Chapter 2

Reliability of neuromuscular transmission and how it is maintained

CLARKE R. SLATER *

Institute of Neuroscience, Faculty of Medical Sciences, University of Newcastle upon Tyne, UK

2.1. An introduction to neuromuscular transmission

An important function of all nervous systems is to control muscle contraction so that movements of the body are appropriate to promote survival. In most species specialized motor neurons convey the signals that represent the neural commands for contraction from the central nervous system to the muscle fibers. These signals are transmitted to the individual muscle fibers at highly differentiated neuromuscular junctions (NMJs). The process of neuromuscular transmission involves the rapid release of multimolecular “quanta” of acetylcholine (ACh) from the nerve, the binding of the ACh to ligand-gated cation channels (ACh receptors, AChRs) in the surface of the muscle fiber, the opening of those channels and the flow of current into the muscle fiber leading to depolarization and opening of voltage-gated sodium channels causing initiation of an action potential (AP) and contraction of the muscle cell.

In most vertebrates, each motor neuron provides the sole innervation for each of many muscle fibers. A fundamental design principle of neuromuscular systems in higher vertebrates, including humans, is that every nerve impulse in a motor neuron should evoke contraction of every muscle fiber innervated it. Thus each motor neuron, and the muscle fibers it innervates, operates as a “motor unit”. For this principle to be realized in practise, it is essential that the process of neuromuscular transmission should be reliable, operating unfailingly over a wide range of functional demands.

The reliability of transmission at the NMJ sets it apart from most other chemical synapses. In the central nervous system (CNS) the impact of individual synapses on the postsynaptic cell must generally be small, to allow many inputs to influence the final output. At the NMJ just the opposite is true. To ensure that each nerve impulse triggers muscle contraction, its impact on the muscle fiber is usually significantly greater than required to trigger an AP. For this reason, the process of neuromuscular transmission is said to have a big “safety factor”. This results from specializations of both pre- and postsynaptic components. While the immediate molecular processes of neuromuscular transmission are qualitatively similar to those at central synapses, the distinctive functional organization of the NMJ is what accounts for its essential reliability. To understand the basis of the reliability of neuromuscular transmission, it is therefore just as important to know how NMJs differ from central synapses as how they resemble them. This understanding provides the necessary foundation for any effort to interpret how impaired transmission arises in human disease.

A detailed account of the cellular, subcellular and molecular organization of the NMJ is given in Chapter 3. This Chapter describes the process of neuromuscular transmission. It emphasizes how the properties of the NMJ normally ensure the reliability of transmission in a wide range of biological circumstances and how changes to those properties may lead to impaired transmission in disease. After a brief review of the main features of neuromuscular transmission (Section 2.1), the essential presynaptic (Section 2.2) and postsynaptic (Section 2.3) aspects of neuromuscular transmission are considered in more detail. Section 2.4 describes how the safety factor has been defined and measured. Section 2.5 considers

*Correspondence to: Professor (Emeritus) Clarke R. Slater, Institute of Neuroscience, Faculty of Medical Sciences, University of Newcastle upon Tyne, Framlington Place, Newcastle upon Tyne NE2 4HH, UK. E-mail: c.r.slater@ncl.ac.uk, Tel: 44-191-222-5732, Fax: 44-191-222-5227.
some of the many modulating influences on the safety factor during normal use. Section 2.6 describes several important aspects of the “biology” of the NMJ; how it varies within and between muscles and species, and how its efficacy arises during development and is maintained in old age. Section 2.7 describes the remarkable adaptive plasticity of the NMJ, triggered by trauma or intoxication, which promotes maintenance of the effective neural control of muscle. Finally, Section 2.8 shows how the principles of the functional organization of the NMJ, developed in the earlier sections, can inform efforts to understand impaired neuromuscular transmission in disease. This account of neuromuscular transmission and its reliability is based primarily on studies of a small number of laboratory species. The emphasis is on mammals, including humans. While neuromuscular transmission in different species has much in common, there are also many important differences. It is therefore always necessary to use caution when trying to interpret findings in one species on the basis of knowledge of another.

2.1.1. The organization of muscle innervation

2.1.1.1. The muscle fiber

The aim of neuromuscular transmission is to cause muscle contraction. The contractile cells, or muscle fibers, that make up muscles are highly specialized to generate mechanical force under the control of the nervous system. At a molecular level, muscle contraction results from the interaction between myosin and actin molecules. These are organized into distinct thick (myosin) and thin (actin) filaments, which are further organized into overlapping arrays, the sarcomeres. These form the functional units of contraction in skeletal muscles.

Muscle contraction is regulated by the level of free Ca\(^{++}\) in the cytoplasm. This, in turn, is controlled by the sarcoplasmic reticulum (SR), a specialized form of ER, which contains energy-dependent Ca\(^{++}\) transporters that allow it to maintain the free Ca\(^{++}\) at a very low level. Ca\(^{++}\) is released from the SR if the surface membrane of the muscle fiber is adequately depolarized. In vertebrates, this membrane is generally excitable and, like neurons, can generate APs. This membrane property results from the presence of a distinct class of voltage-gated Na\(^{+}\) channels. The specific class of these channels in adult mammalian (including human) muscle is known as Na\(_v\)1.4 (Goldin et al., 2000). During an AP, the depolarization of the surface membrane is carried into the cell interior along invaginations of the surface membrane known as T-tubules. These make contact with the SR at sites called triads.

At these sites, voltage-dependent Ca\(^{++}\) channels in the T-tubules (dihydropyridine receptors) interact with Ca\(^{++}\)-release proteins in the SR (ryanodine receptors) to trigger Ca\(^{++}\) release into the cytoplasm, thus triggering contraction.

Skeletal muscle fibers possess many nuclei. This is a result of the fusion of many mononucleated myoblasts during myogenesis to make individual muscle fibers. The diversity of mature muscle fibers reflects distinctive patterns of gene expression by the nuclei in muscle fibers of different functional types. Within each muscle fiber, the great majority of nuclei away from the NMJ express the same pattern of genes. However in mammals, including man, the 5–10 nuclei closest to the NMJ express a distinctive set of genes that encode key proteins of the NMJ, e.g. AChRs. The manner in which this specialization of gene expression is controlled plays an important part in ensuring the efficacy of neuromuscular transmission (see below).

Each muscle fiber is surrounded by a sheath of basal lamina (BL). This is a thin, but mechanically strong, layer of extracellular matrix. Within the BL sheath are a number of mononucleated myogenic cells known as satellite cells. Following damage to the muscle, the satellite cells may become activated and divide and then fuse to form new muscle fibers within the original BL sheaths.

There is considerable diversity in the properties of different vertebrate skeletal muscle fibers, even within the same muscle in the same animal. Some muscle fibers are specialized to produce relatively slow, well-maintained contractions while others produce fast but brief contractions. This diversity results from the expression of specialized isoforms of many of the proteins that give rise to and regulate contraction. It allows patterns of contraction ranging from the sustained contractions needed to maintain posture to the rapid forceful contractions needed to cause sudden movements of limbs.

2.1.1.2. The motor neuron

Motor neurons are among the largest neurons in the nervous system. Their cell bodies are located in the ventral horn of the spinal cord or the cranial nerve nuclei in the brainstem. Each motor neuron receives synaptic contact from many sensory and interneurons. When the summed depolarization resulting from the activity in these cells reaches the threshold, action potentials are initiated in the most proximal part of the axon. Each motor neuron has a single myelinated axon. After leaving the CNS, the motor axons generally remain unbranched until they reach their target muscle. There,
within the intramuscular nerve bundles, they send off branches to the numerous muscle fibers they innervate. Like muscle fibers, motor neurons have different functional properties (Kernell, 2006). In particular, different motor neurons tend to generate APs with characteristic temporal patterns. These are determined by the “intrinsic excitability” of the neuron, which reflects its size and the particular ion channels in its membrane (Gardiner and Kernell, 1990). Motor neurons innervating slow muscle fibers tend to be relatively small, to have a low threshold and to fire continuously at a low frequency (e.g. 20 Hz), while those innervating fast fibers tend to be larger, to have a higher threshold, and to fire in brief high frequency (e.g. 100 Hz) bursts (Henneman et al., 1965; Hennig and Lomo, 1985). The ability of the nerve terminal to sustain ACh release is matched to these different intrinsic patterns.

### 2.1.1.3. The motor unit

To take advantage of the diverse functional properties of skeletal muscle fibers, it is essential that the nervous system can excite specific sets of muscle fibers with particular properties. Within its target muscle, each motor neuron innervates a number of muscle fibers. This number may be only a few, in small muscles of laboratory rodents, or more than 1000 in large muscles of humans (Cooper, 1966). All the muscle fibers innervated by a given motor neuron have very similar properties (Burke et al., 1973) which are well-matched to those of the motor neuron and NMJs, ensuring that each motor unit produces a contraction suited to a particular need. An important feature of this pattern of innervation is that every nerve impulse in the motor axon should reliably excite every muscle fiber in the motor unit.

### 2.1.2. The NMJ and the nature of the reliability of neuromuscular transmission

The normal reliability of neuromuscular transmission is the result of factors operating at many different levels of organization. These range from the detailed atomic structure of the proteins that make up the essential ion channels to the multimolecular complexes that mediate vesicle exocytosis and finally to the multieellular complex that is the NMJ itself. In any situation where impaired reliability is present, all these levels must be considered when trying to understand the basis of that impairment.

#### 2.1.2.1. Structure of the NMJ

A detailed account of the structure and cell biology of the NMJ is given in Chapter ???. Here only certain key features will be reviewed to provide a basis for the following treatment of neuromuscular transmission. When the motor axon makes contact with its target muscle fibers, it loses its myelin sheath and branches repeatedly to form the presynaptic nerve terminals of the individual NMJs (Fig. 2.1A, B). These terminals are capped by processes of the several terminal Schwann cells associated with them (Fig. 2.1A, D, E). Instead of myelin, these terminal Schwann cells elaborate thin layers of cytoplasm that cover and insulate the nerve terminal.

The precise form of nerve terminal branching, and the sites of synaptic contact with the muscle formed by the branches, varies between species. In many cases, including humans, the terminal consists of numerous spot-like regions of synaptic contact, where the axon enlarges to a width of 3–5μm, linked by lengths of axon with a finer caliber (Fig. 2.1B). In other cases the regions of synaptic contact are merged to varying degrees to make a more or less continuous band which, as in the frog, may extend several 100s of μm along the muscle fiber.

In all cases, transmitter release occurs at specialized “active zones” (AZs) in the presynaptic membrane. These consist of highly ordered arrays of protein molecules including both the structural molecules that promote exocytosis and the Ca$^{2+}$ channels that regulate it. Different forms of AZ occur in lower and higher vertebrates (see Section 2.6.1.2). In both groups, the AZs are located opposite the openings between the postsynaptic folds.

The postsynaptic membrane is distinguished by the presence of infoldings, which lie opposite the AZs (Fig. 2.1D). The AChR and Na$_V$1.4 proteins occupy separate postsynaptic domains (Fig. 2.1C, E). The AChRs are concentrated at the crest of the folds, near the nerve, while the Na$_V$1.4 channels are concentrated in the depths of the folds. A thin mesh of extracellular matrix, the basal lamina (BL) surrounds each muscle fiber and motor axon. A single common BL is present in the synaptic cleft between the nerve and the muscle. A number of molecules important for the short and long term activities of the NMJ are bound to this synaptic BL including acetylcholinesterase (AChE), which terminates ACh action, and agrin, which plays an essential role in the development and maintenance of postsynaptic differentiation.

#### 2.1.2.2. Principles of neuromuscular transmission

In all vertebrates neuromuscular transmission involves the release of ACh from the nerve terminal and its action on the muscle to trigger its excitation and contraction. The NMJ is highly specialized to ensure that the main events in this complex process take place within less than a millisecond. Within the cytoplasm
of the presynaptic nerve terminal ACh is contained in membrane-bound vesicles. Each vesicle is believed to contain about 10,000 ACh molecules, which can be released within 100 μs or so following a nerve impulse. These multi-molecular packets, which are roughly uniform in size and effect at any given NMJ, are the elementary units of evoked ACh release. They are often referred to as “quantal” release of ACh, by analogy with the elementary units of electromagnetic energy, and their release as “quantal” release. This release is normally triggered by an increase in the concentration of Ca$^{2+}$ in the nerve terminal, which results from the opening of voltage-gated Ca$^{2+}$ channels in the nerve terminal membrane by the nerve impulse. Normally a single nerve impulse causes 20–200 of these vesicles to release their contents, depending on the species and muscle.

The mechanism for ACh release from the nerve is complemented by a high density of AChRs in the postsynaptic membrane of the muscle fiber. These ligand-gated ion channels open briefly when ACh binds to them. This causes an influx of positive ions and a transient local depolarization of the muscle membrane. Since the NMJ is sometimes referred to as the endplate, the signals associated with this charge influx and subsequent depolarization are known as the endplate current (EPC), and the endplate potential (EPP), respectively (Fig. 2.2A). The analogous much smaller signals that result from the impact of spontaneously released single quanta of ACh are known as miniature EPCs and EPPs (Fig. 2.2). The EPP typically rises to a peak of some 25–45 mV (from the resting potential of about −75 mV) in about 1 ms. As it does so, it causes the opening of a second class of ion channels in the muscle fiber, the voltage-gated NaV1.4 channels, thus initiating an action potential in the muscle fiber (Fig. 2.2B).
The action of the ACh is rapidly terminated by the action of the AChE associated with the synaptic BL, splitting ACh into acetate and choline. The choline is subsequently transported by a high affinity uptake system into the nerve terminal where it is eventually used to make more ACh.

2.1.2.3. The safety factor

The EPP that results from the summed effect of all the ACh released by a single motor nerve impulse normally depolarizes the muscle fiber more than is required to reach the electrical threshold for triggering an AP (Fig. 2.2C) (Wood and Slater, 2001). The significance of this safety factor is seen during intense activity when individual muscle fibers must be activated repeatedly at high frequency. In these conditions, there is a temporary depletion of vesicles available for rapid release in the nerve terminal, resulting in a reduction of the amount of ACh release by individual nerve impulses. An adequate safety factor ensures that even in such circumstances, the EPP will still be able to activate the muscle fiber reliably.

2.1.3. Evidence for reliability at the human NMJ

Neuromuscular transmission is extremely reliable in healthy humans. It appears to be virtually impossible to bring about failure of transmission as a result of voluntary exertion (Bigland-Ritchie et al., 1978, 1982). Instead, normal fatigue during intense exertion...
2.2.1. Introduction to the quantal release of ACh

ACh quanta are released from the motor nerve terminal within 100 μs or so of the arrival of the nerve impulse (Katz, 1969; Van der Kloo and Molgo, 1994). The events that allow the conversion of depolarization into secretion are complex. At the normal mammalian NMJ, they result in the release of more than enough ACh to ensure a postsynaptic depolarization that evokes an action potential in the muscle fiber (Wood and Slater, 2001). This section presents an account of the main events in the release process.

2.2.1.1. Early evidence for quantal release

Early studies of neuromuscular transmission using extracellular electrodes revealed that a motor nerve impulse is followed by a local depolarization of the muscle fiber, the EPP (Eccless et al., 1942). The introduction of intracellular recording techniques allowed the true amplitude and time course of the EPP to be analyzed (Fatt and Katz, 1951). During this analysis much smaller events, with an amplitude of about 0.5–1 mV, were observed to occur spontaneously at a frequency of about 1 Hz (in the frog, Fig. 2.3A) (Fatt and Katz, 1952). These had a similar time course to EPPs and, like them, could only be recorded near the NMJ. Furthermore, like EPPs, they were blocked by the drug curare, which blocks the postsynaptic response to ACh. These similarities to EPPs led them to be called miniature EPPs (mEPPs).

A second key observation linked the spontaneous mEPPs to evoke release. In solutions containing low Ca$^{++}$ (e.g., 0.2 mM instead of the usual 2 mM), and/or raised Mg$^{++}$, neuromuscular transmission was blocked because the EPP was too small to reach the action potential threshold. When the distribution of amplitudes of EPPs at a NMJ in low Ca$^{++}$ was studied, it was found that some stimuli failed to evoke any response in the muscle. The responses that were observed had certain preferred amplitudes, which were integral multiples of a fundamental value (Fig. 2.3B, C) (del Castillo and Katz, 1954a; Boyd and Martin, 1956). This fundamental value had the same mean as the amplitude of the mEPPs at the same NMJ.

The behavior of the NMJ in low Ca$^{++}$ led to the hypothesis that EPPs result from the near simultaneous occurrence of a number of mEPPs. The mEPPs, in turn, were assumed to result from the action of a nearly constant number of prepackaged ACh molecules (see below). The analogy between fundamental units of chemical transmission and of electromagnetic energy led to the practise of referring to the ACh packets as “quanta”. According to this view, each nerve-evoked EPP is caused by the release of an integral number of ACh quanta. This number is referred to as the “quantum content” of the EPP. In low Ca$^{++}$, fewer quanta are released (i.e., the quantum content is reduced) but the effect of the individual quanta is unchanged. It is important to realize that the term “quantum content” does not refer to the amount of ACh in a quantum, or to the number of quanta contained in a single nerve terminal.

Ultrastructural investigation of the NMJ soon suggested a likely structural basis of the ACh quanta. Membrane bound vesicles with a diameter of about 50 nm were seen in the motor nerve terminal (Riger, 1958; Birks et al., 1960) (see Chapter ??). It was suggested that each quantum represented the ACh molecules in a single such vesicle and that, somehow, the nerve impulse caused the near simultaneous fusion of a number of these vesicles with the nerve membrane, allowing them to discharge their contents into the
synaptic cleft (del Castillo and Katz, 1956a). Although there is often a correlation between the frequency of these spontaneous mEPPs and the quantum content of the evoked response, they respond quite differently to changes in the concentration of divalent cations. As a result, it is never safe to use mEPP frequency as a proxy measure of evoked release.

Numerous efforts have been made to establish the number of ACh molecules in a quantum. Probably the most accurate estimate was obtained by comparing the effect of nerve-derived ACh quanta on the frog NMJ with that of calibrated amounts of ACh ejected iontophretically from micropipettes onto the exposed postsynaptic membrane in the same preparations (Kuffler and Yoshikami, 1975; Hartzell et al., 1976). This study found that fewer than 10 000 ACh molecules were required to mimic the effect of natural quanta. It is now generally accepted that a quantum usually contains 5000–10 000 ACh molecules.

2.2.1.2. Spatial aspects of quantal release

Subsequent experiments in the frog showed that ACh release occurred from many discrete sites located along the branches of the motor nerve terminals (del Castillo
and Katz, 1956b). This conclusion was based on micro-electrode recordings of the currents flowing in the extracellular medium (Fig. 2.4A). These currents are highly localized and their site of origin can be determined to within 5–10 \(\mu\)m. In subsequent modifications of this approach, recordings were made simultaneously with 2 or 3 electrodes to provide even greater spatial resolution (Fig. 2.4B, C) (Benish et al., 1988; Zefirov and Cheranov, 1995). These showed that quanta are released from sites similar in size, shape and distribution to AZs (Fig. 2.4D). These electrophysiological findings were consistent with ultrastructural studies of frog NMJs frozen within a few milliseconds of nerve stimulation, which showed what were apparently the profiles of recently fused vesicles closely associated with the AZs (Heuser et al., 1979). These findings are described more fully in Chapter ??.

### 2.2.1.3. Temporal aspects of quantal release

The process of \(\text{Ca}^{++}\)-mediated exocytosis at the NMJ occupies less than a millisecond. Electrophysiological methods have been used to investigate the kinetics of quantal release. Initial studies were made in the frog, where individual branches of frog nerve terminals are up to 150 \(\mu\)m long. As a result, the action potential may reach the most distal end up to 0.3 ms after arising at the proximal end. This clearly complicates the investigation of the time course of release. To circumvent this problem, the method of extracellular recording was refined to restrict release to a small region of the frog nerve terminal (Katz and Miledi, 1965). This approach revealed that in the frog at 20°C, there was a period of about 0.5 ms between the peak active phase of the nerve action potential (indicated by the negative deflection of the extracellular record) and the earliest detectable release.

Analysis of a large sample of evoked uniquantal events, recorded in low \(\text{Ca}^{++}\), showed that there is considerable variation in the time of their occurrence, relative to the peak of the action potential, even at a single site along the nerve terminal (Katz and Miledi, 1965). While most responses occurred about 1 ms after the peak of the nerve action potential, some occurred after only 0.5 ms and others as much as 2 ms later (Fig. 2.5). This temporal dispersion was interpreted as reflecting the time course of a transient increase in the probability of release of individual quanta of ACh, triggered by the increase in the concentration of free \(\text{Ca}^{++}\) in the cytoplasm.

When considering the entire NMJ, a further source of temporal dispersion in quantal release is introduced by the fact, referred to above, that the action potential invades the most distal part of the frog terminal as much as 0.3 ms later than the most proximal part. There is some evidence that this effect is partly compensated by a greater synchrony of quantal release in the most distal parts of the frog nerve terminal, though the basis of this is not yet clear (Bukharaeva et al., 2000). Because the linear extent of normal mammalian motor nerve terminals is much less than that of frogs, all regions of the nerve terminal probably become maximally depolarized by a nerve impulse within 100 \(\mu\)s. In some abnormal situations, in which the nerve terminal becomes elongated as a result of sprouting induced by inactivity, much longer distances and delays may be involved (see Section 2.7).

#### 2.2.2. Depolarization of the nerve terminal by the action potential

It is now generally agreed that evoked quantal release is triggered by an increase in free \(\text{Ca}^{++}\) in the nerve terminal, which results from the opening of voltage-gated \(\text{Ca}^{++}\) channels in the nerve membrane when it is depolarized by an action potential.

The action potential in the motor axon travels to the NMJ by means of saltatory conduction. This mode of conduction results from the fact that the voltage-gated sodium channels responsible for the action potential are only present at the nodes of Ranvier, small (1 \(\mu\)m), widely separated (ca 1 mm), spots along the axon. These active regions are separated from each other by myelin, the insulating layers of plasma membrane of the Schwann cells associated with the axon. As a result, the active phase of the action potential “jumps” from one node to the next.

When the axon reaches the NMJ, both the highly restricted regions of excitability and the myelin sheath are lost. The motor nerve terminal contains many different classes of ion channel that may influence its excitability (Meir et al., 1999). Available evidence indicates that the excitability of the terminal axon differs between frogs, where the most detailed studies have been made, and mammals.

#### 2.2.2.1. Frogs

The small diameter of the presynaptic axon terminals makes it difficult to make intracellular recordings of the action potential. However, important information can be gained by recording with extracellular electrodes, placed close to the nerve terminal (Fig. 2.5). In frogs, which have nerve terminals up to several 100 \(\mu\)m long, extracellular records indicate that the action potential propagates actively virtually to the end of the terminal. Further, blocking the invasion of the terminal by the action potential by local application of tetrodotoxin...
Fig. 2.4. Extracellular recording of quantal release. A. Simultaneous extracellular recordings of spontaneous quantal events from two spots about 10 μm apart on a frog motor nerve terminal. Note that the events recorded by the two electrodes have very different amplitudes, indicating the very focal nature of the quantal currents. B. Diagram of recording situation using three electrodes to determine the site of origin of individual quantal events by triangulation. A single muscle fiber with the last node of Ranvier (black) and a single branch of the nerve terminal, together with the numbered positions of the three electrodes. C. Diagram showing outline of nerve terminal (parallel dashed lines) and positions of recorded quantal events determined by triangulation (dots). Note that these form four bands perpendicular to the nerve. D. Electron micrograph of freeze fracture replica of frog nerve terminal showing positions of AZs. Magnification of C and D is the same. (A from del Castillo and Katz, 1956b, with permission. B from Benish et al., 1988, with permission. C from Zefirov and Cheranov, 1995, with permission. D from Heuser, 1976, with permission.)
Fig. 2.5. Measurement of synaptic delay. Synaptic delay recorded at a frog NMJ. To prevent contraction and to restrict release to the recording site the preparation was bathed in a low Ca$^{++}$ medium and Ca$^{++}$ then restored locally at the recording site from a CaCl$_2$-filled micropipette. A. Synaptic delay is defined as the elapsed time from the negative peak of the nerve AP, indicating the time of maximum inward current, to the onset of the evoked EPC in the muscle. B. Records of several trials show variation in synaptic delay. C. Distribution of delays recorded in many trials. The minimum is about 0.5 ms and the mode is about 0.9 ms. (From Katz and Miledi, 1965, with permission.)
(TTX) at the end of the last myelin segment completely abolishes the EPP (Katz and Miledi, 1967). Thus in frogs, active propagation of the action potential along the length of the nerve terminal is essential for normal neuromuscular transmission.

