CHAPTER II

PHYSIOLOGY OF NEUROMUSCULAR TRANSMISSION

MUSCLE EXCITATION - PRE-SYNAPTIC AREA - THE NEUROMUSCULAR JUNCTION - ACETYLCHOLINE RECEPTOR - THE MUSCLE FIBRE

Muscle Excitation:

The resting muscle fibre normally has potential difference of approximately 90 mV between the two sides of the surface membrane, the inner surface being negative with respect to the outer. It is therefore said to be in a state of polarization. This resting potential has been attributed mainly to the difference between the intracellular and extracellular concentrations of potassium and sodium ions. Stimulation of the muscle fibre indirectly through its motor nerve, or directly by a cathodal (outward) current applied to the fibre, results in the inner surface of the membrane becoming less negative; in fact it becomes approximately 10 mV positive with respect to the outside of the fibre. The cell membrane is said to have become "depolarized" although it actually becomes polarized in the reverse direction. There is evidence that this is due to temporary increase in permeability of the muscle membrane to sodium ions, which enter the cell from the
extracellular fluid, resulting in the inner surface of the membrane becoming more positive. This localized area of depolarization produces an outward current flow in the adjacent polarized membrane resulting in its depolarization. Thus a wave of depolarization is propagated along the muscle membrane. It is believed that repolarization is accomplished initially by an outward flow of potassium ions, followed by an active process in which sodium ions are removed from the cell and potassium ions are restored to the cell. The muscle fibre is then again ready for muscle excitation. This propagated wave of depolarization and repolarization is known as the muscle action potential.

**Pre-junctional or Pre-synaptic Area:**

The conduction of an impulse from nerve to muscle is not an electrical response but relies on the release of acetylcholine at the nerve ending to bridge the gap. This is the chemical theory of neuromuscular transmission as propounded by Dale et al (1936).

The area of nerve ending is obviously very important because at this point the formation storage and finally the liberation of acetylcholine have a very special significance.
a) **Synthesis of Acetylcholine:**

Acetylcholine is formed by the acetylation of choline with the help of the enzyme choline acetylase. In normal circumstances there is an abundance of both choline and the enzyme available at the nerve terminals because large quantities of choline can be absorbed from the extracellular space. (Birks and Macintosh 1957 and 1961).

b) **Storage of Acetylcholine:**

The advent of electron microscopy has revealed the presence of vesicles at the nerve-ending. These packets, or quanta are believed to contain molecules of acetylcholine. The number of quanta available may vary but the size of the packets remains constant. Acetylcholine is probably also formed in the neurone and transported to the presynaptic region by axonal transport.

c) **Release of the transmitter at the neuromuscular junction:**

The vesicles are congregated in the portion toward the junctional surface and microtubules,
mitochondria and other support structures are aligned toward the opposite side. More pertinent is the arrangement of the vesicles. They are ordered in a repeating pattern of triangular arrays with the apex of each triangle enclosing a small electron dense thickened area is a cross-section of a transverse band that runs across the width of the synaptic surface of the nerve ending. Because the bands seem to be part of a system that orients the vesicles in the nerve ending and controls their site of release, they are referred to as "active zones". The active zone is about 50 mm wide and has about 50 vesicles close to it and many more vesicles in the array above it. There are between 500 and 1000 active zones in a nerve ending (Peper et al 1974).

An action potential is the normal activator for the release of transmitter but per se, it does not trigger the release of transmitter. That function belongs to Calcium flux initiated by the action potential (Matz and Miledi 1965, 1967, 1969 a,b). Neither sodium flux nor depolarization will produce the release of transmitter if calcium is not present and addition of calcium to a nerve ending, as by microinjection or via exogenous calcium ionophores inserted into the membrane,
will release transmitter even if the nerve is not depolarized (Miledi 1973, Ito and Miledi 1977). Moreover, the number of quanta released by a stimulated nerve is greatly influenced by the concentration of ionized calcium in the extracellular fluid. The change in the quantal content of an e.p.p. is proportional to the fourth power of the change in calcium concentration, that is doubling the calcium results in a 16-fold increase in quanta (Dodge and Rahamimoff, 1967). The concentration of calcium is not only responsible for the release of transmitter but also the length of time during which the calcium flows into the nerve ending, that is the quantal content of e.p.p. is a function of the total number of calcium ions in the ending after a nerve is stimulated.

