choice for the symptomatic treatment of this neuromuscular disorder.

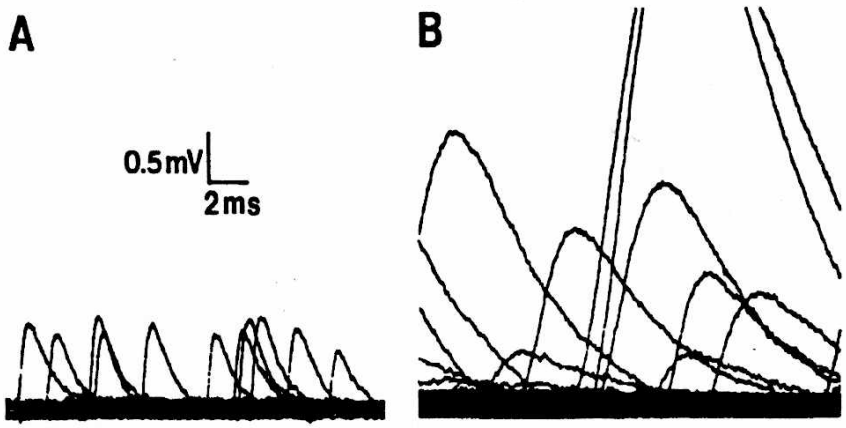

Fig. 7
Examples of spontaneous miniature endplate potentials in a single fibre from the rat extensor digitorum longus muscle (A) and from a similar muscle (B) in which neuromuscular transmission was blocked 13 days previously by botulinum toxin type A. Note the appearance of giant or slow-rising potentials in the blocked muscle (from Thesleff 1985).

Another interesting observation in botulinum toxin paralyzed muscles was the appearance of unusually large spontaneous postsynaptic potentials, so called giant or slow rising miniature endplate potentials (Thesleff *et al.* 1983, Kim *et al.* 1984). Fig. 7 illustrates such potentials in a botulinum toxin type A paralyzed rat muscle. We showed that these potentials were caused by large amounts of acetylcholine released from the same presynaptic pool of transmitter which caused normal miniature endplate potentials and impulse evoked endplate potentials (Lupa *et al.* 1986). Furthermore, the acetylcholine seemed to be of synaptic vesicular origin since drugs which blocked vesicular uptake of acetylcholine also blocked the appearance of giant potentials (Tabti *et al.* 1986). The release of acetylcholine causing these potentials was uninfluenced by calcium ions (Thesleff *et al.* 1983), and could therefore not be evoked by nerve stimuli. Similar potentials, but at a much lower frequency, have previously been observed at normal unparalyzed junctions by Liley (1957). Such large potentials are also common at newly formed endplates with regenerating nerve terminals (Bennett *et al.* 1973, Colméus et al. 1982). The same applies to neuromuscular junctions in which transmission has been blocked for a prolonged period of time by curare or tetrodotoxin (Ding *et al.* 1983, Gundersen 1987). The potentials disappear when proximo-distal axoplasmic transport is blocked by cooling or by colchicine (Thesleff *et al.* 1983, 1990). That points to the possibility that the potentials are somehow related to neurotrophic functions. Large dense-core synaptic vesicles, containing both acetylcholine and neuropeptides, which are transported from the cell soma to the nerve terminals, are common under

the conditions just mentioned (Lüllmann-Rauch 1971, Jirmanová and Thesleff 1976). Such vesicles release their content in a calcium-insensitive way (Matteoli *et al.* 1988). We have therefore suggested (Thesleff *et al.* 1989, 1990) that the giant potentials reflect the exocytosis of the content of such vesicles. Of special interest is that this type of neurosecretion is selectively stimulated by certain drugs. 4-aminoquinoline evokes a population of calcium-insensitive giant potentials in normal muscles and when they are already present, the drug enhances their frequency (Molgó and Thesleff 1982). Of even greater interest is our observation that tacrine, a tetrahydroaminoacridine derivative. has this action (Thesleff *et al.* 1990) as shown in Fig. 8. Tacrine is in that respect 10 times more potent than 4-aminoquinoline and acts at a concentration of a few micromoles. Tacrine is a drug which has recently achieved international interest because of claims that it relieves some of the symptoms characterizing the Alzheimer type of dementia (Summers *et al.* 1981, 1986). If indeed tacrine selectively stimulates the release of trophic neuropeptides, it could explain the favourable effects obtained with the drug in Alzheimer's disease.