2.2.2.2. Mammals
Mammals have much smaller terminals than frogs. Initial studies concluded that the action potential does not invade the terminal actively (Brigant and Mallart, 1982) and that active Na\(^+\) currents are present only in the most proximal part of the terminal axon. In contrast, repolarizing currents due to the efflux of K\(^+\) are present throughout the terminal. While subsequent studies have found some evidence of active Na\(^+\) currents in the distal terminal (Konish and Sears, 1984; Konishi, 1985) these Na\(^+\) currents are apparently not essential for normal quantal release. Thus it appears that in mammals quantal release is triggered largely by passive depolarization of the terminal by current drawn from the opening of Na\(^+\) channels in the most distal nodes of Ranvier and the last “heminode”.

2.2.2.3. Repolarization of the nerve terminal
The repolarization of the nerve terminal plays an important part in limiting the duration of Ca\(^{++}\) entry and thus preparing the NMJ for a further nerve impulse. This repolarization is primarily mediated by K\(^+\) channels in the nerve. These include both voltage- and Ca\(^{++}\)-activated channels. The impact of these channels on quantal release is revealed when they are blocked by drugs such as 3,4-diamino pyridine (DAP) and its analogs (Katz and Miledi, 1979). These drugs can greatly increase the quantum content, making them useful both experimentally and clinically.

2.2.2.3.1. Calcium channels in the nerve terminal
Studies of the effect of Ca\(^{++}\) on quantal release imply that 3–5 Ca\(^{++}\) ions must bind to a target to trigger release (Dodge, Jr. and Rahamimoff, 1967). Observations of Ca\(^{++}\) concentration in the frog motor nerve terminal, using fluorescent indicator dyes, confirm that depolarization of the nerve terminal leads to the brief, highly localized, influx of Ca\(^{++}\) (Wachman et al., 2004). This influx is mediated by voltage-gated Ca\(^{++}\) channels present in the nerve terminal membrane (see Chapter ??).

While Ca\(^{++}\) channels of many different types are present at NMJs (Day et al., 1997) two of these, N and P/Q, predominate in vertebrate motor nerve terminals. Their activities can be distinguished by using highly specific natural toxins as selective blockers (Katz et al., 1995; Protti et al., 1996; Katz et al., 1997; Urbano et al., 2002). In frogs and lizards, the main type is N, while in mammals it is P/Q. P/Q-type channels require less depolarization to open than N-type and less hyperpolarization to inactivate (Catterall et al., 2005). These functional differences may be related to the differences in active propagation of the action potential into the nerve terminal in frogs and mammals.

Fluorescent studies with a variety of specific labels have confirmed that Ca\(^{++}\) channels are concentrated in the motor nerve terminal (Robitaille et al., 1990; Sugiura et al., 1995; Day et al., 1997). In the frog, Ca\(^{++}\) channels are strategically placed immediately opposite the sites of highest AChR density in the postsynaptic membrane (Robitaille et al., 1990). Since this corresponds to the location of the AZs in the nerve terminal, it is generally assumed that the Ca\(^{++}\) channels are integral components of the AZs, and correspond to some or all of the intramembranous particles seen in freeze-fracture preparations. Detailed electrophysiological studies support this view (Yazejian et al., 2000).

2.2.3.2. Dynamics of calcium within the nerve terminal
The relationship between Ca\(^{++}\) entry into presynaptic terminals and quantal release is a topic of much current interest (Gentile and Stanley, 2005). Some studies of synapses in the CNS have suggested that many Ca\(^{++}\) channels must open, causing a generalized increase in Ca\(^{++}\) concentration in the presynaptic terminal, to bring about the release of an individual quantum. A different situation seems to apply at the frog NMJ. Following a single nerve impulse, Ca\(^{++}\) increases in the nerve terminal at a small number of discrete sites (Fig. 2.6A) (Wachman et al., 2004). The position of these sites changes from one response to the next. This suggests that only a small fraction of the Ca\(^{++}\) channels in the nerve terminal open in response to each action potential. As yet, comparable studies on mammalian NMJs have not been reported.

In order that neuromuscular transmission can operate reliably at high frequency, it is essential that the level of free Ca\(^{++}\) in the nerve terminal returns rapidly to its normally low level after each nerve impulse. A variety of mechanisms, including diffusion, binding to cytoplasmic proteins and uptake into intracellular organelles all appear to play a part in this process (Zucker and Regehr, 2002). During high frequency activation, there is often an increase in the quantum content. This is likely to result from the summed effect of the calcium that enters with each new impulse and residual calcium from previous stimuli that persists near the release sites (Katz and Miledi, 1968; Zucker and Regehr, 2002) (see Section 2.5.1).
2.2.4. Exocytosis

2.2.4.1. Molecular basis of exocytosis at the NMJ

Much has been learned in recent years about the molecular events leading to exocytosis in general and at synapses in particular (Sudhof, 2004). It is now clear that the process of exocytosis involves interactions of one set of proteins in the membrane of the synaptic vesicles (V-SNARES including: synaptotagmin, and synaptobrevin) and another in the membrane of the nerve terminal (T-SNARES including syntaxins, and SNAP-25) (Fig. 2.7). An initial set of interactions leads to the “docking” of vesicles at the AZ as recognized by structural criteria, followed by an ATP-dependent step that leaves the vesicles in a “primed” state, poised for release. The conversion of this “primed” state into full fusion is triggered when the concentration of free Ca$^{2+}$ in the cytoplasm reaches 10–20 μM (Stanley, 1997). This leads to Ca$^{2+}$ binding to the vesicle protein synaptotagmin, which saturates at a Ca$^{2+}$ concentration of about 10 μM (Hui et al., 2005). This, in turn, triggers a conformational change that results in further interactions between vesicle and terminal proteins that are believed to draw the vesicle toward the terminal membrane and cause fusion. Further details of the proteins involved in this process are described elsewhere in this volume (Chapter ??).

2.2.4.2. Calcium dynamics and exocytosis

Following the voltage-gated opening of Ca$^{2+}$ channels, the Ca$^{2+}$ ions that enter the nerve terminal must diffuse to their binding sites on synaptotagmin before release can occur. The earliest release events to occur after the nerve action potential therefore shed light on the distance between the sites of Ca$^{2+}$ entry and release. The probability of release begins to increase within 200 μs of the opening of these channels (Stanley, 1997; Gentile and Stanley, 2005). During this time, the region in which the Ca$^{2+}$ concentration reaches 10 μM extends about 25 nm from the opened channel. On the assumption that both the Ca$^{2+}$ channels and their targets are components of the AZ, this distance is so short as to indicate that the Ca$^{2+}$ entering the nerve terminal through a single open Ca$^{2+}$ channel acts mainly on vesicles docked at the AZ of which the channel is a part.

It is generally thought that the intramembranous particles (IMPs) at AZs represent ion channels. At a mammalian AZ, there are usually about 20 IMPs in total (Fukunaga et al., 1983; Fukuoka et al., 1987), only some of which are likely to be Ca$^{2+}$ channels, the others are probably K$^+$ channels. Assuming that two vesicles are docked at each AZ, and the AZ is 50–100 nm long, only some of the Ca$^{2+}$ channels are likely to be close enough to each vesicle to play a part in its exocytosis. Thus fewer than 10 Ca$^{2+}$ channels, and possibly only one (Meriney et al., 1985; Stanley, 1997), may need to open to cause the release of a vesicle.

2.2.5. Determinants of quantum content

2.2.5.1. Number of release sites and efficiency of release

The local events described above, including Ca$^{2+}$ entry into the nerve terminal and the vesicle fusion events it triggers, form the basis of our understanding of the process of quantal release. However, to understand how it is that the nerve releases enough quanta to ensure reliable neuromuscular transmission, we...
Fig. 2.7. Steps in synaptic vesicle exocytosis. Speculative model for the functions of SNARE proteins (synaptobrevin, SNAP-25, syntaxin 1/2) and synaptotagmins in exocytosis. In docked vesicles (A), SNAREs and synaptotagmins do not interact directly. During priming (B), SNARE complexes form, complexins (green) are bound to fully assembled complexes, and synaptotagmins constitutively associate with the assembled SNARE complexes. The synaptic vesicle membrane and plasma membranes are forced together by SNARE complex assembly, which results in an unstable intermediate. Ca$^{++}$ influx (C) further destabilizes the fusion intermediate by triggering the C$_2$ domains of synaptotagmin to partially insert into the phospholipids. This action is proposed to cause a mechanical perturbation that opens the fusion pore. (From Sudhof, 2004, with permission.)
must consider the whole population of AZs in the nerve terminal and the efficiency of release from them.

Frog NMJs have been studied in the greatest detail. Their AZs occur at intervals of about 1 μm along the nerve terminal (Pawson et al., 1998). Since the average total length of the nerve terminal branches is about 1000 μm, there are about 1000 AZs per NMJ. Since the quantum content of the whole NMJ is about 100–200, this means that the probability of release from a single AZ is about 0.15, although estimates up to 0.75 have been suggested (Meriney et al., 1985). It is estimated that there are 20–30 docked vesicles at each AZ (Meriney et al., 1985), or about 25 000 per NMJ, implying that fewer than 1% of the docked vesicles are released per nerve action potential.

In mammals, the AZs are distributed apparently at random throughout the presynaptic membrane. Their density in mice and humans is 2.6 AZ/μm (Fukunaga et al., 1983; Fukuoka et al., 1987). At a typical rat or mouse NMJ, with an area of synaptic contact of about 400 μm² (Lyons and Slater, 1991; Wood and Slater, 1997), this implies that there are about 1000 AZs. Each AZ is believed to harbor no more than two docked vesicles (see Section 2.6.1.2, Fig. 2.22), making a maximum of 2000 per NMJ. The quantum content of EPPs in such a muscle is about 100, or about 0.1 per AZ. This means that about 5% of the docked vesicles are released by each nerve action potential. A similar conclusion applies to human NMJs. In each case, the number is substantially greater than in the frog. The explanation for this difference is not yet clear, but it is likely to be related to the different relationships between vesicles and AZ particles in frogs and mammals.

In spite of the differences in the "efficiency" of release of docked vesicles in different species, the number of quanta released per unit area of presynaptic membrane is roughly constant. Although there are clear exceptions, many NMJs release between 0.15–0.35 quanta/μm². This principle was first recognized in frogs, where a rough correlation between NMJ size and quantum content was found (Kuno et al., 1971). At NMJs such as those in humans where the nerve terminal is formed from many boutons about 3 μm in diameter, each bouton has about 50 AZs and 100 docked vesicles, of which 1–3 are typically released by a single nerve impulse.

At first, it seems strange that so reliable a process as neuromuscular transmission should be founded on such a low probability of release of individual docked vesicles. One possible explanation is related to the fact that muscle fiber activation, particularly of fast mammalian muscles (Hennig and Lomo, 1985) often consists of a burst of nerve impulses at a frequency of around 100 Hz. If each impulse causes the release of a small fraction of docked vesicles "at random", this would ensure that there is always an adequate supply of vesicles ready to be released by following impulses. In any case, it is clear that an understanding of the reliability of release from the nerve terminal as a whole must take into account these spatial features of release.

2.2.5.2. Vesicle pools in the nerve terminal

The vesicles released by a single nerve impulse are a very small fraction of all the vesicles contained in the presynaptic terminal. In a variety of mammals, this total has been estimated at 100 000 to 300 000, e.g. Reid et al. (1999). Thus there is an enormous reserve capacity of vesicles potentially available for release. However, studies using repetitive nerve stimulation have shown that subpopulations, or "pools", of vesicles differ in their actual availability (Rizzoli and Betz, 2005). The docked and primed vesicles represent a "readily releasable" pool which can be replenished rapidly from a "recycling" pool. In addition, there is a much larger "reserve" pool in which vesicles are believed to be tethered to elements of the cytoskeleton. The properties of these pools, and how vesicles move from one pool to another, are topics of much current interest. This topic is discussed more fully in Section 2.5.

2.2.6. Statistical aspects of quantal release

An important aspect of the initial studies of quantal release was the effort to formulate statistical models that could describe the fluctuations in the quantum content (m) of the EPP in different situations (del Castillo and Katz, 1954a; Katz, 1969). These models are relevant here because they have played an important part in many subsequent efforts to quantify quantal release in different situations (see Section 2.4.3.3). The general approach was to assume that within the nerve terminal there are a large number (n) of "units" (provisionally quanta) each with a finite probability (p) of being released by a given nerve impulse. Thus:

\[ m = np \]

For such a system, it should be possible to describe the distribution of quantum contents in a large series by a binomial distribution. Efforts to do this have been of limited value because it is unclear exactly what the variable n represents.

Many of the key observations on which the modeling was based were made in the presence of a low
Ca\(^{++}\) concentration in which the EPP was very small. Because the amplitude of the mEPPs was found not to be reduced, indicating that the size of the individual quanta did not change, it was assumed that the effect of reducing the concentration of Ca\(^{++}\) was to reduce \(m\).

In these experiments, there was either no response at all (a “failure”), or a response whose amplitude was approximately an integral multiple of the mEPP amplitude. Under these conditions the distribution of EPP amplitudes of a large number of events can be described by the Poisson distribution:

\[
p_x = \frac{m^x}{x!} e^{-m}
\]

where \(p\) is the probability of occurrence of a response resulting from \(x\) quanta and \(m\) is the mean number of quanta per response, or quantum content (del Castillo and Katz, 1954a).

A useful feature of a Poisson distribution is that the entire distribution of responses can be predicted from a single variable. For example, if one determines the fraction of trials in which there is no response because zero quanta have been released, it is possible to determine the remaining fractions in which one, two or more quanta will be released, and therefore the value of \(m\). This property is the basis for a widely used method for estimating \(m\) (the “failures” method). An alternative approach is based on the fact that the mean of a Poisson distribution (i.e. \(m\)) is inversely related to the variance. Thus, even in circumstances where individual quanta (mEPPs) cannot be recorded, \(m\) can be estimated from the variance of the response amplitudes (the “variance” method). An important limitation of the use of the Poisson distribution is that it is only valid when \(p\), and hence \(m\), are very small (<3). This makes it invalid to use the Poisson distribution as the basis for estimating \(m\) in conditions of normal release (discussed further in Section 2.4.3.3). A failure to recognize this limitation in some early studies of quantal release in mammals led to estimates of \(m\) that are now recognized as having been much too high.

### 2.2.7. Endocytosis and vesicle recycling

Following a nerve impulse, the membrane of the vesicles that fuse by exocytosis is added to the membrane of the nerve terminal, which therefore gets bigger. This process is balanced by recovery of the membrane by a process of endocytosis (see Chapter ??). The process of endocytosis, and the subsequent trafficking of the recovered vesicles, can be followed using fluorescent lipophilic dyes, most commonly FM1–43 (Cochilla et al., 1999), that become incorporated into the membranes of the endocytotic vesicles. Studies of this sort have revealed much about the properties of the vesicle “pools” within the nerve terminal (Rizzoli and Betz, 2005). These pools play an important part in ensuring that the number of vesicles ready for release is normally adequate. This topic is discussed in more detail in Section 2.5.1 in the context of how the NMJ responds to repetitive activity such as occurs in vivo.

### 2.2.8. Conclusions

The number of vesicles released by a given nerve impulse is a very important determinant of the efficacy of transmission. It is clear that it depends on a large number of factors, reflecting the complexity of the release process (Atwood and Karunanithi, 2002). These factors may be classed into those that influence Ca\(^{++}\) entry into the terminal and those that influence how much ACh that Ca\(^{++}\) causes to be released. The former class includes: action potential amplitude and duration, determined by the number and types of Na\(^+\) and K\(^+\) channels in the membrane of the terminal, and the density, distribution and properties of the voltage-gated Ca\(^{++}\) channels. The latter class includes: synapse size, AZ density, the number of docked vesicles per AZ, and the fraction of docked vesicles that are primed for release. Additional factors probably include vesicle size and the concentration of ACh within them.

### 2.3. Postsynaptic aspects of neuromuscular transmission

#### 2.3.1. Dynamics of ACh in the synaptic cleft

##### 2.3.1.1. Diffusion of ACh in the cleft

The rising phase of the EPC lasts about 0.2 ms. During this time, ACh released from the nerve must diffuse across the synaptic cleft, bind to AChRs, which must then undergo a conformational change (“opening”), and Na\(^+\) ions must diffuse into the muscle fiber to generate the EPC. The ACh released from a single vesicle diffuses rapidly in the synaptic cleft, with an estimated diffusion coefficient of \(1.4 \times 10^{-6}\) cm\(^2\)s\(^{-1}\) (Krnjević and Mitchell, 1960). In 0.2 ms, the mean diffusion distance of an ACh molecule from the site of release = \(\sqrt{2Dt}\) (where \(D\) is the diffusion coefficient and \(t\) is the time) is about 0.25 μm. For a quantum of 10 000 molecules, in a synaptic cleft 50 nm wide, this would result in an average concentration of ACh of >0.5 mM, immediately adjacent to a region of postsynaptic membrane within 0.25 μm of the site of release.
2.3.1.2. Impact of the basal lamina and AChE on ACh dynamics

One may wonder whether all the ACh in a quantum would really reach the postsynaptic membrane, given the high concentration of AChE associated with the synaptic BL. The explanation lies in the fact that there are some 10 times fewer AChE molecules in the synaptic space encountered by a quantum of ACh than there are ACh molecules in a quantum (Anglister et al., 1994). As a result, something like 10% of the ACh rapidly occupies all the AChE molecules while 90% passes through the BL to reach the postsynaptic membrane. As the bound ACh molecules gradually dissociate from the AChRs during a period of a few ms, they are rapidly bound and hydrolyzed by the AChE, limiting both the temporal and spatial extent of action of each quantum of ACh (see Section 2.3.7).

2.3.2. Structure and function of the AChR

2.3.2.1. General structure of AChRs

The AChRs in the postsynaptic membrane of the vertebrate NMJ are of the class blocked by nicotine, and are therefore referred to as nicotinic (e.g. nAChRs). Other AChRs blocked by muscarine (mAChRs) are present on the presynaptic side of the NMJ and may act to modulate quantal release (see Section 2.5.4.1). Each postsynaptic AChR molecule is a cylindrical complex of 5 subunits (Fig. 2.8A). The long axis lies perpendicular to the plane of the membrane and both surrounds and gates a central cation-selective channel. At mature mammalian NMJs, the stoichiometry of the subunits is \( \alpha_2\beta\delta\epsilon \). At immature or regenerating NMJs the \( \epsilon \)-subunit is replaced by a \( \gamma \)-subunit, which is also commonly found in more primitive vertebrates. This replacement alters the kinetic properties of the cation channel (see below).

2.3.2.2. Properties of ACh-gated channels

Each AChR molecule has two ACh binding sites in the interfaces between the a/e and a/d subunits. At the concentration of ACh present in the synaptic cleft following a nerve impulse, binding to the AChRs occurs within microseconds. When both subunits are occupied the probability of the associated channel opening increases dramatically (Fig. 2.8B). The transition from closed to open occurs in less than 100 \( \mu \)s (Matsubara et al., 1992). The open channels are approximately equally permeable to Na\(^+\) and K\(^+\) (Takeuchi and Takeuchi, 1959) and are also slightly permeable to Ca\(^{++}\). The flow of ions through an open channel is determined by the instantaneous value of the relevant electrochemical gradients.

At the normal resting potential the electrochemical driving force on Na\(^+\) is much greater than that on K\(^+\). The net result of opening AChR channels is to allow a positive charge in the form of Na\(^+\) ions to enter the

![Fig. 2.8. From AChRs to mEPCs. A. Schematic diagram of an AChR molecule in the membrane. The five subunits (2\( \alpha \), \( \beta \), \( \delta \), \( \epsilon \)) span the membrane and enclose the ion pore. B. View along the axis of the pore of how the orientation and conformation of the subunits may change when the channel opens (kindly provided by Nigel Unwin). C. Examples of current flowing through a single open channel during three opening events. (From Mishina et al., 1986, with permission.) D. The mEPC results from the near simultaneous opening of a number of channels (only 6 of the 1000 or so that make up a mEPC are shown) followed by their gradual closure.](image-url)
muscle fiber. The conductance of these channels when open is about 40–60 pS. At a normal resting potential of −90 to −70 mV, this corresponds to an inward current of some 4–5 pA. If the membrane potential is more positive than the resting potential, the driving force on Na$^+$ decreases and that on K$^+$ increases, causing the net current to decrease. At about 0 mV, the two currents balance each other and there is no net current through the open channels. If the membrane potential becomes more positive than this “reversal potential”, the net current becomes outward, i.e. the efflux of K$^+$ is greater than the influx of Na$^+$.

2.3.2.3. Impact of subunit composition on channel properties
At immature NMJs, the AChRs present contain a γ-subunit in place of the ε-subunits present at NMJs in the adult (see Section 2.6). The presence of the γ-subunit reduces the conductance from 40–50 pS to about 30 pS but prolongs its mean open time from 1 to 4 ms. The net result is that more charge enters the muscle fiber per opening through a γ-subunit containing AChR, but it does so over a longer period of time, than an ε-subunit containing AChR.

2.3.3. Generation of quantal currents
2.3.3.1. ACh binding to AChRs
The AChR density in the postsynaptic membrane is of the order of 10 000 per μm$^2$ (Matthews-Bellinger and Salpeter, 1983). Thus, within the region to which the 10 000 or so ACh molecules of a quantum diffuse in 0.2 ms, they would encounter about 2500 AChR molecules, each of which has two ACh binding sites. Because the concentration of ACh in the cleft (0.5 mM) is substantially higher than the K$^p$ for the ACh–AChR interaction (3–8 × 10$^{-7}$ M), ACh binds rapidly to the AChRs, saturating most of those present within the diffusion distance of about 0.25 μm (Land et al., 1981).

2.3.3.2. Time course of the EPC
The ACh molecules contained in a single quantum normally cause the opening of about 2000 AChR-associated channels (mouse) within less than 100 μs (see above). Following an opening event, each channel at a mature mammalian NMJ stays open for an average of 1 ms, excluding the very brief closures that characterize channel “bursting”. Each channel has a conductance of about 60 pS. Initially, when all these channels are open, the total conductance increase is therefore about 100 nS. In physiological conditions, this gives rise to a peak mEPC of 3–5 nA (Fig. 2.2).

RELIABILITY OF NEUROMUSCULAR TRANSMISSION

The individual channels opened by a single quantum close after a mean “open time” of about 1 ms. However, random variations in the open time mean that the number of channels still open at any time after the initial activation declines along an exponential time course. As it does so, the mEPC declines in parallel. These factors explain how the shape of the mEPC – a very rapid rise followed by a slower, exponential decline – arises from the summed effects of many “rectangular” single channel events (Fig. 2.8D).

2.3.4. From quantal currents to the EPC
Following a nerve impulse, each of the many separate quanta of ACh released acts on a small region of postsynaptic membrane. This region has a diameter of about 0.5 μm and contains about 2000 AChRs (see Section 2.3.1.1), or about 0.01% of all the AChRs at the NMJ (1–2 × 10$^7$). As a consequence of the fact that the probability of release of a vesicle at each AZ is low, the release sites are on average separated by about 2 μm. There is thus rarely any significant overlap of the “saturated disks” of AChRs associated with adjacent release events. As a result, each quantum acts independently on the postsynaptic membrane. For a quantum content of 50–100, the number of ACh-gated channels opened in response to a single nerve impulse is about 1% of those at the NMJ.

2.3.5. From the EPC to the EPP
2.3.5.1. Introduction to the cable properties of the muscle fiber
The effect of opening ACh-gated channels is to allow the entry of positive charge into the muscle fiber, giving rise to the EPC. The conversion of the EPC into the EPP is determined primarily by the passive cable properties of the muscle fiber, i.e. those that do not involve the voltage-gated Na$^+$ channel. These properties of the muscle fiber as a whole are determined by the electrical conductivity of the cytoplasm, the specific resistance and capacitance of the membrane (that is the resistance or capacitance per unit area), and by the diameter of the muscle fiber (Katz, 1966; Jack et al., 1975).

Current entering the fiber at a point, such as the NMJ, can flow both longitudinally in the cytoplasm and transversely across the membrane (Fig. 2.9A). However, only the latter pathway leads to a change in the membrane potential. The fraction of the current that takes each route depends on the relative resistance each pathway presents. When the diameter is relatively large, current can flow more easily along the interior of the fiber and is less likely to “leak” out across the membrane causing a change in membrane potential.
When the diameter is small, the opposite is true so the peak change in membrane potential caused by a given current is greater. For a cylindrical cell such as a muscle fiber, the peak voltage change caused by a continuous current entering the cell at a point is determined by the “input resistance” of the cell. Typical values for vertebrate muscle fibers are $0.2–1 \times 10^6$ Ohm. Other things being equal, the input resistance of a muscle fiber is inversely proportional to (diameter)$^{3/2}$ (Katz, 1966).