The calcium current begins about the time the action potential approaches its maximum depolarization and persists until the membrane is returned to its normal potential by the outward fluxes of Potassium. Because potassium efflux normally ends the calcium current, the flow of calcium can be prolonged by drugs such as 4-aminopyridine, which slow or prevent the potassium current (Thesleff 1980).
Calcium is presumed to enter the nerve via special proteins that form channels through the nerve membrane. These channels are opened by the action potential, either directly (Katz and Miledi 1969) or by CAMP formed during the action potential (Standaert and Dretchen 1979, 1981). Calcium may also return in an exchange with sodium via an antiporter, a system that carries sodium in one direction and calcium in the other. In the resting nerve the antiporter carries sodium into the cytoplasm and calcium out of it (Blaustein and Oborn 1975), but at the peak of the action potential the system can reverse to carry sodium out and calcium in. (Mullins 1976).

The concentration of free calcium ions inside a nerve normally is about $10^{-7}$ mol. litre$^{-1}$ (Baker, 1978) about 1/10,000 of that outside the nerve. This says that there is a very strong gradient to drive the ion into the nerve, but the low internal concentration of free ion also says that there are very efficient processes for trapping calcium that does enter and far sequestering it until it is extruded. One consequence is that free calcium ions do not diffuse very far in the cytoplasm (Ilinas and Heuser 1977) and therefore they must either act very close to where they enter, or act indirectly
by binding to a protein, most likely the one known as calmodulin, which initiates a sequence of reactions leading to discharge of transmitter (De Lorenzo 1980).

The exact mechanism by which calcium causes release of transmitter is not yet known, but the presence of the ion in the area of active zone seems to initiate a process in which the vesicle membrane fuses with the cell membrane and thereby connects the interior of the vesicle to the extra cellular space of the junctional cleft; transmitter leaves the opened vesicle and crosses the junctional cleft to react with cholin receptors or to be destroyed by ChE, or both.

The Neuromuscular Junction:

On leaving the pre-synaptic or pre-junctional area (i.e. the nerve ending) the acetylcholine molecules traverse a minute gap before arriving at the post-junctional (post synaptic) area on the muscle membrane. The term "neuromuscular junction" therefore includes both pre and post synaptic areas. The post synaptic area, however, is sometimes also referred to as the motor end plate.
As the myelinated motor nerve fibre approaches the muscle fibres, it divides into numerous non-myelinated terminal branches. Each of these fibres runs parallel to the axis of the muscle fibre it supplies and lies embedded in a shallow "gutter" or depression in the muscle surface.

At the myoneural junction the nerve fibre is covered by a membrane complex, sometimes referred to as the Schwann, axoplasmic or perineural membrane. The precise nature of this membrane is unclear, however it forms an anatomical barrier separating the endplate from the Extra Cellar Fluid.

At regular intervals these layers are folded inwards to make indentations towards the muscle fibre. These junctional folds (secondary clefts) are important because they pass close-to and actually indent the muscle "basement membrane" itself. It is in this region that there is a high concentration of cholinesterase. Robertson (1956) has summarised the importance of this area as follows:-
"The folds of the junction are admirably constructed to bring a very great increase in the total area of the muscle surface membrane complex in contact with the nerve-endings. Since it seems likely that acetylcholine is secreted in discrete packets or quanta at the endings, one might expect to see something analogous to secretion granules of acetylcholine either in axoplasm, sacroplasm or within the junctional membranes. It seems reasonable to speculate that the tubular or vesicular bodies of terminal axoplasm might represent such packets of ACh. Electron microscopic studies of this region demonstrate the close proximity of the vesicles (containing Ach) on one hand and these junctional folds on the other.

a) Function of the Neuromuscular Junction:

