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SYMPOSIUM REVIEW

Organization and function of transmitter release sites at the neuromuscular junction

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Abstract The neuromuscular junction is known as a strong and reliable synapse. It is strong because it releases an excess of chemical transmitter, beyond what is required to bring the postsynaptic muscle cell to threshold. Because the synapse can sustain suprathreshold muscle activation during short trains of action potentials, it is also reliable. The presynaptic mechanisms that lead to reliability during short trains of activity have only recently been elucidated. It appears that there are relatively few calcium channels in individual active zones, that channels open with a low probability during action potential stimulation and that even if channels open the resulting calcium flux only rarely triggers vesicle fusion. Thus, each synaptic vesicle may only associate with a small number of calcium channels, forming an unreliable single vesicle release site. Strength and reliability of the neuromuscular junction emerge as a result of its assembly from thousands of these unreliable single vesicle release sites. Hence, these synapses are strong while at the same time only releasing a small subset of available docked vesicles during each action potential, thus conserving transmitter release resources. This prevents significant depression during short trains of action potential activity and confers reliability.

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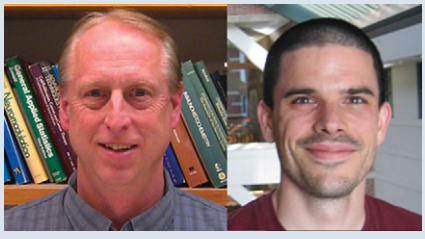
Abbreviations: AP, action potential; MCell, Monte Carlo Cell.

Introduction

As 'giant' synapses go, the neuromuscular junction (NMJ) is one that has been extensively studied for over 70 years (Feng, 1941; Katz, 1969). The size and peripheral accessibility of this model synapse have permitted detailed investigation of many conserved synaptic mechanisms (for review, see Grinnell, 1995), and provided the framework for many subsequent studies at CNS synapses (for review, see Bekkers, 1994). Two features of neuromuscular

Stephen Meriney (left) received his PhD in Physiology/Neuroscience from the University of Connecticut with Guillermo Pilar. He then moved to the Jerry Lewis Neuromuscular Research Centre at UCLA to perform postdoctoral work with Alan Grinnell where he studied synaptic mechanisms at the frog neuromuscular junction. He is currently Professor of Neuroscience and Psychiatry at the University of Pittsburgh. **Markus Dittrich** (right) received his PhD in Physics with Klaus Schulten from the University of Illinois at Urbana-Champaign. He began working with computer models of neuromuscular synapses as part of his postdoctoral work with Joel Stiles at the Pittsburgh Supercomputing Center. He is currently the Director of the National Resource for Biomedical Supercomputing at the Pittsburgh Supercomputing Center and Carnegie Mellon University. For the past 12 years, the Meriney and Stiles/Dittrich laboratories have collaborated to study presynaptic mechanisms at the neuromuscular junction.



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synapses that are critical to their function are strength and reliability. Nervous system-evoked patterned activation of specific skeletal muscles needs to be strong and reliable to allow efficient and consistent movement of body parts in a finely tuned manner. NMJs are strong because they release a large amount of chemical transmitter that is usually more than sufficient to excite postsynaptic muscle cells and lead to their contraction (reviewed by Wood & Slater, 2001). Of course, the NMJ of a given muscle may need to be repeatedly activated during sustained contractions or repetitive movements. The presynaptic mechanisms that contribute to the reliable activation of the NMJ during repeated stimulation have recently been studied at the frog NMJ using a combination of electrophysiology, calcium (Ca^{2+}) imaging and computer modelling. These studies have led to the hypothesis that transmitter release at the NMJ is controlled by the assembly and function of thousands of unreliable single vesicle release sites (Tarr *et al.* 2013), similar to what has previously been called the 'synaptosecretosome' (O'Connor *et al.* 1993; Bennett, 1996).

