Quantal Analysis of Endplate Potentials in Mouse Flexor Digitorum Brevis Muscle

Richard R. Ribchester

1Euan MacDonald Centre for Motor Neurone Disease Research, University of Edinburgh, George Square, Edinburgh, Scotland, United Kingdom

ABSTRACT

The isolated flexor digitorum brevis (FDB) muscle from mice is extremely well suited to rapid acquisition of data and analysis of neurotransmitter release and action at neuromuscular junctions, because the muscle and its tibial nerve supply are simple to dissect and its constituent muscle fibers are short (<1 mm) and isopotential along their length. Methods are described here for dissection of FDB, stimulation of the tibial nerve, microelectrode recording from individual muscle fibers, and quantal analysis of endplate potentials (EPPs) and miniature endplate potentials (MEPPs). Curr. Protoc. Mouse Biol. 1:429-444 © 2011 by John Wiley & Sons, Inc.

Keywords: neuromuscular junction ● endplate potential ● intracellular recording ● electrophysiology ● quantal analysis

Neuromuscular junctions (NMJs) in skeletal muscles of vertebrates are highly accessible experimental objects, favorable for discovery and analysis of fundamental principles of synaptic structure and function (Katz, 1996). In the 1950s, intracellular recordings with glass microelectrodes and electron microscopy laid the foundation for understanding the mechanism of transmission by exocytosis of neurotransmitter molecules and their action on specific receptors (Katz, 1969). Such analysis was subsequently shown to explain synaptic transmission at all chemical synapses, in every part of the nervous system, in every species examined experimentally. More recently, the advent of transgenic technology and various advanced forms of microscopy, as well as the blossoming of optical techniques for making physiological measurements, have broadened the horizons for research into the principles governing neuromuscular connectivity (“connectomics”) and the regulation of synaptic survival and strength (Livet et al., 2007; Lu et al., 2009; Nagwaney et al., 2009; Ribchester, 2009; Wong et al., 2009; Ruiz et al., 2011). However, quantal analysis of neuromuscular transmission still forms the basis of many studies into the physiology of neuromuscular development, maintenance, repair, aging, and disease (Gillingwater et al., 2002; Ribchester et al., 2004; Slater et al., 2006; Slater, 2008; Zitman et al., 2008). Standard preparations that permit data to be obtained on spontaneous and evoked neurotransmitter release and action efficiently are therefore both desirable and useful.

The mechanism of neuromuscular transmission was originally established from studies of the frog sartorius muscle, but there are many disadvantages to this preparation, stemming mostly from the thickness of the muscle and the time-consuming nature of positioning the recording microelectrode in the vicinity of an NMJ. The cutaneous pectoris muscle is a better alternative in this species because this muscle is very thin and the location of the motor nerve terminals can more readily be gauged using dark-field illumination and a standard dissecting microscope or a compound microscope (Blioch et al., 1968; Betz and Bewick, 1993). Standard neuromuscular preparations for electrophysiological analysis of neuromuscular transmission in mice include the fast-twitch extensor digitorum longus.
muscle, the slow-twitch soleus muscle, and the hemidiaphragm (Harris and Ribchester, 1979a,b). While these preparations have the principal merit of being well characterized, they are not the best for making routine electrophysiological recording or analysis of synaptic (endplate) potentials. The principal disadvantages are their thickness—making precise visualization of the NMJs problematical and the risks of hypoxia greater—and the time taken for careful placement of a recording microelectrode tip at the NMJ, which is required for high-fidelity recording of spontaneous and evoked postsynaptic responses in cases where the muscle fibers are relatively long.

Thin preparations that facilitate visualization of murine NMJ under a dissecting microscope include, for example, levator auris longus, triangularis sterni, or transversus abdominis (McArdle et al., 1981; Angaut-Petit et al., 1987; Gillingwater et al., 2002). Good dissection is relatively difficult, but paramount for setting up these preparations, and this is relatively time consuming. These preparations also require careful placement of the electrode tip in the near vicinity of the NMJ, or else the recordings are attenuated by the “cable properties” of the muscle fibers (particularly the time constant and length constant) with excessive leakage of synaptic current occurring if the microelectrode is located more than ~200 μm distant from the current source at the endplate (Auerbach and Betz, 1971; McLachlan and Martin, 1981). These requirements are especially disadvantageous if data throughput is of high priority.

The isolated flexor digitorum brevis (FDB) of rodents is extremely well suited to rapid acquisition of data and quantal analysis neurotransmitter release and action at neuromuscular junctions. The principal advantages of this preparation are as follows:

a. The muscle fibers of mouse or rat FDB are short: ~300 to 800 μm in length and about 30 μm in diameter. This property renders the fibers “isopotential” along their length: that is, current generated from a source located at any point along the fiber (including its NMJ) can be faithfully recorded by a microelectrode located anywhere else in the fiber (Bekoff and Betz, 1977a,b). Thus, it is unnecessary to deliberately place a recording microelectrode near an NMJ in order to obtain high-fidelity recording of either spontaneous MEPPs or evoked EPPs: the fibers can be impaled at random spots and maximal, unattenuated, endplate potentials with a rapid time course will be observed (Ribchester et al., 1995, 2004; Gillingwater et al., 2002).

b. The muscle is located superficially on the plantar side of the foot and it is therefore quite easy, after just a few attempts, for a student or other junior researcher who has reasonable manual dexterity to dissect an undamaged FDB muscle with its tibial/medial-plantar nerve supply intact.

c. The muscle fibers have a pennate (feathered) organization. This feature is very ‘forgiving’ during the training period when dissection technique is being learned, especially if self-taught or with minimal instruction or supervision. Damage to part of the muscle during dissection, for example, still leaves many intact fibers in other parts of the muscle, from which good resting membrane potentials (around −70 mV) and MEPPs, at least, may still be observed and recorded.