2.3.5.2. Space constant
An important consequence of the “leakiness” of the muscle fiber membrane is that the change of membrane potential caused by current entering at a point decays with distance along the fiber away from that point. The decay of potential from a site of current injection, (e.g. the NMJ) can usually be approximately described by a single exponential (Fig. 2.9B). For many vertebrate muscle fibers the space constant of the decay, that is the distance required for the potential to drop to 37% ($1/e$) of its value at the origin, is 0.5–2 mm. It is because of this that the ACh released from the nerve has little direct effect on the membrane potential more than a few mm away from the NMJ.

2.3.5.3. Effects of membrane capacitance
When current flows across a cell membrane, it causes the reorganization of charged molecules in the membrane. This takes time and energy. In response to an abrupt step change in current, the voltage changes to a new value along an approximately exponential time course. For a muscle fiber, the “charging time constant” is typically 2–5 ms (Fig. 2.9C). When a current transient such as an EPC occurs which is brief relative to the charging time constant of the muscle, the peak voltage change does not reach that predicted by Ohm’s Law (Fig. 2.9C). Thus, for an EPC of 250 nA, and an input resistance of $0.4 \times 10^6$ Ohm, Ohm’s Law predicts that the EPP should have an amplitude of 100 mV. In reality, the amplitude is usually only about half of that value.

2.3.5.4. Impact of cable properties on EPP
These biophysical factors have a major impact on the ability of ACh released from the nerve to trigger an action potential in the muscle fiber. The combined effect of the input resistance and capacitance of the muscle determines how big a mEPP will result from a quantum of ACh (Katz and Thesleff, 1957). Input resistance is particularly influenced by muscle fiber diameter. The effect of membrane capacitance is relatively constant in different muscles. Its importance as a determinant of synaptic efficacy is seen in situations in which the decay time constant of the mEPCs and EPCs vary, as during normal development or in a variety of pathological situations (see below). In such cases, the longer the decay time of the EPC, relative to that of the muscle fiber charging time constant, the greater the potential change caused by the EPC will be (Fig. 9D).

2.3.5.5. Non-linear summation of quanta
The amplitude of the EPP is also influenced by the value of the membrane potential. As the membrane potential approaches the reversal potential for the ACh-induce current ($-10$ to 0 mV), less current flows through the opened channels. As a result, when the quantum content increases, and with it the peak amplitude of the EPP, more quanta must be released to cause a further unit increase in EPP amplitude. This feature of the EPP is often referred to as “non-linear summation” of the effects of ACh quanta (McLachlan and Martin, 1981). Its importance relates to the relationship between the quantum content and the amplitude of the EPP and will be discussed in more detail in Section 2.4.3.3.

2.3.6. Initiation of the action potential
2.3.6.1. The threshold
In the great majority of mammalian muscles, the motor neurons activate the skeletal muscle fibers by generating an AP. As in all excitable cells, this requires reducing the internal negativity of the cell to the point where there are enough voltage-gated Na$^+$ channels...
open to cause a regenerative entry of positive charge into the cell (inward current). Subthreshold depolarizing currents have the effect of driving positive charge out of the cell (outward current). Only when the depolarization is great enough to cause the opening of voltage-gated Na\(^+\) channels can it lead to the inward current that causes excitation.

A muscle fiber is normally excited by current flowing at a single site, the NMJ. A result of the cable properties of the muscle fiber (see above) is that when an EPC is just great enough to cause some Na\(^+\) channels to open at the NMJ, the “active” region is surrounded by less depolarized membrane in which the induced current is still outward. At threshold, the net positive charge that is carried into the fiber by the inward current at the NMJ is equal to that carried out by the outward current in the surrounding membrane (Jack et al., 1975). When the depolarization is above threshold, the positive charge entering at the NMJ is greater than that leaving in the surrounding region. This excess of charge depolarizes the adjacent membrane to threshold, setting in train the regenerative process that underlies the action potential.

The depolarization required to reach threshold is usually determined experimentally by passing current into a muscle fiber from an intracellular microelectrode and measuring the resulting change in voltage with a second electrode placed nearby (cf. Fig. 2.9A). Conventionally this is done in a region of membrane away from the NMJ. For a typical mammalian muscle in experimental conditions, an action potential is generated when the membrane is depolarized from a resting level of about −80 mV to a new level of about −55 mV, i.e. a depolarization of 25 mV (Wood and Slater, 1994). In the muscles of laboratory rodents the EPP, measured after blocking the muscle action potential with the cone snail toxin m-conotoxin GIIIB (µCTx, see below), is about 35 mV, clearly greater than needed to reach the action potential threshold (Wood and Slater, 1997).

2.3.6.2. Threshold at the NMJ

The postsynaptic region of vertebrate NMJs contains a much higher density of Na\(^+\) channels than is present away from the NMJ (Fig. 2.1C) (Haimovich et al., 1984; Flucher and Daniels, 1989; Ruff, 1992; Ruff and Whittlesey, 1992; Wood and Slater, 1998a). Analogy with the nodes of Ranvier in myelinated axons suggests that the threshold for action potential generation should be lower at the NMJ than in the non-junctional region, where threshold is conventionally measured. This prediction has been confirmed by determining the action potential threshold in response to EPPs. To do this, rat muscles were exposed to a critical level of D-tubocurarine to create near-threshold EPPs (Wood and Slater, 1997). In this study, EPPs with a peak amplitude greater than about 10 mV triggered muscle action potentials (Fig. 2.10). Thus the depolarization of the immediate postsynaptic membrane required to trigger an action potential is less than half that required in the non-junctional region.

2.3.6.3. Folds and their electrical effects

An additional influence on the threshold at the NMJ is the geometry of the postsynaptic folds and the distribution of ion channels within them (Vautrin and Mambrini, 1989; Martin, 1994). The high concentration of AChRs at the crest of the folds (see Fig. 2.1D, E) means that during the EPC, positive charge enters the muscle fiber only at the crests of the folds (Fig. 2.11A). The onward “path of least resistance” for this current is through the cytoplasm of the junctional folds rather than across the folded membrane. However the sheet of cytoplasm within the folds, which may be as thin as 100 nm, offers a resistance some 10 times greater than the input resistance of the fiber as a whole (Fig. 2.11B) (Martin, 1994). During the flow of a single mEPC there is thus a potential gradient of about 10 mV between the tops of the folds and the bulk of the cytoplasm of the muscle fiber, where the voltage can be sampled by an intracellular electrode. Put another way, the amplitude of a typical mEPP, which is 0.5–1 mV when measured with a conventional electrode, would be about 10 mV if it could be measured at the tops of
the folds. The NaV1.4 channels lining the lower portions of the junctional folds and the troughs of the synaptic clefts thus experience a greater depolarization from the EPC than if they were present on an unfolded membrane, as in the non-junctional region.

These two factors, the high local density of NaV1 channels and the folding of the postsynaptic membrane, cooperate to lower the effective threshold at the NMJ. The observed magnitude of this effect is well-accounted for by the appropriate model of the postsynaptic region proposed by Martin (Martin, 1994). By lowering the effective threshold, the folds and the NaV1.4 channels in them amplify the effects of the ACh released from the nerve. As a result, fewer ACh-gated ion channels need to be opened to generate an action potential than in the absence of these factors.

2.3.7. Termination of ACh action

The action of a quantum of ACh is terminated by a combination of diffusion of ACh molecules within the synaptic cleft and their enzymatic destruction by the AChE associated with the synaptic BL. If ACh were cleared from the cleft solely by diffusion, it would take many milliseconds for an average ACh molecule to diffuse out of reach of any AChRs. During this time, the molecules would be free to bind to further AChRs and thus prolong the EPC well after the initial muscle fiber action potential has terminated.

The AChE in the synaptic cleft greatly speeds up the process of ACh clearance. As described above, most of the ACh molecules released from the nerve bind rapidly to underlying AChRs, where they remain bound for about 1 ms. During this time, the ACh molecules that bind initially to AChE are cleaved and the AChE becomes available. As ACh molecules unbind from the AChRs, they quickly encounter and bind to AChE and are cleaved. The net effect of the AChE is to limit both the lateral spread of ACh molecules in a quantum and the duration of their action (Fig. 2.12) (Hartzell et al., 1975). In physiological conditions, it is believed that as a result, most AChRs open only a single time in response to a quantum of ACh. If AChE is absent, or its effectiveness is impaired, both the duration and magnitude of the effect of a quantum are increased.

2.3.8. Conclusions

Each quantum of ACh released from the nerve acts on a discrete “response domain” in the muscle fiber surface. This consists of the AChR molecules at the crests of the 1–2 folds adjacent to the site of release, the high resistance sheets of cytoplasm within the junctional folds, and the NaV1.4 channels contained in the membrane of the folds themselves. Each response domain occupies an area of about 0.2 \( \mu \text{m}^2 \) of the postsynaptic surface in contact with the nerve, or about 0.05% of the total synaptic surface at a typical mammalian NMJ. As a result, even when the quantum content is as much as 100, there is little probability that the ACh molecules from one released quantum will compete for AChRs with those from an adjacent quantum.

An important concept developed in recent years concerns the role of the postsynaptic folds. These previously enigmatic structures are now believed to act as amplifiers of ACh action. As such, they play an important role in ensuring the reliability of neuromuscular transmission.

2.4. Safety factor of neuromuscular transmission: definition and measurement

The previous sections have described the two core aspects of neuromuscular transmission: the quantal
release of ACh by the nerve impulse and the action of that ACh on the muscle fiber to initiate an action potential. In practice, it is the balance between these two processes that determines whether the neural activation of muscle is successful. Many studies have shown that in a wide variety of conditions more ACh is released by each nerve impulse than is required to excite the muscle fiber. The terms “safety factor” or “safety margin” of neuromuscular transmission are used, more or less interchangeably, to describe this excess. For the clinician, an important implication of this safety factor is that by the time clinical weakness becomes apparent, considerable impairment of neuromuscular transmission may already have occurred.

This section addresses the questions of how the safety factor has been experimentally defined and its size estimated. Consideration of these topics is important because it gives insight into the various methods that have been used and the particular limitations they bring to the estimates of safety factor derived from them. It also provides a quantitative basis for understanding the normal reliability of neuromuscular transmission and how that may be altered in disease. This material has been reviewed in detail elsewhere (Wood and Slater, 2001).

2.4.1. Origin of the concept of a safety factor in excitable cells

The concept of a safety factor for cell excitation was initially developed in the context of AP propagation in unmyelinated axons (Rushton, 1937). Subsequent studies of myelinated axons suggested that the longitudinal current flowing within an axon from an active to an inactive node of Ranvier was 5–10 times greater than that required to initiate an AP (Hodgkin and Rushton, 1946). Further studies pointed out that the critical factor for the initiation of an AP is that
the charge transferred, and the membrane depolarization it causes, should be distributed in such a way that enough sodium channels are opened to allow a net influx of positive charge (Jack et al., 1975), thus setting in train the regenerative events that constitute the AP (see Section 2.3.6.1).

Studies of isolated nerve-muscle preparations have established that during normal neuromuscular transmission more ACh is released from the nerve, causing more positive charge to enter the muscle at the NMJ, than is required to depolarize the muscle fiber to the threshold for AP generation (Wood and Slater, 1997). The safety factor of neuromuscular transmission is thus an expression of how much greater an effect the nerve has on the muscle fiber than is required to generate an AP (reviewed in (Wood and Slater, 2001)).

2.4.2. Definitions of the safety factor for neuromuscular transmission

A number of different working definitions of the safety of neuromuscular transmission have been proposed. The differences between them generally reflect the type of experiment carried out. In pharmacological studies, the safety factor was defined as the fraction of AChRs that could be blocked before AP generation was prevented (Paton and Waud, 1962; Chang et al., 1975). In electrophysiological studies, the safety factor has often been defined as the ratio of the estimated peak amplitude of the EPP to the threshold depolarization required to generate an AP (Harris and Ribchester, 1979a; Kelly and Robbins, 1983; Engel, 2004). Another approach has been to estimate the magnitude of the postsynaptic current flowing in response to a nerve impulse (Magleby, 1994), defining the safety factor as the excess current generated in response to a nerve impulse over that required to reach the AP threshold. Since the quantum content of the EPP is the feature of neuromuscular transmission that varies most during normal neuromuscular activity (see Section 2.5.1), still another approach is to define the safety factor as the ratio of the number of transmitter quanta released to the number required to excite the muscle fiber (Wood and Slater, 1997). This is the approach which will be emphasized in this section.

2.4.3. Estimating safety factor at the NMJ

To estimate the safety factor in terms of the number of quanta, it is necessary to estimate both the number of quanta released per nerve impulse and the number required to initiate an AP. In general this requires using intracellular microelectrodes to record both quantal and nerve-evoked synaptic events. To make this possible, it is first necessary to block the generation of muscle fiber APs, both to allow the effect of the transmitter to be measured in the absence of the AP and to prevent the mechanical disturbance resulting from contraction. This leads to a fundamental difficulty in determining the safety factor; it is inherently impossible to determine both the amount of transmitter normally released by a nerve impulse and the amount required to trigger an AP in the same experimental conditions. Many different approaches have been taken to resolve this problem, each with its own limitations. A number of these are considered below. Further details can be found elsewhere (Prior et al., 1993; Isaacson and Walmsley, 1995).

2.4.3.1. Blocking action potential generation

Numerous methods have been used to block AP generation. Reducing the ratio of Ca$^{++}$ to Mg$^{++}$ concentrations in the bathing solution reduces transmitter release to levels that are too low to generate an AP (del Castillo and Katz, 1954b). This method has the obvious limitation that it does not allow the full quantum content to be measured. Nonetheless, its simplicity makes it potentially useful for assessing the relative values of quantum content in different situations (Alshuaib and Fahim, 1990; Lyons and Slater, 1991). However, it must be borne in mind that even this use depends on the assumption that the sensitivity to Ca$^{++}$ is constant in the situations being compared, and this may not be so.

An alternative that was used in many early studies was to partially block the response of the muscle to ACh with $\alpha$-tubocurarine to reduce the amplitude of the EPP to a sub-threshold level (Fatt and Katz, 1951). Using this approach in mammals, 80% or more of the AChRs must be blocked to ensure that contraction is fully abolished and intracellular recordings can be made (Paton and Waud, 1962; Chang et al., 1975). As a result the mEPPs are usually too small to measure. Therefore, when a measure of the quantum content is required, indirect estimates based on the analysis of variation of EPP amplitudes must be used. These are inherently inaccurate in any situation that allows normal release (see Section 2.2.6) (Martin, 1955; Slater et al., 1992). A further limitation is that $\alpha$-tubocurarine may also reduce the amount of transmitter released by interacting with presynaptic nicotinic autoreceptors (see Section 2.5.4.1) (Glavinovic, 1979b; Magleby et al., 1981; Bowman et al., 1988; Ferry and Kelly, 1988).

An approach used successfully in a number of studies is to damage the muscle fibers, usually by cutting them some distance from the NMJ (Barstad and Lilleheil, 1968; Glavinovic, 1979c; Maselli et al., 1991; Slater et al., 1992). This causes depolarization of the
membrane, usually to a resting potential of about 
−40 mV. This, in turn, leads to inactivation of the 
voltage-gated sodium channels, thereby blocking AP
generation in the muscle. This has the advantage that no
drugs are required. However there are several disadvan-
tages. The low resting membrane potential and reduced
input resistance of the muscle fibers caused by the
damage means that both EPPs and mEPPs are small and
therefore difficult to record. This effect can be partially
overcome by injecting a steady current into the muscle
through a second intracellular electrode to restore
the resting potential locally to a more negative value.
A further complication is that extensive damage to the
muscle results in the leakage of K⁺ into the extracellular
space. Unless care is taken to wash the muscle thor-
oughly, this may depolarize the intramuscular nerve
branches, leading to a failure of propagation of the
nerve impulse into the presynaptic terminal. In spite
of these limitations, this approach has provided much
useful information.

Recently, a natural toxin has become available
which, in appropriate circumstances, blocks APs in the
muscle but not in the nerve. In principle, this is currently
the best available approach to the study of evoked
release. μ-conotoxin GIIIB (μCTX) is a component of
the venom of the cone snail Conus geographus. In initial
studies, it was found that μCTX blocks rat muscle fiber
sodium channels while having no effect on those from
nerve or brain (Cruz et al., 1985; Moczydlowski et al.,
1986). In subsequent studies, concentrations of μCTX
were found which blocked action potentials in muscles
of guinea pig (Muraki et al., 1991), mouse (Gonoi
et al., 1989), rat (Plomp et al., 1992) and frog (Sosa
and Zengel, 1993) but not in their nerves. However, it
was subsequently shown that at high concentrations,
μCTX blocks sodium currents in mouse nerves (Braga
et al., 1992). While it is generally believed that the
relative specificity for Nav1.4 channels allows the use of
μCTX to block all vertebrate NMJs, this is unfortu-
nately not the case. In some human and chicken tissues,
μCTX blocks the nerve impulse at concentrations
lower than that required to block muscle APs (Plomp et al.,
1995; Wood and Slater, 1998b). This raises the possi-
Bility that in some circumstances, the use of μCTX
may lead to partial block of the nerve impulse and the
reduction of transmitter release.

2.4.3.2. Estimating transmitter release
using electrophysiology

The effect of the released transmitter on the muscle
can be measured and expressed in several ways. The
most straightforward is to record and measure the peak
amplitude of full-sized EPPs in a situation in which
the muscle fiber action potential has been blocked.

To make accurate recordings of synaptic events with
intracellular electrodes, it is important to place the
electrodes close (<100 μm) to the NMJ. Otherwise,
the passive cable properties of the muscle membrane
result in the amplitude of the EPP declining as the
recording electrode is placed increasingly far from
the NMJ (Fatt and Katz, 1951; Betz et al., 1984). This
effect is best overcome by making recordings in a
situation where the NMJ can be directly visualized.
The can be done most directly by using fluorescent dyes
such as 4-di-2-asp (a mitochondrial dye; (Magrassi
et al., 1987)) or FM1–43 (see Section 2.2.7). In favor-
able conditions, it may also be possible to visualize the
NMJ without fluorescent dyes, simply using bright
field or Nomarski interference contrast optics. As an
alternative, recordings may be limited to those where
the rise time of the EPP is faster than some preset value
(e.g. 1 ms), since EPP rise time is a function of distance
from the NMJ (Fatt and Katz, 1951; Betz et al., 1984).

While the amplitude of the EPP provides a measure of
transmitter effect, it is not an accurate reflection of the
amount of transmitter released from the nerve. This is
because of the non-linear relationship between the
amount of transmitter released and the resulting depolar-
ization of the muscle fiber membrane (see Section
2.3.5.6). As a result, the amplitude of the EPP generally
underestimates the number of quanta that give rise to it
(McLachlan, 1978; Slater et al., 1992). This confounding
factor becomes greater as the quantum content increases.

Various formulas for adjusting the EPP amplitude to
take account of this “non-linear summation” have been
devised (Martin, 1976; Stevens, 1976; McLachlan and
Martin, 1981), such as:

\[ v' = v/(1 - v/E) \]

where \( v' \) is the adjusted EPP amplitude, \( v \) is the
recorded EPP amplitude and \( E \) is the difference
between the resting potential and the reversal potential
for transmitter action, usually between 0 and −10 mV.
In practise there are considerable uncertainties
involved in using these corrections. This is because,
in addition to the effect of non-linear summation, the
magnitude of the peak depolarization of the recorded
EPP is also influenced by the capacitative properties of
the muscle fiber membrane (see Section 2.3.5.3).
Efforts have been made to account for this, for exam-
ple by introducing an additional variable \( f \) into some
of the equations (Martin, 1976) as follows;

\[ v' = v/(1 - fv/E) \]

Unfortunately, the value of \( f \) can only be determined
empirically, greatly reducing the usefulness of the
whole approach.
RELIABILITY OF NEUROMUSCULAR TRANSMISSION

A better way around the difficulties raised by non-linear summation is to make recordings of synaptic currents rather than potentials. To do this, two micro-electrodes need to be inserted within 100 μm of the NMJ and used to “voltage-clamp” the muscle fiber. EPCs can then be recorded over a range of membrane potentials (Takeuchi and Takeuchi, 1959; Magleby and Stevens, 1972; Glavinovic, 1979c; Slater et al., 1992). This method provides a more direct estimate of transmitter release since the amplitudes of the recorded currents are linearly related to the number of ion channels opened by the released ACh and hence to the amount of ACh released (McLachlan, 1978).

2.4.3.3. Estimating quantum content

To estimate the safety factor in terms of the number of quanta normally released by a nerve impulse, it is necessary to determine the quantum content. The most direct way to do this is to determine the ratio between the peak amplitude of the EPCs and the mEPCs. Since there is statistical variation in both the effect of individual quanta and of the number of quanta released, even at the same NMJ in the same experiment, mean amplitudes of a suitable number of events must always be used in these calculations. Another important consideration is that the quantum content varies depending on the pattern and frequency of activity (see Section 2.5.1). In isolated nerve-muscle preparations from frogs and mammals, stimulation at frequencies greater than about 0.2–1 Hz results in a frequency dependent reduction in quantal release (see below). In what follows, values for quantum content have been derived from studies in which the frequency of stimulation was not greater than 1 Hz. It must be emphasized that while this approach allows comparison of quantum content in differing situations, it does not represent the values expected to apply during natural patterns of activity.

The amplitude of synaptic events depends on the membrane potential at which they are recorded. It is therefore important either to record both spontaneous and evoked events at the same membrane potential, or to make some correction for differences in membrane potential. One approach is to use two intracellular electrodes in a “current clamp” configuration to maintain a constant membrane potential that is sufficiently negative to allow mEPPs to be recorded even from cut fiber preparations (Slater et al., 1992). In many species, spontaneous quantal events occur at a frequency of about 1 Hz and 50–100 events can be recorded with no difficulty. In some cases, such as normal humans or immature rodents, the frequency is much lower—of the order of a few per minute. One way around this, which is effective in human nerve-muscle preparations, is to stimulate the nerve at high frequency (50 Hz) for up to 10 s. This results in a great increase in the frequency of quantal events, which lasts from 10–20 s (Magleby, 1994). In our experience, the mean amplitude of mEPPs recorded this way does not differ significantly from those recorded without stimulation, though this may not be true for more exhaustive stimulation (Van der, 1991; Glavinovic, 1995).

Once the mean amplitudes of mEPCs and EPCs have been determined the quantum content (m) can be calculated as:

\[ m = \text{EPC amplitude} / \text{mEPC amplitude} \]

This “direct” method can also be used with EPPs and mEPPs but then corrections must be made for both variations of resting membrane potential and non-linear summation (see Section 2.3.5.6) and these are likely to lead to inaccuracies in the final value:

\[ m = \text{NLS corr EPP amplitude} / \text{mEPP amplitude} \]

where “NLS corr EPP amplitude” is the amplitude of the EPP corrected for non-linear summation. Reported values of quantum content obtained in comparable conditions using the “direct” method, with either potential or current recording, are generally from 20–200.

Many early estimates of quantum content were made in situations where quantal events could not be resolved so direct estimates of quantum content were not possible. In the absence of a measured value of quantum size, the assumption was made that the responses could be described by the Poisson distribution. On this basis, the mean quantum content could be estimated from a measure of the variance of the amplitude of the EPP (see Section 2.2.6). While this seems an attractive method to be used in preparations blocked by d-tubocurarine, it overlooks the fact that the Poisson approximation is only justified in situations where the probability of release from the nerve terminal, and hence the quantum content, is very low. When, as in solutions of normal Ca\(^{2+}\) concentration, this is not true, the “variance” method significantly overestimates the quantum content (Fig. 2.13) (Martin, 1965; McLachlan, 1978; Slater et al., 1992).

For a long time, the use of the “variance” method to estimate quantum content led to the belief that the quantum content at mammalian, including human, NMJs was around 200, similar to that in frogs (Ginsborg and Jenkinson, 1976). Figures such as this may still be found in common text books. It is now clear, however, that at a stimulus frequency of 1 Hz, the true value at rat NMJs is between 50–100 (Glavinovic, 1979a; Catterall and Coppersmith, 1981; Plomp et al., 1992; Wood and Slater, 1995) while in humans it is lower still, 20–50 (Fig. 2.21, Section 2.6.1.1) (Cull-Candy et al., 1980; Engel et al., 1990; Slater et al., 1992; Plomp et al., 1995).
In summary, the most accurate way to estimate quantal release is to record EPCs and mEPCs from muscle in which action potentials have been blocked and to use the direct method to calculate quantum content. In species where mCTX cannot be used, or when quantal events cannot be resolved, it may be necessary to resort to other less direct approaches as described above.