The frog NMJ as a model synapse

We have known for many years that the frog NMJ has hundreds of presynaptic transmitter release sites (about 700 'active zones'). Each active zone is characterized by two rows of 20–40 docked synaptic vesicles positioned laterally to 200–250 intramembraneous particles (as identified in freeze-fracture replicas and interpreted to represent presynaptic transmembrane proteins) arranged in a precisely organized, long linear array of two parallel double rows (Heuser *et al.* 1974, 1979; Pawson *et al.* 1998; Harlow *et al.* 2001; see Fig. 1*B*). Therefore, the entire frog NMJ may have a total of 14,000–28,000 docked vesicles ready for release from these 700 active zones. However, following single action potential (AP) stimulation, only about 350 vesicles are released (Katz & Miledi, 1979). In other words, on average each of the 700 active zones has only one synaptic vesicle fusion event following every other AP stimulation. Until recently, it was not clear why the transmitter release probability from active zones in the frog NMJ was so low. Ca^{2+} imaging at this synapse revealed significant variability in Ca^{2+} entry during repeated low-frequency stimulation (Wachman *et al.* 2004). Using an analysis of variability in Ca^{2+} entry into these active zones during single AP stimuli at low frequency, Luo *et al.* (2011) provided evidence that there were relatively few Ca^{2+} channels in these active zones (about 30–50). This represents only a small fraction of the active zone proteins thought to make up the double rows of particles identified in freeze-fracture studies (Pumplin *et al.* 1981). Interestingly, this number of Ca^{2+} channels roughly matches the number of synaptic vesicles thought to be docked at active zones. Furthermore, Luo *et al.* (2011) predicted that the probability of a Ca^{2+} channel opening during a single presynaptic AP was very low (about 0.2). Thus, this study showed that during a single AP only about 6–10 Ca^{2+} channels open in each long linear active zone. Considering that on average only a single vesicle is released following every other stimulus, it follows from these recent studies that there must be a very low probability of vesicle fusion following the opening of a Ca^{2+} channel within these active zones. In the rare instances when Ca^{2+} entry does trigger vesicle fusion, it appears that the flux through one or only very few open channels is responsible (Yoshikami *et al.* 1989; Shahrezaei *et al.* 2006).

To aid in the interpretation of these data, and to advance new hypotheses that could be tested experimentally, Dittrich and colleagues (Ma *et al.* 2010, 2011; Dittrich *et al.* 2013) developed a detailed computer model of a frog NMJ active zone and investigated it using spatially realistic Monte-Carlo diffusion–reaction simulations via the computer program MCell (Monte Carlo Cell; Kerr *et al.* 2008). Their model included a realistic active zone geometry (Fig. 1*C*) with synaptic vesicles, voltage-gated Ca^{2+} channels, Ca^{2+} buffer and Ca^{2+} sensor sites on synaptic vesicles representing synaptotagmin molecules (Chapman, 2002). Upon stimulation with an AP waveform, voltage-gated Ca^{2+} channels opened stochastically and allowed Ca^{2+} ions to enter the presynaptic space. Ca^{2+} ions then diffused within the terminal, bound to Ca^{2+} buffer and/or vesicle-associated Ca^{2+} sensor sites (synaptotagmin), and eventually triggered vesicle fusion and subsequent release of neurotransmitter.

Using the availability of extensive experimental data (based on years of NMJ study), Dittrich *et al.* (2013) were able to tightly constrain their model, making it both quantitative and predictive in nature. They could then use their model to test experimental hypotheses and gain detailed insight into biological events inaccessible to experimental study, enabling the design of new experiments. This modelling approach was also important in the validation of a recent single-pixel optical fluctuation analysis study of presynaptic Ca^{2+} transients (Luo *et al.* 2011). Recently, this synergistic use of computer modelling and experimental techniques allowed Dittrich *et al.* (2013) to predict the Ca^{2+} binding stoichiometry and dynamics that underlie transmitter release from the frog NMJ. They showed that in the presence of an excess of Ca^{2+} binding sites (modeled after recent data on synaptotagmin copy number), only some of which needed to bind Ca^{2+} to trigger vesicle fusion, their model accurately predicted normal synaptic physiology. In the future, such a model can be used to provide novel insight not only into the nature of Ca^{2+}-triggered synaptic vesicle release, but also into many other currently open questions regarding synaptic structure and function. For example, as these MCell simulations allow the investigator to follow the diffusion of individual Ca^{2+} ions within the modelled

presynaptic space, it becomes straightforward to predict how many Ca^{2+} ions from each open voltage-gated Ca^{2+} channel contribute to the release of a given synaptic vesicle. This, in turn, enables the study of nanodomain *versus* microdomain coupling of Ca^{2+} channels to vesicle release. Simulation results for the frog NMJ active zone have already shown the highly localized nature of the interaction of Ca^{2+} ions from voltage-gated Ca^{2+} channels with nearby binding sites on vesicles (nanodomain coupling; Ma *et al.* 2011; Dittrich *et al.* 2013).

Taken together, the frog NMJ, a classic model system that has been used for over 70 years, continues to serve as a powerful tool with which to elucidate mechanistic details of chemical synaptic transmission. Recent work described above, which combines results from experimental and modelling work, provides significant evidence that active zones at the frog NMJ are assembled from thousands of unreliable single vesicle release sites that each consist of a single vesicle and a small number of closely associated voltage-gated Ca^{2+} channels.