Although imperfect for whole mounts, the FDB muscle, particularly in mice, is also suitable for subsequent morphological analysis of NMJ using conventional or confocal microscopy, for instance after staining endplates with fluorescent conjugates of α-bungarotoxin (Gillingwater et al., 2002). FDB muscles can also be quite readily dissected or dissociated into single fibers using collagenase, then either studied acutely using a variety of physiological methods, or after culture in vitro (Bekoff and Betz, 1977a,b; Gillespie and Ribchester, 1988; Lupa and Caldwell, 1991; Yeung et al., 2002; DiFranco et al., 2011; Nocella et al., 2011).
NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Materials

- Physiological saline solution (see recipe)
- Mouse (any laboratory strain of any age/gender; e.g., C57B16)
- μ-conotoxin GIIIB (Bachem)
- 4 M potassium acetate
- Dissection tools (iris scissors, spring scissors, fine forceps)
- Sylgard-lined Petri dish (see recipe)
- Fine minutien pins (Fine Science Tools)
- Suction electrode (see recipe)
- Micromanipulators (e.g., Leica or Sutter Instruments)
- Recording chamber: the author’s laboratory uses a Perspex chamber made in-house, but a Sylgard-lined Petri dish (see recipe) will suffice
- 1-mm glass capillary tubing containing and internal welded glass filament (1.5 mm O.D., 0.84 mm I.D. standard wall borosilicate glass with filament; World Precision Instruments, cat. no. 1B150F-6)
- Electrode puller: Among the most popular electrode pullers with electrophysiologists is the Brown-Fleming puller manufactured by Sutter Instruments
- Syringe with tip drawn out after warming the plastic in a Bunsen burner flame
- Microelectrode amplifier (Axoclamp 2B, Axon Instruments)
- Silver-silver chloride wire or pellet (0.8 mm diameter × 20 mm Ag/AgCl electrode; World Precision Instruments, cat. no. EP08)
- Mains interference filter (Digitimer Humbug; optional)
- Low-pass (<2 kHz) filters (Neurolog, Digitimer, UK)
- Pulse train generator (Digitimer D4030 Programmer)
- Isolated stimulator (Digitimer DS2)
- Dissecting microscope
- Light source for dissecting microscope (dark-field condenser or flexible fiber-optic light)
- Recording display device (oscilloscope, or computer running WinWCP)
- WinWCP software program (Strathclyde Electrophysiology Software; http://spider.science.strath.ac.uk/sipbs/showPage.php?page=software_ses)
- Data acquisition unit (CED micro 1401, Cambridge Electronic Design, http://www.ced.co.uk/)
- Additional reagents and equipment for sacrifice of the mouse (Donovan and Brown, 2006)

Perform dissection

1. Make up one liter of physiological saline solution.
2. Sacrifice a mouse using an authorized, legal method approved by the institution where the research is to be conducted. Use large scissors to remove one or both hind limbs near the hip.

   Stunning, immediately followed by cervical dislocation (Donovan and Brown, 2006) is suitable and swift.

3. Use iris scissors to make a skin incision along the anterior of the amputated limb and dorsum of the foot. Grip the skin firmly and strip it in one continuous motion from the leg and foot.
4. Using fine minutien pins, pin the isolated, skin-stripped limb to the base of a Petri dish lined with Sylgard, and containing enough physiological saline to keep the exposed tissue submerged during the dissection.

The plantar surface of the foot and medial side of the calf should be pinned uppermost.

5. Expose the tibial nerve by separating the superficial calf musculature and carefully cutting through connective tissue fascia and fat using miniature spring scissors. Cut the tibial nerve close to the knee and then dissect it along its length into the foot, taking special care where the nerve traverses the heel, where it can easily be damaged. Cut the lateral plantar branch of the tibial nerve and continue the dissection of the medial plantar nerve into the foot, passing alongside the FDB muscle.

A side branch of the medial plantar nerve enters the muscle midway along its length.

Although it may appear aesthetically pleasing to clean away all the connective tissue adhering to the nerve and the medial side of the FDB muscle, there is no real need to do so; attempting this can result in stretching or other form of damage to the nerve, sufficient to block nerve conduction.

6. Cut the proximal tendon of the FDB at the heel and dissect connective tissue from both the lateral and medial sides. Reflect the nerve-muscle preparation towards the toes and cut the three distal tendons, close to their insertions in the underlying calcaneus.

This involves, in passing, cutting through the belly of superficial lumbrical muscles originating on the distal tendons of FDB.

7. Secure the isolated FDB muscle with attached tibial/medial-plantar nerve to the Sylgard-lined dish with fine minutien pins inserted through the proximal tendon and all three distal tendons. Clean away connective tissue and superficial nerve branches running over the distal third of the muscle that are cutaneous branches of the medial plantar nerve.

The preparation should now resemble the one shown in Figure 1A.