### 2.4.3.4. Measuring the “threshold quantum content”

Having obtained a measure of transmitter release per nerve impulse, it is then necessary to estimate how much transmitter is “required” to generate an action potential. This involves first determining the threshold at the NMJ, as described in Section 2.3.6.2, and then estimating the number of quanta that must be released to reach that threshold. The number of quanta that would have to act to reach threshold can be estimated from the mean amplitude of threshold EPCs recorded during partial block by d-tubocurarine (Wood and Slater, 1997). This amplitude must then be divided by the mean amplitude of mEPCs recorded in the absence of d-tubocurarine to arrive at an estimate of the threshold quantum content. For both the “fast” EDL and the “slow” soleus muscles of the rat, the value of threshold quantum content is about 13. This is about 25% of the normal quantum content at these NMJs.

### 2.4.3.5. Comparison of reported values of safety factor at rat NMJs

The concept of a safety factor for neuromuscular transmission was first discussed in detail nearly 40 years ago (Paton and Waud, 1967). Since then there have been remarkably few efforts to assess it quantitatively and those that have been made have used a variety of methods and preparations and are not, therefore, strictly comparable. Nonetheless, they provide a general consensus that at NMJs in most mammalian limb muscles, studied in vitro, the safety factor is between 2 and 10 (Table 2.1).

Some of the highest estimates of safety factor come from pharmacological experiments at mammalian NMJs (Paton and Waud, 1962; Chang et al., 1975). By blocking increasing fractions of AChRs with d-tubocurarine, it was found that 80–90% of the receptors could be blocked before there was any failure of indirectly evoked contraction. These studies suggest that, at low frequencies, 5–10 times more ACh-gated channels are normally opened than is required to generate an action potential. Such experiments also established that there is a substantial excess of AChRs at the NMJ (Ginsborg and Jenkinson, 1976). However, the distributed nature of transmitter release and the short distance over which the ACh in a single quantum acts (see Section 2.3.8) means that in spite of this nominal excess, most of the AChRs are out of range of the ACh released by any given nerve impulse.

Many studies have used intracellular recording techniques to estimate the safety factor. In their classic study of the EPP in mammalian muscle, Boyd and Martin (1956) estimated that the EPP, in the absence of an action potential, would be about 35–40 mV while a depolarization of only 10–20 mV would be required to generate an action potential. Thus, a safety factor of 2–4 can be derived from their data. In other studies, the amplitude of the full-sized EPP was estimated from the product of the quantum content and the mEPP amplitude and then “corrected” for non-linear summation (Kelly, 1978; Harris and Ribchester, 1979b). In these studies, the quantum content was estimated from the variance of EPPs recorded at NMJs blocked with d-tubocurarine. Since the variance method substantially overestimates the quantum content (Section 2.4.3.2) this approach is likely to overestimate the EPP amplitude. In the same studies, the action potential threshold was measured by passing rectangular current pulses through the membrane some distance from the NMJ. This is likely to overestimate the threshold (Section 2.3.6.2). Since both components of the safety factor ratio are overestimated, the errors generated tend by chance to cancel each other out.

A value for safety factor has been defined based on how many more quanta are released per nerve impulse than are required to trigger an action potential. Using the methods described in Section 2.4.3, values within the range observed by others were obtained (Wood and Slater, 1997). In rat soleus and EDL, the normal
quantum content is 46.3 and 65.1, respectively while the corresponding threshold quantum contents are 13.3 and 13.0. Thus, the calculated safety factors are 3.5 for soleus and 5.0 for EDL.

2.4.4. Conclusions

Whatever methodological approach has been used, studies of the safety factor at normal mammalian NMJs stimulated in vitro at or near 1 Hz suggest a typical value of about 4 (Table 2.1). While some estimates appear to be based on more firm methodological foundations than others, it is important to reiterate that all must be considered imperfect estimates of the true value \textit{in vivo}, where varying patterns of activity and the actions of many modulating factors may play an important part in determining the safety factor at any moment. The most important of these modulating factors are considered in the following section.

2.5. Modulation of safety factor during normal use

Why is the safety factor for neuromuscular transmission so high? During natural use of the neuromuscular system, the pattern and amount of activity change over a wide range. The ability of the nerve to release transmitter varies significantly as the frequency of activation changes. In particular, during high frequency activity, the quantum content declines significantly. Most published estimates of quantum content that have been used to estimate safety factor (Table 2.1) were determined at low frequencies (0.1–1 Hz) and are assumed to represent close to the maximum release of which the NMJ is capable in physiological conditions. During normal usage, the quantum content is almost certainly lower.

There are many factors in the nerve terminal that influence its response to different activity patterns (Van der Kloot and Molgo, 1994; Atwood and Karunanithi, 2002). Most important are the dynamics of Ca\(^{2+}\) concentration and the synaptic vesicle “pools” in the nerve terminal, and the action of chemical modulators on the nerve terminal and the Schwann cells associated with it. Although our understanding of these factors, and the interactions between them, is still very incomplete, it is likely that they play an important part in regulating the efficacy of neuromuscular transmission.

2.5.1. The effects of repetitive activity on release

When isolated nerve-muscle preparations are stimulated repetitively at frequencies much above 0.1 Hz,
the quantum content varies in ways that depend both on the frequency, duration of stimulation, and the species and type of muscle. These changes in release involve both increases and decreases, each of which may occur on several different time scales (Fig. 2.14A) (Magleby, 1994; Zucker and Regehr, 2002).

### 2.5.1.1. Decreases in quantum content

With mammalian nerve-muscle preparations in physiological concentrations of Ca\(^{2+}\) (e.g. 2mM), the predominant effect of repetitive stimulation at 1–100 Hz is a progressive reduction in quantum content that often has several components that vary in time course and magnitude (Liley and North, 1953; Elmqvist and Quastel, 1965; Kamenskaya et al., 1975). The fastest component of depression generally lasts for 10–20 stimuli so that at 50–100 Hz, this phase thus lasts <0.5 s (Fig. 2.14B). During this time the quantum content typically falls to 30–80% of its value at 1 Hz, depending on the frequency.

It is generally believed that the decline in quantum content during high frequency stimulation reflects the depletion of the population of vesicles docked at the AZs (Rizzoli and Betz, 2005). It is likely that the

---

**Fig. 2.14.** Changes in neuromuscular transmission during repetitive activation. A. The effect of 90 s stimulation at 100 Hz on the amplitude of the EPP, relative to that during an initial period of stimulation at 0.1 Hz, at a partially curarized (dTC, 3 μM) frog NMJ. An initial increase due to facilitation of ACh release is followed by a decline due to depression of release. When the 100 Hz stimulation stops, there is a further phase of increased EPP amplitude reflecting post-activation potentiation of ACh release. (From Magleby, 1994, with permission. B.) The quantum content at NMJs in human intercostal muscle declines during repetitive activation. (From Kamenskaya et al., 1975, with permission.) Note that there is little evidence of facilitation at mammalian NMJs when the quantum content is normal, in contrast to frog NMJs.
docked vesicles exist in different states of readiness for release. In particular they may be in various stages of “priming”, in the sense that they have undergone ATP-dependent associations with the release proteins of the nerve membrane that represent the first steps in exocytosis (Fig. 2.7). The process of priming is complex and involves members of the Munc and Rab protein families (Sudhof, 2004). Current estimates of the priming process suggest that it takes place on a timescale of a few seconds (Rettig and Neher, 2002). These primed vesicles are considered to form a “readily releasable pool” (RRP, see Section 2.5.2).

Only a small fraction of the vesicles in the terminal are docked at AZs at any time. For a typical mammalian NMJ, the total number of vesicles is about 250 000–350 000. Of these, no more than about 1000–2000 can be docked (2/AZ, 2.6 AZ/μm², synaptic area 200–400 μm²). The probability of any one docked vesicle being given nerve impulse is about 0.05–0.1 at rest, giving a quantum content of about 50–100. In the frog, if the RRP is rapidly depleted, it refills within a few seconds (Kalkstein and Magleby, 2004). In any physiological situation, the instantaneous quantum content is therefore likely to reflect a balance between the rates of depletion and refilling of the RRP. The higher the frequency of simulation, the more fully the RRP is depleted and the lower the instantaneous quantum content.

Prolonged stimulation causes further, slower, components of reduced quantum content on timescales of seconds and minutes (Magleby, 1994). The first of these (~5 s) is believed to reflect the “priming” of vesicles already close to the AZ into a releasable state. The slowest component of depression is believed to reflect the movement of vesicles from a reserve pool held at some distance form the AZs to the vicinity of the AZs (see below) (Rizzoli and Betz, 2005).

### 2.5.1.2. Increases in quantal release

In addition to processes that lead to decreases in quantum content, others do the opposite. At least three distinct phases of increasing quantum content have been defined: facilitation (<1 s), augmentation (5–10 s) and potentiation (10 s of seconds to minutes) (Kamenskaya et al., 1975; Magleby, 1979). Facilitation has long been believed to result from the persistence of "residual Ca²⁺" in the nerve terminal after an initial nerve impulse (Katz and Miledi, 1968). If a second impulse arrives before the effect of the Ca²⁺ that entered during the first has fully subsided, then the residual Ca²⁺ is expected to sum with that entering during the second impulse and “facilitate” quantal release (Zucker and Regehr, 2002).

In bathing solutions with normal Ca²⁺ concentration (2 mM), facilitation is not a marked feature of the response at normal mammalian NMJs. This is because the amount of Ca²⁺ entering the opened Ca²⁺ channels is already enough to cause a near maximal effect. However, as the external Ca²⁺ is lowered, or Ca²⁺ entry is reduced by a pathological process, the response falls below the maximal and the facilitating effect of any residual Ca²⁺ becomes obvious. Thus there is often an inverse relationship between the low frequency quantum content and the amount of facilitation. An important clinical example of this is seen in the Lambert–Eaton Myasthenic Syndrome (see Section 2.8).

The factors accounting for augmentation and potentiation are less clear. All phases of enhanced quantal release are associated in time with the accumulation of Ca²⁺ in the nerve terminal (Zucker and Regehr, 2002). One likely source of the prolonged increase in Ca²⁺ associated with potentiation is the slow release from mitochondria of Ca²⁺ taken up during intense activity (David and Barrett, 2003) (see below).

### 2.5.1.3. Relationship to clinical EMG protocols

How do the many components of frequency-dependent modulation of quantal release, described above, interact to determine the real instantaneous quantum content and safety factor in vivo? This question is of great importance to the clinical neurophysiologist who needs to devise tests that can reveal the functional state of the nerve terminals in patients. One of the most common tests uses the response of the compound muscle action potential (CMAP) to nerve stimulation at 3–5 Hz to test for the efficacy of neuromuscular transmission. At this frequency, facilitation is low so depression can be readily detected.

### 2.5.2. Vesicle pools and their dynamics

Underlying much of our understanding of the modulation of quantal release by activity is the notion that synaptic vesicles exist in different states within the nerve terminal. These are defined both by the location of the vesicles, in particular their proximity to the AZs, and by their functional state. Studies of the various temporal phases of depression and enhancement of release during repetitive stimulation have led to the notion that vesicles exist in distinct “pools”, and that they can move from one pool to another with definable kinetics (Fig. 2.15) (Rizzoli and Betz, 2005). Recent investigation of the dynamics of these pools has utilized the activity-dependent labeling of vesicles with fluorescent dyes, most notably steryl dyes including FM1-43 and analogs of it (Cochilla et al., 1999).
The vesicles of the RRP represent only a small fraction (\(<1\%) of all the vesicles in a motor nerve terminal. It is depleted within less than 1 s by stimulation at 10–100 Hz. Most vesicles (80–90\%) are located some distance from the AZs and are believed to be tethered to the actin cytoskeleton by phosphorylation-dependent bonds with synapsin 1 (Rizzoli and Betz, 2005). Periods of stimulation lasting minutes are required to release vesicles from this “reserve pool”. Between the reserve pool and the RRP are vesicles that are available for rapid docking, the so-called “recycling pool”. This pool typically contains 10–20\% of the vesicles in the terminal and vesicles from it are released by stimulation lasting more than a few seconds. While it is clear that movement of vesicles between these pools can occur in experimental conditions, little is known about their dynamics in vivo.

### 2.5.3. Mitochondria as modulators of quantal release

As mentioned above, many of the short-term changes in quantum content induced by repetitive activity appear to reflect changes in the concentration of free Ca\(^{2+}\) in the nerve terminal. A number of factors influence how, and at what rate, the increase in Ca\(^{2+}\) concentration in the nerve associated with a single nerve impulse is restored to the resting level. The mitochondria, which are prominent components of the presynaptic cytoplasm (Fig. 2.1D), are one of these factors.

The most familiar role of mitochondria is to provide ATP to power a wide variety of cellular activities, such as the complex and demanding processes of exocytosis and ionic regulation during intense activity. However, mitochondria also act to sequester Ca\(^{2+}\), and are therefore well placed to play a key role in Ca\(^{2+}\) homeostasis in the nerve terminal (Alnaes and Rahamimoff, 1975; David et al., 1998). Depolarization of mitochondria by drugs such as CCCP (m-chlorophenyl-hydrazone) blocks Ca\(^{2+}\) uptake into the mitochondria and thus allows a build up of Ca\(^{2+}\) in the nerve terminal during intense activity. This, in turn, increases the rate of spontaneous mEPPs that is very pronounced during and after high frequency stimulation (David and Barrett, 2003). When NMJs poisoned by CCCP are studied at normal Ca\(^{2+}\) concentration, intense stimulation (50 Hz for 10 s) leads to a much greater decline in quantal release than in normal medium (Fig. 2.16A, C). This effect is paradoxical in that an increase in Ca\(^{2+}\) within the nerve terminal would be expected to increase evoked release, as during facilitation (see above). The explanation seems to lie in a great increase in the frequency of spontaneous quantal release (Fig. 2.16B). This increase in “asynchronous” release is so great that it rapidly depletes the RRP, thus reducing evoked release.

It is possible to visualize and measure the levels of Ca\(^{2+}\) in the nerve terminal using Ca\(^{2+}\)-sensitive fluorescent dyes, injected into the terminal (David et al., 1997). During bursts of high frequency stimulation, the level of Ca\(^{2+}\) increases first in the cytoplasm and then in the mitochondria. After such a burst, the elevated Ca\(^{2+}\) persists in the mitochondria after it has declined in the cytoplasm. If mitochondrial function is blocked, using drugs that abolish the mitochondrial proton gradient, the increase in Ca\(^{2+}\) in the cytoplasm is greatly exaggerated (David and Barrett, 2003). Thus, the observed changes in release all have parallels in the levels of cytoplasmic Ca\(^{2+}\) and these
Fig. 2.16. Role of mitochondrial function in maintaining quantal release. Mouse NMJs were stimulated at 50 Hz for 10 s. A. EPPs recorded at the beginning (EPP 1–5) and end (EPP 496–500) of the train of stimuli. The lower traces show the much greater decline of EPP amplitude when mitochondria are depolarized by CCCP than in the control solution. Note also the great increase in mEPP frequency at the CCCP treated NMJ. B. Changes in quantal release per second due to evoked (“phasic”) release and spontaneous (“asynchronous”) release during the train. Note that in CCCP blocked preparations, asynchronous release rapidly outstrips evoked release. C. Changes in quantum content during and after the train. Note the greater and more prolonged reduction at NMJs when the mitochondria had been blocked. (From David and Barrett, 2003, with permission.)
appear to be closely regulated by the mitochondria. The effects of specific mitochondrial blockers show that these relatively short-term effects of the mitochondria are dependent on the state of the proton gradient rather than on the ability of the mitochondria to supply ATP (David and Barrett, 2003).

It is clear from these studies that mitochondria can exert a modulatory influence on quantal release at the NMJ. While it is difficult to assess the significance of this effect in vivo, it seems possible that the general metabolic state of an animal could have a real impact on the efficacy and reliability of neuromuscular transmission.

2.5.4. Effects of chemical modulators on release

There is extensive evidence that quantal release of ACh at the NMJ is subject to modulation by a variety of naturally occurring chemical mediators (Van der Kloot and Molgo, 1994). The history of this field is complex, partly because many of the effects are on a modest scale, partly because it is difficult to establish the site of action of bath applied drugs, and partly because of real differences in the properties of NMJs in frogs on the one hand and the mammals that have been studied. Nonetheless, there is now extensive evidence of negative modulation of quantal release both by ACh itself and by ATP (or its metabolite adenosine) released along with ACh, and of positive modulation by noradrenaline. In principle, these modulatory effects could reduce the safety factor during normal activity or enhance it in times of stress (“fight or flight”).

2.5.4.1. Autocrine effects on release: cholinergic

For many years there has been evidence that ACh acts presynaptically as well as postsynaptically at the NMJ. Early pharmacological studies suggested that ACh exerted a direct depolarizing effect on the nerve terminals that acted to decrease quantum content (Hubbard and Wilson, 1970; Ferry and Kelly, 1988). This effect was believed to be blocked by both D-tubocurarine and atropine, leaving doubts about the nature of the cholinergic receptors involved. Subsequent studies have provided clear evidence that muscarinic AChRs (mAChRs) are present at mammalian NMJs and that bath-applied muscarine reduces quantum content at mouse NMJs maintained in a physiological concentration of Ca\(^{+}\) (0.4 mM and high Mg\(^{+}\) (6–8 mM) to reduce the EPP amplitude and thus abolish contraction and stimulated at 0.3 Hz. (From Minic et al., 2002, with permission.)

Similar biphasic effects have been described at frog NMJs, where it has also been shown that the enhancement of release caused by activation of M\(_2\) mAChRs is associated with an increase in the amplitude of the presynaptic Ca\(^{++}\) current (Slutsky et al., 1999, 2001). No change in Ca\(^{++}\) current was observed following activation of M\(_1\) mAChRs. Since M1 and M2 mAChRs act via different G-protein coupled pathways, it is clear that the balance between the effects of these two mAChR types is likely to be influenced by many aspects of the physiology of the nerve terminal.

2.5.4.2. Autocrine effects on release: purinergic

Synaptic vesicles contain and release ATP as well as ACh (Silinsky and Hubbard, 1973; Silinsky, 1975). Once in the synaptic cleft, ATP is broken down by ectonucleotidases, releasing adenosine. There is evidence that both exogenous ATP and adenosine can depress quantal release and that purinergic receptors for both compounds are present at the NMJ (Deuchars et al., 2001; Moores et al., 2005). As with cholinergic modulation of release, the effects of activation of these receptors are complex. Exposure of isolated nerve-muscle preparations to ATP reduces quantal release. It seems likely that this is due in part to direct effects of ATP on P\(_2\) receptors (Hong and Chang, 1998; Sokolova et al., 2003) and in part to the effects of adenosine released following ATP breakdown.

In physiological conditions, exogenous adenosine inhibits quantal release (Ginsborg and Hirst, 1972),
an effect mediated by P1A1 purinergic receptors (Silinsky, 1980). At mouse NMJs, this effect is accompanied by a reduction in the presynaptic Ca\(^{++}\) current (Fig. 2.18A, B) (Silinsky, 2004). However, activation of P1A2 receptors, detected after inhibiting the downstream effects of P1A1 receptors, increased ACh release (Silinsky et al., 1989). To add further to the complexity of purinergic modulation of quantal release at the NMJ, there is also evidence of cross-talk between the muscarinic and purinergic systems (Oliveira et al., 2002).

### 2.5.4.3. Effects of reactive oxygen species

The events associated with neuromuscular transmission and muscle contraction require a constant and immediate supply of ATP. Reactive oxygen species (ROS) such as superoxide, H\(_2\)O\(_2\) and the hydroxyl radical are generated as consequences of ATP synthesis by mitochondria. Recent studies have provided evidence that ROS exert a negative effect on quantal release (Giniatullin and Giniatullin, 2003; Kovyazina et al., 2003). This may serve to limit ACh output during intense activity.

### 2.5.4.4. Schwann cells and modulation of quantal release

While it is clear that multiple effects of ACh and ATP/adenosine can be demonstrated at mammalian and frog NMJs, it is difficult to know where the receptors mediating these effects are located. Although it is often assumed that these “presynaptic” receptors are on the nerve terminals, they could also be on the terminal Schwann cells (Minic et al., 2002). There is considerable evidence from the frog NMJ that Schwann cells play an active role in the regulation of quantal release (Auld et al., 2003). The terminal Schwann cells express functional muscarinic and P2X and P2Y purinergic receptors. Activation of these receptors leads to an increase in cytoplasmic Ca\(^{++}\), probably as a result of release of Ca\(^{++}\) from intracellular stores (Jahromi et al., 1992; Robitaille, 1995; Robitaille et al., 1997). This increase in Schwann cell Ca\(^{++}\) is associated with a parallel increase in quantal release from the nerve. However, the link between these events is unclear.

Both mAChRs and P2Y purinergic receptors act via G-protein coupled pathways. The effects on quantal release of direct injections of non-hydrolysable GTP and GDP analogues into the Schwann cells, designed to mimic receptor activation and inhibition respectively, have therefore been investigated (Robitaille, 1998). Injection of GTP\(_S\), a non-hydrolyzable analog of GTP, which activates G-proteins, led to a significant reduction in quantal release in response to high frequency stimulation (Fig. 2.19). By contrast, injection of GDP\(_S\), which antagonizes G proteins, had no such effect. It thus seems that in the frog, the terminal Schwann cells may play an active part in regulating quantal release from the motor nerve terminal. While it is less clear that the same effects occur at mammalian NMJs, it has been shown that activation of mAChRs leads to increases in cytoplasmic Ca\(^{++}\) in mammalian Schwann cells (Rochon et al., 2001).

It is thus clear that there is a rich network of potential autoregulatory presynaptic effects at the NMJ. These bear similarities to regulatory pathways at CNS synapses, and to the involvement of astrocytes as modulators of synaptic strength. What remains unclear is how important these effects are at normal NMJs in vivo. Many of them are most prominent at high frequencies of stimulation, and may serve to protect against the deleterious effects of excessive ACh action on the postsynaptic region (see below).

### 2.5.4.5. NO as a modulator of release

Nitric oxide synthase (NOS) is concentrated at NMJs in frogs and mammals (Kusner and Kaminski, 1996; Descaries et al., 1998) although its cellular distribution remains controversial (Roth et al., 2005). There is considerable evidence that its product, NO, inhibits quantal release at frog NMJs (Lindgren and Laird, 1994; Thomas and Robitaille, 2001; Etherington and Everett, 2004). The mechanism of the effects of NO appears to depend on the frequency of stimulation.

![Fig. 2.18. Adenosine reduces quantal release. Exogenous adenosine (1 mM) reduces both the Ca\(^{++}\) current in the nerve terminal at mouse NMJs (A) and the amplitude of the EPP (B). (From Silinsky, 2004, with permission.)](image)
Thus at low frequency they are cGMP-dependent but at high frequency they are not (Thomas and Robitaille, 2001; Etherington and Everett, 2004). While the presence of NOS at mammalian NMJs suggests that NO may be a modulator of transmission in mammals, this has not been directly demonstrated.

2.5.4.6. Adrenergic modulation of quantal release

In addition to the potential modulatory effects mediated by ACh and ATP released at the NMJ, there are other effects mediated by circulating factors. The best known of these is the action of noradrenaline. It has long been recognized that stimulation of the sympathetic nerve supply can potentiate neuromuscular transmission in fatigued frog muscles (Orbeli, 1923). Modern micro-electrode studies agree that this effect involves an increase in the amplitude of the EPP, but there has been little consensus about its origin. Thus increases in both mEPP size (Van der and van der Kloot, 1986) and quantum content (Hutter and Loewenstein, 1955; Krnjevic and Miledi, 1958; Jenkinson et al., 1968; Vizi, 1991) have been suggested. Recently, a further effect of noradrenaline on frog NMJs has been reported (Bukchareva et al., 1999). Using focal extracellular recording (see Fig. 2.4 above), it has been found that bath applied noradrenaline increases the synchrony of quantal release (Fig. 2.20). This allows more effective summation of the effects of the individual quanta and results in a larger EPP. This effect is mediated by a signaling pathway involving activation of protein kinase A and an increase in cAMP concentration (Bukharaeva et al., 2002).

