A Quantitative Description of the Interaction of Enhancement and Depression of Transmitter Release at the Neuromuscular Junction

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A QUANTITATIVE DESCRIPTION OF THE INTERACTION OF ENHANCEMENT AND DEPRESSION OF TRANSMITTER RELEASE AT THE NEUROMUSCULAR JUNCTION

By

Alice M. Holohean

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A QUANTITATIVE DESCRIPTION OF THE INTERACTION OF ENHANCEMENT AND DEPRESSION OF TRANSMITTER RELEASE AT THE NEUROMUSCULAR JUNCTION

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Synaptic transmission alters the strength of the postsynaptic potential, through a process called short-term synaptic plasticity (STP). In this study, endplate potentials (EPPs) from the frog neuromuscular junction were used to resolve and quantify the presynaptic components involved in enhancement and depression of transmitter release during repetitive stimulation under normal quantal release conditions (2 mM Ca$^{2+}$, 1mM Mg$^{2+}$). During trains of stimulation given between 10 - 200 Hz, the amplitude of the EPPs first increased then decreased; a maximum increase of 77% was produced after 2-4 stimuli. EPP amplitudes began to increase at ~ 20 Hz, were maximal at ~ 55 Hz, and thereafter, decreased as the rate of stimulation increased. The integrated total release after 25 stimuli was little changed across frequencies between 10 – 100 Hz. EPPs ran down in two phases: a fast phase, attributed to the depletion of a readily releasable pool (RRP) of synaptic vesicles, followed by a slow phase, attributed to the depletion of vesicles from a depot pool (DP). Depletion of the readily releasable pool of synaptic vesicles (RRP) was determined by quantifying release under the fast and slow time rundown and subtracting the number of vesicles associated with mobilization to the RRP from the total number of vesicles released during stimulation trains of 50 impulses. Impulses were delivered at 12 different rates ranging from 50 to 200 /s. Estimates of the number of vesicles released
from the RRP increased with frequency of stimulation until maximal depletion levels of 5500 - 6000 vesicles were reached at stimulation rates between 90-130/s, assuming a control quantal content of 200 vesicles released per impulse. Depletion was less at lower frequencies when the number of stimuli delivered was identical. When the RRP maximally depleted, release was inversely related to stimulation rate, as would be expected if mobilization from the depot pool was the sole determinate of release during the slow phase. An equation constructed from four known components of enhancement and two components of depression - the depletion of vesicles from a readily releasable pool (RRP) and from the depot pool (DP) that refills the RRP, was used to fit and then simulate EPPs obtained during trains using different patterns of stimulation and varying amounts of extracellular Ca^{2+}; the decay time constant parameters of enhancement, numerically derived from the observed data, were fixed at $\tau \sim 46, 220, 1600, \text{ and } 20000$ ms. The number of components of enhancement necessary to approximate the data decreased, from four in low (0.14 - 0.2mM) extracellular Ca^{2+}, to one ($\tau \sim 46$ ms) in 2.0 mM extracellular Ca^{2+}, but four components of enhancement were necessary to fit the data when the amplitude of the EPP was not depressed below the control amplitude. This model was able to predict within ~3 % EPP amplitudes over a 10-fold range of frequency and Ca^{2+} concentration.
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CHAPTER 1. OVERVIEW OF THE PROBLEM

INTRODUCTION

Chemical synaptic transmission (Langley, 1907) at the neuromuscular junction (NMJ) is characterized by stimulation-induced enhancement and depression of endplate potential (EPP) amplitudes (Betz, 1970; Del Castillo and Katz, 1954; Dittman et al., 2000; Dobrunz and Stevens, 1997; Elmqvist and Quastel, 1965; Feng, 1941; Katz and Miledi, 1968; Kusano and Landau, 1975; Liley and North, 1953; Magleby, 1973; Magleby and Zengel, 1982; Mallart and Martin, 1968; Takeuchi, 1958; for reviews see Magleby, 1987; Regehr and Stevens, 2001; Zucker and Regehr, 2002). Short-term changes in synaptic strength have been hypothesized to be the result of separate processes occurring simultaneously during a train of stimulation that alter the amount of evoked transmitter release (Magleby and Zengel, 1982; Stevens and Wesseling, 1999a; Zucker and Regehr, 2002). Evidence that multiple independent components in the presynaptic terminal contribute to stimulation-induced alterations in the amount of transmitter released has been provided by experiments using kinetic and pharmacological approaches to isolate these factors (Atluri and Regehr, 1996; Brain and Bennett, 1995, 1997; David et al., 1997; Lin et al., 1996, 1998; Regehr et al., 1994; reviewed by Magleby, 1987; Regehr and Stevens, 2001; Rettig and Neher, 2002; Zucker and Regehr, 2002). Under non-physiological conditions of low quantal release, four components of enhancement have been identified and quantitatively defined (Magleby and Zengel, 1982; Zengel et al., 1980; Zengel and Magleby, 1977, 1981). However, under normal quantal release conditions and dependent on the frequency of stimulation, depression can dominate the post-synaptic response during a train of impulses (Betz, 1970; Del Castillo
and Katz, 1954; Dobrunz et al., 1997; Dobrunz and Stevens, 1997b; Hubbard, 1963; Liley and North, 1953; Rosenthal, 1969; Stevens and Wang, 1995; Takeuchi, 1958; Thies, 1965; Wu and Betz, 1998). My study examines the interaction of factors producing enhancement and depression at the frog NMJ where the release and recovery of synaptic vesicles has been well studied (reviewed by Rizzoli and Betz, 2005; van der Kloot and Molgó, 1994), and the components of enhancement have been isolated, described and quantified (Bennett et al., 2007; Magleby and Zengel, 1982; Mallart and Martin, 1968; Takeuchi, 1958; Zengel et al., 1980; Zengel and Magleby, 1977, 1981). The aim of this work is to account for depression under physiological conditions using a realistic model of synaptic release.

A quantitative model previously described that accounts for transmitter release in low quantal conditions (Magleby and Zengel, 1982) is expanded to include depression, incorporated as a depletion of synaptic vesicles during repetitive stimulation. The model includes four pharmacologically identified components of enhancement with different kinetic properties (Magleby; 1987; Zucker and Regehr, 2002), referred to as the first and second components of facilitation, F1 and F2, augmentation (A), and potentiation (P). Stimulation patterns are designed to resolve the fast components of enhancement (F1 and F2) and are applied under various [Ca^{2+}]_o conditions to evoke changes in the amount of quantal release that include components of depression. The goal of this work is to gain insight into the mechanisms of short-term synaptic enhancement and depression under physiological conditions of normal quantal release.

Current knowledge regarding evoked synaptic release is first reviewed with an emphasis on the frog NMJ in order to understand possible underlying mechanisms.
involved in short-term plasticity. This review is followed by examples of short-term synaptic enhancement and depression and a mathematical description of the hypothesis tested in this thesis. It is found that by expanding a quantitative model that accounts for enhancement of quantal release in low quantal release conditions, to include the depletion of vesicles from a readily releasable pool (RRP) refilled by mobilization of vesicles from a depot pool (DP), then enhancement and depression can be accurately predicted for data obtained over a wide range of Ca$^{2+}$ concentration and stimulation frequency.

SYNAPTIC RELEASE

Evoked synaptic transmitter release with an emphasis on the frog neuromuscular junction

Evoked synaptic transmitter release at the frog NMJ occurs when Ca$^{2+}$ entering voltage sensitive Ca$^{2+}$ channels drives the near synchronous release of ~200 quanta in ~0.8 ms. The coordination of multiple factors such as Ca$^{2+}$ channel activation, the amount of Ca$^{2+}$ entering the terminal, the location of Ca$^{2+}$ buffers, the rate of the Ca$^{2+}$ transient, the quantal content of a vesicle, the activation of proteins involved in vesicle docking, priming and release, the number of synaptic vesicles available for release, and the rate of recycling and/or docking and priming of reused or new vesicles for release all contribute to transmitter release that generates the EPP. A change in any of these processes could occur during repetitive stimulation, resulting in short-term synaptic enhancement or depression. This section reviews the mechanisms involved in evoked transmitter release.

Evoked release is synchronous

The number of quanta released per impulse per unit area of presynaptic membrane at the NMJ is relatively constant across species (for stimuli given at 1 Hz, 0.15 – 0.30 quanta/μm$^2$, Slater et al., 1992; Wood and Slater, 1997; reviewed in Wood and Slater,
An action potential depolarizing the motor nerve terminal releases ~ 200 vesicles at the frog NMJ. In mammals this value is lower, ~ 50 to 100 in rats and ~ 20 to 50 in humans (reviewed by Wood and Slater, 2001). The relationship between quantal transmitter release, the probability of release, and the number of available quanta for release has been expressed by del Castillo and Katz (1954) with the equation:

\[ m = pq \]

where \( m \) is the average number of quanta released, \( p \) is the average release probability and \( q \) is the number of available quanta (Katz, 1969).

**Nerve stimulation activates release through Ca\(^{2+}\) entry into the nerve terminal**

Axon depolarization activates multiple voltage-gated processes in the NMJ nerve terminal. Two of these mechanisms have been implicated in evoked synaptic vesicle release: Metabotropic acetylcholine (ACh) autoreceptor conformation changes from a high affinity to a low affinity state (Ben-Chaim et al., 2006; Parnas and Parnas, 2007), and presynaptic Ca\(^{2+}\) channels open, resulting in an influx of Ca\(^{2+}\) into the terminal (Borst and Sakmann, 1996; Schneggenburger and Neher, 2000). The time between the action potential and the maximal Ca\(^{2+}\) current is ~ 0.3 ms (Meinrenken et al., 2003).

Two mechanisms have been proposed to explain the synchronous release of synaptic vesicles upon depolarization; neither theory is exclusive of the other. The high affinity voltage sensitive ACh metabotropic autoreceptor has been hypothesized to act as a lock that prevents docked and primed vesicles from asynchronous release (Ben-Chaim et al., 2006; Khanin et al., 2006). Depolarization is the key that alters the conformation
of the autoreceptor from a high affinity state to a low affinity state, thereby allowing vesicles to be released simultaneously by the influx of Ca$^{2+}$.