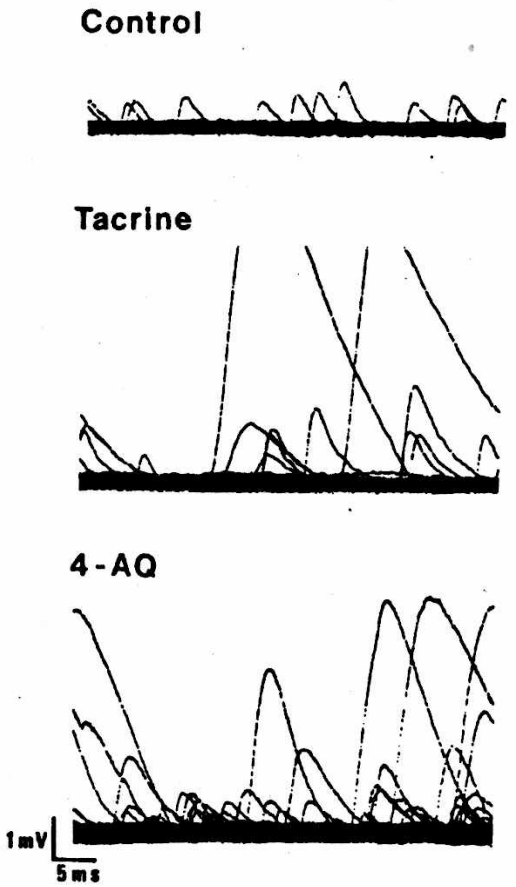

Fig. 8
Superimposed oscilloscope tracings showing spontaneous miniature endplate potentials in the mouse nerve-hemidiaphragm preparation: (a) in an untreated muscle, normal sized, fast-rising potentials, (b) in the presence of tacrine (20 μM), and (c) in the presence of 4-aminoquinoline (4-AQ, 200 μM), illustrating the appearance of a population of giant or slow-rising potentials (from Thesleff *et al.* 1990).

Let me also mention another aspect of my neuromuscular research. In the beginning of the 70ties, Lee in Taiwan showed that certain snake toxins (alfabungarotoxin) bound selectively and irreversibly to the nicotinic cholinergic receptor in skeletal muscle. Such toxins subsequently became valuable tools for the isolation and characterization of this receptor. Naturally, I wanted to use this new technique in studies of cholinergic receptors in innervated and in denervated muscles. We used the tritiated alfa-toxin from the Siamese cobra to look at its binding to innervated and to chronically denervated mouse muscles (Libelius 1974). As expected, we observed a high amount of binding in the denervated but not in the innervated muscle as shown in Fig. 9. To our surprise the binding, however, did not saturate but increased with time of incubation. This increase was blocked at low temperature which indicated to us that it might be due to endocytotic uptake of the toxin molecule into the muscle fibre. This could be confirmed by the use of conventional extracellular marker molecules like ^{3}H-inulin and horseradish peroxidase (Libelius *et al.* 1978, 1979, Tagerud and Libelius 1984, Libelius and Tagerud 1984). The uptake in denervated muscle was restricted to a spindle shaped segment closely associated with the former endplate as shown by Fig. 10 (see Plate 1) (Tagerud *et al.* 1986a). We have also shown that similar endocytotic activity is present in myotubes before innervation (Tagerud *et al.* 1990) and in muscle fibres where neuromuscular transmission had been blocked for a prolonged period of time by botulinum toxin (Tagerud *et al.* 1986b). Since membrane uptake by endocytosis is coupled to exocytotic insertion of the membrane, we believe, as a working hypothesis, that our observations of endocytosis in denervated or in paralyzed muscles are in fact reflections of secretory activity from the endplate, possibly of growth factors for motor neurones. That could explain the remarkable amount of nerve sprouting seen in partially denervated or in chronically paralyzed skeletal muscles.

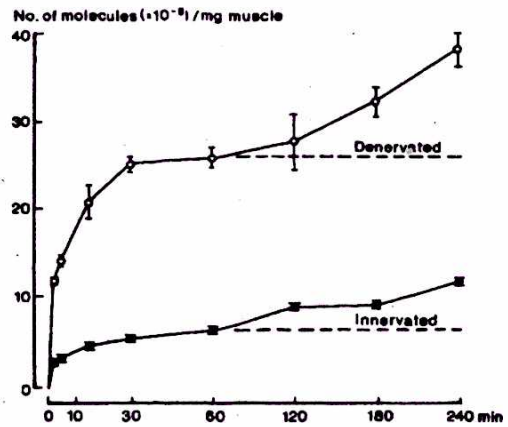

Fig. 9
Uptake of ^{3}H-labelled cobra neurotoxin to the innervated (solid symbols) and to the 6-days denervated (open symbols) extensor digitorum longus muscle of the mouse. Mean values ± S.E.M. Toxin concentration was 5 μg/ml (modified from Libelius 1974).

I have now taken the reader through a 40 years' journey in the neuromuscular junction. For me that journey is close to its end because I retired on

July 1, 1990. However, I have kept my laboratory also after my retirement and as every good soldier I aspire to die with my boots on, that is in the lab.

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Plate 1 – Thesleff

Fig. 10
High endocytic activity in the endplate region of denervated muscle fibres. a) 12-days denervated mouse hemidiaphragm 2 h after an intravenous injection of horseradish peroxidase. Arrow denotes segments with high endocytotic activity which occur in the endplate region of the denervated muscle. Bar 2 mm. b) Longitudinal section of 14-days denervated mouse tibialis anterior muscle 2 h after a similar injection of horseradish peroxidase. Peroxidase staining appears with a spindle-like distribution in one of the fibres. Bar = 100 μm. c) Transverse section from a muscle similar to b). One fibre contains peroxidase staining with a ring-like distribution. Bar = 50 μm (by courtesy of R. Libelius and S. Tagerud).