The potential biological significance of an enhancement of quantal release mediated by activation of adrenergic receptors presumably lies in its ability to ensure effective muscle activation in times of stress. Since most of the experimental evidence for such a modulatory pathway comes from work on frogs, its significance to mammalian and human physiology is unclear. Ephedrine, a sympathomimetic, has been used to treat patients with impaired neuromuscular transmission due to myasthenia gravis and several congenital myasthenic syndromes (see Section 2.8.7.1). When tested on canine NMJs in vitro, ephedrine has mixed effects, involving both enhanced quantal release and blockage of ACh-gated ion channels (Shinnick-Gallagher and Gallagher, 1979; Sieb and Engel, 1993; Milone and Engel, 1996). At concentrations associated with clinically beneficial effects in humans, it had little effect in vitro. One possible explanation of these findings is that ephedrine has some central effects in humans (Molenaar et al., 1993).

2.5.4.7. Modulation of quantal release by peptides and proteins

An influence of peptides on neuromuscular transmission activity is common in invertebrates and in the enteric nervous system of vertebrates (Shaw, 1996). At the vertebrate NMJ, there is some evidence that a variety of proteins or peptides may also exert a modulating effect. Many of these studies have been made on cultured nerve and muscle cells in vitro and have concentrated on the
Effects of neurotrophins have also been shown to influence quantal release in mammalian nerve-muscle preparations. BDNF and NT-4 both increase neuromuscular transmission in the rat diaphragm (Mantilla et al., 2004). A number of neurotrophins and related compounds were tested on nerve-muscle preparations from neonatal mice (Ribchester et al., 1998). While GDNF increased mEPP frequency, none of the other seven compounds tested had any effect. The significance of peptidergic modulation of neuromuscular transmission in adult mammals thus remains unclear.

Other peptides or proteins are released from motor nerve terminals along with ACh (Van der Kloot and Molgo, 1994). These include CGRP and agrin. Both these substances have effects on NMJ development and/or maintenance. However, there is no convincing evidence that they, or any other proteins released from the nerve have direct effects on quantal release.

2.5.5. Effects of muscle length on transmission

Contracting muscles generally change their length. Motor nerve terminals are mechanically coupled to the muscle fiber through adhesive interactions of both cells with the basal lamina. As a result, changes in muscle length have an impact on the length of the nerve terminal. In the frog, muscle stretch causes a significant increase in both spontaneous and evoked quantal release (Grinnell et al., 2003). Because frog terminals consist of straight branches running along the long axis of the muscle fiber, they are likely to experience a fractional change in length similar to that of the muscle fiber. The sensitivity of quantal release to muscle length is mediated by integrins present at the surface of both pre- and postsynaptic elements of the NMJ.

There is little evidence for sensitivity of mammalian neuromuscular transmission to muscle length. Although integrins are present at mammalian NMJs (Martin et al., 1996), studies of the rat diaphragm revealed no obvious sensitivity of quantal release to stretching (Grinnell et al., 2003). Whether this is due to the relatively compact and symmetric mechanical arrangement of mammalian NMJs or to the lack of the molecular components that transduce length changes is not known.

2.5.6. Conclusions

It is clear that many factors can influence the short-term efficacy of neuromuscular transmission at mammalian NMJs. Those that have been investigated most extensively are the pattern of activity, Ca\textsuperscript{++} sequestration by mitochondria and the effects of a wide variety
of possible chemical modulators. Nearly all of these factors exert their effects on quantal release from the nerve. There are very few well-documented examples of short-term changes that influence the postsynaptic effect of ACh once it has been released.

Each of these many factors may be viewed as influencing the safety factor of neuromuscular transmission. Thus, each has an impact on the likelihood that a given nerve impulse in a motor neuron will cause contraction of the muscle fibers it innervates. However, with the exception of those activity-related effects, which appear to have their origins in the inherent properties of the release mechanism, the real physiological significance of many of the potential modulating factors that have been detected in vitro remains unclear.

2.6. Biological aspects of safety factor

The description presented so far of the factors that determine the reliability of neuromuscular transmission has been concerned almost entirely with the mature NMJs of normal adult animals. Further, although some mention has been made of differences between the NMJs of frogs and mammals, little attention has been paid to how NMJs in different species, or even within the same species, might differ. The first part of Section 2.6 will consider how NMJs vary between and within species. While a comparative approach may at first seem inappropriate or redundant to some clinical readers, it will become apparent that an appreciation of how different species achieve reliability has provided valuable insight into the way human NMJs achieve the same end.

The second part will consider how the factors that determine reliability in the adult come about, and how those factors change as individuals age. It will focus almost entirely on NMJs in mammals, including humans.

2.6.1. Variation between vertebrate species

There are substantial differences in the NMJs in different vertebrate species. At the level of the whole NMJ, some are much bigger than others and have different “shapes”. Within the NMJ, there are also important variations in the details of both the pre- and postsynaptic specializations. These variations all have an impact on the efficacy of neuromuscular transmission.

2.6.1.1. NMJ size and shape

The NMJ of the frog is often presented as the “typical” NMJ. Yet it differs in important ways from those in many other vertebrates in its conformation, overall size, and detailed structure. The frog motor nerve terminal consists of 5–10 elongate branches that run along the length of the muscle fiber. The overall length is typically 300 μm but the summed length of all the branches is typically about 1000 μm (Nudell and Grinnell, 1982). The axonal branches are overlaid by terminal Schwann cells. At intervals of about 1 μm, small finger-like projections of the Schwann cells wrap around the axon, intruding into the synaptic cleft. These effectively divide the axon into functional units about 1 μm long.

In mammals, including humans, a similar subdivision of the nerve terminal exists. As mentioned above (Section 2.1.2.1), mammalian terminals often consist of a series of roughly circular spot-like boutons, interconnected by fine axon branches (Fig. 2.1A, B). The area and volume of these boutons are roughly similar to the longitudinal units of the frog NMJ. In rats and mice, the boutons are sometimes fused, forming elongate terminal segments that are nonetheless much shorter than the major branches in the frog. The overall area of the NMJ in rats and mice is typically less than 25% of that in frogs. In humans, the NMJs are usually made up of clearly separated spot contacts and are even smaller than in rats and mice.

The functional significance of the natural variation in NMJ size is revealed when one compares the quantum content in the different species (Fig. 2.21). In frogs, the quantum content is typically 100–200 while in humans it is only 20–50, with rats and mice somewhere in between. When one compares the quantum content/unit area, however, all species are much more similar. Thus, there appears to be a relatively fixed “intrinsic release density” of about 0.2 quanta/μm² (Wood and Slater, 2001). At the same time, there is real variation around this basic value even in the same species and when NMJs on muscle fibers of similar size, and input resistance, are compared. This is likely to reflect adaptation of the release system to the specific functions of different motor units.

2.6.1.2. Release mechanism

In addition to the differences in the overall conformation or shape of the NMJs in different species, there are also differences on a smaller scale that influence quantal release. Of particular importance is the difference in AZ structure. In frogs, where the first detailed structural analysis of AZs was made (Hirokawa and Heuser, 1982; Harlow et al., 2001), the AZ consists of two parallel linear arrays of membrane proteins about 1 μm long and separated by about 50 nm. About 30 docked vesicles are normally associated with each AZ, lying on either
Each docked vesicle is contacted on one side by 2–4 lateral "ribs" that extend from the particle arrays (Harlow et al., 2001). By contrast, in mammals, the AZs are much smaller, consisting of a total of about 20 particles in two parallel arrays about 0.1 μm long (see Section 2.6.1.2 and Chapter ??). Each mammalian AZ can accommodate no more than two docked vesicles that lie between the paired arrays.

The possible functional significance of these differences in AZ structure is seen when the probability of release of individual docked vesicles in frogs and mammals is compared. In frogs, less than 1% of the 30 000 or so docked vesicles at each NMJ are released by a single action potential, whereas in mammals, about 5% are released. While the explanation for this is not yet certain, it seems likely to be related to the greater number of contacts between each vesicle and AZ particles seen in mammals.

Like the AZs as a whole, the Ca\(^{2+}\) channels within them also differ between frogs and mammals. In lower vertebrates, including fish, amphibia and reptiles, activity-dependent release is regulated by N-type Ca\(^{2+}\) channels whereas in mammals it is P/Q channels (see Section 2.2.3.1). Differences in the number and properties of the Ca\(^{2+}\) channels may contribute to the greater efficiency of release at mammalian NMJs.

In summary, mammalian NMJs appear to be designed to allow more efficient quantal release than those of frogs. When release/area is compared, mammals release 0.2–0.4 quanta/μm\(^2\)/nerve impulse, while for frogs the comparable number is 0.05–0.1. Because of this, mammalian NMJs achieve reliable transmission with smaller NMJs than frogs.

### 2.6.1.3. Postsynaptic specializations

The quantum content at human NMJs is so low that at first it is hard to understand how the safety factor can be large enough to ensure reliable transmission. Comparison with different species suggests that there must be compensating factors that ensure adequate transmission in humans. Insight into the nature of such factors has come from examination of electron micrographs (Fig. 2.23). These reveal significant differences in the extent of postsynaptic folding (Wood and Slater, 2001). In frogs, the folds are not very extensive and increase the postsynaptic area about 2-fold. In rats and mice, the folds are more extensive, and in humans they increase the area about 8-fold (Slater et al., 1992). Thus in these species, there is an inverse relationship between quantum content and folding.
The functional significance of these anatomical relationships appears to be related to the amplifying effect of the folds, described above (Section 2.3.6.3). At human NMJs, the very extensive folds would provide greater amplification than the shallower folds of frogs and might thus compensate for the relatively low quantum content and size of the EPP. The inverse relationship between quantum content and folding (Fig. 2.23) implies that different species achieve an adequate safety factor in different ways, depending on different balances between presynaptic and postsynaptic factors. Frogs have large nerve terminals that release many quanta and need little postsynaptic amplification. For humans the reverse is true.

What might account for the particular balance between pre- and postsynaptic factors that has evolved in each species? There is at present no definitive answer. One possibility is related to the fact that in mammals, and possibly other groups, when large and small species are compared the number of muscle fibers increases with body size much more than the number of motor neurons (Cooper, 1966). Thus each motor neuron innervates more muscle fibers in larger animals. Considerations of cellular economy may demand that as the number of NMJs per motor neuron increases, the volume of each nerve terminal is kept small. According to this view, human NMJs are small because humans are relatively large. Unfortunately, there is little appropriate information about the NMJ in other large species to allow a good test of this hypothesis. Nonetheless, it forms a useful starting point for the interpretation of the functional implications of some examples NMJ pathology (see below Section 2.8).

2.6.2. Variation between NMJs in the adult

There is considerable variation of NMJ properties within the same species, and even within the same muscle in the same individual. These differences are related to the range of tasks that muscles must perform, from maintaining posture, to allowing delicate manipulations of individual digits, to rapid forceful movements of the whole body. As motor units range from slow, low threshold units with relatively small caliber muscle fibers that develop relatively little force to fast units with large caliber fibers that develop much force, the properties of the NMJs vary in parallel with the muscle fibers.

2.6.2.1. Matching NMJ size to muscle fiber size

It has long been clear that NMJs on small muscle fibers are themselves smaller than those on large fibers.
2.6.2.2. Matching of functional properties of NMJs to activity patterns

Motor units in mammalian muscles differ not only in the properties of the muscle fibers they contain, but also in their patterns of activity. In rats, where such patterns have been most carefully studied (Hennig and Lomo, 1985), motor neurons innervating slow, low threshold units tend to fire in long trains at about 10–20 Hz that last many seconds. By contrast, motor neurons innervating fast, high threshold units tend to fire in short bursts of 5–10 impulses with a mean frequency of about 100 Hz.

The release properties of the NMJs innervating these two types of motor unit are adapted to the appropriate pattern of activity (Fig. 2.25) (Reid et al., 1999). Thus, NMJs in the predominantly slow soleus muscle of rats are better at maintaining quantum content during prolonged stimulation than are those of the predominantly fast EDL. At the NMJs of the fast EDL, where the quantum content “runs down” relatively rapidly, a larger fraction of docked vesicles is released per impulse than in the slow soleus (Reid et al., 1999). This suggests that fast NMJs are specialized to release more quanta per impulse, thus ensuring adequate activation of the muscle during short high frequency trains of impulses. It may be this very specialization that prevents fast NMJs from maintaining high quantal output during prolonged stimulation in an experimental situation.

In addition to the differences in release properties, NMJs in fast and slow rat muscles differ in some postsynaptic features. In fast muscle fibers of the diaphragm, the folds are deeper and more numerous than in the slow fibers (Padykula and Gauthier, 1970). Although it is not clear that this difference is a feature of fast and slow NMJs in all rat muscles (Wood and Slater, 1997), it suggests that the extent of postsynaptic amplification may be matched to the activity pattern.

The result of the distinct features of fast and slow NMJs in rats is a high safety factor in both types of motor unit (Wood and Slater, 1997). By achieving this reliability in slightly different ways, the properties of
transmission are effectively matched to the patterns of activity different motor units usually experience. This presumably helps to strike a balance between reliability and economy.

2.6.2. Activity-dependent plasticity of the NMJ

How is the matching of NMJ properties to functional demand achieved? One possibility is that the NMJ responds to different patterns of activity by adjusting its own properties so as to be able to support the prevailing pattern. An experimental approach to this question has been to subject NMJs to imposed patterns of activity by means of stimulating electrodes implanted close to the peripheral nerves (Reid et al., 2003). When rat EDL nerves were stimulated with a pattern of activity characteristic of the slow soleus muscle for several weeks, their release properties became much more like those of the soleus (Fig. 2.26). Conversely, when the soleus nerve was stimulated with a “fast” pattern quantal release from the soleus NMJs became more like that from the normal EDL. Activity-dependent changes in NMJ structure also take place that are consistent with transformation from fast to slow and vice versa (Waerhaug and Lomo, 1994). While the mechanisms of these changes are not known, it is clear that adult mammalian NMJs can undergo long-term changes that adapt them to the pattern of imposed activity.

2.6.3. Development of the mammalian NMJ

The distinctive features of the adult mammalian NMJ arise during a series of developmental stages that lasts several weeks (Sanes and Lichtman, 1999). At each of these stages, even when the NMJs are still structurally and functionally very immature, they function in a way that is appropriate for the degree of maturity of the animal. The products of this developmental process are NMJs that are not only reliable in a general sense, but are well matched to the functional requirements of the motor units of which they are a part. The following account of NMJ development is based on studies of rats and mice.

2.6.3.1. Development of the motor neuron

Motor neurons are among the earliest nerve cells to be “born”, i.e. undergo their final round of DNA synthesis and begin to extend an axon and dendrites (Jacobson, 1970). In mammals, the motor neurons that innervate an individual muscle are usually grouped into a longitudinally oriented “column” that occupies 2–3 spinal segments. Their axons grow out of the spinal cord or brain stem even before muscles have formed. As they grow, they select paths that lead to the muscles they are destined to innervate. This selection involves decisions that are based on local cues. The ability of an immature motor neuron to make such decisions indicates that it has some “knowledge” of its identity, and
that different motor neurons have different identities. Once contact with the appropriate pre-muscle mass has been established, but not before, the axons branch extensively. In rats and mice, functional contacts with newly formed muscle are first present around E14 (embryonic day 14), a week before birth. A similar stage occurs in humans at week 9 of gestation (Hesselmans et al., 1993).

Motor neurons in neonatal rats and mice fire at a uniformly low frequency. The first evidence of faster, more adult-like firing patterns, and of differences between the firing patterns of different motor neurons, is seen about 2 weeks after birth (Vrbova et al., 1985; Personius and Balice-Gordon, 2001). The onset of these more adult-like firing properties of motor neurons coincides with the time of myelination of the most distal intramuscular nerve branches (Slater, 1982). It is likely that before this happens, the small caliber unmyelinated branches would be unable to fire at high frequencies and would therefore filter out any high frequency activity.

2.6.3.2. Development of the muscle
Mammalian skeletal muscle fibers arise during development from the fusion of many mononucleated myogenic cells (often called myoblasts). Fusion is just one stage of a process that involves withdrawal of proliferating myoblasts from DNA synthesis, alignment of the spindle shaped myoblasts into "strings" of cells in the premuscle masses and then their fusion to form the myotubes. The myotubes incorporate further myoblasts, as they grow into mature muscle fibers. In adult muscle fibers, there is typically 1 nucleus for every 10 µm of length. Thus a single fiber in a large human muscle, such as vastus lateralis, which has fibers up to 20 cm long, has up to 20,000 nuclei.

The distinctive properties of muscle fibers of different functional types are determined by the pattern of gene expression. Since the nuclei away from the NMJ all express the same set of genes, an important developmental question concerns how that homogeneity of nuclear gene expression within a given fiber comes about. There is evidence for two very different, though not at all mutually exclusive, mechanisms. On the one hand, there is evidence that myoblasts differ in their properties from a very early stage in development. Thus, there may be "fast" and "slow" myoblasts before myotube formation occurs (Nikovits Jr et al., 2001), and these may fuse more or less selectively to make myotubes containing nuclei with intrinsically similar properties. On the other hand, there is very strong evidence, particularly in mammals, that the properties of adult muscle fibers can be fundamentally changed by imposing on it different patterns of activity e.g. (Lomo, 1989). This indicates that the properties of a muscle fiber may be modified after innervation by the activity pattern of the particular motor neuron that innervates it. Both schemes lead to important questions about how the innervation of muscle fibers arises so as to result in motor units in which all the muscle fibers have the same properties.

2.6.3.3. Synapse formation and elimination
Muscle fibers become innervated very soon after they first form. In rats and mice signs of innervation can already be detected within a day or two of myotube formation. At about this time, clusters of AChRs form...
in the muscle fiber membrane at the sites of nerve contact, followed a few days later by the appearance of AChE in the basal lamina (Sanes and Lichtman, 1999).

An important feature of the early motor innervation of vertebrates is that several motor neurons initially innervate each muscle fiber (Jansen and Fladby, 1990). This innervation occurs at a single postsynaptic site that is contacted by the terminal axons of several motor neurons (Fig. 2.27A). Over a period of several weeks (rats, mice), a process of local competition between these branches results in the contacts with all but one motor neuron being eliminated (Fig. 2.27B). Although the nature of this process of synapse elimination has been extensively investigated, its details remain largely obscure. There is evidence that each branch of a motor axon competes locally at a given NMJ (Kasthuri and Lichtman, 2003) and that axons with the strongest input to a given NMJ are more likely to survive the competitive process than weaker ones (Buffelli et al., 2003). However neither of these findings fully explains the final outcome of the competition in which a single motor axon, with appropriately matched properties, innervates each muscle fiber.

Neuromuscular transmission is effective throughout the processes of synapse elimination and NMJ maturation. In the early stages, when polyneuronal innervation is present, quantum content and mEPP frequency are both very low (Diamond and Miledi, 1962). However, because the muscle fibers have a small caliber at this time, their input resistance is very high. As a result, very little current is required to bring the membrane potential to the action potential threshold. Thus, even spontaneous mEPPs may sometimes trigger muscle fiber action potentials (Jaramillo et al., 1988). As a result, at the peak of polyneuronal innervation, each of the several motor neurons innervating a muscle fiber is able to trigger its contraction (Jansen and Fladby, 1990).

An important consequence of the process of synapse elimination is that each motor neuron ends up innervating muscle fibers with similar properties. Clear signs of functional homogeneity of the muscle fibers within

---

**Fig. 2.27.** Development of muscle innervation. A. Comparison of polyneuronal innervation of muscles in newborn rats or mice (left) with the adult state (right). B. Changes in the number of axon terminals innervating NMJs in rat diaphragm with age. (From Bennett and Pettigrew, 1974, with permission.)
As these structural changes take place, immature forms of different motor units are clearly developed (Fladby and Jansen, 1988). It therefore seems unlikely that differences in activity patterns between motor neurons play a decisive role in either survival selection or in matching the properties of motor neurons to the muscle fibers they innervate. A possible alternative is that the initial matching of nerve and muscle cells is achieved by a molecular recognition system that involves activity-dependent expression of surface and/or diffusible molecules that interact to promote survival of the most compatible pairs at each developing NMJ. Such a mechanism could depend on activity as a driving force, without the pattern of activity determining the specific outcome of the competition.

2.6.3.4. Structural maturation of the developing NMJ

As NMJ maturation progresses, and the elimination of supernumerary synapses comes to completion, a number of structural and molecular changes take place that have the combined effect of enhancing the speed of neuromuscular transmission and matching its efficacy to the enlarging muscle fibers. Soon after synapse elimination is complete, the terminals of the sole surviving axon begin to enlarge (Fig. 2.28) (Slater, 1982; Marques et al., 2000) and the quantum content increases. The postsynaptic specializations, most notably the accumulation of AChRs and AChE, become restricted to the region of the muscle fiber in immediate contact with the expanding nerve terminals.

The postsynaptic folds begin to develop soon after birth (Fig. 2.29) (Kelly and Zacks, 1969; Matthews-Bellinger and Salpeter, 1983; Bewick et al., 1996). It appears likely that the folds form by the addition of a new membrane to their depths. However the factors that control the distribution and growth of the folds are poorly understood. It has been suggested that the opening of the fold represents a site of reduced nerve–muscle adhesion, possibly related to the presence of the AZs in the nerve (Marques et al., 2000). The structural maturation of the NMJ is largely complete 3–4 weeks after birth (Slater, 1982).

2.6.3.5. Molecular maturation of the developing NMJ

As these structural changes take place, immature forms of three key classes of ion channel, CaV1, AChRs and NaV1, are replaced by adult ones. The CaV1 channels that control evoked quantal release from motor nerve terminals in rat embryos are mostly of the N-type. During the first week after birth, these are replaced by the P/Q-type (Rosato and Uchitel, 1999). Since the P/Q-type channels require less depolarization to open (see Section 2.2.3.1) this apparent “recapitulation of phylogeny” (see Section 2.6.1.2) may help to ensure that passive depolarization of the enlarging nerve terminal causes adequate numbers of channels to open in response to each nerve impulse.

Two distinct forms of the AChR protein are expressed by mammalian muscles. The form that predominates at immature NMJs has α(2), β, γ, and δ subunits. In the adult form, the γ subunit is replaced by an ε subunit. The two forms differ in their channel properties: in humans, the immature form has a mean open time of about 7–8 ms and a conductance of 44 pS while the adult form has a mean open burst time of 1–2 ms and a conductance of 60 pS (Sine et al., 2002). As a result, a single opening of average duration of an immature AChR channel allows 3–4 times as much charge to enter the cell as an adult channel. This makes the immature channels more efficient at converting bound ACh to charge entry.

Immature muscle fibers usually have a small diameter and therefore a high input resistance. This results in a greater depolarization per unit charge. However, the increase in input resistance leads to an increase in the passive charging time constant of the fiber (see Section 2.3.5.5) which tends to reduce the depolarization due to a brief inflow of current (see Fig. 2.9C, D). The slower kinetics of the currents mediated by AChRs with a γ-subunit are thus better matched to the passive cable properties of the immature fibers than those in the adult. On the other hand, because of their slower kinetics, they are less well adapted to transmit the high frequency repetitive activity that occurs in the mature animal. It is thus relevant that the change in AChR properties occurs about the same time that the adult firing patterns of the motor neuron, and the myelination of the most distal axons, which allows those patterns to reach the NMJ, appear.

Conversion between the two AChR forms occurs during the period when synapse elimination is nearing completion. In rats and mice the conversion begins about the time of birth and is complete at the end of the second week after birth. Studies in adult animals show that expression of the immature form is suppressed by the normal activity of the muscle (Lomo and Rosenthal, 1972; Goldman et al., 1988). In contrast, expression of the ε-subunit that characterizes the adult form is insensitive to muscle or nerve activity, as it must be to persist at the adult NMJ. Even when the nerve is cut at birth, expression of ε-AChR mRNA is initiated in the first week after birth as usual (Brenner et al., 1990).

A similar isoform switch of NaV1 also occurs during NMJ maturation (Lupa et al., 1993; Stocksley et al., 2005). The immature NaV1.5 form differs from...
the adult Na\(_V\)1.4 form in that it is opened at more negative membrane potentials. As a result, less depolarization is required to trigger an action potential in an immature than a mature muscle. Although the initial accumulation of Na\(_V\)1 channels at developing NMJs occurs later than that of AChRs, the isoform switch occurs during the same period of 2–3 weeks postnatal. Thus newborn rats have nearly all Na\(_V\)1.5 channels, while by 1 month most channels are Na\(_V\)1.4 (Lupa et al., 1993; Stocksley et al., 2005).