The arrival of sufficient acetylcholine molecules at the end-plate to cause a threshold depolarization, will trigger off a mechanism for the whole of the muscle fibre so that a wave of depolarization spreads outwards along its entire length, causing a mechanical contraction in its wake. The acetylcholine molecules are destroyed by a specific enzyme—Cholinesterase—almost as rapidly
as they are produced. The distance between the nerve membrane as the motar-end plate is in the region of 1 \( \mu \text{m} \). This short range enables the acetylcholine molecules to excite the end-plate in the fraction of a millisecond before being hydrolysed by cholinesterase. The close association of cholinesterase with the post-synaptic membrane has led to the suggestion that cholinesterase is itself a part of the receptor molecule (Miledi et al 1971).

b) **The Resting End-Plate Potential:**

When a micro-electrode is inserted at random into the middle of a resting muscle fibre, a steady potential of about 60-90 millivolts (inside negative) will be recorded. If the electrode is moved gradually towards the end-plate region a point is finally reached where small potential changes of about 0.5 millivolts are recorded. These miniature end-plate potentials represent the release of the small packets of acetylcholine as each vesicle ruptures. Such miniature potentials occur at fairly regular intervals (about one per second) and are only observed in resting state.
The amplitude of one of these miniature potentials is 0.5 - 2 mV and is insufficient to reach the threshold necessary to trigger off depolarization of the end-plate itself.

c) Margin of safety on Neuromuscular Transmission (Receptor Occupancy)

In the normal patient there is a large margin of safety in neuromuscular transmission. Over 70 percent of the cholinergic receptors are needed to be occupied before any signs of paresis takes place due to curare (Paton and Waud 1967). Some skeletal muscles require a higher percentage of receptor occupancy than others before signs of paresis are evident, e.g. it has been suggested that the diaphragm (clinically very resistant muscle) requires 90 percent occupancy before its function starts to fail. Both depolarizing and competitive neuromuscular blocking drugs would be expected to alter the safety factor. In the case of depolarizing drugs, slight depolarization makes the fibre more excitable than normal i.e. the safety factor has increased. On the other hand, deeply depolarized fibres become less excitable. Competitive neuromuscular blocking drugs
should also reduce the safety factor, in this case by raising the threshold to ACh.

d) The Role of the Inorganic Ions in Neuromuscular Transmission:

Magnesium blocks neuromuscular transmission, probably by interfering with the pre-synaptic release of acetylcholine (del Castillo and Engback 1954). If given in increasing quantities, magnesium finally produces complete blockade. Similarly withdrawal of calcium produces the same effect. The role of calcium is to oppose the neuromuscular blocking action of the magnesium and these two ions appear to be antagonistic. Glucose is also necessary for the formation of acetylcholine since this is an active metabolic process. (Feldberg 1945).

e) Removal of Acetylcholine:

Acetylcholine molecules only have a minute distance (1 μm) to travel before reaching their target. Their subsequent fate, however, is a little more obscure. The majority are certainly hydrolysed by the specific enzyme – cholinesterase – to acetic acid and choline.
The molecules of choline are then available for resynthesis by the nerve terminals. Nevertheless some of the Acetylcholine molecules probably diffuse into the interstitial space and are then no longer available to take part in the resynthesis process. Evidence for this diffusion process is based on the finding that acetylcholine molecules can be collected from the perfusion fluid of a nerve muscle preparation that has been protected from hydrolysis by an inhibitor such as eserine (Brown et al 1936).
ACETYLCHOLINE RECEPTOR:

The ACh receptor (ACh-R) is the best known membrane-bound receptor protein.