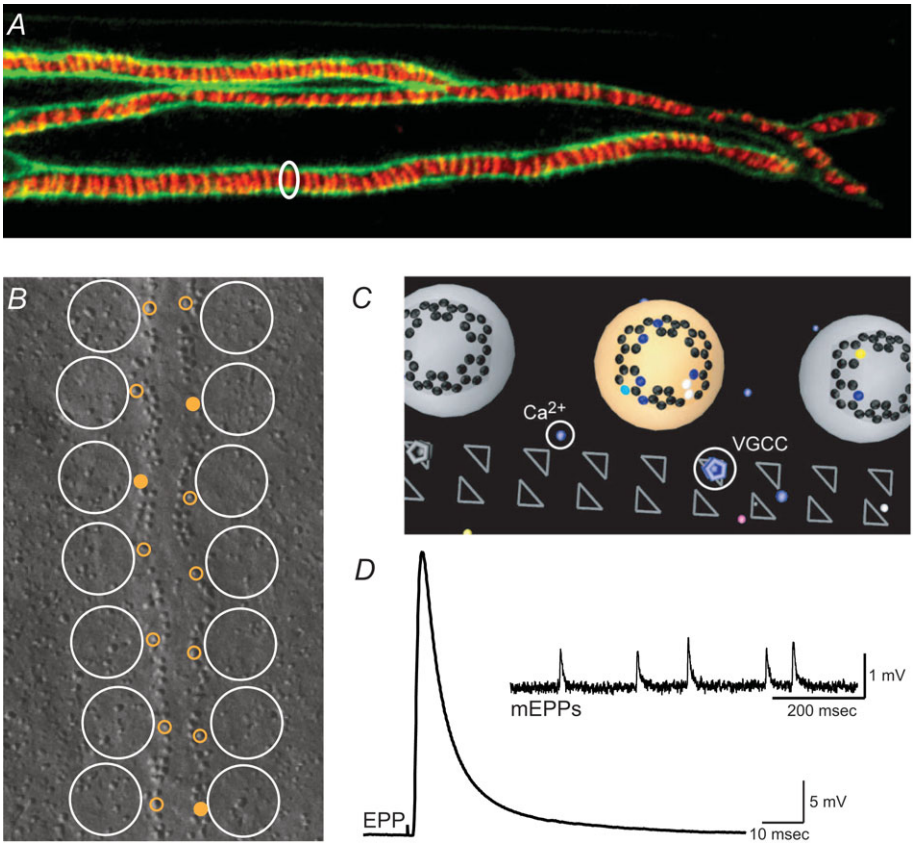

Figure 1. Structure and function of the frog neuromuscular junction
A, portion of a large frog neuromuscular junction stained using FITC-labelled peanut lectin to decorate Schwann cell extracellular matrix (green) and Alexa594-labelled α-bungarotoxin to identify the location of postsynaptic receptor folds (red) immediately opposite presynaptic active zones. A single acetylcholine receptor band that represents the predicted position of one active zone is circled. Image obtained by S.D.M. following the protocols of Ko (1987) and Reddy *et al.* (2003). *B*, graphical depiction of the spatial organization of docked synaptic vesicles (large white circles) and presynaptic Ca^{2+} channels (small yellow circles; filled circles represent the fraction of channels that open on average during an AP stimulus) overlaid onto a freeze-fracture replica of about half of a frog neuromuscular junction active zone (Heuser *et al.* 1979). Graphic adapted from Luo *et al.* (2011). *C*, graphical representation of a small portion of the MCell computer model of the frog neuromuscular junction active zone (bottom view). In this graphic, synaptic vesicles are large grey or yellow spheres, synaptotagmin binding sites are represented as an array of black dots at the base of synaptic vesicles (binding sites with bound Ca^{2+} are coloured according to the Ca^{2+} channel contributing the ion), triangles represent the position of presynaptic active zone proteins (some of which are occupied by voltage-gated Ca^{2+} channels; VGCC), and coloured dots represent Ca^{2+} ions (Ca^{2+}), colour coded based on the voltage-gated Ca^{2+} channel of origin. *D*, endplate potential (EPP) recorded from a single frog neuromuscular junction following exposure to 4 μM μ-conotoxin PIIIA to block selectively postsynaptic sodium channels (average of 10 sweeps). *Inset*, spontaneous miniature endplate potentials (mEPPs) recorded in the absence of nerve stimulation. Data in *D* obtained by S.D.M. from the cutaneous pectoris nerve-muscle preparation following the protocol of Shon *et al.* (1998).