Alternatively, a saturable, cooperative Ca$^{2+}$ sensor with 5 binding sites and high affinity (Kd ~ 5 μM with saturation at 10 μM; (Wölfel and Schneggenburger, 2003)) has been proposed to be sufficient for activated release within a short (temperature and preparation dependent) delay of 50-500 μS. Evidence for this hypothesis is shown by an increase in presynaptic capacitance (a measure of vesicle fusion) during a brief Ca$^{2+}$ transient produced by photolysis of caged Ca$^{2+}$ (measured in the Calyx of Held). Fusion is detected at ~ 1-2 μM [Ca$^{2+}$]$_i$ and simulated evoked release at 5 μM [Ca$^{2+}$]. Saturation occurs at 20 μM [Ca$^{2+}$]. Gaussian Ca$^{2+}$ transients from the flash last 400 μS – 500 μS. The rapid decay reflects the large Ca$^{2+}$ buffering capacity of the terminal. (Bollmann et al, 2000; Meinrenken et al., 2003; Schneggenburger and Neher, 2000; reviewed by Sudhof, 2004). This model of evoked release requires multiple Ca$^{2+}$ buffers and uptake and extrusion systems including a fixed endogenous buffer (of which there are many including synaptotagmin, BK channels, and docking proteins such as Munc), a diffusible buffer (eg. ATP with a lower limit of 1.0 mM available for Ca$^{2+}$ binding), and Ca$^{2+}$ uptake (mitochondria) and extrusion (Na/Ca$^{2+}$ exchangers) systems (also see Regehr, 1997; Suzuki et al., 2002).

**Synchronous release is driven by Ca$^{2+}$**

A rise in presynaptic intraterminal Ca$^{2+}$ activates an as yet unclear number of presynaptic proteins that result in an increase in the probability of synaptic vesicle fusion with the presynaptic terminal membrane (Augustine et al., 1985; reviewed by Augustine, 2001; Augustine et al., 1987; Sudhöf, 2004; Zucker, 1989; Zucker and Regehr, 2002).
The synaptic vesicle and cytoplasmic membrane and presynaptic cytoplasm contain proteins involved in release (reviewed by Sudhöf, 2004). The synaptic vesicle membrane has an average diameter of ~ 50 nm in the frog (van der Kloot et al., 2002) and the membrane of the synaptic vesicle can hold ~ 200 protein molecules (Jahn and Sudhöf, 1993; also see van der Kloot, 2003), a portion of which are accounted for by transport proteins such as the proton pump (80 kD) that provides the energy to drive neurotransmitter uptake into the vesicle through the transmitter transporter (Forgac, 1999).

**Synchronous release is controlled release**

SNARE (derived from soluble N-ethylmalamide-sensitive factor attachment receptor) proteins provide the necessary machinery for vesicle fusion. A core SNARE complex is formed by four α-helixes contributed by three SNAREs: three α- helixes from presynaptic membrane proteins, one α- helix from syntaxin (anchored to the presynaptic membrane by the C-terminal domain), two α- helixes from SNAP-25 (anchored to the presynaptic membrane through several cysteine-linked palmitoyl chains), and one α-helix from the vesicle membrane protein synaptobrevin (anchored by a C-terminal domain in the synaptic vesicle membrane).

The metastable SNARE complex overcomes the repulsive forces between the vesicle and presynaptic membranes and is reversibly stabilized by a soluble protein called complexin (Chen et al, 2002; Giraudo et al., 2006). Complexin binds in an antiparallel α-helical groove between the synaptobrevin and syntaxin helices to stabilize the SNARE complex.
In knockout preparations, the loss of complexin dramatically reduces exocytosis (Reim et al., 2001), whereas the loss of a vesicle protein called synaptotagmin increases spontaneous fusion events (Pang et al, 2006), leading to the suggestion that synaptotagmin acts as a clamp on SNARE complexes stabilized by complexin. Ca\(^{2+}\) bound synaptotagmin unclamps complexin from the metastable SNARE complex and vesicle fusion occurs (Pang et al., 2006; Tang et al., 2006). Rapid fusion takes place when Ca\(^{2+}\) binds to the Ca\(^{2+}\) sensor synaptotagmin anchored to the vesicle membrane (Sorensen et al., 2003). Ca\(^{2+}\) triggered fusion pore opening takes place in 0.5 ms (measured in the Calyx of Held, Borst et al., 1995; Borst and Sakmann, 1998).

**The synapse has Ca\(^{2+}\) sensors**

Synaptotagmin is a Ca\(^{2+}\) sensor for fast synaptic transmission (Augustine, 2001; Brose et al., 1992; Geppert and Sudhof, 1998; Perin et al., 1990, 1991a, 1991b) but is not required for Ca\(^{2+}\)-independent release, as synaptotagmin knockout mice lack synchronous but not asynchronous release (Geppert et al., 1994, 1997). Two adjacent motifs in synaptotagmin, C2A and C2B, with five conserved aspartate residues apiece, coordinate Ca\(^{2+}\) (Shao et al., 1996, 1998; Sutton et al., 1995; Ubach et al., 1998). When bound with Ca\(^{2+}\), synaptotagmin can bind to negatively charged phospholipids (Bai et al., 2004; Li et al., 1995; Mackler et al., 2002; Zhang et al., 1998) and other SNARE proteins (Bennett et al., 1992; Chapman et al., 1995). Ca\(^{2+}\) also promotes oligomerization of synaptotagmin (Bai et al., 2004; Sugita et al., 1996). Ca\(^{2+}\) sensitive synaptotagmins contain two Ca\(^{2+}\)- and phospholipid- binding domains (with a total of 5 Ca\(^{2+}\) binding sites) that act cooperatively, such that binding of Ca\(^{2+}\) to one site raises the binding affinity to the other sites. The apparent Ca\(^{2+}\) binding affinity for the double C2A – C2B domains in
synaptotagmin-1 is 4.4 ± 0.5 μM (Nagy et al., 2006), with a 2-fold increase in the order of Ca$^{2+}$ dependence if the C2A domain is mutated (Sorensen et al, 2003; also see Stevens and Sullivan, 2003).

There are 13 different isoforms of synaptotagmin, of which 6 can bind Ca$^{2+}$ (Bhalia et al., 2005; Li et al., 1995; Sugita et al., 2002). Synchronous release can be mediated by 3 of these isoforms – synpatotagmin-1, -2, and -9, all of which have mixed anatomical locations and distinct kinetic properties in triggering synaptic vesicle release (Geppart et al., 1994, Xu et al., 2007). Synaptotagmin-1 is thought to be evolutionarily conserved and is functional in *Drosophila* NMJs (Mackler et al., 2002; Yoshihara and Littleton, 2002). Synaptotagmin-1 and -2 have similar Ca$^{2+}$ affinities and Ca$^{2+}$ cooperativity (Nagy et al., 2006), whereas synaptotagmin-9 has lower affinity for Ca$^{2+}$ (Shin et al., 2004). Millar et al. (2005) suggest that differences in the release characteristics at crayfish tonic and phasic synapses, where the size of the readily releasable pool is inversely related to the release rate, is due to differing Ca$^{2+}$ sensitive release sensors such as synaptotagmin.

**Evoked release is quantal and can be measured postsynaptically**

Synaptic vesicles contain quantal packets of approximately 6,000 – 10,000 transmitter molecules. Given an interior diameter of 42 nm, one vesicle contains ~ 0.5 M ACh in the frog (van der Kloot et al., 2002). When fused to the membrane, a quantal packet of transmitter is released into the synaptic cleft (Katz and Miledi, 1965a; reviewed by van der Kloot and Molgó, 1994). The relationship between quantal transmitter release (m), the probability of release (p), and the number of vesicles available for release (N) is given by $m = pN$. 

Evoked release is quantal and can be measured postsynaptically
A spontaneously released quantal packet of transmitter from a single synaptic vesicle in the presynaptic terminal of the nerve gives rise to a miniature endplate potential (mEPP), produced by opening non-selective cation ACh channels, in the postsynaptic terminal on the muscle membrane. The size of the mEPP is determined by the number of channels activated by transmitter-bound postsynaptic receptors, the duration of binding and activation, the input resistance and capacitance of the muscle fiber, and the resting membrane potential of the muscle (Fatz and Katz, 1951). A single presynaptic action potential produces ~ 200 mEPPs, which sum to generate the extracellular endplate potential (EPP) of the NMJ (Miledi and Thies, 1971).

The time course of evoked release varies with temperature (Q_{10} > 4, Barrett and Stevens, 1972) but not with extracellular Ca^{2+} concentration or with the number of vesicles released (Barrett and Stevens, 1972; Katz and Miledi, 1965b; Meinrenken et al., 2002). This implies that all vesicles that are released by an action potential are docked, primed and ready to be released and that increasing Ca^{2+} increases the number of vesicles released in parallel. Estimates of the latency of vesicle release for the frog synapse following a stimulus, derived by deconvolution of points on an average intracellularly recorded end plate current (EPC), where an EPC produced by single vesicle quantal content (mEPC) is represented by an instantaneous peak with an exponential decay, give peak quantal release at 1.4 ms, decaying with a time constant of 1.2 ms at 10°C (Cohen et al., 1981; van der Kloot and Molgó, 1994). The average time course of vesicle fusion and release is relatively invariant and the time course of an excitatory post synaptic current (EPSC) is stable even when the number of released quanta is reduced more than 00-fold (Borst and Sakmann, 1996; Isaacson and Walmesley 1995; van der Kloot, 1988).
The role of pools of synaptic vesicles

Birks and MacIntosh (1961) first postulated separate pools of ACh, a surplus pool and a depot pool in series with a “readily releasable” pool, to explain the decrease in the amount of ACh that remains in a cat ganglion after prolonged stimulation (for the early development of these models in the synapse see Betz, 1970; Elmqvist and Quastel, 1965). In bipolar cells of goldfish, which do not have action potentials, stimulation produces three kinetic components of membrane dye FM 1-43 fluorescence (this styryl dye provides a measure of increased membrane in the presynaptic terminal and, hence, exocytosis); a fast phase of ~ 1500 vesicles released in 10 ms, a slower phase of 4400 vesicles released over 1 sec, and a continuous phase of 1010 vesicles / s (Neves and Lagnado, 1999). Three pools of vesicles have been postulated: a readily releasable pool (RRP) that is docked and primed, a slowly releasable or recycling pool and a reserve pool (Rizzoli and Betz, 2005; Zucker and Regehr, 2002). The recycling pool and the reserve pool are differentially affected by the frequency of stimulation. Two types of release competent vesicles have been observed in the Calyx of Held: Comparatively slow-to-release vesicles (fusion rate constant of ~ 6 / s) that are thought to have loosely associated trans-SNARE complexes and fast-to-release vesicles (fusion rate constant ~ 80 / s) with tightly associated release complexes (Heinemann et al., 1993; Sakaba and Neher, 2001; Voets et al., 1999; Voets 2000).