Stimulate and record EPPs

8. Connect a suction electrode (mounted on a coarse micromanipulator) to the nerve. Aspirate the tibial nerve into the pipet tip and apply a brief (0.2 msec) maximal (nominally 10 V) stimulating pulse to the nerve.

The muscle should twitch. If the intention is then to record action potentials, the next step can be skipped. However, if the aim is to perform a quantal analysis the muscle action potential and the twitch must first be blocked.

9. Expel the nerve from the suction pipet. Incubate the muscle in a small volume of physiological saline containing 2 μM μ-conotoxin GIIIB for 20 to 30 min.

This should be sufficient to block the NaV1.5 channels in the muscle fibers while leaving presynaptic action potentials and evoked release of transmitter unaffected.

10. Transfer the isolated nerve-muscle preparation to a recording chamber. If using gas-equilibrated, bicarbonate-buffered physiological saline, ensure that the chamber is perfused with this solution at a rate of about 1 ml/min in order to maintain the pH.

If the experiments are to be conducted at ambient temperature (up to 25°C), as assumed here, no perfusion should be necessary when using thoroughly oxygenated or aerated HEPES-buffered physiological saline.

11. Pull glass microelectrodes from 1-mm glass capillary containing an internal welded glass filament. Back-fill the electrodes (take care not to touch or break the tips) with 4 M potassium acetate using a syringe whose tip has previously been drawn out after warming the plastic in a Bunsen flame.
Figure 1  Isolated tibial nerve/FDB muscle preparation and intracellular recordings of EPPs. (A) Typical appearance of a satisfactory working dissection of the FDB muscle and its attached tibial nerve supply. The tendon of origin at the heel (top) and the three distal tendons are pinned to a Sylgard-lined dish with minutien pins. (B) Superimposed digital sweeps during nerve stimulation at about 1/sec from an approximate location in the muscle indicated in A. Dotted lines and arrows show properties of the EPP that are routinely measured, for instance, using WinWCP software. The recording was made after blocking muscle fiber action potentials using μ-conotoxin GIIIB.
The dimensions and shape of the electrode tips should confer electrical resistances (or impedances) of about 30 MΩ, which can be checked using a built-in current source in most microelectrode amplifier systems.

12. Connect the filled electrode to the high-impedance headstage of a suitable amplifier, for example an Axoclamp 2B, mounted on a micromanipulator. Micromanipulate its tip into the solution bathing the isolated FDB. Ground the solution in the recording chamber with a silver-silver chloride wire or pellet. Adjust the DC offset on the amplifier to read zero millivolts and check the electrode resistance. Connect other shielding wires from manipulators, chamber holder, microscope stand, and other equipment to a common earthing point, to reduce or abolish ‘ground loops.’

A ‘Humbug’ (Digitimer) mains interference filter is useful and usually effective for eliminating any residual 50-Hz noise (60 Hz in the U.S.A.) if attempts to minimize earth loops by grounding to a common point are only partially successful. Low-pass (< 2 kHz) filters (for example, Neurolog, Digitimer) are also important for optimizing the fidelity of recordings with minimal attenuation of the biological signals, while removing other types of high-frequency noise.

13. Aspirate the tibial nerve into the suction pipet/electrode connected to the isolated stimulator.

The timing of the stimulus pulses should be triggered with an appropriate device: for example, a Digitimer 4030 Programmer connected to a DS2 stimulator (Digitimer). Set rectangular pulse duration to 0.2 msec and stimulating voltage to between 1 and 10 V. Stimulation frequency should initially be 0.5 to 1 Hz.

14. Use a dissecting microscope and micromanipulator to bring the microelectrode tip into the vicinity of a muscle fiber. Monitor the voltage at the microelectrode tip simultaneously using one channel with direct coupling (DC) at low gain (20 mV/cm on a digital oscilloscope) and another channel in parallel with alternating coupling (AC) at higher gain (1 mV/cm or higher).

15. Use the micromanipulator to lower the tip of the electrode to just above the surface of the preparation while illuminating the preparation through a dark-field condenser or an appropriately-angled fiber-optic light source to help visualize the approximate location of the electrode tip. Gently tap the micromanipulator while carefully manipulating the microelectrode tip onto the surface of a muscle fiber—if the electrode tip is sharp enough, whiplash-penetration of a fiber will register a clean step to around −70 mV on the DC recording. Note this resting membrane potential.

Spontaneous MEPPs should be visible on the AC recording, provided the resting potential is more negative than about −45 mV. These will occur unpredictably at a mean frequency between about 0.2-2.0/sec.

16. Trigger the recording display device (oscilloscope or computer running WinWCP; see step 17) and apply a stimulus to the nerve (0.2 m pulse duration; nominally 1- to 5-V pulse amplitude), delaying the stimulus pulse for about 5 msec.

An action potential may be observed if the dissection is good and the resting potential is more negative than about −60 mV. The muscle may contract and dislodge the microelectrode, causing subsequent loss of resting potential. If μ-conotoxin has been administered correctly, however, the recording should be stable and response should be a large (>20 mV) EPP. The muscle should not contract. Repetitive stimulation at 1/second should evoke EPPs resembling those shown superimposed in Figure 1B. These repeated responses may be quite uniform in amplitude if the quantal content is high, but fluctuate more in amplitude from stimulus to stimulus if the quantal content is relatively low.
Perform quantal analysis

17. Capture trains of EPPs using a computer running a public-domain software package, free to academic users, called WinWCP (Strathclyde Electrophysiology Software; (http://spider.science.strath.ac.uk/sipbs/showPage.php?page=software)).