The techniques of interference optics makes possible visualization of the motar-end-plates, facilitating many morphological and electrophysiological experiments. Freeze fracture and freeze-etching techniques have brought a much better view of the ultrastructure of the pre and post synaptic membrane. With immunohistochemical and autoradiographic electron microscopy, the exact localization and the density of ACh-R in the post-synaptic membrane could be established. Biochemists succeeded in isolating and characterizing the receptor protein. Antibodies against ACh-R could be produced and they induced an experimental myasthenia in animals. In frog the unmyelinated nerve terminal is elongated with several branches of microns. Synaptic transmission occurs over the whole length of the nerve terminal. The mammalian neuromuscular junction is relatively compact and extends about 10 μm above the muscle membrane, justifying the name "end-plate". As revealed by ionophoretic application of agonists, the ACh-R are mainly restricted to this "end-plate" region in normal muscles.
**FIG. 1:** Schematic drawing of the ultrastructure of the frog neuromuscular junction in longitudinal section. Thin fingers from a Schwann cell, overlaying the nerve terminal, often completely embrace the nerve terminal in regions between the junctional folds, thus dividing the terminal into compartments. Numerous synaptic vesicles often congregate near specialized presynaptic structures, called "active zones". These are located just opposite the openings of the junctional folds. Synaptic vesicles fuse at the active zones. Ach-receptors are located in a high density mainly at the crests of the junctional folds. The distance between the folds is about 1 μm. The same structures are found in mammalian motor end plates, but their arrangement is not as regular as in the frog neuromuscular junction.
The pre-synaptic membrane is characterized by specialized structures, called "active zones" which are believed to be the release sites of the ACh-filled vesicles. These "active zones" are located just opposite the openings of the junctional folds. (as shown in figure).

An important step in the characterization of ACh-R properties was the finding of the snake venom bungarotoxin (C-Bu Tx), a polypeptide which binds specifically and almost irreversibly to nicotinic receptors on skeletal muscles (Lee. 1972). This toxin competes with cholinergic agonists and antagonists for the same binding sites on ACh-R. Using radionuclide-or peroxidase-labelled C-Bu Tx, it could be shown by electron microscopy that the ACh-R are sharply localized to the crests of the functional folds just beneath the active zones. The lower value of the particle density compared with the C-Bu Tx binding sites density can be explained by the fact that two to four C-Bu Tx molecules bind to one ACh-R. The specific AChE, which destroys the transmitter in the synaptic cleft, is mainly located in the basement membrane and evenly distributed over the whole subsynaptic membrane including the depths of the folds.
On normal muscle fibres the ACh-R density decreases markedly outside the end-plate area being about 1000-fold less in extrasynaptic membrane more than 200 μm away from the end-plate. However, in developing or denervated muscle ACh-R called extrajunctional ACh-R, exists over the entire muscle membrane.

a) Biochemistry of ACh-receptor:

Use of the snake venom α-BuTx facilitated the purification and biochemical analysis of the ACh-R from the postsynaptic membrane of the electric organs of the electric fishes. The ACh-R is an integral membrane protein which extends about 5 nm into the extracellular and about 1.5 nm into the intracellular space. (Ross et al 1977). The receptor has a molecular weight of about 250000. It consists of five glycoprotein subunits, two of them of 40,000, the others of 50,000, 60,000 and 65,000 apparent molecular weight. The 40,000 peptides carry the binding sites for both the acetylcholine and for α-CBuTx (Heidmann and Changeux 1978, Dolly-1979). The location of the ion channel within the protein complex has not been identified.
b) **Agonist-Receptor Interaction:**

The ACh-R have two functions in the physiological chain of events that leads to the contraction of muscle fibres after nerve stimulation - first, the recognition of neurotransmitter molecules, and second the formation of an open ion channel which results in a membrane permeability change.

The ion channel controls the flow of small cations like Na\(^+\), K\(^+\), to a lesser extent, Ca\(^{+2}\) through the membrane. The opening of the channel thus short-circuits the membrane potential, depolarizing the end-plate region of the muscle fibre. At all physiological membrane potentials Na\(^+\) will tend to enter the cell from the extracellular space (where its concentration is high) and K\(^+\) will tend to leave the cell from cytoplasm. At the resting potential (or any other \(-\)ve membrane potential) the net current through the ion channel flows in the inward direction. The amount of current is directly proportional to the number of open channels, so the membrane current is the most direct response of the agonist-receptor interaction which one can measure at present. For this it is necessary to keep the membrane potential constant which can be done by voltage clamp technique.
The Muscle Fibre:

A striated muscle fibre possesses three principle components:

i) The myofibril which contains the contractile element

ii) The mitochondria and nuclei

iii) The cell membrane and intercommunicating systems.