The regulation of expression of the two isoforms of Na\(_V\)1 has close similarities to that of AChRs. Thus expression of Na\(_V\)1.5 is repressed by imposed activity (Awad et al., 2001) whereas that of Na\(_V\)1.4 is insensitive to activity. The insensitivity of the expression of the adult forms of AChR and Na\(_V\)1 to activity ensures that they persist in adequate numbers on active muscle fibers. However it leaves open the question of the nature of the developmental switch that initiates their expression.

A likely candidate for a factor regulating the expression of adult channels at mammalian NMJs is agrin (McMahan, 1990). Agrin is synthesized by motor neurons and released from their terminals, after which it

---

**Fig. 2.28.** Structural maturation of mouse NMJs. Changes in overall structure of NMJs in postnatal mouse EDL muscles. A. Nerve terminals, labeled with ZIO. a. newborn, b. 1 week. c–f. 2 weeks. g. 3 weeks. h. adult. B. AChRs labeled with R-\(\alpha\)-bungarotoxin. a. newborn. b. 1 week. c–e. 2 weeks. f. 3 weeks. g. adult. Note loss of polyaxonal innervation, enlargement of surviving nerve terminal and increasing segregation of AChR cluster. (From Slater, 1982, with permission.)
binds to the synaptic BL. From there, it can interact with a muscle specific receptor kinase (MuSK) in the muscle membrane (Glass et al., 1996). Activation of MuSK promotes many aspects of the differentiation of the postsynaptic membrane. These include the accumulation of AChR, AChE and NaV1 molecules at the NMJ and expression of the genes that encode their mature forms by the 5–10 myonuclei closest to the junction (Cohen et al., 1997; Meier et al., 1997).

The switch to expression of adult NaV1.4 channels occurs at the same time as the development of the postsynaptic folds, during the first 2–3 weeks after birth. It is likely that the folds form by the incorporation of new membrane into the depths of the folds. At the time that this is happening, the mRNA encoding the NaV1.4 is concentrated in the postjunctional region (Stocksley et al., 2005). It is therefore likely that the membrane being added to the folds already bears a high concentration of NaV1.4.

There is some evidence that as the density of NaV1 channels at the NMJ begins to increase, those channels are at least partially excluded from the crest of the folds where the concentration of AChR is already very high (Bailey et al., 2003). It may be that the complex of molecules that supports the junctional AChR cluster forms a barrier to NaV1 channel entry. Such a barrier might thus account for the sharp boundary between the main postsynaptic ion channel domains (Winckler et al., 1999).

2.6.3.6. Functional consequences of NMJ maturation

The many changes in the structural and molecular properties of the NMJ that occur during its maturation adapt it for reliable high-frequency activation of mature muscle fibers. These changes are matched to complementary changes in the nerve and the muscle. Their overall effect is the conversion of an immature system that is good at generating slow muscle contractions in response to low frequency activity in the nerve to a much faster system, adapted to the needs of a freely moving and increasingly independent animal. For example, in the hind limb muscles of rats and mice, the timing of these changes corresponds to the time when the animal can first support its hind quarters against gravity and begin to walk. While the factors influencing the reliability of neuromuscular transmission change during development, at all stages, the process remains well-suited to the tasks it needs to perform.

The events that give rise to the mature NMJ are part of a coherent developmental program that defines the patterns of expression of a number of proteins, such as ion channels, that play central roles in neuromuscular transmission. In addition it determines the size and conformation of the NMJ. Both aspects of the program have important consequences for the efficacy and reliability of the mature NMJ. This program can be reactivated in the adult in circumstances where neuromuscular transmission is impaired, as during normal aging (see below) or in response to cellular damage or chemical intoxication of the NMJ (see Section 2.7).

2.6.4. Changes in the NMJ affecting safety factor during normal aging

It is well-known that muscle power is reduced in humans during old age. Changes in muscle innervation make an important contribution to this phenomenon. There is good evidence from clinical neurophysiological studies of a decrease in the number of motor units,
particularly in distal muscles, after the age of 60 (Campbell et al., 1973; Sica et al., 1974; Galea, 1996) and for some deterioration in the efficacy of neuromuscular transmission (Larsson and Ansved, 1995; Galea, 1996). Anatomical studies show that the number of large cell bodies in the ventral horn is reduced (Kawamura et al., 1977; Tomlinson and Irving, 1977), while electrophysiological estimates of motor unit numbers (Galea, 1996) also point to a loss of motor neurons. Thus single fiber EMG (SFEMG) studies show an increase in fiber density and macro EMG studies show an increase in amplitude of motor unit potentials (Stalberg and Trontelj, 1979; Stalberg and Fawcett, 1982). These findings are all consistent with the death of some motor neurons during old age and a compensating reinnervation of the denervated muscle fibers by newly formed branches of the surviving axons. More direct evidence for such a phenomenon is presented below.

Anatomical studies in animals and humans suggest that the structure of NMJs changes with age (Fig. 2.30) (Barker and Ip, 1966; Tuffery, 1971; Bennett and Stenbuck, 1980; Bennett and Davis, 1982; Arizono et al., 1984; Oda, 1984; Cardasis and LaFontaine, 1987; Diaz et al., 1989; Wokke et al., 1990; Rich et al., 1998). In most cases, these changes involve increased local complexity of axonal branching within individual NMJs (Oda, 1984; Andonian and Fahim, 1989; Prakash and Sieck, 1998), which may (Waerhaug, 1992), or may not (Fahim and Robbins, 1982; Fahim et al., 1983), be associated with an overall change in the total area of the NMJ. Some studies have also found an increase in preterminal branching (Barker and Ip, 1966; Tuffery, 1971; Arizono et al., 1984; Oda, 1984; Wokke et al., 1990), though this has not been seen in all such studies (Wokke et al., 1990). On the postsynaptic side, a number of studies have reported an increase in the complexity and branching of the regions of specialized postsynaptic membrane, which parallels the presynaptic changes. In addition, there is an increasing amount of specialized postsynaptic membrane that is not in contact with the nerve terminal, suggesting a partial withdrawal of the nerve (Arizono et al., 1984; Cardasis and LaFontaine, 1987). These changes all suggest that there is considerable structural remodeling of mature NMJs, particularly in association with senescence.

In light of the general impact of NMJ structure on its function, it seems likely that changes in neuromuscular transmission would accompany the remodeling seen in old age. While this is suggested by the SFEMG data referred to above, there is little information about the detailed functional properties of the NMJ in elderly humans. From animal studies, mostly in rats and mice, it seems that an adequate safety factor of transmission is generally maintained in spite of the structural changes that accompany old age. In rat diaphragm, the safety factor was reported to increase steadily from about 4 to 10 during the first 9 months of life and then to decline (Kelly, 1978; Kelly and...
RELIABILITY OF NEUROMUSCULAR TRANSMISSION

Robbins, 1983). The reported changes in safety factor were accompanied by similar changes in quantum content (though the values given are very high due to the use of the “variance” method (Section 2.4.3.3) to determine them). On the basis of existing evidence, it seems likely that the changes in quantum content are a major determinant of the changes in the safety factor during aging.

All these observations suggest that a gradual process of NMJ remodeling becomes much more intense during old age as a fraction of motor neurons die. Recent studies have shown that the terminal Schwann cells play an important part in the induction of nerve terminal sprouting following partial denervation (Son et al., 1996) and it is likely that they also influence the remodeling of the presynaptic terminal nerve during aging. So long as this process maintains the total extent of the nerve terminals and synaptic contact, it is likely that the safety factor would be maintained. Eventually, however, the presence of “unoccupied” postsynaptic membrane suggests that this endeavor fails. While the increasing complexity of the postsynaptic surface may represent an effort to enhance the effect of a reduction of quantum content, the overall effect may be a decline of safety factor in old age.

2.6.5. Conclusions

Patterns of muscle use vary widely between species and muscle types, and at different stages in an animal’s life. The properties of NMJs are adapted to these patterns in order to ensure reliable activation of muscle throughout life. These adaptations occur at every level of organization, from molecular (e.g. CaV1, AChR and NaV1 isoforms) to “system” (e.g. poly- to mononeuronal innervation). An important focus of current investigation is the effort to understand how this adaptation is controlled at a molecular level. Experimental studies of how NMJs respond to, and recover from, traumatic injury and toxic assault have done much to reveal the main processes that underlie this ability of the NMJ to adapt to changing circumstances so as to maintain reliable muscle activation.

2.7. Response of the neuromuscular junction to trauma or intoxication

The vertebrate neuromuscular system, including the NMJ, is extremely good at repairing itself and restoring function after many sorts of damage. Several different types of cellular mechanism contribute to this ability and help to ensure that effective and reliable activation of muscle is maintained or re-established. Thus motor axons regenerate well and can form new presynaptic terminals in a few days. For their part, muscle fibers can regenerate after complete destruction and accept and respond to new innervation on a similar time scale. This Section reviews some of the key experiments that have revealed the processes that underlie this important ability of the NMJ.

2.7.1. Nerve transection and regeneration

2.7.1.1. Degeneration of severed axons

Damage to peripheral nerves is a common occurrence and may result from sports activities, domestic, motor or industrial accidents or a wide variety of other causes. Whatever be the nature of the damage, the distal segments of any severed axons degenerate. Paradoxically, the first part of the isolated axon segment to degenerate is that furthest from the site of injury, the presynaptic nerve terminal (Slater, 1966; Miledi and Slater, 1970). This is followed by degeneration of the remainder of the axon by a complex process known as Wallerian degeneration (Coleman, 2005).

2.7.1.2. Axon regeneration

Peripheral nerves are capable of extensive regeneration. The success of this regeneration, in terms of the restoration of effective muscle control, depends on whether continuity of the BL and connective tissue sheaths surrounding the nerve and its axons are left intact. If they are, the axons can regenerate within them and make their way back to their original target muscle fibers and reinnervate them at the site of the original NMJ. Under these circumstances, regeneration occurs in mammals at a rate of 2–5 mm/day (Hoffman and Lasek, 1980). If the sheath is cut through, although regenerating axons may eventually return to the denervated muscles, they are unlikely to return to the muscle of origin, much less to the same muscle fibers and synaptic sites (Bennett et al., 1973). As a result, even if new synaptic contacts are made, they may be of little use. Thus the connective tissue sheath, which appears to play only a passive “supporting” role, is, in fact a key factor in ensuring re-establishment of effective motor innervation.

2.7.1.3. Structural reinnervation of the muscle

Once an axon regenerates to the site of an original NMJ, differentiated presynaptic terminals form rapidly and effective synaptic contact is re-established within a few days (Bennett et al., 1973). This appears to be due in part to the presence of molecules associated with the synaptic basal lamina, which survive axon degeneration and act as “stop signals”, arresting growth of the regenerating axon and triggering presynaptic differentiation (Glicksman and Sanes, 1983).
These are likely to include agrin (see Section 2.6.3.5) (Sanes, 1989; McMahan, 1990; Campagna et al., 1995; Ruegg and Bixby, 1998). If a regenerating motor axon makes contact with a muscle fiber away from the region of normal innervation, there is a strong tendency for it to grow along the muscle until it makes contact with the site of the original NMJ and re-establish synaptic contact there (Bennett et al., 1973).

In some circumstances, most notably after denervation of the “slow” soleus muscle of rats and mice, implanted “foreign” nerves can make new NMJs at ectopic sites on the muscle fiber away from the original NMJs. Most of the experiments on ectopic innervation have been made on the rat soleus muscle, normally innervated by the tibial nerve, by implanting a branch of the peroneal nerve away from the original NMJ. If the normal innervation is then cut or blocked, the foreign nerve makes new functional NMJs within 1–2 weeks (Fex and Thesleff, 1967; Jansen et al., 1973; Lomo and Slater, 1978). The ability of the foreign axons to make such ectopic NMJs is triggered by inactivity: if the denervated muscle is kept active by direct stimulation, then ectopic NMJs fail to form (Jansen et al., 1973; Lomo and Slater, 1978). One mediator of this effect may be the nerve–cell adhesion molecule (NCAM). NCAM is normally present in a higher concentration at the NMJ than elsewhere. Following denervation its expression away from the NMJ is greatly increased (Covault and Sanes, 1985).

NCAM may help to bring nerve and muscle close together and thus facilitate subsequent interactions in synaptogenesis (Walsh et al., 2000).

2.7.1.4. Response of the muscle to denervation

Acquiring an increased responsiveness to innervation is one of a number of changes in the muscle that are triggered by inactivity and promote the restoration of effective innervation. One of the best studied of these is the expression of AChRs. Functional AChRs are normally expressed primarily in the region of the NMJ, with lower levels also present at the myotendinous junctions (Miledi and Zelena, 1966). Elsewhere, that expression is largely inhibited by muscle activity (Lomo and Rosenthal, 1972). Within a few days of denervation, AChRs appear all along the muscle fiber (Lomo and Slater, 1978). These new AChRs resemble those at developing NMJs in having slow kinetics, characteristic of those containing a γ- rather than an ε-subunit (Brenner and Sakmann, 1978). Although their density (500/µm²) is much less than at the normal NMJ (10 000/µm²), it is much higher than in normally active muscles. The presence of AChRs away from the original NMJ allows growing motor axons to depolarize the muscle fiber membrane, and this may help to initiate the events leading to new NMJ formation.

At the original NMJ, the density of AChRs remains much higher than in the extrajunctional region even after denervation or paralysis. However, activity of AChE, particularly of the asymmetric form most specifically associated with the mammalian NMJ, declines significantly (Hall, 1973). This has the effect of enhancing the activity of any ACh released from immature regenerating nerve terminals.

Several other changes in the muscle fiber induced by inactivity have the effect of increasing muscle excitability. These include a reduction of membrane potential, typically from −80 mV to −60 mV, which brings the resting potential close to the AP. This probably occurs as a result of decreased permeability to K⁺ (Nicholls, 1956). In addition, Naᵥ₁.5 channels, which open at a less depolarized membrane potential than the Naᵥ₁.4 channels of active muscle, are expressed both at the NMJ and in the extrajunctional region (Caldwell and Milton, 1988; Kallen et al., 1990; Catterall, 1995; Awad et al., 2001). Finally, inactivity generally causes muscle atrophy. The reduction of fiber caliber, together with that of K⁺ permeability, gives rise to an increase in input resistance (see Section 2.3.5.2). As a result, less synaptic current is required to achieve a given change in membrane potential. All these factors cooperate to increase the excitability of the muscle fiber, so less charge needs to enter the fiber to initiate contraction. Thus loss of normal activity as a result of nerve transaction triggers a constellation of changes in the muscle that promote restoration of effective innervation.

2.7.1.5. Functional reinnervation of the muscle

The functional properties of reinnervated mammalian NMJs often return to near normal values (Bennett et al., 1973; McArdle and Albuquerque, 1973; Tonge, 1974b; Slack and Hopkins, 1982; Katz et al., 1996). During the first few weeks after damage by nerve crush, which leaves the nerve sheath intact to promote reinnervation, the quantum content increases progressively from an initially low value to near normal (Fig. 2.31). In the early stages, when quantum content is low, evoked release is sensitive to blockers of both P-type Ca²⁺ channels, as normal, and to L-type channels (Katz et al., 1996). With time the contribution of the L-type channels is lost. N-type channels, which contribute to evoked release at NMJs in early development, do not appear to be re-expressed during nerve regeneration.

2.7.2. Partial denervation

2.7.2.1. Axon sprouting and muscle fiber type conversion

There are many situations in which only a fraction of fibers in a muscle lose their innervation. Such "partial
denervation” may result from trauma, normal aging, or a variety of diseases including most notably amyotrophic lateral sclerosis (ALS). In these cases, new intramuscular sprouts grow out from the surviving motor axons and often reinnervate the NMJs on the denervated muscle fibers (Brown et al., 1981; Gordon et al., 2004). This may lead to a local increase in the number of fibers innervated by individual motor axons that can be detected as an increase in fiber density during SFEMG investigation in a clinical setting. It is often the case that the sprouting axon is from a motor unit whose properties are different from those of the muscle fiber it comes to innervate. In such cases, the nerve may impose its “type” on the muscle fiber, leading to the phenomenon of “fiber type grouping” (Banker and Engel, 2004).

The influence of the nerve on the “type” of adult muscle fiber is mediated by the pattern of activity (Salmons and Vrbova, 1969; Windisch et al., 1998). If a denervated muscle is stimulated directly with implanted electrodes its type, based largely on the pattern of expression of the genes encoding myosin heavy chains, is determined by the pattern of imposed activity (Windisch et al., 1998). The fact that new patterns of innervation resulting from axonal sprouting lead to new patterns of muscle fiber type indicates that the new synaptic connections are sufficiently effective to stimulate muscle activity. In most cases, it seems that these new connections are established at the sites of the original NMJ. This raises the question of how axonal sprouts “find” those sites.

**Fig. 2.31.** Recovery of neuromuscular transmission during reinnervation of mouse muscle Mouse soleus muscles show the first signs of functional reinnervation 10 days after nerve crush in the thigh. The efficacy of innervation increases to normal over the next 2–3 weeks. (From Tonge, 1974b, with permission.)

### 2.7.2.2. Schwann cells promote axon sprouting

A key factor in the guidance of axonal sprouts to denervated NMJs is the Schwann cell. When muscle fibers are denervated by a nerve crush, the terminal Schwann cells extend long sprouts along the muscle fibers, similar in appearance to those that grow out from axons in partially denervated muscles (Reynolds and Woolf, 1992). The Schwann cells guide regenerating axons back to the original NMJs. In some cases the axons “overshoot” the NMJs and grow along the Schwann cell sprouts, occasionally innervating other muscle fibers (Fig. 2.32).

In a partially denervated muscle, Schwann cell sprouts grow out only from the denervated muscle fibers (Fig. 2.33). These sprouts then appear to guide axonal sprouts from intact motor neurons to the denervated NMJs (Son et al., 1996). The signaling pathways that trigger Schwann cell sprouting are unclear. If a muscle is completely denervated, there is extensive Schwann cell sprouting from all NMJs, suggesting that inactive muscle fibers may release molecules that promote Schwann cell growth. On the other hand, in partially denervated muscles, Schwann cell sprouts appear to make “bridges” preferentially with NMJs that are still innervated and active (Love and Thompson, 1999). One possibility is that Schwann cell sprouts grow out initially at random and are then withdrawn (Reynolds and Woolf, 1992) unless they make contact with an innervated NMJ (Love and Thompson, 1999).

### 2.7.3. Muscle damage

The integrity of the NMJ may also be disrupted by damage to the muscle. This may happen as a result of traumatic injury or the action of natural myotoxins, or occur as a feature of inherited muscle diseases such as muscular dystrophies. In one frequently used experimental model intramuscular injection of notexin, one active component of the venom of the snake *Notechis scutatus scutatus*, into the rat soleus muscle causes destruction of the muscle fibers but leaves intact the population of myogenic satellite cells (Harris and Johnson, 1978). These cells proliferate rapidly following muscle damage. Within 2 days of the complete destruction of the rat soleus muscle, each satellite cell divides about three times and these myoblasts fuse to make new myotubes, one in each original BL sheath (Fig. 2.34A). If the nerve is undamaged, or only its terminals are damaged, the nerve reoccupies the original postsynaptic sites within a few days (Jirmanova and Theleslev, 1972; Grubb et al., 1991). The first signs of neuromuscular transmission appear within less than a week of the return of the nerve (Fig. 2.34B).
and quantum content returns to normal within 3 weeks (Fig. 2.34C) (Grubb et al., 1991).

The rapid functional reinnervation of damaged muscle implies that both the pre- and postsynaptic components of the NMJ can be reassembled within a few days. A number of studies indicate that this process is facilitated by the presence of molecular signals associated with synaptic basal lamina. The best studied of these is agrin (see Section 2.6.3.5). Indeed, much of the early evidence indicating the presence of persistent signals at the NMJ that might direct its own regeneration were made on regenerating muscles (Burden et al., 1979; McMahan and Slater, 1984; Brenner et al., 1992; Jo and Burden, 1992). These studies show that such signals direct both the structural and molecular differentiation of the postsynaptic membrane and the expression of synapse specific genes in the newly formed myonuclei that accumulate at the regenerating NMJ.

Particularly striking demonstrations of these properties of the synaptic BL come from experiments in which all the cellular constituents of the NMJ were destroyed and then selected cells (e.g. nerve or muscle) were allowed to regenerate (Sanes et al., 1978; Burden et al., 1979; Glicksman and Sanes, 1983; McMahan and Slater, 1984). In each case, local differentiation of the regenerated cell occurred in the absence of its normal synaptic partner, presumably as a result of the signals persisting in the synaptic BL.

Although agrin remains fixed to the BL after muscle damage, the NMJs that form on regenerating muscle fibers often differ structurally from the original NMJs at the same site. For example, after damage induced in mouse muscle by the myotoxin from *Dendroaspis jamesoni* (Duchen et al., 1974) or that occurring in *mdx* mice, which have an inherited form of muscular dystrophy (Nagel et al., 1990; Lyons and Slater, 1991; Personius and Sawyer, 2005), the reformed NMJs consist of a collection of distinct spot contacts rather than the normal bands of synaptic contact (Fig. 2.35). This suggests that the nerve is able to lay down agrin at new sites, and that agrin at sites unapposed by a nerve may break down.

### 2.7.4. Toxins and the NMJ

#### 2.7.4.1. Introduction to the toxins

There are many natural toxins that attack the nervous system in general and the NMJ in particular. In addition to their clinical significance, a number of these toxins have been used extensively in an experimental context to explore the covert plasticity of the mature NMJ. In contrast to the CNS, where the blood–brain barrier protects synapses from many foreign molecules, the NMJ is much more easily accessible to molecular attack. Bacteria, plants and both invertebrate and vertebrate animals all produce neurotoxins that act on the NMJ. These toxins can be classified...
according to their site and mode of action. Thus, toxins may act preferentially on the presynaptic nerve terminal or postsynaptically on the muscle fiber, and they may act to block ion channels, to disrupt critical cellular processes such as exocytosis, or to cause cell damage. In addition to these natural toxins, there is a wide variety of man-made "environmental" toxins that have a deleterious effect on the NMJ.

The great diversity of natural toxins, both in terms of their origins and their modes of action, make the toxicology of the NMJ a rich field in its own right, that goes well beyond what is appropriate for this Chapter. The reader is referred to more specialized articles (e.g. Van der Kloot and Molgo, 1994; Schiavo et al., 2000; Harris et al., 2003) and texts on neurotoxicology. Here the responses of the NMJ to several toxins that have been extensively investigated, and which exemplify different modes of action, are described. They are of particular interest because of what they reveal about the regenerative capacity of the NMJ.
Fig. 2.34. Innervation of regenerating muscles. The response of rat soleus muscles to a single perimuscular injection of notexin (2 μg). A. Histological preparations (H&E) showing that the muscle fibers undergo breakdown within 1 day and that new fibers are present 2–3 days later. By 28 days, the fibers have reached normal size but retain centrally located nuclei. B. Typical EPPs recorded from normal muscles (control) and at an early stage of innervation of the regenerating muscle fibers (4 days). Note that the EPP is much slower at the reinervation NMJ, both because γ-AChRs are present and because AChE activity is reduced. C. Mean quantum content at NMJs on regenerating muscles. The earliest EPPs are seen 4 days after intoxication and the normal level of release is reached a week later. (From Grubb et al., 1991, with permission.)
2.7.4.2 Presynaptic toxins that block release

Numerous toxins block the activation of voltage-gated Na$^+$ and Ca$^{++}$ channels. The best known of these is the puffer fish toxin, tetrodotoxin, which blocks NaV1 channels and thus prevents action potentials from depolarizing the nerve terminal. A variety of toxins derived from invertebrates exist that block Ca$^{++}$ channels in the nerve terminal. Among these are ones that specifically block the various different types of Ca$^{++}$ channel that may be present at mammalian NMJs, e.g. N-, P/Q- and L- (Uchitel, 1997). The effect of these toxins on release depends on which Ca$^{++}$ channels are present and in what relative abundance. This, in turn, depends on the biological state of the NMJ (see Section 2.6).

Perhaps the best known, and most clinically relevant, presynaptic toxins are those produced by the anaerobic bacterium Clostridium botulinum. These toxins bind to specific polysaccharide groups on the motor nerve terminal (Jahn, 2006) by their heavy chains and are taken into the cytoplasm where their light chains catalyze the cleavage of one or more SNARE proteins involved in quantal release from the nerve terminal (see Section 2.4.1) (Schiavo et al., 2000; Montecucco and Molgo, 2005). This leads to profound blocking of quantal ACh release and the paralysis that characterizes clinical botulism. There are seven different serotypes of botulinum neurotoxin (BoNT/A–G), which differ in the particular SNARE proteins they cleave.