A suitable A/D interface is required, such as the CED micro 1401 (Cambridge Electronic Designs). Analysis of stored data can be performed offline for measurement mean amplitude, rise time, and time to half decay of the EPPs as their principal characteristics. Expect EPP rise times to be 1 to 2 msec; amplitudes to be in excess of 20 mV, and half-decay times on the order of 2 to 4 msec.

18. Carry out a quantal analysis using the ‘variance method’ built into WinWCP.

This method is based on the Poisson equation. Quantal content, m, is given by the reciprocal of the square of the coefficient of variation of EPP amplitudes, after correction for nonlinear summation (see Commentary). Check EPPs for drift using the facility in WinWCP to graph EPP amplitude against stimulus number. Quantal analysis should only be applied to EPPs that fluctuate around a stationary mean. Using the last 10 to 30 EPPs in a train may be sufficient to meet this criterion.

The software routines in WinWCP will calculate the quantal content of EPPs after correction for nonlinear summation according to an empirically derived formula based on voltage-clamp experiments (McLachlan and Martin, 1981; see Commentary under Equation 2). For relatively long muscle fibers such as those in classical preparations like EDL, soleus, diaphragm, or triangularis sterni, the value of 0.8 is about appropriate for f. For FDB muscle fibers, however, enter an “f” factor of 0.3 to 0.4. The mean amplitudes of MEPPs (either measured or estimated by eye) can be entered to compare the “direct” calculation of quantal content with the variance method. Quantal contents of around 50 are typical for mouse FDB muscle fibers.

The variability in the EPP amplitude can be increased, with a concomitant reduction in mean amplitude, by exchanging the physiological saline for one in which Mg2+ concentration is increased to 3 to 4 mM and Ca2+ concentration is reduced to 1 mM. Under such conditions, the mean quantal content, m, may be reduced to about 3 or less, and either the direct method or the method of failures may be used to estimate m (see Commentary).

Analyze MEPPs

If there is interest only in recording spontaneous transmitter release, then resting frequency of MEPPs can be increased if desired, by increasing the temperature of the bathing solution from ambient to 32°C to 37°C, although be aware that as the temperature rises to within the physiological range, more effective perfusion with oxygenated saline may be required, to counteract hypoxia in the motor nerve terminals. Alternatively, e.g., at room temperature, MEPP frequency can be elevated by increasing the K+ ion concentration in the bathing medium to 10 to 15 mM. Also expect depolarization of the resting membrane potential of the muscle fibers to about −50 mV, with this level of elevated potassium ions (for an elegant simulator of the effects of changing the concentration of sodium, potassium and chloride ions on the resting potential, see http://www.nernstgoldman.physiology.arizona.edu/). Adding lanthanum chloride (2 μM) also elevates basal MEPP frequency (Curtis et al., 1986; Ribchester et al., 1998).

19. Appraise the stochastic nature of occurrences of MEPPs by counting the number of MEPPs in successive time intervals (sweeps) of 1- to 10-sec duration.

The distribution of frequencies (0,1,2,...; etc.) per sweep normally conforms to a Poisson distribution:

\[ P(x) = \frac{e^{-m}m^x}{x!} \]

Equation 1
where \( P(x) \) is the observed frequency of \((x)\) MEPPs per sweep, and \( m \) is the mean frequency per sweep. The observed and predicted distributions may be compared statistically using a \( c^2 \) test.

20. Derive and measure properties of spontaneous MEPPs, including their amplitude, rise time, decay time, mean frequency, and interval distribution, offline, from data files of continuous records using a commercial software program for PCs called Mini Analysis (Synaptosoft).

The program incorporates data-conversion software for several industry-standard hardware interfaces and data file formats, including Axon Instruments and CED1401 series of interfaces.

**REAGENTS AND SOLUTIONS**

*Use deionized, distilled water in all recipes and protocol steps.*

**Physiological saline solution**

*For 1 liter:*

- 40 ml 3 M NaCl
- 5 ml 1 M KCl
- 10 ml 0.2 M CaCl\(_2\)
- 5 ml 0.2 M MgCl\(_2\)
- 2 ml 0.2 M Na\(_2\)HPO\(_4\)
- 938 ml H\(_2\)O.

Mix and add 2 g NaHCO\(_3\) and 1 g D-glucose, then bubble through tubing or an aspirator with a 95% O\(_2\)/5%CO\(_2\) gas mix for at least 10 min before use.

As an alternative, omit NaH\(_2\)PO\(_4\) and NaHCO\(_3\) and instead add HEPES to a final concentration of 5 mM, and adjust pH to 7.2 to 7.4. Bubble with either air or 100% O\(_2\).

**Suction electrode**

This electrode can be manufactured from 2 mm capillary glass, narrowed in a Bunsen flame, cut with a serrated blade or diamond knife, and flame-polished to a tip diameter about 50% larger than the diameter of the tibial nerve at its tip. This pipet should then be fitted with an internal silver or platinum wire and a similar but longer, external wire coiled around the capillary glass to its tip. Connect the wires via appropriate plugs to an isolated stimulator and connect a small syringe, via silicone tubing, to the back of the suction electrode/pipet. Mount the whole assembly onto a coarse micromanipulator.