A closer study of the myofibril reveals that it contains a series of two important overlapping bands - the A bands and the I bands. The I bands are attached to another important structure which runs transversely round the fibre and is called the Z line. The area between two Z lines is referred to as a sarcomere. The A band, on the other hand is darker and contains the important enzyme adenosinetriphosphatase (ATPase) which is essential for the break down of ATP in order to release energy. A muscle contraction takes place when two types of protein - actin of the I band and myosin of A band - interdigitate to produce a shortening of the fibre length (Huxley and Taylor 1958, Huxley. 63). A closer look at the Z line using electron microscope reveals that it is really a transverse aqueous channel connecting the cell membrane
with the interior of the fibre. The mouth of this system lies on the surface where it merges with the cell membrane and is open to the extracellular medium (Page 1968). This channel is called the transverse tubular or "T" System. On passing to the interior, it comes very close to another aqueous channel system - the sarcoplasmic reticulum. The "T" System and the sarcoplasmic reticulum system do not actually join but in certain areas they lie in very close proximity. The sarcoplasmic reticulum is a much larger system and runs vertically between two Z bands.

Thus when a motor-end-plate becomes depolarized the excitatory disturbance is believed to spread into the interior of the fibre along the "T" system (Huxley and Taylor 1958). Here it comes into contact with the terminal sacs of the sarcoplasmic reticulum. There is considerable evidence to support the view that the tubules of this system act as an intracellular store for calcium ions which are released on depolarization. The resulting rise in calcium ion concentration of the sarcoplasmic triggers the activity of the enzyme ATPase, thereby initiating the breakdown of the stored adenosine triphosphate to release energy and produce a contraction (Weber et al 1964). Relaxation occurs when the calcium
ions re-accumulate within the sarcoplasmic reticulum by means of a calcium pump (Page 1968). The process is repeated again and again as the contraction wave spreads along the length of fibre.

Heat, Energy and Oxidative Phosphorylation in Muscle:

The method of producing heat and energy in muscle is a complex one involving a series of reductions and oxidations within respiratory chain. At each stage in the chain a certain amount of heat and energy is produced. At three stages this energy is made available for the oxidative phosphorylation of adenosine diphosphate (ADP) to form adenosine triphosphate (ATP) with the help of the enzyme adenosine triphosphatase (ATPase) which is associated with the respiratory chain of the Kreb's cycle.

The process could be presented as follows:-

a) within the mitochondrion

Energy from respiratory chain + ADP + P\text{ATPase in} \frac{\text{ATP}}{- \text{ATPase in}} - \text{respiratory chain}
b) within the cytoplasm

\[
ATP + H_2O \xrightarrow{ATPase associated with Myosin} ADP + P + \text{Energy of muscle contraction}
\]

In muscle, adenosine triphosphate act as the principle store of energy which can be released on contraction. Calcium ions in muscles are believed to stimulate the activity of the enzyme myosin ATPase thereby releasing energy for contraction. It has also been shown (Snodgrass and Piras 1966) that an excess of calcium ions can cause an uncoupling of oxidative phosphorylation. The most likely effect of this mechanism is that the calcium ions depress the activity of the enzyme ATPase in the respiratory chain. This results in a fall in the level of ATP in the cytoplasm and a rise in the concentration of ADP in the mitochondrion whilst the energy of respiratory chain is released as heat. In these unusual circumstances it is theoretically possible to release vast amounts of heat very rapidly. Another interesting observation is that ATP not only act as a store of muscle energy but it also causes relaxation of the muscle fibre. Thus, in the event of uncoupling of oxidative phosphorylation the fall in ATP level causes the muscle fibre to become stiff as is witnesses in
Rigor mortis and in malignant hyperpyrexia. Creatinine phosphate is another high energy store that is available as a reserve for the production of ATP with the aid of the enzyme creatinine phosphokinase (CK) viz.

\[
\text{Creatinine P + ADP} \xleftrightarrow{\text{Creatinine phosphokinase}} \text{ATP + Creatinine}
\]

In many myopathies, in malignant hyperpyrexia and case of cell destruction, a raised level of the creatinine kinase (CK) can be detected.