These toxins, in particular BoNT/A, are of considerable therapeutic importance because they can be used to effect partial paralysis of selected muscles in the treatment of a variety of dystonias and spasticity. In addition, BoNT/A is used by large numbers of people as a cosmetic agent that reduces myogenic facial wrinkling. In many situations the NMJ is eventually able to recover, at least partially, from BoNT/A on a timescale of several months. This necessitates multiple treatments to maintain a useful effect. Understanding how the NMJ regains reliable function is thus of interest both because of what it reveals about the mechanisms for maintaining reliable neuromuscular transmission, and for what it may reveal about the effects of repeated exposure to BoNT/A in a clinical setting.

Recovery from botulism occurs at a rate that depends on the particular serotype involved. It seems that the duration of action depends, at least in part, on the particular SNARE protein cleaved, and even on the site of cleavage within that protein. For example, BoNT/A and BoNT/E both cleave SNAP-25, though at different sites (Fig. 2.7). However, recovery from BoNT/E is much faster than from BoNT/A. Following BoNT/A intoxication of rat or mouse muscles, neuromuscular transmission recovers on a timescale of weeks. Functional recovery from BoNT/A is associated with the outgrowth of axonal sprouts from the motor nerve terminal which occurs as early as a week after exposure to BoNT/A (Fig. 2.36A) (Duchen, 1970; Tonge, 1974a; Juzans et al., 1996; de Paiva et al., 1999; Santafe et al., 2000). Some of these sprouts make new synaptic contacts with the muscle fiber, complete with high concentrations of AChRs and AChE, at varying distances from the original NMJ. This recovery is associated with a steady increase of quantum content back to its normal value (Fig. 2.36B) (Tonge, 1974a; de Paiva et al., 1999; Santafe et al., 2000). This is a dramatic example of the rerunning of the developmental program that brings about postsynaptic differentiation in response to the inductive influence of the nerve.

There are varying accounts in the literature of the natural history of the sprouts and new synaptic contacts induced by BoNT/A. Some authors have reported that the ectopic contacts persist and the original nerve terminals eventually withdraw, leaving a highly abnormal innervation pattern (Duchen, 1970). Other accounts describe the eventual withdrawal of the ectopic contacts and the simultaneous recovery of quantal release at the original NMJs (de Paiva et al., 1999; Meunier et al., 2002). Still in doubt is how much the new contacts contribute to the overall functional recovery of the NMJ and the question of whether the NMJs that eventually result from the recovery process are fully normal.

A different pattern of recovery is seen when BoNT serotypes with a shorter duration of action are used

Following the shortest acting form, BoNT/E, recovery occurs in a few days and does not involve the formation of axonal sprouts. With BoNT/F, functional recovery takes about 3 weeks and is accompanied by a brief period of sprout formation. Thus, it seems that paralysis lasting more than 1–2 weeks elicits a process of sprouting from the nerve that appears to be aimed at bringing about functional recovery by the formation of new synaptic contacts which may, or may not, persist long after BoNT administration.

The signaling pathways that trigger axonal sprouting after BoNT are not known. Early studies showed that imposing activity on muscle paralyzed by BoNT/A reduced the extent of sprouting (Brown et al., 1977). This suggests axonal sprouting is suppressed by normal activity. A number of studies have tried to identify molecules whose expression by the muscle is activity-dependent and which suppress axonal sprouting. Although some candidates, such as IGF1 (Caroni and Schneider, 1994), have been implicated it is not clear that any single signaling pathway can account for the wide range of events that occur after BoNT intoxication.

What is clear from studying both recovery from BoNT, and the response to partial denervation, is that the NMJ is capable of a high degree of “adaptive plasticity”, which allows it to recover useful function after long term block. Nonetheless, a number of important questions about that recovery remain unanswered. One such question is: how complete is recovery? In a clinical setting, movement of a small fraction of all muscle fibers may lead to recovery of useful function. Further, at the level of the individual NMJ, a quantum content several-fold lower than normal may be adequate to elicit some contraction. Thus assessment of clinically detectable recovery of function may not be a good indicator of recovery at the level of the NMJ. A second important question is: what is the effect of repeatedly blocking the NMJ? There have been few detailed studies of the recovery after repeated BoNT/A injections, and none of these have been done in situations where the structural and functional properties of the NMJ could be adequately assessed. When these two issues are taken together, considerable uncertainty remains about the possible cumulative effects of repeated BoNT/A injections.

2.7.4.3. Presynaptic toxins that damage the nerve

A second class of presynaptic toxins are those that damage the nerve terminal. These toxins are exemplified by β-bungarotoxin (β-BgTx), a phospholipase A2 component of the venom of kraits of the genus Bungarus sp. (Prasarnpun et al., 2005). Both in vivo and in vitro, β-BgTx causes acute (<1 h) block of neuromuscular transmission. This results from impaired release of ACh and is associated with a profound reduction in the number of synaptic vesicles in the nerve terminal. When the damage to the nerve is local, the terminal can begin to regenerate within a few days and functional innervation is restored within a week.

A second class of presynaptic toxin is exemplified by the α-latrotoxin (αLTX) from the black widow spider Latrodectus mactans (Ushkaryov et al., 2004). This toxin has a complex mode of action, but its most dramatic effect is a great increase in the rate of spontaneous release of ACh quanta from the nerve. This is a result of binding of the toxin to neurexins in the
motor nerve terminal and then forming tetrameric Ca$^{++}$ permeable pores in the membrane. It is the flow of Ca$^{++}$ into the nerve through these pores that enhances spontaneous release. In addition, αLTX binds to latrophilins in the nerve terminal. This leads to activation of a G-protein mediated cascade that also promotes spontaneous quantal release. During the action of αLTX, evoked release of ACh quanta is impaired. Ultimately, the action of αLTX leads to destruction of the nerve terminal, but this damage may be quite local. As with β-BgTx, regeneration of the terminal can occur starting a few days after intoxication, and substantially full recovery is possible (Robbins et al., 1990).

2.7.4.4. Postsynaptic toxins that block AChRs

Much of what is known about the action of ACh at the NMJ is owed to the existence of natural compounds that interfere with AChR function. The first of these to be discovered was curare, a concoction of substances extracted from plants such as Strychnos toxifera, used in the production of poisoned darts by various South American indigenous peoples. The principal active component, the alkaloid α-tubocurarine (dTC), is a reversible competitive blocker of AChRs at the NMJ and is now used clinically as a muscle relaxant. When dTC binds to the AChR it prevents their activation by ACh and thus reduces the sensitivity of the postsynaptic membrane to released ACh. This reduces the amplitude of the mEPPs and EPPs. In sufficient doses it reduces the EPP to below the threshold for activation of the muscle, causing paralysis. At a critical dose, transmission may be effective at low frequencies of stimulation but fail at higher frequencies when the quantum content is depressed (see Section 2.5.1.1).

A second AChR blocker of great historical importance is α-bungarotoxin (αBgTx). This small (MW 8000 Daltons), highly stable protein is a component of the venom of the banded krait Bungarus multicinctus and is an example of the postsynaptically active toxins of elapid and hydrophiid snakes. It is an irreversible blocker of the ACh binding site of the AChRs at the NMJ. It is easily conjugated to fluorescent or electron-dense groups or made radioactive through conjugation to $^{125}$I. The irreversible nature of its binding has made αBgTx extremely useful in a wide variety of experimental situations, not least in the purification and visualization of the AChRs. Its effect on neuromuscular transmission is similar to that of dTC with the notable exception that it is virtually irreversible.

2.7.4.5. Postsynaptic toxins that damage the muscle

Numerous myotoxins are known that cause catastrophic breakdown of muscle fibers, usually as a result of phospholipase activity. Mention has already been made of notexin and how the muscle and NMJ respond to it (see Section 2.7.3).

2.7.4.6. Environmental toxins

The toxicity of man-made compounds may be intentional, as in insecticides, or incidental. Of the intentionally toxic compounds, some of the best known are the compounds that act by blocking the AChE at the NMJ (Blain, 1999). Examples are sarin and the organophosphate pesticides. Since these compounds block AChE activity, they allow ACh released from the nerve to persist in the synaptic cleft longer than usual. This leads to repeated opening of the AChRs and a prolongation of the decay of the EPC. In the short term, this potentiates neuromuscular transmission but soon the high concentration of ACh in the cleft leads to depolarization of the postsynaptic membrane, which inactivates the Na$_{K1}$ channels responsible for the action potential. Further, desensitization of the AChRs may occur as a result of persistent binding of ACh. In addition, the entry of Ca$^{++}$ into the muscle fiber through the open AChRs may cause local activation of lytic processes in the cell that lead to breakdown of the postsynaptic apparatus (Leonard and Salpeter, 1979).

2.7.5. Conclusions

The ability of the NMJ to recover from a wide variety of mechanical and chemical assaults indicates the evolutionary importance of effective and reliable neuromuscular transmission. The BL, and the presence in it of molecules that can direct the rapid reconstruction of the NMJ after its constituent cells have been damaged, play an important part in this recovery. In many circumstances, the reconstruction of the NMJ in an adult mammal occurs more rapidly than its original development. Finally, it is increasingly clear that the Schwann cells play an important part in facilitating the adaptive plasticity of the nerve terminal.

2.8. The impact of disease on safety factor

2.8.1. Overview

The efficacy and reliability of neuromuscular transmission depend on many factors operating at numerous levels of functional organization. As a result many molecules could be targets of pathogenic processes that might interfere with the reliability of neuromuscular transmission. It is perhaps surprising, therefore, that diseases in the NMJ are rare. For example, myasthenia gravis, the most common disease affecting the NMJ
directly, has a prevalence in Western countries of only 10–20/100 000 (Flachenecker, 2006). Since the NMJ is so important for survival, it is likely that many such conditions would be lethal if expressed during early development, and this is the case for many engineered mutations of key NMJ molecules in mice. On the other hand, the remarkable ability of the NMJ to respond adaptively to interference may result in adequate functional compensation so that some conditions remain unrecognized. However rare, conditions leading to impaired neuromuscular transmission are highly debilitating and represent a significant clinical problem. Other chapters in this Handbook deal with these individual diseases in detail. Here, the aim is to consider more generally the ways in which the main functional components of the NMJ are influenced by disease processes.

Two broad classes of disease, distinguished by their etiology, account for most of the recognized conditions affecting neuromuscular transmission. The first class to be recognized contains the acquired autoimmune disorders, including myasthenia gravis (MG), the Lambert–Eaton Myasthenic syndrome (LEMS), neuromyotonia (NMT) and the Miller–Fisher variant of the Guillain–Barré syndrome (MFS). The second class contains the inherited conditions known collectively as Congenital Myasthenic Syndromes (CMS) (Beeson et al., 2005). Many of these conditions involve attack on, or abnormalities of, molecules that play a central role in the immediate events of neuromuscular transmission, such as voltage-gated ion channels, AChE and the AChR. However it has become clear in recent years that a number of other conditions result from direct structural damage to the NMJ (e.g. MFS, Overell and Willison, 2005) or abnormalities of the events that account for the development and maintenance of the normal structure of the NMJ (e.g. Chevessier et al., 2004; Slater et al., 2006). It is thus important, when trying to understand the basis of impaired neuromuscular transmission in any particular case, to keep in mind the broad aspects of the cell biology of the NMJ as well as the immediate events and molecules that mediate transmission.

Regardless of their etiology, diseases affecting neuromuscular transmission may be distinguished by whether the initial effect on transmission is positive or negative. In some cases, such as myasthenia gravis or the congenital AChR deficiencies, the primary effects are to impair transmission. In others, such as neuromyotonia, the slow channel syndrome or AChE deficiency, the initial effects are to exaggerate transmission. In the longer term, the increased transmitter effect associated with these conditions may lead to structural damage to the NMJ that contributes to symptoms (see Section 2.7.4.6).

2.8.2. Conditions influencing transmitter release

A number of conditions are known in which the amount of ACh released from the nerve terminal is less than normal. This can be either because the amount of ACh per vesicle is reduced, or because the number of vesicles released by a nerve impulse is low.

2.8.2.1. Impaired ACh synthesis

The events leading to quantal release of ACh begin with ACh synthesis from acetylCoA and choline. This is mediated by the enzyme choline acetyltransferase (ChAT), which is present in the cytoplasm of the nerve terminal. When the gene encoding ChAT is completely inactivated in mice, the result is lethal at birth and there is no evidence of ACh synthesis, indicating that there is no effective alternative pathway for ACh synthesis (Misgeld et al., 2002).

Pathogenic mutations of the gene encoding this enzyme have been found to be the basis of a form of CMS known as CMS with episodic apnea (CMS-EA, formerly referred to as Familial Infantile Myasthenia) (Ohno et al., 2001). In this condition, neuromuscular transmission is close to normal at rest. However, abnormal fatigue is apparent during sustained exertion. When NMJs from these patients are studied in vitro, a decline in EPP amplitude is seen only after repetitive stimulation (10 Hz) for 5 min. This decline is a result of decreasing mEPP amplitude and is not accompanied by any change in quantum content. It is, however, associated with a reduction in size of the vesicles, consistent with the view that the amount of ACh/vesicle is lower than normal in these patients (Mora et al., 1987). Thus it appears that the safety factor of transmission is reduced in CMS-EA because the abnormally low rate of ACh synthesis is not able to keep up with intense demand.

Once synthesized, ACh must be taken up into synaptic vesicles before it can be released. So far, there are no reported conditions in which this uptake process is impaired.

2.8.2.2. Reduced quantum content of the EPP

Quantum content can be reduced for a number of reasons. For example, failure of the nerve impulse to depolarize the nerve terminal leads to greatly reduced release. An example of this is seen in the naturally occurring mouse mutant motor endplate disease (med) (Harris and Pollard, 1986). In this mouse, a profound impairment of quantal ACh release results from a mutation in the gene encoding the voltage-gated sodium channel in the nerve (SCN8a) (Burgess et al., 1995; Kohrman et al., 1996). No similar condition has yet been described in humans.
RELIABILITY OF NEUROMUSCULAR TRANSMISSION

However, at least four conditions are known in humans in which the safety factor is reduced because the number of quanta released is too small. These are LEMS, “limb-girdle myasthenia” (LGM), congenital endplate AChE deficiency (Engel et al., 1977) and botulism. In LEMS, autoantibodies are present that react with the voltage-gated Ca\(^{2+}\) channels in the nerve terminal (Vincent et al., 1989). Binding of these antibodies at the extracellular face of the channels leads to a decrease in the number of Ca\(^{2+}\) channels in the nerve terminal, presumably as a result of cross-linking and subsequent endocytosis, and disruption of the AZs (Fukuoka et al., 1987). As a result, the amount of Ca\(^{2+}\) entering the nerve in response to a nerve impulse is less than normal. This, in turn, results in fewer quanta being released than normal. This effect is typically great enough so that transmission fails at many NMJs and the CMAP at rest is much smaller than normal. During exercise, however, the weakness associated with LEMS may be reduced. This is assumed to be a result of the build-up of residual Ca\(^{2+}\) in the nerve terminal, leading to enhanced quantal release (see Section 2.5.1.2) and a marked increase in the amplitude of the CMAP. However, this does not last long after exercise stops. This effect forms the basis for a clinical test for LEMS.

LGM is a condition characterized by weakness primarily of the proximal muscles with little involvement of facial or eye muscles (see below). The reduction of quantum content in LGM has a completely different basis from that in LEMS, and therefore provides an interesting comparison. In LGM, the average quantum content is about 50% of normal but the size of the mEPPs is only slightly reduced. The average size of the NMJ is also about 50% of normal. Thus, the quantal release per unit area is normal, indicating that the reduction in ACh output is not primarily a result of an impairment of the release mechanism per se, but rather a “secondary” consequence of the reduction in NMJ size (Slater et al., 2006).

In congenital endplate cholinesterase deficiency the amount of AChE at the NMJ is severely reduced (Section 2.8.3.5). The quantal release from the nerve is also reduced, and this is associated with a reduction in size of the nerve terminals, many of which are completely or partially encased by Schwann cells. In botulism, as described above (Section 2.7.4.2), one or more proteins that play a key role in the process of exocytosis are cleaved by the toxin and thus inactivated. As a result the quantum content is dramatically reduced.

The comparison of the first three conditions points out the important influence of structural factors as determinants of the reliability of neuromuscular transmission.

2.8.2.3. Prolonged release due to impaired repolarization

In addition to conditions in which quantal release is impaired, there is at least one condition in which the opposite is true. In neuromyotonia (NMT), as in myogenic myotonia, muscle relaxation is slowed after contraction. In NMT this results from slowed repolarization of the nerve terminal membrane after a nerve impulse rather than from a similar effect in the muscle cell. Repolarization is normally mediated by the opening of voltage-gated K\(^{+}\) channels (see Section 2.2.2). In at least some forms of NMT, autoantibodies are present (Shillito et al., 1995; Newsom-Davis, 1997) that recognize the K\(^{+}\) channels in the nerve terminal, presumably causing them to be removed by endocytosis. As a result, the density of K\(^{+}\) channels is less than normal, the repolarizing current is reduced and the period of repolarization extended. This, in turn, extends the period of Ca\(^{2+}\) entry into the nerve terminal and with it, the release and action of ACh. The resulting prolonged EPP causes the generation of multiple action potentials in the muscle fiber and persisting contraction after individual motor nerve impulses.

2.8.2.4. Lytic damage to nerve terminals by autoantibodies

Destruction of the nerve terminal is the most extreme way of impairing ACh release. In the Miller–Fisher variant of the Guillain–Barré Syndrome (MFS), autoantibodies are elaborated, often in response to bacterial infections, which are specific for polysaccharides of the GQ1b class. These groups are present on motor nerve terminals, and are bound by the circulating antibodies. This, in turn, leads to local recruitment of the complement system, and lysis of the nerve terminal (O’Hanlon et al., 2002). In this sense, their action resembles that of certain presynaptically active natural toxins, e.g. β-bungarotoxin (see Section 2.7.4.3). In both cases, the ready access of substances in the blood to the NMJ is an important aspect of the pathogenic process.

Following acute lytic attack on the nerve terminal, axonal sprouting and the reinnervation of the original NMJ normally lead to recovery on a timescale of weeks to months. Indeed, it is in recovery from diseases of this kind that the real value of the adaptive plasticity of the NMJ is seen. During this recovery phase, it is likely that the reliability of neuromuscular transmission is reduced, as during reinnervation following mechanical or toxic injury.

2.8.3. Conditions influencing transmitter action

Once ACh is released from the nerve, it must bind to the AChRs and cause their channels to open before
there is any excitatory effect on the muscle. Two important classes of NMJ disorders are associated with changes in the number or properties of the AChRs in the postsynaptic membrane.

2.8.3.1. Abnormal ACh-gated channel function

Mutations of the genes encoding the subunits of the AChR can influence either the abundance or the functional properties of the AChR, or both. An important group of CMSs are those associated with abnormal function of the AChRs.

A number of mutations in different AChR subunits, and different domains of the individual subunits, result in changes in the rate of closing of the opened AChR ion channel (Engel and Sine, 2005). When the channel closes too rapidly, the amount of positive charge entering the muscle fiber per nerve impulse is reduced and with it, the amount of depolarization associated with the action of each quantum of transmitter. As a result, the EPP is smaller than normal and the safety factor correspondingly reduced. There is evidence in some of these diseases of increased expression of AChRs containing a $\gamma$-subunit (Milone et al., 1998). These may act to compensate for the loss of normal $\varepsilon$-subunit function, and may thus represent an important compensatory strategy that allows toleration of some mutations in the $\varepsilon$-subunit. This strategy is not available for compensation from mutations in the other AChR subunits, and this probably accounts for the relative predominance of $\varepsilon$-AChR mutations in patients with CMS caused by mutations in AChR genes.

A second group of AChR mutations, resulting in slowing of closure of the ion channel, underlie the “slow-channel syndromes” (SCS). Initially such mutations would be expected to increase the safety factor since more positive charge than normal would enter the muscle fiber following each channel opening event. However, the weakness seen in these patients results from a paradoxical impairment of transmission. This is associated with focal degeneration of junctional folds and loss of acetylcholine receptors in the membrane (Engel et al., 1982) as well as a depolarization block developing in the course of physiologic activity. Degeneration of the junctional folds is attributed to excessive $\text{Ca}^{++}$ entry during the prolonged synaptic response; the $\text{Ca}^{++}$ conductance of the mutant slow-channels is not increased (Fucile et al., 2006). Degeneration of the junctional folds is also associated with block of AChE activity by chemical toxins (e.g. sarin, organophosphates, see Section 2.7.4.6) or inherited deficiency of AChE.

2.8.3.2. Inherited AChR deficiency

A reduction in the local density of AChRs in the postsynaptic membrane most often occurs either as a result of a mutation in one of the AChR subunit genes in various forms of CMS (usually the $\varepsilon$-subunit), or as a result of the action of autoantibodies specific for the AChR, as in myasthenia gravis (MG, see below). Mutations in the AChR genes that affect the promoter or disrupt the reading frame can result in severe inherited deficiencies in expression of the subunit in question. Most such mutations have been found in the gene encoding the $\varepsilon$-subunit, since this is the only subunit that can be replaced by an analogous subunit ($\gamma$-AChR) (Engel and Sine, 2005).

A frequent feature of inherited AChR deficiencies is an abnormally elongated NMJ (Fig. 2.37) (Vincent et al., 1981; Slater et al., 1997). This appearance suggests that in these cases, the nerve terminal has undergone a phase of sprouting and formation of new synaptic contacts. As in the response to partial denervation or BoNT/A (see Sections 2.7.2.1 and 2.7.4.2), this remodeling is presumably induced by some consequence of the reduced muscle activity. It is noteworthy, however, that in these cases of AChR deficiency, this adaptive plasticity is inadequate to reestablish the normal level of reliability of the NMJ. The explanation for this is unknown.

The accumulation of AChRs in the postsynaptic membrane depends on a complex set of interactions
involving numerous proteins in addition to the AChRs themselves (see Section 2.6.3.5). Of particular importance is rapsyn, a 43 kD protein, which is essential for AChR clustering (Gautam et al., 1995). A number of patients with a severe form of CMS have recently been found to harbor mutations in the gene encoding rapsyn (Ohno et al., 2002; Engel and Sine, 2005). In these patients, both the density of AChRs and the intensity of postsynaptic folding at the NMJ are greatly reduced. In addition, the NMJs have a strikingly elongate conformation. Thus, the NMJs in these patients are similar to those in patients with mutations of the AChR itself. In both cases, it is obvious that the reliability of neuromuscular transmission is profoundly compromised by the reduction in AChR density.

MuSK is a key component of the signaling cascade by which neurally-derived agrin induces a high density of AChRs in the postsynaptic membrane (see Section 2.6.3.5) (Strochlic et al., 2005). As with rapsyn, this molecule must be phosphorylated by MuSK to cause AChR clustering, mice in which the gene for MuSK has been knocked-out are unable to form functional NMJs and therefore die at birth (DeChiara et al., 1996). A number of patients with CMS have now been found with mutations in the gene encoding MuSK (Chevessier et al., 2004). Although little is known about the functional properties of the NMJs in these patients, their conformation is abnormal, with some similarities to that in other cases of AChR deficiency.

These patients with defects of rapsyn and MuSK exemplify the point that due to the highly interactive nature of the processes, which ensure the normal reliability of the NMJ, there are many molecular targets for pathogenic attack.

2.8.3.3. Acquired AChR deficiency

The most familiar disease that has its primary impact on the reliability of neuromuscular transmission is myasthenia gravis. In most patients with this condition, the pathogenic agents are autoantibodies to the AChR. The majority of these do not act as functional blockers of the AChR or its channel. Rather, they bind to and cross-link the AChRs causing them to be internalized and degraded. The result is a profound reduction in the density of AChRs in the postsynaptic membrane, a reduction in the amplitude of mEPPs and EPPs (Elmqvist et al., 1964; Cull-Candy et al., 1980) and an attendant loss of efficacy and reliability of neuromuscular transmission. In addition to the down-regulation of AChRs, there is also a disorganization of the postsynaptic apparatus, with a widening of the folds (Engel and Santa, 1971). This is presumably caused by the lytic process associated with complement binding to the AChRs in the postsynaptic membrane. In light of the current view of the function of the folds (see Section 2.3.6.3), it is likely that their disruption exacerbates the effects of the primary deficiency of AChRs on transmission.