**Sylgard-lined Petri dishes**

Using the Dow Corning Sylgard 184 silicone elastomer kit, mix 1 ml of silicone elastomer curing agent with 9 ml of silicone elastomer base and pour into a 60 × 15-mm plastic petri dish. Leave on a level surface to cure (may take 2 to 3 days to cure at room temperature). Alternatively place in a 37°C oven overnight.

**COMMENTARY**

**Background Information**

Intracellular recording of synaptic potentials using glass microelectrodes connected to high-impedance amplifiers was introduced in the late 1940s and energetically applied to the analysis of chemical synaptic transmission at neuromuscular junctions since the early 1950s, initially by B. Katz and his colleagues at University College London. Their electrophysiological analysis of synaptic transmission at neuromuscular junctions in frog skeletal muscle quickly established the “quantal hypothesis” of synaptic transmission. Subsequent morphological analysis of early transmission electron micrographs translated this concept to a “vesicle
mainly influx of Na current is the result of the net flux of cations, initiating a transient, net inward current. This ligand-gated ion channels transiently open, to postsynaptic acetylcholine receptors. These few microseconds, a proportion of them bind diffuse across the synaptic cleft and, within a ("quantum") of transmitter molecules. These of SNARE proteins, releases a discrete bolus through voltage and Ca-dependent interaction with the presynaptic terminal membrane, acetylcholine. Thus, fusion of a single vesicle in a motor nerve terminal stores 5,000 to 10,000 molecules of the neurotransmitter acetylcholine. Thus, fusion of a single vesicle with the presynaptic terminal membrane, through voltage and Ca-dependent interaction of SNARE proteins, releases a discrete bolus ("quantum") of transmitter molecules. These diffuse across the synaptic cleft and, within a few microseconds, a proportion of them bind to postsynaptic acetylcholine receptors. These ligand-gated ion channels transiently open, initiating a transient, net inward current. This current is the result of the net flux of cations, mainly influx of Na\(^+\) and efflux of K\(^+\) ions through the transmembrane pores formed by five subunits of ACh receptors (2a, 1b, 1g/e and 1d). The summed activation of the set of ACh receptors/channels bound by the ACh molecules released from one synaptic vesicle produces a transient inward current, called the miniature endplate current (MEPC). This generates a transient depolarization, \(\sim 1 \text{ mV}\) in amplitude, with a rise time of about 1 msec and a 50% decay time of about 2.5 msec, called the miniature end-plate potential (MEPP). MEPPs can be quite easily recorded using sharp-tipped (<1 \(\mu\)m diameter) glass microelectrodes filled with concentrated electrolyte (typically 3 M KCl or 4 M potassium acetate) connected via a high-impedance headstage, through high-pass and low-pass filters, amplified and displayed either on an oscilloscope screen or digitized through an A/D converter into a personal computer.

**Miniature end-plate potentials and quantal size**

MEPPs occur intermittently and unpredictably in resting muscle fibers (without nerve excitation) at a frequency averaging about 1/second (Fig. 2A). The intervals between MEPPs vary randomly and follow an exponential distribution, analogous to the random decay of radioactive nuclides (Figure 2B). They have a time course very similar to that of the EPP but are considerably smaller in amplitude, varying from just greater than the noise level to about 2 to 4 mV (Figure 2C,D). Occasionally, ‘giant’ MEPPs greater than 5 mV may be observed. Measurement of the mean amplitude of a series of spontaneous MEPPs (excluding ‘giants’) establishes the “quantal size” for that neuromuscular junction (Slater, 2008).

The overall incidence of MEPPs can be increased by tonic depolarization of nerve terminals, most simply by elevating extracellular potassium ion concentration, although the disadvantage of this method is that the amplitude of MEPPs is reduced, because muscle fiber depolarization also reduces the inward “driving force” on the permeant ions that carry positive ionic current. A judicious combination of modestly elevated K\(^+\) (10 to 15 mM) plus a low concentration of lanthanum ions (La\(^{3+}\), 2 \(\mu\)M) can produce an enhancement of MEPP frequency without substantially compromising their amplitude (Ribchester et al., 1998).

**Evoked end-plate potentials and quantal content**

Nerve excitation, as indicated above, transiently elevates intracellular Ca\(^{2+}\) and, within about a millisecond, this promotes fusion of many docked vesicles at active zones (Nagwane et al., 2009). The result is exocytotic fusion of several tens of vesicles with the presynaptic membrane, releasing their contents into the synaptic cleft within microseconds. The resulting multi-quantal bolus of ACh activates a multiple of the receptors activated by the contents of a single vesicle. How many vesicles fuse as a result of excitation of the nerve terminal depends on the Ca\(^{2+}\) influx, mainly through voltage-gated P/Q type Ca-channels, opened by the presynaptic action potential (Urbano et al., 2002).
The number of vesicles undergoing nerve-evoked exocytosis is called the "quantal content" of the evoked endplate current (EPC) or corresponding endplate potential (EPP). Normally, an EPP is substantially larger in amplitude than that required to trigger an action potential in the muscle fiber. Thus, in order to observe the EPP, unadulterated by the regenerative effects of voltage-activated Na or K channels, it is necessary to suppress the postsynaptic, muscle-fiber action potential. In mammalian muscle this can most effectively be achieved using the marine snail toxin, μ-conotoxin GIIB at concentrations of 1 to 2 μM (Braga et al., 1992; Wood and Slater, 1997; Gillingwater et al., 2002). At this concentration, the toxin binds reversibly to the Nav1.5 isoform of voltage-gated Na channel normally present in skeletal muscle fiber membranes, while leaving the presynaptic action content.
potential unscathed, since this arises from Na-channels that are much less sensitive to μ-conotoxin GIIIB. A classical, but inferior alternative method is to partially block the postsynaptic response with curariform drugs such as d-tubocurarine. However, this method also reduces the amplitude of the MEPPs, rendering accurate measurements of quantal size virtually impossible. A third method is the simple expedient of cutting the ends of the muscle fibers, leaving the endplates intact (Auerbach and Betz, 1971; Harris and Ribchester, 1979a,b). This causes a large depolarization of the muscle fibers (down to about −30 mV or less), inactivating voltage-gated Na channels and thus blocking muscle fiber action potentials. However, because of the reduced voltage driving force, the MEPPs are also normally obscured by the baseline noise in the recordings, which again makes accurate measurement of quantal size unfeasible.