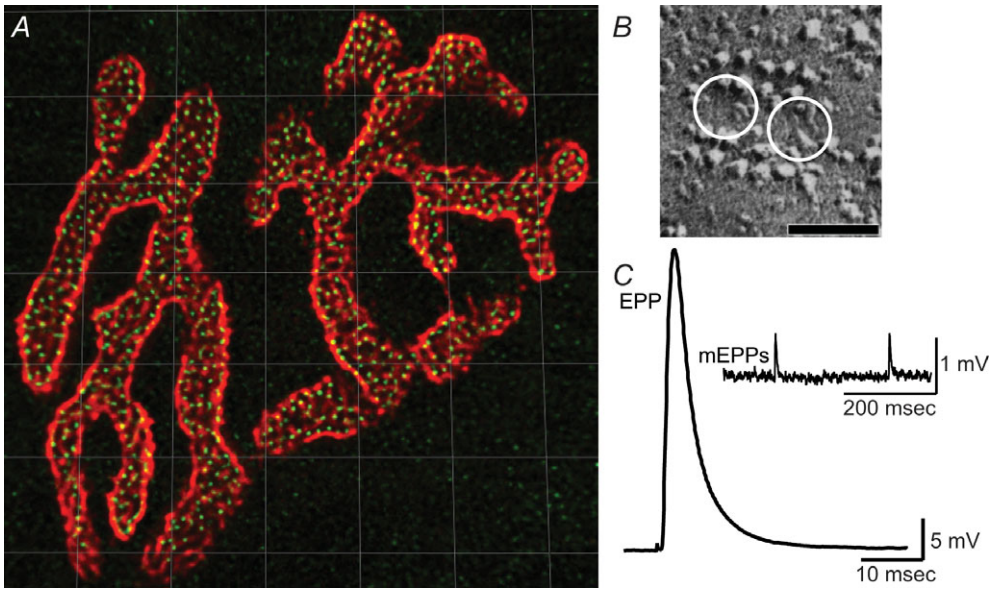

Figure 2. Structure and function of the mouse neuromuscular junction
A, an entire single mouse neuromuscular junction stained with Alexa 594 α-bungarotoxin to label postsynaptic acetylcholine receptors (red), and Alexa 488 bassoon antibody to label presynaptic active zones (green spots). Confocal brightest projection image obtained by S.D.M. following the protocol of Nishimune *et al.* (2004), and processed for deconvolution. Grid lines = 5 μm. *B*, freeze-fracture replica of a single mouse neuromuscular junction active zone. White circles represent the predicted position of docked synaptic vesicles. Adapted from Nagwaney *et al.* (2009). Scale bar = 50 nm. *C*, average endplate potential (EPP) recorded from a single mouse neuromuscular junction following exposure to 1 μM μ-conotoxin GIIIB to block selectively postsynaptic sodium channels (average of 10 sweeps). *Inset*, spontaneous miniature endplate potentials (mEPPs) recorded in the absence of nerve stimulation. Data in *C* obtained by S.D.M. from the epitrochleoanconeus nerve-muscle preparation following the protocols of Urbano *et al.* (2003) and Rogozhin *et al.* (2008).

The mammalian NMJ

Recent evidence from the study of mammalian NMJs suggests that these synapses are also constructed using unreliable single vesicle release sites. In contrast to frog, active zones at the mouse NMJ contain only very *short* linear arrays (100–200 nm long) of presynaptic transmembrane proteins (Fig. 2*B*; Nagwaney *et al.* 2009). These short active zones are tightly associated with about two synaptic vesicles that are positioned *between* active zone