Frequency dependent recycling of multiple pools of synaptic vesicles

Presynaptic uptake and release of vesicles at the frog NMJ, studied using styryl dyes with fast and slow partitioning properties (due to different hydrophobicities) to selectively stain vesicular membranes during endocytosis, indicate frequency of
stimulation as a factor mobilizing release from the different pools (Richards et al., 2003; Wu and Betz, 1996). Low frequency (2-5 Hz) stimulation-induced release is almost entirely from the RRP and maintained by recycling released synaptic vesicles. High frequency stimulation (30 Hz) depletes both the RRP ($\tau$ of depletion $\sim$ 5 s, with recycling refilling the pool with a $\tau$ of recovery $\sim$ 20 s) and the reserve pool ($\tau$ of depletion $\sim$ 35 s with budding from cisternae replenishing the pool with a $\tau$ $\sim$ 5 - 8 min). Different modes of recycling at the frog NMJ have been proposed previously, including local recycling (Ceccarelli et al., 1973) and clathrin vs non-coated recycling (Heuser and Reese, 1973) as well as Ca$^{2+}$ mediated recycling (Ceccarelli and Hurlbut, 1980). Multiple recycling mechanisms have been shown to be present in many systems: the snake NMJ is reported to have Ca$^{2+}$ mediated enhancement of a single recycling route (Teng et al., 2005 and see Teng et al. 2007); *Drosophila* photoreceptors appear to have multiple pools of vesicles (Koenig and Ikeda, 1996); goldfish bipolar cells have two components of membrane retrieval (von Gersdorff and Matthews, 1994); hippocampal neurons include an additional, what appears as a “kiss and run,” mechanism consisting of transient flickering fusion pores (Gandhi and Stevens, 2003); and the Calyx of Held has loosely and strongly release-coupled vesicles with multiple recycling strategies (Voets, 2000, also see Rettig and Neher, 2002). In the frog, release and re-supply appears to be sequential with the RRP exocytosed first and the reserve pool second (Richards et al., 2003). This could due to the rapid release of RRP vesicles (7-fold faster) or vesicles queuing in line at release sites (Henkel et al., 1996). Recovery from depression depends upon the rate of vesicle recycling (Pyle et al., 2000; Richards et al., 2003), indicating depression arises from depletion.
**Ca\(^{2+}\) alters vesicle recycling**

A train of stimuli in normal external Ca\(^{2+}\) can result in a decrease in the amount of transmitter released per impulse (Dobrunz et al., 1997; Dobrunz and Stevens, 1997, 1999; Takeuchi, 1958; Thies, 1965). These results have been interpreted as a depletion of the RRP (Zucker and Regehr, 2002). If this is true, then the rate of refilling the synaptic vesicle pool determines, in part, the amount of depression observed. Repetitive stimulation at 10 Hz has been shown to activate a Ca\(^{2+}\) dependent recycling mechanism in hippocampal autapses that doubles the refilling rate of a pool of vesicles released with a hypertonic solution, from a \(\tau \approx 6\) s to a \(\tau \approx 3\) s (Stevens and Wesseling, 1998). Similar results have been observed for stimuli given at low frequency after intense stimulation at the Calyx of Held, where an increase, from 10 stimuli at 100 Hz to 30 stimuli at 300 Hz, changed a single exponential recovery with a \(\tau\) of \(\sim 5\) s to a double exponential recovery with \(\tau\)'s of \(\sim 86\) ms and 3.4 s that was blocked by Cd\(^{2+}\) or EGTA (Wang and Kaczmarek, 1998). However, Stevens and Wesseling (1999a) note that recovery from depletion cannot be accurately measured using low frequency stimuli after a high frequency train of stimuli because the decay of augmentation (multiexponential fusion efficiency) is superimposed on a single exponential time constant for the recovery of the RRP. The time constant of recovery for synaptic vesicles in the snake NMJ with clathrin-dependent endocytosis decreases from 7 s to 3 s when extracellular Ca\(^{2+}\) is raised from 0 mM to 7.2 mM (Teng and Wilkinson, 2003). Raising free intracellular Ca\(^{2+}\) to 300 nM also doubles the rate of recovery of clathrin coated vesicles (Teng and Wilkinson, 2005). At the frog NMJ, for a stimulation rate of 30 Hz, the rate of endocytosis is not correlated with residual Ca\(^{2+}\) (\(\leq 1\) μM). But, vesicle pools were not isolated in this early work using
FM1-43 fluorescence and the majority of release during 30 Hz stimulation was from the reserve pool, not from the recycled pool. (Betz et al., 1992; Wu and Betz, 1998). Ca\(^{2+}\) concentrations accelerating vesicle recovery appeared to be higher (\(> 20 \mu M\)) than those for vesicle release (\(< 20 \mu M\)), depending on the synapse (reviewed by Wu, 2004). High levels of Ca\(^{2+}\) have also been shown to inhibit recycling (von Gersdorff and Matthews, 1994).

**SHORT-TERM SYNAPTIC ENHANCEMENT AND DEPRESSION**

**Enhancement of synaptic vesicle release**

The relationship between frequency of stimulation and probability of vesicle release is often studied using experimental conditions of low extracellular Ca\(^{2+}\). Under these conditions, the number of quanta released per stimulus is reduced to the extent that release is not limited by the number of synaptic vesicles available (Magleby, 1973). Four Ca\(^{2+}\)-dependent components of enhanced vesicular release have been described based on different kinetics and time constants of decay at low extracellular Ca\(^{2+}\) (Magleby and Zengel, 1982): first and second components of facilitation, F1 (\(\tau \sim 50 \text{ ms}\)) and F2 (\(\tau \sim 300 \text{ ms}\)) (Bennett et al., 1997, 2000, 2007; Elmquist and Quastel, 1965; Magleby, 1973; Mallart and Martin, 1967a; 1967b; Suzuki et al., 2002; Younkin, 1974); augmentation, A (\(\tau \sim 7 \text{ s}\)) (Erulkar and Rahaminoff, 1978; Kalkstein and Magleby, 2004; Magleby and Zengel, 1976 a, b and c; Stevens and Wesseling, 1999a); and potentiation, P (\(\tau \) of tens of seconds to minutes) (Nicoll and Malenka, 1995; Magleby and Zengel, 1975; Magleby and Zengel, 1976 b and c; Rosenthal, 1969). Stimulation-induced Ca\(^{2+}\)-dependent increases in the probability of synaptic vesicle release are thought to underlie enhancement of transmitter release (Atluri and Regehr, 1996; Brain and Bennett, 1995,
Depression of synaptic vesicle release

When Ca$^{2+}$ is raised from low levels to physiological levels, transmitter release during repetitive stimulation decreases and after stimulation recovers within seconds to minutes (Betz, 1970; Del Castillo and Katz, 1954; Hubbard, 1963; Liley and North, 1953; Takeuchi, 1958; Thies, 1965). The decrease is referred to as depression and three components have been described: ‘very fast’ ($\tau_{\text{recovery}} < 0.5$ s; Dobrunz et al., 1997; Dobrunz and Stevens, 1997b; Stevens and Wang, 1995); ‘fast’ ($\tau_{\text{recovery}} \sim 5$-6 s, Takeuchi, 1958; Wu and Betz, 1998) and ‘slow’ ($\tau_{\text{recovery}}$ of minutes; Rosenthal, 1969). Several mechanisms have been hypothesized to contribute to depression, including the release of modulatory substances, such as adenosine and phorbol esters during repetitive stimulation (Redman and Silinsky, 1994; Robitaille et al., 1999; Searl and Silinsky, 2003; but see Christensen and Martin, 1970; Lin et al., 1998), autoinhibition (Barstad, 1962), desensitization of postsynaptic receptors (Auerbach and Akk, 1998; Katz and Thesleff, 1957, Trussel and Fischback 1989; Trussel et al., 1993), the inactivation of presynaptic Ca$^{2+}$ channels during repetitive stimulation (Forsythe et al., 1998; Xu and Wu, 2005; for review Xu et al., 2007), the loss of release elements in the presynaptic terminal (Wölfel et al., 2007), the loss of synaptic vesicles available for release (del Castillo and Katz, 1954; Rosenmund and Stevens, 1996) and a reduction in the number of molecules of ACh transported into a vesicle (Debanne et al., 1996; Glavinovic, 1987, van der Kloot et al., 2002; reviewed in van der Kloot, 2003). During high frequency repetitive stimulation
lasting ~ 10 min, neither mEPC or mEPP amplitudes at the NMJ change in amplitude (Magleby and Pallotta, 1981; Zengel and Magleby, 1981; Zengel and Sosa, 1994), therefore, at these stimulation rates, there was no indication of postsynaptic receptor desensitization or a change in vesicular ACh content (reviewed by van der Kloot and Molgó, 1994). It is unlikely that Ca\(^{2+}\) channel inactivation through repeated depolarization (action potentials) is involved in depression at the NMJ because short-term repetitive stimulation patterns in low Ca\(^{2+}\) solutions produce only enhancement (Magleby and Zengel, 1982), but not depression. The loss of release elements or the release or activation of modulatory substances, such as adenosine activating autoreceptors in a negative feedback loop, have not been ruled out as a mechanism for depression (Robitaille et al., 1999; Searl and Silinsky, 2006).