**Nonlinear summation of EPPs**

Unfortunately, it is not possible to ascertain the number of vesicles released by a nerve stimulus, under normal physiological conditions, simply by dividing the amplitude of the evoked EPP by the mean amplitude of the MEPP (the so-called ‘direct method’). This is because quantal components sum nonlinearly—that is, the depolarization (i.e., the EPP) caused by instantaneous action of the contents of, say, 50 vesicles on ACh receptors is much less than 50 times the depolarization caused by a single vesicle, each of which alone would produce a depolarization equivalent to a MEPP. To illustrate this, suppose the average amplitude of a series of MEPPs recorded at an NMJ is 1 mV. An EPP generated by two vesicles would produce an EPP of about 2 mV. But an EPP with a quantal content of 50 will not depolarize the endplate by 50 mV; it is more likely to be about 30 to 40 mV (depending on several other properties including the resting potential and other intrinsic biophysical properties of the muscle fiber). Nonlinear summation arises principally because the effect of ACh is to gate the nons-elective cationic permeability of the ion channel that is integral to the receptor. Thus, in the theoretical limit, an infinite amount of ACh (or an infinitely high quantal content) could only produce depolarization to the net equilibrium potential (“reversal potential,” or “transmitter null potential”) for the permeant ions. This theoretical limiting membrane potential is about −5 mV (inside negative). Equations have been derived that compensate for nonlinear summation before estimating quantal content, but none is entirely satisfactory, and the best way to avoid this problem altogether is to voltage-clamp the endplate, normally using a two-electrode method, and to measure miniature and evoked synaptic currents, which sum linearly (McLachlan and Martin, 1981; Wood and Slater, 1997; Thyagarajan et al., 2010). However, this technique is more difficult than single-microelectrode recording, limiting the number of recordings in any one session, and, thus, the yield of data is correspondingly smaller. McLachlan and Martin’s formula for correcting an observed mean EPP amplitude, \( v \), for nonlinear summation is:

\[
v' = \frac{v}{1 - f v/E_m - E_v - E_m}
\]

**Equation 2**

where \( E_v \) is the ACh null potential, \( E_m \) is the resting membrane potential, and \( f \) is the ‘fudge’ factor necessary to align the endplate current with the EPP as a function of transmitter released.

One way to avoid the problem of nonlinear summation altogether is to reduce the amount of transmitter released by a presynaptic action potential (i.e., quantal content) to small multiples of the quantal size. This is most simply achieved by reducing the extracellular Ca\(^{2+}\) concentration below its normal physiological level of 2 mM (e.g., to 1 mM) or increasing the extracellular Mg\(^{2+}\) from its normal level of 1 mM (e.g., to 4 mM), or both. Under these conditions, the probabilistic nature of exocytosis can be observed from the marked fluctuation of EPP amplitudes—from no response (‘failure’) to quantal multiples up to 10 times the mean MEPP amplitude. As originally calculated by Katz and his colleagues, the distribution of EPP amplitudes (and therefore, quantal contents) should resemble a binomial distribution:

\[
P(x) = \frac{n!}{x!(n-x)!} p^x q^{n-x}
\]

**Equation 3**

where the quantities \( n, p, \) and \( q (=1-p) \) represent number of quanta (or release sites) available for release and their individual release probabilities. Both quantities represent unknowns that cannot be measured independently. However, in the limiting case where probability of observing individual components of the EPP is low and these components occur independently of one another, then
the binomial distribution reduces to a Poisson distribution:

\[ P(x) = \frac{e^{-m} m^x}{x!} \]

**Equation 4**

where the number of quantal components of any given EPP, \( x \), can take any integer value or zero, and \( m \) is the mean quantal content. This equation can be exploited in various ways in practice to estimate quantal content. For instance, the method of failures is based on a straightforward count of the number of times there is no response (‘failure’) during a series of test stimuli. No amplitude measurements on either EPPs or MEPPs are necessary because, according to the Poisson equation, when \( x = 0 \):

\[ P(0) = \exp(-m) \]

**Equation 5**

and, since \( P(0) \) is simply the ratio of failures to tests, taking natural logarithms of both sides of the equation and re-arranging for \( m \) yields:

\[ m = \ln(\text{Tests}/\text{Failures}) \]

**Equation 6**

This is usually in very good agreement with estimates made directly by dividing the mean EPP amplitude by the mean MEPP amplitude. So, for instance, if the mean MEPP amplitude is 1 mV and the mean EPP amplitude 3.5 mV, then 3.5 would be the mean quantal content. From the above formula, this mean quantal content would predict that there should be about three ‘failures’ in a train of responses to 100 stimuli. Put the other way, if there were three failures in response to 100 stimuli, then, according to the above formula, the mean quantal content would be 3.51.