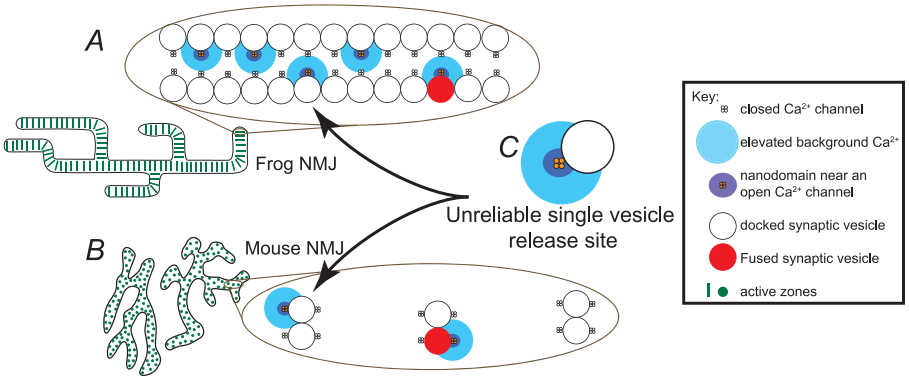

Figure 3. Organization of neuromuscular junction active zones based on assembly of unreliable single vesicle release sites
A, each of hundreds of active zones in the frog neuromuscular junction (NMJ) is hypothesized to be constructed using a long linear double array of unreliable single vesicle release sites (*C*). *B*, each of hundreds of active zones in the mouse neuromuscular junction are separated from one another by about 500 nm, and are hypothesized to be constructed using a short linear array of only two unreliable single vesicle release sites. *C*, the basic building block of neuromuscular junctions is hypothesized to be an unreliable single vesicle release site. Graphic adapted from Tarr *et al.* (2013).

proteins (as opposed to along the lateral edges of active zones in frog NMJs; Nagwaney *et al.* 2009; see Fig. 2*B*). The large pretzel-shaped mouse NMJs have been shown to contain 600–800 of these small active zones, evenly spaced about 500 nm from one another (Chen *et al.* 2012; Fig. 2*A*). Because each active zone has been shown to have an average of two docked synaptic vesicles, the entire mouse NMJ may have 1200–1600 docked vesicles ready for release during an AP stimulus (Nagwaney *et al.* 2009). Despite the large number of available vesicle release sites, following a single AP stimulation, only about 60–80 vesicles are released (Wang *et al.* 2004; Ruiz *et al.* 2011). Thus, on average these large synapses only release transmitter from about 10% of available active zones with each AP.

Interestingly, when Wang *et al.* (2010) used a statistical approach, based on variability in postsynaptic currents at the mouse NMJ, to estimate the probability of release and number of release sites, they predicted a probability of release of 0.6–0.9 from a total of 50–70 release sites (when measured at 1–2 mM extracellular Ca^{2+}). Given the large number of total available active zones (600–800) at this synapse, these data imply that only a small subset of active zones contributes to vesicle release during stimulation while the others are inactive. This conclusion was supported by initial studies using pH-sensitive probes for vesicle fusion that revealed a heterogeneous distribution of areas of nerve terminal that participate in releasing transmitter at the mouse NMJ during low-frequency stimulation (Tabares *et al.* 2007; Wyatt & Balice-Gordon, 2008). Together, these data lead to the intriguing conclusion that only a subset of available vesicle release sites are used. One confound of this conclusion is that the consistent and restricted use of a small number of available release sites would lead to significant short-term depression even during short trains of AP activity, which is not seen experimentally.

Alternatively, each mouse active zone may be constructed using unreliable single vesicle release sites that contain a small number of presynaptic Ca^{2+} channels, each with a low probability of opening, as was shown at the frog NMJ (Luo *et al.* 2011). Constructing mammalian NMJs using a large number of spatially distributed release sites, each with a low probability of releasing a synaptic vesicle during single AP activity, is also consistent with previous anatomical and electrophysiological data (Wang *et al.* 2004; Ruiz *et al.* 2011). Under this scenario, the specific active zones that participate in vesicle fusion at the mouse NMJ during each AP vary randomly, determined by the stochastic nature of presynaptic Ca^{2+} channel gating. In preliminary computer modelling using MCell, Ma *et al.* (2010) found that assembly of mouse active zones using single vesicle release sites designed based on frog data resulted in a mouse active zone model in good agreement with experimental results. If one considers the expected low probability that Ca^{2+} flux through a single Ca^{2+} channel would trigger vesicle fusion, one would predict that during any given AP only a small subset of mouse active zones would experience Ca^{2+}-triggered vesicle fusion. Further analysis of Ca^{2+}-triggered vesicle fusion at the mammalian NMJ using tools with increased sensitivity, in combination with more advanced computer models, will be required to determine the sub-active zone mechanisms that control transmitter release at this large synapse.

Summary

Combining experimental and computational approaches, there is significant evidence for the hypothesis that the strength and reliability of frog and mouse NMJs are due to the assembly of large numbers of unreliable single vesicle release sites (Fig. 3; Tarr *et al.* 2013). Reliability is derived from the low probability of transmitter release from any given site (of which there are thousands), thus ensuring a dependable supply of releasable vesicles under sustained activity. On the other hand, synaptic strength is achieved by assembling sufficiently large numbers of single vesicle release sites into NMJs to bring the postsynaptic muscle cell to threshold in response to a single AP.

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Additional information

Competing interests

None.

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