**Enhancement and depression occur concurrently**

Depression and enhancement have been shown to occur concurrently (Cuttle et al., 1998; Dittman et al., 2000; Kalkstein and Magleby, 2004; Klyachko and Stevens 2006; Magleby, 1973; Varela et al., 1997; Zengel et al., 1994), but little is known quantitatively about the interaction between depression and components of enhancement during continuous stimulation at normal levels of transmitter release in physiological solutions (but see Abbott, 1994; Abbott et al., 1997; Markram and Tsodyks, 1996; Thomson and Deuchars, 1994; Varela et al., 1997). The overlapping component of fast depression has been reported to mask augmentation at the frog neuromuscular junction, as both have similar time courses (Kalkstein and Magleby, 2004; Klyachko and Stevens, 2006). Increased internal Ca\(^{2+}\), while essential for facilitation, has also been shown to accelerate recovery from depression (Sakaba & Neher, 2001).
Models describing synaptic plasticity

To describe the interaction of multiple processes involved in short-term synaptic enhancement and depression, a number of models have been developed (Abbott and Regehr, 2004; Bennett et al., 1997, 2000, 2007; Dittman et al., 2000; Hennig et al., 2006; Khanin et al., 2006; Krausz and Friesen, 1977; MacLeod et al., 1998; Magleby and Zengel, 1982; Martin, 1976; Matveev et al., 2002, 2006; Millar et al., 2005; Rettig and Neher, 2002; Sen et al., 1996; Tang et al., 2000; Trommershäuser et al., 2003; Tsodyks and Markram, 1997; Varela et al., 1997). However, none have been completely successful in predicting the observed changes in the amount of transmitter released during repetitive stimulation under different conditions of quantal release. Variables used to construct these models include: Ca$^{2+}$ channel activation and inactivation, presynaptic intraterminal Ca$^{2+}$ concentration, Ca$^{2+}$ binding sites on presynaptic proteins, Ca$^{2+}$ buffers, activation of autoreceptors, two or more vesicle pools, docked and primed sites, Ca$^{2+}$ sinks and extruders such as mitochondria, transporters and pumps, postsynaptic receptor desensitization, retrograde signals, vesicle mobilization and recycling, vesicle priming, Ca$^{2+}$ mediated priming of the release site, transmitter metabolism, and quantal content. It has also become increasingly clear that different types of synapses respond differently to repetitive stimulation and models have been developed to account for variations in responses in tonic or phasic synapses within the same organism (Millar et al., 2005).

HYPOTHESIS TESTED

The hypothesis of this dissertation is based on specific elements reviewed in the previous section and tested using experimental results and quantitative modeling (described in detail in Part 5). In sum: The nerve terminal has a large number of synaptic
vesicles, only some of which are readily available for evoked release, referred to as the readily releasable pool (RRP) of synaptic vesicles (Zucker and Regehr, 2002). Vesicles depleted from the RRP are replaced by vesicles mobilized from a larger DP and the DP itself is replenished by recycled vesicles or by the budding of new vesicles from the cisternae of the endoplasmic reticulum (Wu and Betz, 1996). Repetitive stimulation produces an increase in the probability that each release-ready vesicle is released by a nerve impulse, as reflected by an increase in the amount of quantal release. When the rate of release of synaptic vesicles from the RRP occurs faster than the rate of mobilization of new vesicles into the RRP from a depot pool (DP) of vesicles, the RRP becomes partially depleted and depression develops (Betz, 1970; Dobrunz et al., 1997, Dobrunz and Stevens, 1997; Kusano and Landau, 1975; Magleby, 1987; Takeuchi, 1958; Zucker and Regehr, 2002). Enhancement is accounted for by an increase in the probability of vesicle release from the RRP and depression is due to the inability of the rate of vesicle mobilization into the RRP to keep up with the rate of vesicle release from the RRP.

At the frog neuromuscular junction the RRP comprises ~10,000 synaptic vesicles. A single action potential in the presynaptic nerve terminal releases 200 quanta, ~ 2% of the RRP. On this basis, the average probability of releasing a vesicle in the RRP is about 0.02 for a single action potential. This can be described by

\[
\text{Release}_0 = \text{Probability}_0 \times \text{RRP}_0
\]
where, in the absence of repetitive stimulation, Release$_0$ is the number of synaptic vesicles released, Probability$_0$ is the chance of each vesicle being released, and RRP$_0$ is the initial number of vesicles in the RRP.

Repetitive stimulation produces an increase in the probability that each vesicle is released, reflected as an increase in the amount of facilitation observed. Thus, during repetitive stimulation,

\[
\text{Release}_t = \text{Probability}_t \times \text{RRP}_t \tag{2}
\]

where release$_t$, probability$_t$, and the RRP$_t$ all change with time $t$. Therefore, the ratio of EPP amplitudes during the train, EPP$_t$ to the control EPP amplitude before the train (EPP$_0$) is

\[
\frac{\text{EPP}_t}{\text{EPP}_0} = \frac{\text{Release}_t}{\text{Release}_0}
\]

\[
= \left[\frac{\text{Probability}_t}{\text{Probability}_0}\right] \times \left[\frac{\text{RRP}_t}{\text{RRP}_0}\right] \tag{3}
\]

where RRP$_t$ / RRP$_0$ is defined as the fractional amount of vesicles in the readily releasable pool.

Four components of enhancement, F1, F2, A, and P, are each defined as the fractional increase in release, such that each given component $= (\text{EPP}_t - \text{EPP}_0) / \text{EPP}_0$ when the other components and depression are zero.

Changes in Probability$_t$ of release reflect a dynamic integration of F1, F2, A, and P. On this basis, the change in probability with repetitive stimulation is given by
\[
\text{Probability}_t / \text{Probability}_0 = (F_1^* + F_2^* + 1)^n (A+1) (P+1) \quad (4)
\]

where \( n \) is the power relationship between \( \text{Ca}^{2+} \) and transmitter release (Dodge and Rahamimoff, 1967) and \( F_1^* \) and \( F_2^* \) are the residual substances that give rise to \( F_1 \) and \( F_2 \). From Eqs.1-4, the ratio of EPP amplitudes during the train to the control EPP amplitude before the train is

\[
\text{EPP}_t / \text{EPP}_0 = [(F_1^* + F_2^* + 1)^n (A+1) (P+1)] x \left[ \frac{\text{RRP}_t}{\text{RRP}_0} \right] \quad (5)
\]

After each stimulus, a depleted RRP is refilled by mobilization of vesicles from a depot pool (DP). The mobilization of vesicles into the RRP occurs between impulses and is defined by the expression

\[
\text{Mobilization}_{\text{RRP}} = \left[ \frac{\text{DP}_t}{\text{DP}_0} \right] x \left[ \text{RRP}_0 - \text{RRP}_t \right] (1 - e^{-\Delta t / \tau_{\text{RRP}}}) \quad (6)
\]

where \( \Delta t \) is the time between impulses. Vesicles mobilized into the RRP are lost from the depot pool but the depot pool can be replenished by new vesicles either by endocytosis or vesicles budding from the cisternae. Replenishment of the depot pool occurs between impulses and is defined as

\[
\text{Replenishment}_{\text{DP}} = \left[ \text{DP}_0 - \text{DP}_t \right] (1 - e^{-\Delta t / \tau_{\text{DP}}}) \quad (7)
\]

where \( \Delta t \) is the time between impulses.
Transmitter release during the train is computed using iterative numerical methods because parameters can change with time and frequency of stimulation. This working hypothesis is tested by predicting data obtained experimentally using varying stimulation patterns under different \([\text{Ca}^{2+}]_o\) conditions.

Because facilitation has a much faster time course relative to the other processes involved in synaptic plasticity, the experiments can kinetically resolve facilitation (F1 and F2) and the RRP and provide a test of proposed mechanisms for the relationship between facilitation and the RRP.
CHAPTER 2. EXPERIMENTS

PART 1. ENHANCEMENT AND DEPRESSION OF QUANTAL RELEASE DURING STIMULUS TRAINS

PART 1. METHODS

Determining the size of the EPP during a train of stimulation

The sartorious muscle from Northern grass frogs (Rana pipiens) was used throughout this study. Animals were cooled on ice to the point of non-responsive eye blink, decapitated, and then pithed according to methods approved by the University of Miami Miller School of Medicine Animal Use and Care Committee. The sartorious muscle with attached nerve and tendons was removed and stretched slightly by pinning the tendons to the bottom of a chamber filled with modified Ringer’s solution containing in mM: 115 NaCl, 2 KCl, 2 CaCl2, 1 MgCl2, 5 HEPES, 5 glucose and 0.03 choline chloride; pH was adjusted to 7.3. Experiments were performed at room temperature, which was kept within a physiological range for the frog (18-21°C). In some experiments the concentration of CaCl2 was changed as indicated in the text. To minimize the variance produced by quantal fluctuation (McLachlan, 1981; Slater et al., 1992), evoked extracellular end-plate potentials (EPPs), which correlate with averaged intracellular recordings (Magleby, 1973), were used as a measure of transmitter release. The EPP is a function of both the magnitude and time course of current through non-selective channels activated by the release of ACh and the resistance and capacitance of the muscle membrane. The rising phase of the EPP reflects the increase in the number of positive ions flowing into the muscle fiber membrane within a few millimeters of the end plate and the decay phase represents the movement of charge down the fiber, away from the endplate. Under the conditions of the experiments that follow, transmitter release
measured by either the mEPC (Magleby and Pallotta, 1981) or the mEPP (Zengel and Magleby, 1981) remain constant, indicating postsynaptic sensitivity and size of the quanta remain constant (reviewed by van der Kloot and Molgó, 1994; but see van der Kloot, 2003).

A galvanically isolated stimulation unit was used to deliver suprathreshold stimulus pulses of 0.1 msec duration to the attached nerve placed in a polyethylene suction electrode with an AgCl wire. EPPs were recorded with a fire polished glass capillary tube (~ 0.5 mm diameter) filled with Ringer’s and positioned over the muscle endplate region where the endplate potential was found to be maximal. Whereas a sharp extracellular electrode placed at the endplate measures currents, the surface electrode used in this study measures the extracellular response from many endplates and, acknowledging that the potential being measured is not the intracellular EPP, the extracellular EPP has been found to be an accurate measure of the intracellular EPP (Magleby, 1973). To stabilize the response and minimize the effect of nonlinear summation (McLachlan and Martin, 1981), muscle contraction was prevented and EPP amplitude was reduced well below threshold to < 5 – 10 mV, by the addition of the reversible nicotinic acetylcholine receptor blocker curare (~5 µM) to the Ringer’s solution.