Another method based takes advantage of the equivalence of mean and variance in the Poisson distribution and hence the variance method for calculating mean quantal content from measurements of the amplitudes of a train of EPPs:

\[ m = \left( \frac{\bar{v}}{s_v} \right)^2 \]

**Equation 7**

where \( \bar{v} \) is the mean EPP amplitude and \( s_v \) the standard deviation.

Like the method of failures, one benefit of the variance method is that it is unnecessary to have accurate (or any) measurements of MEPP amplitude.

The above methods for calculating quantal content are somewhat oversimplified, although adequate for most comparative purposes: for instance, the formulae given do not take account of high frequency stochastic fluctuations (membrane or electrode ‘noise’) in the recordings. Care should also be taken when applying this method since the assumptions underlying the applicability of a Poisson distribution may not be valid under normal physiological conditions of transmitter release. For a more advanced discussion, see for example Byrne and Roberts (2009) or monographs and review articles on these issues (Hubbard et al., 1969; Christensen and Martin, 1970; Johnson and Wernig, 1971; Bennett and Robinson, 1990; Cooper et al., 1995; Clements and Silver, 2000; Silver, 2003).

**Neuromuscular ‘safety factor’ and synaptic homeostasis**

Estimates of MEPP frequency, amplitude (quantal size), and EPP amplitude (and quantal content) are useful for estimating the ‘safety factor’ for neuromuscular transmission: that is, the excess of neurotransmitter released (or quantal content) over that required to depolarize the muscle fiber sufficiently for action potential generation and muscle contraction. Normally, at least in rodent muscle, the safety factor is about 3 to 5. In conditions where either neurotransmitter is reduced (such as the Lambert-Eaton Myasthenic Syndrome; or mild botulism) or the sensitivity to neurotransmitter is compromised (as in classical myasthenia gravis; or ‘curarization’ during surgical anesthesia), then the safety factor for neuromuscular transmission may be reduced by a significant margin, producing muscle weakness or flaccid paralysis (Wood and Slater, 1997; Slater, 2008). Under some chronic conditions of this nature, poorly understood ‘homeostatic’ mechanisms are activated to restore the safety factor, either by up-regulating post-synaptic sensitivity (quantal size) or by up-regulating quantal content (Plomp et al., 1994, 1995; Plomp and Molemaar, 1996). Conversely, but only demonstrated convincingly so far by genetic manipulations in *Drosophila*, up-regulation of one facet of synaptic transmission (e.g., quantal size) in some instances causes a compensatory down-regulation of the complementary quantity (i.e., quantal content), maintaining the overall amplitude of the evoked voltage response, that is, the EPP.
amplitude (Frank et al., 2006; Bergquist et al., 2010).

Another important factor influencing the magnitude of endplate depolarization is the ‘input resistance’ of the muscle fiber, which is determined by its dimensions, myoplasmic electrical impedance, and passive membrane ionic permeability. Other things being equal, relatively large diameter muscle fibers have a lower input resistance than relatively small diameter muscle fibers. Assuming that the number of molecules of ACh in one presynaptic vesicle is largely independent of the target muscle fiber size (or input resistance), then the endplate current for a given quantal content will be the same on small- or large-diameter muscle fibers (but see Wilkinson et al., 1992). However, by Ohm’s law, the depolarizations (i.e., MEPPs) will depend on the input resistance (\(V = IR\)), so the MEPPs recorded from large-diameter muscle fibers will be smaller in mean amplitude than those recorded from a more slender muscle fiber that has a higher input resistance. Again, there is evidence that homeostatic mechanisms bring about comparability in the mean size of the evoked response. This is at least partly achieved by proportional variation in the area of synaptic contact; motor terminals on large diameter muscle fibers occupy a proportionally larger area of the muscle fiber surface at the endplate than those on smaller diameter fibers. Since the quantal content of EPPs is determined partly by the density and number of active zones, then the larger the area of synaptic contact, the greater the quantal content of the EPPs. Large endplates are found on relatively large-diameter fibers, and so these generally have a higher quantal content than neuromuscular junctions of smaller area typically found on small-diameter fibers. This relationship therefore compensates for the inverse effect of fiber diameter on quantal size and serves to maintain the average amplitude of the EPP, and therefore the safety factor for neuromuscular transmission, independently of muscle fiber size (Harris and Ribchester, 1979a,b; Ribchester et al., 2004; Slater, 2008).

The packing density and length of the postsynaptic folds at NMJ also affects the safety factor for neuromuscular transmission by altering the current density in the synaptic cleft (Martin, 1994; Wood and Slater, 1997). A high packing density of ACh receptors at the crests of the junctional folds and the voltage-gated Na-channels that normally occupy the crypts of the folds thus bring about a higher safety factor for transmission for a given area of synaptic contact and muscle fiber input resistance. This enables, for instance, the characteristically small NMJs of some human muscle fibers (which have a low quantal content, but a high junctional fold density) to nonetheless sustain an adequate safety factor for neuromuscular transmission, with no overt signs of muscle weakness (Slater et al., 1992, 2006).