Axonal sprouting can also occur in autoimmune MG. This is associated with a second form of adaptive plasticity, an increase in the output of quanta per unit area of synaptic contact (Plomp et al., 1995). The magnitude of this increase is inversely correlated with the amplitude of the mEPPs at individual NMJs. The basis of this effect is not known. Possible explanations include a change in the type or density of Ca++ channels or in their modulation by G-protein coupled mediators, or an alteration in the duration of the nerve action potential, possibly by changes in the properties or number of K+ channels in the nerve terminal. As with those examples of inherited AChR deficiency cited above, the adaptive response of the nerve in MG is clearly inadequate to normalize the reliability of transmission.

A significant fraction of patients with MG do not have detectable serum antibodies to AChRs. Some of these “seronegative” patients have antibodies to MuSK in their serum (Hoch et al., 2001; McConville et al., 2004), although there is doubt about whether these antibodies account for the functional defect in any or all of these patients (Selcen et al., 2004).

2.8.3.4. Prolonged ACh action due to AChE deficiency

The action of ACh on the postsynaptic membrane is normally terminated by hydrolysis catalyzed by the AChE associated with the synaptic basal lamina (see Section 2.3.4). A number of patients have been identified in whom the duration of the synaptic potentials is greatly prolonged and virtually all AChE activity is absent (Engel et al., 1977). It has recently been found that these patients have mutations in the gene encoding the AChE molecule, most commonly in the region encoding the collagenous tail (COL Q), which is involved in binding the enzyme to the basal lamina (Ohno et al., 1998). In the absence of normal AChE activity, ACh molecules that unbind from AChR after the initial activation are free to rebind to AChRs and thus to trigger repeated openings. In these circumstances, the termination of ACh action after the release of a quantum of transmitter is determined by the rate at which it diffuses away from the release site, resulting in a decreased local concentration.

The consequences for the NMJ of a profound deficiency of AChE are similar to those of the prolongation of the AChR open time in the SCS. In NMJ AChE deficiency, however, the opening events of the AChR channel are normal but the individual channels open
repeatedly owing to the prolonged lifetime of ACh in the synaptic space. In both cases, excessive entry of Ca$^{++}$ occurs, resulting in local degeneration of the postsynaptic apparatus. In NMJ AChE deficiency, neuromuscular transmission is compromised by the combination of decreased quantal release by nerve impulses from small nerve terminals, many of which are encased by Schwann cells, a depolarization block during physiologic activity, and structural changes in the junctional folds that alter the geometry of the NMJ. The two conditions can be distinguished by the absence of any beneficial response in cases of AChE deficiency. Although anticholinesterase treatment may in principle alleviate the symptoms of SCS, it is obvious that in the long term, this approach would only intensify the pathogenic process.

2.8.4. Conditions influencing muscle excitation

Muscle contraction is normally activated by an action potential that propagates along the full length of the fiber. Unless the muscle fiber action potential occurs, the depolarization of the EPP causes at most a local graded contraction that is of no functional significance. Two classes of disease are known in which the triggering of muscle fiber action potentials at the NMJ is impaired.

2.8.4.1. Abnormal Na\textsubscript{V}1.4 properties

One patient has been described in whom mutations of the gene encoding Na\textsubscript{V}1.4 results in abnormally rapid inactivation of the channels (Tsujino et al., 2003). As a result, the number of Na\textsubscript{V}1.4 channels that are opened by a full-sized EPP is reduced and action potential generation is impaired. In this patient, all aspects of ACh release and action appear to be normal. It is important to realize that Na\textsubscript{V}1.4 is normally expressed only in skeletal muscle. As a result, these mutations do not directly influence the function of nerve or cardiac muscle. Other mutations affecting the structure of Na\textsubscript{V}1.4 have been described and are the basis of the periodic paralyses and myotonias (Jurkat-Rott and Lehmann-Horn, 2005). These mutations generally result in a gain of function, in the sense that the activated channels remain open longer than normal, leading to the generation of multiple action potentials that is the defining feature of myotonias. The rarity of this form of CMS, in which there is a loss of function of Na\textsubscript{V}1.4, presumably reflects the lethality of many other mutations affecting this essential channel.

2.8.4.2. Disturbances of folding

The second class of conditions affecting the generation of muscle fiber action potentials is less well defined, but probably much more common. In these conditions, the characteristic folding of the postsynaptic membrane is disrupted and/or reduced. This may occur as a result of mutations affecting postsynaptic proteins, of attack by autoantibodies as in MG (Engel et al., 1976), or as a result of local degeneration induced by excess Ca$^{++}$ entry through opened AChR channels in SCS or AChE deficiency. Current views about the significance of the folds as amplifiers of ACh action are described above (Section 2.3.6.3).

The cellular processes that account for the formation and maintenance of the folds are poorly understood. It is noteworthy that mutations affecting many postsynaptic proteins, both integral membrane proteins and components of the cytoskeleton, lead to reduced folding (De Kerchove et al., 2002). These proteins include AChR (Engel et al., 1982), rapsyn (Ohno et al., 2002), utrophin (Deconinck et al., 1997; Grady et al., 1997), dystrophin (Torres and Duchen, 1987; Lyons and Slater, 1991), and syntrophin (Adams et al., 2004). A similar reduction in folding has also been seen in cases of limb-girdle myasthenia (Slater et al., 2006). It thus seems likely that the very extensive folding, which is a feature of human NMJs, is sensitive to subtle mechanical properties of the postsynaptic membrane that are influenced by many proteins.

Folding may also be disrupted as a result of antibody-dependent degeneration of the postsynaptic apparatus. In MG, this results from a complement-mediated attack on the postsynaptic membrane (Engel et al., 1976). Associated with this effect on the folds, in both MG and an animal model of it, is a reduction in the number of Na\textsubscript{V}1.4 channels and an increase in action potential threshold (Ruff and Lennon, 1998). Similar effects are likely to be associated with local postsynaptic degeneration in CMS and AChE deficiency, although this has not been directly established in these conditions.

It is thus clear that a wide variety of circumstances give rise to reduced or disorganized folding. In few of these situations has the impact of this effect on muscle fiber action potential generation been quantified. Nonetheless, it seems clear that what may often be “secondary” effects of a pathological process at the NMJ may have significant functional consequences.

2.8.5. Conditions influencing NMJ size

Many of the well-defined pathological conditions that lead to impaired neuromuscular transmission have their primary effect on either the pre- or the postsynaptic component of the NMJ. In some conditions, however, both components are clearly affected. An example of this is limb-girdle myasthenia (LGM) (Slater
et al., 2006). In LGM patients, the effects of individual quanta on the muscle fiber are substantially normal, indicating that the function and local density of AChRs is normal (Fig. 2.38). However there is a reduction in quantum content to about 50% of its normal value. This is associated with, and probably caused by, a reduction in the overall size of the NMJs. At the same time, the extent of postsynaptic folding is also reduced to about 50% of its normal value. It is likely that the reduction in folding raises the effective threshold for action potential initiation in the muscle (see Section 2.3.6.3), thus exacerbating the effect of the reduced quantum content.

It has recently been found that many cases of LGM, including those described immediately above, result from mutations in the gene encoding Dok-7, a protein that modulates the activity of MuSK (Beeson et al., 2006). This is the first gene to be identified that influences the efficacy of neuromuscular transmission primarily by an effect on NMJ size and conformation (“synaptopathy”), rather than on the properties or density of one of the molecules that mediate the immediate events of neuromuscular transmission. Further investigation of the normal action of Dok-7 may shed light on the question of how one of the most distinctive and functionally important features of the NMJ, its size, is determined.

2.8.6. Impaired transmission associated with diseases of the motor neuron

In a number of conditions, the reliability of neuromuscular transmission is compromised as part of a broad decline in the state of health of the motor neuron. A comprehensive review of these conditions would go beyond the remit of this chapter. Nonetheless, it is worth mentioning two examples of this group which, though still poorly described or understood, are of considerable clinical importance. These are the early stages of ALS and the so-called post-poli syndrome. In both these conditions, loss of reliability of neuromuscular transmission occurs before, or in the absence of, overt necrosis of the motor neuron.

2.8.6.1. Early stages of ALS

Several reports document decreased reliability of neuromuscular transmission during the early stages of ALS (e.g. (Stalberg et al., 1975; Denys and Norris, Jr, 1979)). This reduced efficacy of transmission is associated with the expansion of motor units that survive the motor neuron death that defines this condition. In the early stages of the disease, when relatively few motor neurons have died, there are still many surviving motor neurons available to provide innervation for the muscle fibers that have become denervated. It is likely that transmission at the earliest of these re-established NMJs is fully effective. However, as the extent of motor neuron death increases, the surviving motor neurons become increasingly extended. At this stage it is likely that the general level of reliability of transmission declines, leading to clinically significant weakness.

The one detailed study of transmission at NMJs in 10 patients with ALS found a decline in quantum content to about 50% of normal values (Maselli et al., 1993). Whether this was a loss of release efficacy, or a result of a reduction in the size of individual NMJs was not determined. The decline in quantum content was associated with a decline in mEPP amplitude to about 67% of normal. In that study, it was not possible to determine whether the NMJs from which recordings were made were original or newly formed, or how much the axons in question had sprouted. It is therefore unclear whether the observations represent a decline in function of origin NMJs, or a failure of newly formed innervation to reach a normal level of reliability, or a mixture of both.

Some animal studies suggest that changes at the NMJ are among the earliest signs of motor neuron degeneration in some forms of inherited motor neuron disease (Frey et al., 2000). In three strains of mutant mice with motor neuron loss, structural degeneration of some motor nerve terminals was observed before signs of clinical weakness were apparent or there was detectable motor neuron loss. The first nerve terminals to be lost were those of fast motor units. While no functional studies were made, it seems that events leading to overt degeneration of the nerve terminal may occur at an early stage in these conditions. Whether this is also true of the more common sporadic forms of ALS in man is unclear.

2.8.6.2. Post-polio syndrome

A second condition in which decreased reliability of neuromuscular transmission is associated with motor neuron pathology is the post-polio syndrome (PPS) (Trojan and Cashman, 2005). In this condition, patients who have had polio as children experience a new phase of increased weakness typically 35–40 years later. This new weakness appears to be due in large part to impaired neuromuscular transmission. SFEMG studies reveal that the extent of the observed increase in “jitter”, an indication of a decline in the safety factor of neuromuscular transmission, is correlated with the enlargement of the motor unit as indicated by increased fiber density (Maselli et al., 1992). Detailed studies of neuromuscular transmission in isolated biopsy samples have found varying degrees of impairment involving decreases in
Fig. 2.38. Abnormalities of the NMJ in LGM. A. The evoked EPC is reduced in amplitude at LGM NMJs but the amplitude of mEPCs is normal, indicating reduced quantal release from the nerve. B. The NMJs are abnormally small in LGM patients. Top row, nerve terminal visualized after silver impregnation and histochemical demonstration of AChE activity. Middle row, AChRs labeled with R-α-bungarotoxin. Bottom row, histochemical demonstration of AChE activity. C. Both quantal release and NMJ size are reduced in LGM patients (open symbols) compared to controls (filled symbols). (From Slater et al., 2006, with permission.)
both quantum content and mEPP amplitude (Maselli et al., 1995). In the absence of appropriate morphological studies, the basis of these changes is unclear.

The most generally accepted hypothesis concerning the basis of PPS is that motor nerve terminals that have undergone an expansion of their territory experience a time-dependent decline in function that eventually leads to the observed clinical weakness (Wiechers, 1988). The typical age of onset of the renewed phase of weakness in PPS is about 50 years of age, a decade or so earlier than the onset of motor neuron death that occurs in the normal population (see Section 2.6.4).

The biological bases of these aspects of age-dependent decline in motor neuron function are not understood. Nor is it clear how they are related to the normal processes of motor neuron aging and degeneration.

2.8.7. Treatments and their basis

Given the wide variety of conditions that alter the normal reliability of neuromuscular transmission, there are very few drugs available to treat their symptoms. These drugs can be divided broadly into those that alter quantal ACh release, and those that alter ACh action. Practical experience with different treatments is described in the chapters of this Handbook dealing with specific diseases. Here, only some brief general comments about the available drugs and their action will be made. Treatments of the autoimmune basis of the acquired diseases of the NMJ will not be covered.

2.8.7.1. Drugs that alter ACh release

The drug most commonly used to enhance evoked ACh release is 3,4-diaminopyridine (DAP). This drug blocks voltage-gated K⁺ channels in muscle and nerve (see Section 2.2.2.4). Its most relevant action at the NMJ is to prolong the nerve terminal action potential and thus increase the entry of Ca²⁺. This in turn increases the number of quanta released. This drug has been particularly effective in the treatment of LEMS but is also useful in other conditions.

Sympathomimetic drugs have an enhancing effect on neuromuscular transmission (see Section 2.5.4.6), which may be related to their ability to synchronize quantal release. Ephedrine, a plant alkaloid, has sympathomimetic effects but has little effect in vitro on neuromuscular transmission in therapeutically achievable concentrations. It has nonetheless proven useful in some patients with Limb-girdle Myasthenia (Slater et al., 2006) and with AChE deficiency (Bestue-Cardiel et al., 2005). In the latter case the beneficial effect may be related to ephedrine’s action as an open channel blocker with an associated speeding up of AChR channel closure (Milone and Engel, 1996).

2.8.7.2. Drugs that alter ACh action

The drugs used most commonly to treat impaired reliability of neuromuscular transmission are the anticholinesterases. Blocking the activity of the AChE in the synaptic cleft prolongs the period of action of the ACh released from the nerve to remain active until diffusion away from the site of release lowers the local concentration to an inactive level. This allows AChR channels to remain open longer than normal and more positive charge to enter the muscle for every nerve impulse, thus enhancing the chance of generating an action potential in the muscle fiber. In many conditions of impaired transmission, regardless of whether they are pre- or postsynaptic in origin, this type of treatment is potentially effective. Obvious exceptions are AChE deficiencies and SCS, where even if there is a potential short term benefit, the long term effect is absent or negative (see Section 2.8.3).

Blockers of ACh action have potential use in conditions where overactivation of AChRs occurs. Quinidine and related compounds are open channel blockers that hasten the closure of open AChR channels (Sieb et al., 1996). This results in reduced amplitude and rapid decay of the mEPCs and EPPs. Quinidine has been used effectively in cases of SCS (Fukudome et al., 1998). Some patients treated with quinidine develop an allergy to it. In these patients fluoxetine (Prozac), also an open channel blocker (Garcia-Colunga et al., 1997), has been useful (Harper et al., 2003).

2.8.8. Conclusions

There have been enormous advances during the last 50 years in understanding the basic mechanisms that account for the process of neuromuscular transmission and for its great reliability in mammals. This knowledge has provided the essential foundation for efforts to understand the basis of impaired transmission in the many different human diseases in which the NMJ is the primary target. In many cases, it has also allowed the development of effective therapies to treat those conditions. Several important conclusions for the future can be drawn from these efforts.

2.8.8.1. Many levels of organization contribute to the reliability of neuromuscular transmission

The process of fast chemical transmission of signals at synapses is one of the most remarkable products of evolution. The specific form of this process found at mammalian NMJs is one of many, and represents a particular specialization for very fast and reliable transmission. Many different levels of organization...
contribute to this reliability. At one end of the spectrum are the individual molecules involved in transmission such as the key ion channels and the proteins that mediate exocytosis. Many of these molecules are present in multimolecular complexes, such as those regulating exocytosis and the distribution of ion channels in the postsynaptic apparatus. These molecular complexes are strategically located in subcellular units subserving the essential functions of transmission. Thus the AZs and the vesicles immediately associated with them are exactly positioned opposite the opening of the postsynaptic folds and the distinct ion channel domains associated with them, forming what may be thought of as a “unit of transmission”. In many species, these units are in turn associated with distinct regions of the NMJ based on the boutons of the nerve terminal. In humans, there are typically 50 or so such units associated with each bouton. The NMJ as a whole is composed of a number of boutons, or analogous regions.

An important consequence of this hierarchical organization of the NMJ is that the great reliability of neuromuscular transmission can arise from events that individually have low probability. These include the opening of individual Ca\(^{++}\) channels in the nerve, the release of individual docked vesicles, and the activation of individual AChRs in the muscle fiber membrane. As a result of the way the NMJ is organized, the transmission of a single nerve impulse uses only a small fraction of the molecular components present. During repetitive activation each unit of transmission is only used in response to approximately every tenth nerve impulse. This allows time between periods of activity for local recovery processes such as restoration of ion concentrations and uptake of choline into the nerve terminal.

### 2.8.8.3. Different factors may determine reliability in different species and different stages of development

It is clear that different species achieve reliability of neuromuscular transmission in somewhat different ways. This makes it important to be cautious when interpreting observations in one species, e.g. humans, on the basis of findings made in a different species, e.g. frog. One example of this is provided by the differences in the quantal release mechanisms in frogs and mammals. The substantially greater probability of an individual docked vesicle being released at mammalian versus frog NMJs is very likely due to the differences in both the structural features of the AZs and the type of Ca\(^{++}\) channels present. While a detailed explanation of these differences is not yet available, it seems likely that more detailed knowledge of how release occurs at mammalian NMJs will be an important basis for understanding the defects of human transmission.

Another example is provided by the differing balance between pre- and postsynaptic factors in achieving reliability in different species. In frogs the emphasis is on the release of many quanta, with relatively little postsynaptic amplification. In humans, the opposite is true, with small nerve terminals releasing relatively few quanta and extensive postsynaptic folds providing more substantial amplification. The biological factors that account for the particular strategy used in a given species are unknown. Nonetheless it is important, when considering defects at human NMJs, to be aware of the important contribution to reliability of transmission likely to be made by the folds and the numerous molecular disturbances that seem to lead to reduced folding.

Major gaps exist in our understanding of the events leading to the development of the mature transmission mechanism in any species and in mammals in particular. It is clear that the key ion channels supporting transmission all undergo changes in type during NMJ maturation. Less is known about the structural changes at the developing NMJ and the molecular basis for them. For example, little is known about the sequence of events leading to the formation of mature AZs, or when that process occurs in development relative to other recognized milestones. The presence of N-type Ca\(^{++}\) channels at immature mammalian NMJs suggests an example of “ontogeny recapitulating phylogeny”, since N-type channels predominate in frogs and possibly other lower vertebrates. It remains unclear whether these N-type channels at the immature NMJ function in the context of AZs whose structure is characteristically mammalian.

It is tempting to look for common principles of function and organization of the NMJ across many
species. While it is true that the most basic principles of quantal release and ligand-mediated activation of postsynaptic receptors are common to NMJs from nematodes to man, there are also many very important differences. Attention to the origins and significance of these differences is likely to continue to provide important insights into the properties of human NMJs.

2.8.8.4. The potential for adaptive plasticity of the NMJ has limits that are poorly understood

The NMJ has a remarkable ability to respond to conditions that impair transmission so as to compensate for the functional loss. This adaptive plasticity includes both structural plasticity of the nerve and the ability to rerun programs of coordinated molecular expression that operate in the immature animal to promote effective transmission at structurally immature NMJs. It is clear that changes in activity exert a powerful regulatory influence over the adaptive process. Considerable insight has been gained recently into the molecular mechanisms underlying the links between activity and patterns of gene expression, particularly in muscle. However there is still little detailed knowledge about how the key process of axonal growth and sprouting is regulated, either during development or during adaptive responses in the adult.

Given the striking potential for responding in an adaptive way to impaired transmission, it seems odd that in many diseases this response has obviously been inadequate. In some cases, it may be that the adaptive response is so good that the effects of impaired transmission are rendered undetectable. In many other cases, where weakness is all too obvious an outcome, the question arises whether the response mechanism has reached some inherent limits, or is itself defective. In either case, better knowledge of the factors regulating the adaptive plasticity of the NMJ might lead to ways of enhancing the restoration of more normal function.

References


RELIABILITY OF NEUROMUSCULAR TRANSMISSION


Flucher BE, Daniels MP (1989). Distribution of Na+ channels and ankyrin in neuromuscular junctions is complementary to that of acetylcholine receptors and the 43 kD protein. Neuron 3: 163–175.


Haimovich B, Bonilla E, Casadei J et al. (1984). Immunocytochemical localization of the mammalian voltage-
RELIABILITY OF NEUROMUSCULAR TRANSMISSION


RELIABILITY OF NEUROMUSCULAR TRANSMISSION


C.R. SLATER


RELIABILITY OF NEUROMUSCULAR TRANSMISSION


Sine SM, Shen XM, Wang HL et al. (2002). Naturally occurring mutations at the acetylcholine receptor binding site
independently alter acetylcholine receptor sites in rat soleus muscles. Mol Cell Neurosci 28: 694–702.

Slater CR, Young C, Wood SJ et al. (1997). Urophin abundance is reduced at neuromuscular junctions of patients with both inherited and acquired acetylcholine receptor deficiencies. Brain 120: 1513–1531.


Dear Author,

During the preparation of your manuscript for typesetting some questions have arisen. These are listed below. Please check your typeset proof carefully and mark any corrections in the margin of the proof or compile them as a separate list. This form should then be returned with your marked proof/list of corrections to Elsevier Science.

**Disk use**

In some instances we may be unable to process the electronic file of your article and/or artwork. In that case we have, for efficiency reasons, proceeded by using the hard copy of your manuscript. If this is the case the reasons are indicated below:

- Disk damaged
- Incompatible file format
- Virus infected
- Discrepancies between electronic file and (peer-reviewed, therefore definitive) hard copy.
- Other: ...................................................

We have proceeded as follows:

- Manuscript scanned
- Manuscript keyed in
- Artwork scanned
- Files only partly used (parts processed differently:......................................................)

**Bibliography**

If discrepancies were noted between the literature list and the text references, the following may apply:

The references listed below were noted in the text but appear to be missing from your literature list. Please complete the list or remove the references from the text.

Uncited references: This section comprises references which occur in the reference list but not in the body of the text. Please position each reference in the text or, alternatively, delete it. Any reference not dealt with will be retained in this section.

<table>
<thead>
<tr>
<th>Query Refs.</th>
<th>Details Required</th>
<th>Author's response</th>
</tr>
</thead>
<tbody>
<tr>
<td>AU2</td>
<td>reword for sense?</td>
<td></td>
</tr>
<tr>
<td>AU3</td>
<td>Which chapter are you referring to - Engel?</td>
<td></td>
</tr>
<tr>
<td>AU4</td>
<td>Which chapter are you referring to?</td>
<td></td>
</tr>
<tr>
<td>AU5</td>
<td>Which chapter are you referring to?</td>
<td></td>
</tr>
<tr>
<td>AU6</td>
<td>Which chapter are you referring to?</td>
<td></td>
</tr>
<tr>
<td>AU7</td>
<td>Which chapter are you referring to?</td>
<td></td>
</tr>
<tr>
<td>AU8</td>
<td>Author is this OK?</td>
<td></td>
</tr>
<tr>
<td>AU9</td>
<td>Which chapter are you referring to?</td>
<td></td>
</tr>
<tr>
<td>AU10</td>
<td>OK as edited?</td>
<td></td>
</tr>
<tr>
<td>AU11</td>
<td>OK as edited?</td>
<td></td>
</tr>
<tr>
<td>AU12</td>
<td>Which chapter are you referring to?</td>
<td></td>
</tr>
<tr>
<td>AU13</td>
<td>OK as edited?</td>
<td></td>
</tr>
<tr>
<td>AU14</td>
<td>Beeson et al. 2006 - volume and page span?</td>
<td></td>
</tr>
<tr>
<td>AU15</td>
<td>del Castillo and Katz 1954a and b are the same reference</td>
<td></td>
</tr>
<tr>
<td>AU16</td>
<td>van der KW - is this author's full name?</td>
<td></td>
</tr>
<tr>
<td>AU17</td>
<td>Not in ref list; pls. supply</td>
<td></td>
</tr>
<tr>
<td>AU18</td>
<td>Not in ref list; pls. supply</td>
<td></td>
</tr>
<tr>
<td>AU19</td>
<td>Ref not cited in text; OK to delete?</td>
<td></td>
</tr>
<tr>
<td>AU20</td>
<td>Not in ref list; pls. supply</td>
<td></td>
</tr>
<tr>
<td>AU21</td>
<td>Do you mean 1979 a or b?</td>
<td></td>
</tr>
<tr>
<td>AU22</td>
<td>Not in ref list; pls. supply</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>-----------------------------</td>
<td></td>
</tr>
<tr>
<td>AU23</td>
<td>Not in ref list; pls. supply</td>
<td></td>
</tr>
<tr>
<td>AU24</td>
<td>Not in ref list; pls. supply</td>
<td></td>
</tr>
<tr>
<td>AU25</td>
<td>Not in ref list; pls. supply</td>
<td></td>
</tr>
</tbody>
</table>