Computer programming developed in-house (Kalkstein and Magleby, 2004), using a programming interface (DTx-EZ OLE) to a DT-3016 digital-to-analog and analog-to-digital converter (Data Translation, Marlboro, MA), were used to stimulate, display in real-time, and record evoked potentials with 16 bit resolution, which was sufficient to cover the dynamic range of the EPP amplitudes with minimal error. During
a stimulus train, the program recorded the oscilloscope trace of the potential change across time and stored the digital record. EPP peak amplitudes were calculated using a baseline from the extrapolated decay of the previous EPP. All traces were reviewed and trials containing either erratic changes in baseline, usually due to nerve failure, or post-synaptic action potentials during a stimulation train, when in a few cases EPPs reached threshold, were eliminated.

Data analysis was done using a programmed interface, developed in-house, using customized software and Igor Pro (WaveMetrics, Lake Oswego, OR). The control EPP (EPP₀) for a given stimulation trial was calculated from the average of five EPPs obtained before the train using an interstimulus interval of 5-10 s. Thereafter, EPPₜ / EPP₀ was the normalized amplitude of the EPP at time t. Where indicated, multiple trials with the same experimental conditions in the same muscle were averaged across time. An example of the method is shown on Figure 1.
Height of the EPP, is calculated using the projected tail of the previous EPP

Average first five EPPs given at 1/10s to obtain the control response $EPP_0$

Four responses at 100 Hz

Figure 1. Method used to quantify transmitter release. Open circles (o) are the proportional change of the EPPs during the train from the control EPPs ($EPP_t / EPP_0$). The inset (top right) shows the EPPs from the first 8 stimuli. The first five EPPs are the control responses averaged to obtain $EPP_0$; amplitude is shown as the red vertical line on a backwards bracket. The next three responses are the EPPs used to obtain the EPP, for EPP (10ms), EPP (20ms), and EPP (30ms). Because the preceding EPPs do not completely decay before the next stimulus occurs, the tail of the EPP is exponentially extrapolated to obtain the height (in blue) of the next EPP.

High frequency stimulation produces enhancement followed by depression

Trains of stimuli were used to obtain estimates of total transmitter release and the amount of depression at different frequencies ranging from 10 Hz to 100 Hz. Responses to 50 or 25 consecutive stimuli at 20, 25, 50, 67 and 100 Hz are shown on Figure 2.
PART 1: RESULTS

As shown in Figure 2, enhancement of the EPP increased as the frequency of stimulation increased for the first 2-4 stimuli. For stimulation rates between 10 – 100 / s the peak response per preparation occurred at stimulation rates between 50 – 67 /s with an average peak response, EPP\textsubscript{t} / EPP\textsubscript{0}, of 1.77 ± 0.165 (mean ± SEM) at 55.0 ± 12.6 ms (n = 4). Thereafter, quantal release decreased as the number of stimuli increased. The amount of depression seen at frequencies higher than 40 Hz increased with the rate of stimulation; for 25 stimuli the smallest observed mean EPP\textsubscript{t} / EPP\textsubscript{0} was 0.41 ± 0.12 (mean ± SEM, n=4) and occurred at the highest frequency of stimulation tested (100 Hz). The largest mean ratio EPP\textsubscript{t} / EPP\textsubscript{0} was 0.84 ± 0.12 and occurred at frequencies between 20 and 40 Hz. At higher frequencies, depression dominated the response and increased as the rate of stimulation increased. The amplitude of the response after 25 or 50 stimuli is

**Figure 2.** Plots of EPP\textsubscript{t} / EPP\textsubscript{0} vs time during stimulation at 20, 25, 50, 67 and 100 impulses / s, from two different muscles, for (A) 50 consecutive stimuli and (B) 25 consecutive stimuli. Responses after 1400 ms are not plotted in A.
shown in Figure 3 where an increase in depression can be seen for stimulation rates over 40 Hz.

![Figure 3. The size of $EPP_t / EPP_0$ at the 25th (blue) or 50th (red) stimulus in trains given at different frequencies (between 10-100 Hz). Closed and open symbols identify different preparations within each group.](image)

**Measurement of cumulative quantal release**

If every stimulus produced the same amount of transmitter release then with 25 and 50 stimuli the cumulative total transmitter release would be 25 and 50 times $EPP_0$, respectively. The cumulative total release normalized to the amplitude of the first EPP for 25 and 50 impulses was $28.5 \pm 1.22$ and $41.9 \pm 1.89$ (mean ± SEM), respectively. The cumulative normalized release in four different preparations is shown in Figure 4. The amount of release during these short trains increased as the stimulation rate increased until release became maximal at ~ 50 stimuli/s. Release then either decreased with increasing stimulation rate (in two preparations) or remained relatively unchanged.

**PART 1. DISCUSSION**

The results in Figure 4 show that depending on the frequency of stimulation, the cumulative quantal release during short trains varied 25 – 60%. Cumulative release was largest at stimulation rates between 30 and 50 Hz and smaller for rates faster or slower.
Depression increased with frequency > 40 Hz. This result agrees with findings at the frog NMJ where transmitter release and de-staining of vesicles loaded with FM1-43 was slower at 40 Hz than at frequencies between 2 – 30 Hz (Betz and Bewick, 1993) and at the squid giant synapse where depression occurred rapidly when the synapse was stimulated at 50 Hz (Kusano and Landau, 1975).

PART 2. QUANTAL RELEASE AFTER DEPLETION

A working hypothesis for stimulation rate-induced variations in release is that the nerve terminal has a limited number of synaptic vesicles that are readily available for release, called the readily releasable pool (RRP) (Klein and Hardin, 1975; Rettig and Neher, 2002; Rosenmund and Stevens, 1996). In a steady state of release enough time is left between stimuli so that vesicles released from the RRP can be replaced by vesicles mobilized into the RRP from a depot pool (DP) of vesicles (Stevens and Wesseling, 1998; Wesseling and Lo, 2002). If the rate of release exceeds the rate of mobilization of vesicles from the DP, ‘depression’ develops (Betz, 1970, Elmqvist and Quastel, 1965;
This concept is depicted in Figure 5.

Figure 5. In a steady state of stimulation, quantal release is limited by the rate of vesicle replacement into the readily releasable pool (RRP). (A) Diagrammatic description of the replacement of released RRP vesicles with mobilized vesicles. Previous work has shown recovery of depression due to endocytosis to have a \( \tau \sim 6 \text{ sec} \) (Wu and Betz, 1999). (B) If vesicles released are immediately replenished and the probability of release remains constant then EPP size across time would remain constant.

**PART 2. METHODS**

**Mobilization per impulse is inversely proportional to the interstimulus interval in the depleted steady state response**

If the RRP is depleted then, according to the model, the number of vesicles that are able to be mobilized from the depot pool will be determined by the amount of time between stimuli (Wesseling and Lo, 2002). A cartoon of the expected results is shown on Figure 6. If the time between stimuli is doubled then twice the number of vesicles will have time to mobilize; if the time is halved then half the number can mobilize.
Depleted RRP: Transmitter release is from mobilized vesicles

Figure 6. A cartoon of the expected result if release is dependent on mobilization after depleting the RRP. If the stimulation rate is halved, twice the time is available to mobilize vesicles into the RRP; conversely, if the stimulation rate is doubled, half the time is available for mobilization.

This model assumes that, when the RRP is depleted, the rate of mobilization is constant and independent of the stimulation rate. High frequency stimulation rates allow less time between impulses than slower rates for vesicle mobilization. Given a constant mobilization rate, the number of vesicles released will depend on the time within the interstimulus interval.

PART 2. RESULTS

An inverse relationship is observed between stimulation rate and transmitter release during the mobilization phase after depletion of the RRP

To test the hypothesis shown in Figure 6, trains of stimuli were applied at different frequencies and transmitter release was measured after depletion of the RRP. In Figure 7, depleting trains at 62 or 125 Hz were used. After depletion of the RRP, the stimulation rates were doubled or halved to test whether or not the amount of transmitter release was dependent on the rate of stimulation. The amount of release during test trains showed a
slowly developing decrease or increase in the amount of transmitter released per impulse that was dependent on the rate of stimulation.

**Figure 7.** Mobilization accounts for release when the RRP is depleted. After a train of 60 stimuli given at either 62 or 125 Hz, doubling or halving the stimulus frequency results in a reciprocal decrease or increase in the amount of transmitter released. Results are from the same preparation.

Figure 8 shows the last 25-50 impulses of depleting stimulation trains followed by sets of 25 test impulses at different stimulation rates and then 25 impulses at the original depleting stimulation rate. After depletion, for stimulation rates of 62, 71, 83, 100 and 125 Hz, the amount of release was dependent on the amount of time between stimuli.
The EPP amplitudes from the last impulse in the test trains in Figure 8 vs the frequency of stimulation are plotted in Figure 9, where an inverse relationship can be observed between stimulation rate and transmitter release after depletion of the RRP. At the lowest control stimulation frequency (83 Hz), the lower rates of testing stimuli (62 and 71 Hz) produced less release than expected for a linear relationship (see triangles in Figure 9). Measuring depletion of the RRP is the subject of the next section and shows that at 83 Hz the RRP is not depleted (see Figure 11). According to the model, if the RRP is not depleted, then the amount of quantal release is not solely dependent on the time between stimuli.

**Figure 8.** After depletion of the RRP the asymptote of the number of vesicles released by a train of 25 stimuli is inversely related to the stimulation rate. Shown are composites for five different testing trains of 25 stimuli given at rates of 62, 71, 83, 100 and 125 Hz, preceded by depleting trains of 50 stimuli given at either (A) 100 Hz or (B) 125 Hz. Shown are the last 25 impulses of the depleting stimulation train followed by 25 impulses at the different stimulation testing trains and then 25 impulses at the original depleting stimulation rate.
Figure 9. An inverse relationship is observed between stimulation rate and transmitter release during the mobilization phase after depletion of the RRP. The red line depicts theoretical data if the mobilization rate (per unit time) is independent of stimulation rate.