**Termination of ACh action**

The molecular effects of ACh on their receptors are either intercepted or terminated by the action of the hydrolytic enzyme acetylcholinesterase (AChE), embedded in the synaptic basal lamina (Massoulie and Millard, 2009). The affinity and potency of this enzyme ensures that only about 50% of the molecules released into the synaptic cleft cross the synaptic basal lamina to binding sites on their postsynaptic receptors. Unbound ACh molecules that detach from receptors are swiftly hydrolyzed as well. In the presence of inhibitors of AChE, termination of transmitter efficacy is accomplished by a slower process of diffusion of ACh from the synaptic cleft. The main discernible indicator of this is the prolonged time course of repolarization during the EPP (Magleby and Terrar, 1975).

**Troubleshooting**

1. **Problem:** The skin does not strip easily from the foot at the outset of dissection.
   
   This is common in mice older than about 4 months; or in younger mice if the skin is tugged too brusquely. The skin can be dissected away surgically if the stripping method does not work.

2. **Problem:** The isolated FDB muscle does not contract in response to nerve stimulation.
   
   This can arise if the nerve is stretched or damaged, blocking nerve conduction; or if there has been an error in the making-up of the physiological saline; or if the stimulator is not working (check the batteries and make sure that it is switched on); or the triggering device has not been set up correctly; or the wires are not connected correctly to the stimulator; or there is insufficient fluid contacting the internal and/or external wires of the suction electrode; or the stimulating voltage is insufficient in magnitude or duration.

3. **Problem:** The microelectrode signal cannot be adjusted to zero before fiber penetration.
   
   Check that the bath ground and the microelectrode tip are in the bathing medium, and that the amplifier is turned on. Sometimes this

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3. Making microelectrodes and recording muscle action potentials with μ-conotoxin GIIIB. Figure 2 shows typical spontaneous MEPPs and evoked responses with reduced extracellular Ca\(^{2+}\) ions and elevated Mg\(^{2+}\) ions, indicating typical quantized fluctuations and ‘failures.’

4. Offline analysis of quantal size and quantal content from 30 fibers recorded in one muscle may take 1 to 4 hr.

5. Problem: The resting membrane potential is initially quite high (about −65 mV) but then drifts steadily to less negative values.

This may occur when the electrode is too blunt to produce a clean penetration of the target muscle fiber, or significant amounts of chloride ion are leaking from the micropipet tip into the muscle cytoplasm. Check the tip resistance of the microelectrode (it should be about 10 M\(\Omega\) or higher). Change the microelectrode if the problem persists with successive fiber impalements, or change the electrolyte used to fill the electrodes from 3 M KCl to 4 M potassium acetate.

6. Problem: There are no MEPPs.

This can arise if the temperature is low (MEPP frequency is temperature sensitive), or if the resting membrane potentials are very low (less negative than −40 mV), or if there is an acetylcholine receptor blocker, such as d-tubocurarine or α-bungarotoxin present, or if there was some pathological feature of the mouse from which the dissected muscle was obtained.

7. Problem: There are no evoked responses to nerve stimulation, or the evoked responses are much smaller than expected.

This may arise if the electrode has not in fact penetrated the muscle fiber (bending of the electrode tip can produce a spurious voltage deflection that resembles membrane penetration), or if the Ca\(^{2+}/Mg\(^{2+}\) ratio in the bathing fluid is too low, or if the nerve is not responding to stimulation (see point 2, above), or if there was some pathological feature of the mouse from which the dissected muscle was obtained.

8. Problem: Quantal analysis gives a large disparity between failures, variance, and direct methods for estimating quantal content.

This arises if the analysis of quantal content does not take account of the requirement for a stationary mean value around which the random quantal fluctuations vary (check the graph of amplitude against record number), or an incorrect entry has been made for MEPP amplitude, or the value entered for the nonlinear summation ‘fudge’ factor (\(f\)) is incorrect or inappropriate, or if the membrane noise levels exceed the quantal variations in the evoked response, or if uniquantal (MEPP) responses are buried in noise, leading to overestimates of the number of ‘failures’; it may also be the result of some pathological feature of the mouse from which the dissected muscle was obtained.

**Anticipated Results**

A photomicrograph of an isolated FDB muscle and representative recordings of EPPs and MEPPs are shown in Figures 1 and 2. Figure 1 shows an isolated FDB muscle and typical records of several EPPs superimposed, produced by repetitive stimulation after blocking muscle action potentials with μ-conotoxin GIIIB. Figure 2 shows typical spontaneous MEPPs and evoked responses with reduced extracellular Ca\(^{2+}\) ions and elevated Mg\(^{2+}\) ions, indicating typical quantized fluctuations and ‘failures.’

**Time Considerations**

1. Making solutions may take about 30 min.
2. Dissection time by a skilled operator, from killing of the mouse to mounting an FDB nerve-muscle preparation in a recording chamber, can be as little as 15 to 30 min.
3. Making microelectrodes and recording from thirty muscle fibers sufficient for a quantal analysis may take 1 to 4 hr.
4. Offline analysis of quantal size and quantal content from 30 fibers recorded in one muscle may take 1 to 4 hr.
Acknowledgments

The University of Edinburgh is a charitable body, registered in Scotland, with registration number SC005336.

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