PART 2. DISCUSSION

These observations are consistent with a concept that vesicles mobilized from the DP refill the depleted RRP at a constant rate. When more time is available to refill the RRP, more depleted vesicles can be replaced. If the RRP is not depleted, vesicles are available to respond to increased probability of release occurring during faster rates of stimulation. After depletion, vesicles are not available to respond to an increased probability of release and, instead, release is dependent upon the amount of time between stimuli. At rates greater than 83 Hz, the mobilization of new vesicles into the RRP from a DP cannot keep up with the rate of release because the mobilization rate is constant and not dependent on the number of stimuli but on the time between stimuli (also see Curtis and Eccles, 1960). If the stimulation rate was low enough, then there would be time to fill the RRP between stimuli but at lower rates of stimulation the enhancement of the probability of release is less so less release would be expected. Thus, release would not be entirely mobilization limited at 83 Hz, where release is from an undepleted RRP. Consequently, release not directly related to mobilization, is a function of the frequency...
of stimulation where a decreased rate of stimulation decreases the amount of
eenchancement during a train thereby producing a decreased amount of release.

PART 3. ESTIMATING THE SIZE OF THE RRP

PART 3. METHODS

The RRP is defined as the number of vesicles readily available for release.

Vesicles released from the RRP generate the EPP, so that the EPP gives a measure of the
depletion of the RRP by a single impulse such that \( m = k \times (EPP) \), where \( m \) = the number
of quanta released (packets of transmitter released by vesicles) and \( k \) is a proportionality
constant given by \( k = \text{integral of the EPP} / \text{integral of the mEPP} \). Thus, the number of
vesicles released by a single stimulus is \( m_0 = k \times (EPP_0) \). When \( m_0 \) is equivalent to the
release of 200 synaptic vesicles (Wood and Slater, 2001), then the number released over
time \( t \) is \( m_t = 200 \times (EPP_t / EPP_0) \).

As seen in Figures 1, 2, and 7, depression develops during repetitive stimulation.
The data suggest that depression is due to the depletion of vesicles from a finite pool of
vesicles available for release, the RRP. During the stimuli train, depression can be seen
to develop with two time constants, fast and slow (see Figure 10). The fast phase is
attributed to the depletion of the RRP and the slow phase may reflect a decrease in the
number of vesicles mobilized from the depot pool into the RRP as the depot pool is
depleted.

Examples of this concept are shown in Figures 10A and 10B where 100 impulses
were given at stimulation rates of (A) 125 / s and (B) 83 / s. During both trains, quantal
release initially increased with each successive stimulus and then decreased rapidly at
first and then more slowly. In the first example, where the rate of stimulation is 125 /s,
the time constant for the fast decay was 67.5 ms and for the slow decay was 3.30 s. In the second example, a slower stimulation rate of 83 / s gave time constants of 104 ms and 2.82 s.

![Graph](image)

**Figure 10.** The rundown of EPPs evoked during a train is described by two exponentials (represented by the black and cyan lines) with substantially different time constants of decay. Each plot is from a different preparation. The first five points are control responses delivered at 1 every 10 s. Rundown persists during the slow component.

To determine the size of the RRP, the integral of the vesicles released under the slow time constant are subtracted from the total amount of transmitter released during the rundown train. Two examples of this method are shown on Figure 11A and 11B, where EPPs produced by 100 stimuli, delivered at a rate of (A) 50/s or (B) 125/s, are plotted across time (also see Figure 13 for examples for 100 stimuli delivered at 100/s).

Assuming 200 vesicles are released by the first stimulus (Wood and Slater, 2001), the total number of vesicles released during the train from the endplate shown in Figure 11A was 19,600 and in Figure 11B was 16,400. After correcting for mobilization, 3400 vesicles in (A) and 5200 vesicles in (B) were depleted from the RRP. Mobilization
accounted for 83% of the release from the endplate in Figure 11A and 68% of the total release in Figure 11B.

**Figure 11.** Depletion of the RRP was determined by subtracting total mobilization, measured using the integral under the slower exponential in cyan, from total cumulative release. Exponentials were derived from points between the vertical lines. Examples are from two different preparations.  
(A) During 100 stimuli delivered at a rate of 50 Hz, a total of 19,600 vesicles were released assuming 200 quanta were released by an isolated stimulus. 3400 vesicles were from the RRP and 16,200 vesicles were from mobilization. Mobilization accounted for 83% of the total release.  
(B) After 100 stimuli delivered at a rate of 125 Hz, a total of 16,400 vesicles were released assuming a single stimulus releases 200 quanta. 5200 vesicles were from the RRP and 11,200 vesicles were from mobilization. Mobilization was 68% of the total release.

**PART 3. RESULTS**

**Estimates of the depletion of vesicles from the RRP**

Estimates of the depletion of vesicles from the RRP for seven different nerve / muscle preparations are plotted in Figure 12A as a function of the stimulation rate used to deplete the RRP. The number of depleted vesicles varied from ~ 2500 to 9000 for
endplates on different muscles. Figure 12B plots the average responses for all the experiments in Figure 12A. As the frequency of stimulation increased, the mean number of depleted vesicles increased to a plateau level for stimulation rates of 100 to 120 Hz.

**Figure 12.** Estimates of the number of vesicles depleted from the RRP depend on the stimulation rate. The number of vesicles depleted was estimated using trains of 100 stimuli given at the indicated frequencies. (A) The top graph shows the number of vesicles depleted by different frequencies of stimulation. Results are shown in color to indicate different nerve - sartorious muscle preparations. (B) The graph gives the mean ± SEM for the data shown in the top graph plotted by stimulation frequency.

**PART 3. DISCUSSION**

The results provide evidence for a relatively fixed number of vesicles available for release at stimulation rates above ~ 90 /s. The values obtained for the number of vesicles in the RRP are in agreement with those reported by other laboratories using
different techniques. Heuser et al. (1979) using freeze-fracture electron microscopy after
depolarization with high K⁺, obtained a value of ~ 4000 vesicles. Rizzoli and Betz
(2005) estimated ~ 10000 vesicles readily available for release after dye trapping to
fluoresce endocytosed vesicles.

PART 4. RECOVERY FROM DEPLETION OF THE RRP

PART 4. METHODS

Recovery from depression is measured by first applying a conditioning train to
deplete the RRP and then applying a test train identical to the conditioning train to again
deplete the RRP. The ratio of the number of depleted vesicles of the test train to the
conditioning train then gives the fractional recovery of the RRP that occurred in the time
between the two trains. Figure 13 presents an example of 100 impulses at 100 Hz as
conditioning and test trains, with a 20 s gap between the trains; note the data are plotted
by stimulus number. In this example, the proportional loss between the first train and the
second train was 15 %; therefore, within 20 s the RRP recovered 85 % of the vesicles lost
in the first train.

PART 4: RESULTS

Figure 14 shows a semi-logarithmic plot of the mean and standard deviation (N =
7) of recovery and Figure 14B shows a linear plot of the mean. The recovery was
rapid at first and then became slower with time. Recovery of the RRP (Figure 14B)
could be described by the sum of 3 exponentials (τ ~ 185 ms; τ ~ 3.50 s; and τ ~ 10.50
min).
Figure 13. Recovery of the RRP after a conditioning train. 100 impulses at 10 Hz followed by a 20 s gap between the next train, and then a second train of stimulation of 100 impulses at 10 Hz. The conditioning train released a total of 17200 vesicles, assuming a single stimulus releases 200 quanta. 3800 vesicles were from the RRP and 13400 vesicles were from mobilization. Mobilization accounted for 78 % of the total release. The test train released a total of 12400, the RRP depleted 3200 vesicles and mobilization used 9200 vesicles. Notice that the RRP recovered 84 % of its vesicles after a 20 s gap but the Total Pool (RRP + Depot (mobilized) Pool) recovered only 72 % of vesicles lost.

Figure 14. Fractional recovery of the RRP over time. (A) Semi-logarithmic plot of the mean ± SEM for the fractional recovery of an RRP obtained t ms after an identical set of impulses (100 stimuli at 100 Hz). (B) Linear plot of the data is described by three time constants for the recovery of the RRP size; \( \tau \sim 185 \) ms; \( \tau \sim 3.50 \) s; and \( \tau \sim 10.50 \) min.
PART 4. DISCUSSION

The recovery rates reported here (τ ~ 185 ms, 3.50 s, 10.50 min), except for the slowest rate, are similar to those observed at the climbing fiber to Purkinje cell synapses (τ ~ 200 ms, 3 s, and slow) (Dittman and Regehr 1998), and in active synapses in the Calyx of Held (τ ~ 90 ms and 2 s) (Hermann et al., 2007).

Recovery rates may involve the docking and priming of available vesicles (“immature”) in the terminal onto the release site and endocytosed vesicles from clathrin coated and non-clathrin related mechanisms (budding from cisternae and pinocytosis); several models of transmitter release include heterogenous vesicles from different pools (Trommershäuser, 2003). In the frog NMJ, Richards et al. (2003) has observed two types of pools with considerably different recovery rates: an RRP with clathrin coated vesicles that has a fast (6 s) recovery rate and a reserve pool that recovers slowly (min) as vesicles are pinocytosed or bud from the cisternae. In their studies, the RRP pool was refilled almost entirely by recycling, not by mobilization from the reserve pool. However, Betz and Bewick observed that recovered vesicles in the recycling pool take ~ 15-30 s to re-release (internalization time of 60 s subtracted from total recycle time of 75-90 s) (Betz and Bewick, 1992; Betz and Bewick, 1993; Betz and Wu, 1995). Therefore, recycling and reserve pools may account for the fast (3.50 s) and slow (10.50 min) time constants of recovery in my data but are unlikely to be the mechanisms producing the fastest time constant (185 ms).
PART 5. MEASURING ENHANCEMENT DURING STIMULATION IN NORMAL QUANTAL RELEASE CONDITIONS

PART 5. METHODS AND RESULTS

Enhancement produced by a single impulse during repetitive stimulation in the presence and absence of depression

Enhancement and the decay of enhancement from a single impulse during a train of stimuli can be examined by adding or dropping a single impulse during the train (Magleby, 1973; Magleby and Zengel, 1982). As shown in Fig 14, responses to dropped or added impulses every 20th stimulus during a 33 Hz train of stimuli provide a measure of enhancement during depression. A transient increase in release is seen when adding an extra impulse during the train and a transient decrease in release is seen when an impulse is dropped from the train of stimulation. Increases and decreases in release during a stimuli train have been interpreted as enhancement and decay of enhancement, respectively (Magleby, 1973; Magleby and Zengel, 1982; Zengel and Magleby, 1982).

Figure 14. A train of stimulation at 33 Hz with a dropped or added impulse every 20th stimulus. Plotted are the proportional changes in EPP amplitude measured as EPP at time t (EPPt) to the control EPP amplitude (EPP0).

When the amount of enhancement provided by an added stimulus at time t is scaled for the amount of depression during the train, the magnitude of enhancement, as
measured by the fractional change in EPP amplitude, is proportional to the amount of
depression (Figure 15).

![Graph showing the magnitude and decay of enhancement during a train of stimuli.](image)

**Figure 15.** (A) The magnitude of enhancement is proportional to the amount of
depression during a stimulus train (Shown are EPPt / EPP0 from the portion of the train
with an added impulse within an interstimulus interval of 33/s using the mean EPP
amplitude of the four successive impulses immediately before the added impulse
(Control EPP) and 7 impulses after the added impulse (testing impulse). (B)
Enhancement is constant during a train of stimuli that depresses responses. Plotted are
the means and standard deviations of the proportional magnitudes of enhancement when
scaled to correct for depression. The spread of the scaled data is shown by plotting the
enhancement at time t, shown in yellow.

The magnitude and decay of enhancement during a train of stimuli can be
estimated from

\[
F(t - t_{\text{DROP}})_{\text{DROP}} = \frac{(EPP_{0B} - EPP_t)}{EPP_{0B}}
\]

\[
F(t - t_{\text{ADD}})_{\text{ADD}} = \frac{(EPP_t - EPP_{0B})}{EPP_{0B}},
\]

where \( t \) is time, \( EPP_{0B} \) is the amplitude of the imaginary EPP at the intersection of a line
drawn from the amplitude of the impulse at time \( t \), denoted \( EPP_t \), to a projected baseline
as shown in Figure 16B for a dropped impulse.
Using this method, the magnitude of enhancement from an impulse given during stimulation trains obtained in normal (2.0 mM) and low (0.14 mM) extracellular [Ca^{2+}], compared in Figure 17, was determined to be 0.29 and 0.27 for normal and low extracellular Ca^{2+}, respectively. The time constant of decay was faster in normal (\(\tau \sim 40.6\) ms) than in low extracellular Ca^{2+} (\(\tau \sim 93.7\) ms) and can be compared with those from previous studies done in low Ca^{2+}, where \(\tau_{F1} \sim 50\) ms and \(\tau_{F2} \sim 300\) ms (Bennett et al., 1997, 2000, 2007; Elmquist and Quastel, 1965; Magleby, 1973; Mallert and Martin, 1967a, 1967b; Suzuki et al., 2002; Younkin, 1974). The decay of enhancement under normal Ca^{2+} is similar to the time constant of decay for F1, whereas the decay under low
\( \text{Ca}^{2+} \) conditions is somewhat slower and might reflect a contribution of a slowly decaying F2 component.

\[ \text{Figure 17.} \text{ Magnitude of enhancement for dropped and added impulses for a stimulus train at 33 Hz with a dropped or added impulse every 20th stimulus.} (A) \text{ The observed data collected under 2.0 mM } [\text{Ca}^{2+}]_o. (B) \text{ Data observed under low (0.14 mM) } [\text{Ca}^{2+}]_o. (C) \text{ The initial magnitude and time constant of the decay of enhancement extracted from the data obtained during and after dropped or added impulses according to the methods described in the text and on Figure 16 for (C) 2.0 mM } [\text{Ca}^{2+}]_o \text{ and (D) 0.14 mM } [\text{Ca}^{2+}]_o. \]

\text{PART 5. DISCUSSION}

The observations in this section indicate a component of enhancement with a time constant similar to F1 that is present during depression due to repetitive stimulation under conditions of physiological [Ca\(^{2+}\)]\(_o\). Under conditions of decreased [Ca\(^{2+}\)]\(_o\), an additional
component of enhancement with a somewhat longer time constant can be seen. The initial magnitude and time constant of decay of the first component of enhancement appears consistent with F1 facilitation identified in previous studies (Bennett et al., 1997, 2000, 2007; Elmquist and Quastel, 1965; Magleby, 1973; Mallert and Martin, 1967a, 1967b; Suzuki et al., 2002; Younkin, 1974). The somewhat longer time constant of the additional component of enhancement found in low $[\text{Ca}^{2+}]_o$ may arise from F2 facilitation where a slower time constant of decay would be expected, but the experiments described thus far were unable to clearly resolve an F2 component in physiological $\text{Ca}^{2+}$, most likely due to the expected low magnitude of F2 after one impulse.

The F1-like induced change in the magnitude of the EPPs, whether determined at low or high quantal content, appeared to scale with the size of the EPP amplitudes obtained before adding or dropping an impulse to test for enhancement. This suggests a multiplicative relationship between F1 and other components involved in quantal release. A multiplicative relationship between facilitation and other components of enhancement under low quantal release conditions (Magleby and Zengel, 1982; Zengel and Magleby, 1982) or enhancement and depression under normal quantal release conditions (Kalkstein and Magleby, 2004) has been previously observed.

PART 6. QUANTIFYING CONCURRENT ENHANCEMENT AND DEPRESSION OF QUANTAL RELEASE

PART 6. METHODS

In order to quantify components of enhancement, depression and their interaction, numerical methods were used to isolate the components and quantify them using data obtained during different patterns and durations of stimulation. Examples are shown in
Figure 18 for EPPs recorded under physiological Ca$^{2+}$. The patterned stimulation (see Figure 18 legend for details) revealed enhancement and depression superimposed.

Figure 18. EPPs recorded using four different stimulation patterns to reveal enhancement and depression during a train of stimulation. During depression (defined as the progressive decrease in EPP amplitude during repetitive stimulation) the enhancement added by individual impulses can still be observed when stimuli are dropped or added. (A) A stimulation rate of 33 Hz with a dropped or added impulse given every 20$^{th}$ stimulus; (B) Stimulation trains of 40 stimuli delivered at a rate of 40 Hz (interstimulus interval of 25 ms) with 2 s intervals between each train; (C) Stimulation rate of 2.5 Hz (interstimulus interval of 400 ms) with a dropped or added impulse given every 20$^{th}$ stimulus; (D) Alternating trains of 20 stimuli given at a rate of 6.6 Hz (interstimulus interval of 150 ms) and 10 stimuli at a rate of 3.3 Hz (interstimulus interval of 300 ms).
The trains of stimulation (shown in Figure 18) were given at frequencies that produce depression and were patterned to capture facilitation components F1 and F2, if present. In order to identify and measure enhancement and depression, components were identified mathematically by their magnitudes and time constants or by the changes in the estimated number of vesicles in the RRP, respectively (Magleby and Zengel, 1982; Wu and Betz, 1998). Four kinetic components, F1, F2, A and P, were used to model enhancement while two components of depression were used to model how the synapse is depleted of vesicles during repetitive stimulation. Figure 19 illustrates the model used to calculate transmitter release.

**Figure 19.** Diagram of the model of synaptic vesicle release used to study the interaction between enhancement and depression.
F1, F2, A and P are defined as the fractional increase in release, where release is measured by \((EPP_t - EPP_0) / EPP_0\) when the other components including depression are absent (38,43,67). Therefore,

\[
\begin{align*}
F1_t &= (EPP_t - EPP_0) / EPP_0 \\
F2_t &= (EPP_t - EPP_0) / EPP_0 \\
A_t &= (EPP_t - EPP_0) / EPP_0 \\
P_t &= (EPP_t - EPP_0) / EPP_0
\end{align*}
\]

when

\[
\begin{align*}
F2_t &= 0; A_t = 0; P_t = 0; D_t = 0 \\
F1_t &= 0; A_t = 0; P_t = 0; D_t = 0 \\
F1_t &= 0; F2_t = 0; P_t = 0; D_t = 0 \\
F1_t &= 0; F2_t = 0; A_t = 0; D_t = 0
\end{align*}
\]

When the nerve is repetitively stimulated, the RRP is partially depleted of vesicles. In the model, depression arises from the inability of the rate of vesicle mobilization from the DP to keep up with the loss of vesicles from the RRP that occurs during repetitive stimulation. Mobilization of vesicles into the RRP occurs at a rate proportional to the magnitude of the RRP depletion. The DP is replenished at a rate proportional to the magnitude of DP depletion. The source of vesicles for the DP is not defined and can include vesicles coming from an additional pool or those rapidly endocytosed after membrane fusion (Rizzoli and Betz, 2004; also see Ertunc et al., 2007). The model assumes that the DP is replenished between nerve impulses as

\[
\text{Replenishment} = [DP_0 - DP_t] \left(1 - e^{-\Delta t / \tau(DP)}\right)
\]

where \(DP_0\) and \(DP_t\) are the number of vesicles in the DP at time 0 before the stimulation train and time t during stimulation, respectively, and \(\tau(DP)\) is time constant for refilling the DP, and \(\Delta t\) is the interval between nerve impulses. Movement of vesicles from the DP into the RRP (mobilization) between nerve impulses is given by

\[
\text{Mobilization} = \left[\frac{DP_t}{DP_0}\right] \times \left[RPP_0 - RPP_t\right] \left(1 - e^{-\Delta t / \tau(RRP)}\right)
\]
where $DP_t / DP_0$ is the fraction of the DP that is filled, $RRP_0$ and $RRP_t$ is the number of vesicles in the RRP at time 0 before the stimulation train and time t during stimulation, respectively, $\Delta t$ is the interval between nerve impulses and $\tau(RRP)$ is the time constant for refilling the RRP.

A quantitative model that can describe enhancement under low Ca$^{2+}$ conditions (where depression is not seen) is described by (Magleby and Zengel, 1982)

\[ \frac{EPP_t}{EPP_0} = \left[ (F1 + F2 + 1)^n (A + 1) (P + 1) \right]. \]

If enhancement is an increase in the probability of release and depression is a decrease in the number of vesicles available for release, then

\[ \frac{EPP_t}{EPP_0} = \left[ (F1 + F2 + 1)^n (A + 1) (P + 1) \right] \times \frac{RRP_t}{RRP_0} \]  (5)

(Equation 5 in the introduction).

The decay of isolated components of enhancement can be approximated by a single exponential decay (Mallert and Martin, 1967a, 1967b; Rosenthal, 1969; Woodson et al., 1978; Wu and Betz, 1998; Zengel and Magleby, 1977; Zengel et al., 1980). Therefore, the decay of the underlying factors (indicated by *) that give rise to the components can be approximated by

\[ F1^*_t = F1^*_0 \ e^{-t/\tau(F1^*)} \]
\[ F2^*_t = F2^*_0 \ e^{-t/\tau(F2^*)} \]
\[ A^*_t = A^*_0 \ e^{-t/\tau(A^*)} \]
\[ P^*_t = P^*_0 \ e^{-t/\tau(P^*)} \]

If it is assumed that the enhancement from F1 and F2 arise from these underlying factors, then functions describing the underlying factors $F1^*$ and $F2^*$ giving rise to F1 and F2 have been previously described (Magleby and Zengel, 1982) as
\[
\frac{dF_1^*}{dt} = J(t)f_1^* - k_{F_1^*}F_1^*
\]

and

\[
\frac{dF_2^*}{dt} = J(t)f_2^* - k_{F_2^*}F_2^*
\]

where \(J_t\) is a unit impulse function (where \(J_t = 1\) at the time of the nerve impulse and 0 at all other times) used to represent the stimulus train, \(k\) represents the rate constants for the loss of \(F^*\), and \(f^*\) is the incremental increase in \(F^*\) that occurs with each impulse.

The change in the underlying factor \(A^*\) that gives rise to augmentation has been previously described (Magleby and Zengel, 1982; Zengel and Magleby 1981, 1982) by

\[
\frac{dA^*}{dt} = J(t)a^* - k_{A^*}A^*
\]

where \(k_{A^*}\) is the rate constant for the loss of \(A^*\), \(J_t\) is the unit impulse function at the time of stimulation, and \(a^*\) is the incremental increase in \(A^*\) with each impulse. During a stimulus train the magnitude of augmentation added by each impulse, \(a^*\), can increase during the train such that

\[
a^* = a_0^*Z^{ST}
\]

where \(a_0^*\) is the increment added by the first impulse of the train, \(Z\) is a constant that determines the increase in \(a^*\) with each impulse, \(S\) is the stimulation rate, and \(T\) is the duration of stimulation (Magleby and Zengel, 1982).

Repetitive stimulation changes both the magnitude and time course of potentiation (Magleby and Zengel, 1975, 1976b, 1976c, 1982; McNaughton, 1982; Rosenthal, 1969; Woodson et al., 1978). The decay of \(P\) is given by

\[
P_t = P(T)e^{-t/\tau_P},
\]
where \( P_t \) is potentiation at \( t \) seconds after a conditioning train, \( P(T) \) is the initial magnitude of potentiation immediately after a conditioning train of duration \( T \) seconds and \( \tau_p \) is the time constant of the decay of potentiation. The increase in the time constant of decay of \( P_t \) seen during a train of stimulation when the magnitude of \( P \) increases is given by

\[
\tau_p = \tau_p e^{P(T)/B},
\]

where \( \tau_p \) is the time constant of decay for potentiation, and \( \tau_{p0} \) and \( B \) are constants given by a semilogarithmic plot of \( \tau_p \) against \( P \).

The underlying factor \( P^* \) giving rise to potentiation has been described by

\[
dP^*/dt = J(t)p^* - k_{p*}P^*
\]

where \( k_{p*} \) is the rate constant for the loss of \( P^* \), \( J \) is a unit impulse function at the time of each impulse, and \( p^* \) is the incremental increase in \( P^* \) with each impulse (Magleby and Zengel, 1975, 1976b, 1976c, 1982). However, there is an apparent saturation relationship between the observed potentiation, \( P_t \), so that the underlying process giving rise to potentiation can be described by

\[
P = \{(P^* + 1)/[(P^* / G) + 1]\} - 1
\]

where \( G \) is a constant (see Magleby and Zengel, 1982).

**Summary of methods**

Trains of stimulation (shown in Figure 18) at frequencies that produce depression are patterned to capture facilitation components F1 and F2. Equations 8-17 quantify the amount of enhancement contributed by the individual components F1, F2, A and P while the synapse is being depleted of vesicles. The magnitude and time constant of each
component is determined by fitting EPP amplitudes during repetitive stimulation with Eqns. 8 - 17, as stated above (Magleby and Zengel, 1982).

**Estimating the parameters describing the rate of decay for enhancement and depression**

The mean time constants of decay for F1, F2, A and P were calculated from the means of best fit values to data obtained using different stimulation patterns and levels of extracellular Ca\(^{2+}\). The mean value for each time constant was then used to fix the decay rates to reduce the number of free parameters. The mean values were:

\[
\begin{align*}
\tau_{F1} &= 46 \text{ ms} \\
\tau_{F2} &= 220 \text{ ms} \\
\tau_{A} &= 1600 \text{ ms} \\
\tau_{P} &= 20000 \text{ ms}
\end{align*}
\]

These values are consistent with previously published values (Magleby and Zengel, 1982).

Previous studies on the frog NMJ have shown the RRP size to be approximately 10000 vesicles (Rizzoli and Betz, 2005). We carry out our calculations with RRP\(_0\) fixed at 10000. The size of the quantal control (EPP\(_0\)) and the size of the DP were then determined by fitting the data with Eqns. 8 – 17.

**PART 6. RESULTS**

**Results for stimulation trains in 2.0 mM extracellular Ca\(^{2+}\)**

Observations on fitted contributions of the different components of enhancement and of depression in physiological Ca\(^{2+}\) for a drop/add pattern of stimulation superimposed onto a steady stimulation rate of 33 Hz are shown in Figure 20. The depression seen during the train (Figure 20 A) reflects a decline in both the size of the
RRP (Figure 20B) and the depot pool (D). The F1 component of facilitation rises sharply at the start of the train to a steady state and fluctuates up and down in response to the dropped and added impulses (Figure 20C). The potentiation component is very small but rises steadily as the train of stimulation continued (Figure 20F). Zero magnitudes of F2 (Figure 20 E) and A (Figure 20 G) were identified indicating F2 and A are not needed to describe these data. The parameters used in the fit were $f_1^* = 0.53$, RRP$_0 = 10000$, $\tau_{RRP} = 2.1 \text{ s}$, DP$_0 = 69395$, $\tau_{DP} = 30 \text{ s}$, with EPP$_0 = 167 \text{ vesicles}$.

Figure 21 shows the observed and fitted responses for a trial of 7 stimulation trains of 40 impulses given at 25 ms intervals (40 hz) with 2 s intervals between the 7 successive trains. In this example, the data during the trial were well fit using only the F1 component, depletion of the RRP, and depletion of the depot pool (DP). The values were: $f_1^* = 0.29$, RRP$_0 = 10000$, $\tau_{RRP} = 3.8 \text{ s}$, DP$_0 = 58841$, $\tau_{DP} = 47 \text{ s}$, with EPP$_0 = 181 \text{ vesicles}$.

In both of these experiments in physiological (2 mM) conditions of Ca$^{2+}$ (N=8), as well as others not shown, the largest stimulus-induced responses occurred during the first few impulses of the observed data. The model underpredicted these responses. Other models were explored to capture these data. They included an extra immediately releasable pool (IRP) and allowing for both a fast and slow time constant for mobilization of vesicles from the DP to the RRP. Neither of these models resolved the problem, suggesting some other process(es) not included in the model contribute to the greater than predicted enhancement during the first few impulses.
Figure 20. Observed data and fitted data curves for EPPs evoked in 2.0 mM Ca\(^{2+}\) by stimuli given at a rate of 33 Hz with impulses dropped or added impulse every 20\(^{th}\) stimulus; values for the components used in the model are shown separately. 

(A) The observed EPP (in black) for 400 stimuli is overlaid with values predicted by the model (in red).

(B) The amount of depletion predicted by the model for the RRP during the stimulation train, given an initial value of 10000.

(C) Magnitude of the underlying component \(F_1^*\), predicted by the model for the stimulation train used to collect the observed data.

(D) Model predicted size and rate of depletion of the DP. Model predicted magnitude of additional components included in the model for the stimulation train used to collect the observed data are shown in (E) \(F_2^*\), (F) potentiation, \(P^*\) and (G) augmentation, \(A^*\). The values used in the model were: \(f_1^* = 0.53\), \(\tau_{\text{RRP}} = 2.1\) s, \(\text{DP}_0 = 69395\), \(\tau_{\text{DP}} = 30\) s, with EPP\(_0 = 167\) vesicles.
The data from the stimulation patterns discussed so far did not require F2 or A to predict the responses, as the best fits were obtained when the search routine set the magnitudes of F2, A and P to ~ 0. Previous studies define F2 by a time constant of 300
ms (Bennett et al., 1997, 2000, 2007; Elmquist and Quastel, 1965; Magleby, 1973; Mallart and Martin, 1967a; 1967b; Suzuki et al., 2002; Younkin, 1974) so additional patterns using longer interstimulus intervals were designed to better capture the F2 component. In Figure 22, impulses were delivered every 400 ms with a dropped or added impulse every 20th stimulus. In Figure 23, the train of stimulation alternated between 20 impulses every 150 ms and 10 impulses every 300 ms. In both data sets, the components for F1, the RRP and the DP were sufficient to simulate the data obtained in physiological Ca\textsuperscript{2+}: the search routine set F2, A and P to \sim 0.

In 9 of 10 experiments with, F2, A and P were not needed to accurately predict the observed data obtained in 2.0 mM extracellular Ca\textsuperscript{2+}. However, in one experiment with a stimulation rate of 33 Hz and dropped and added impulses every 20th stimulus (identical to the pattern used in Figure 20), all four components of enhancement (F1, F2, A and P) as well as a RRP and a DP were present in the fit. This experiment is shown in Figure 24. One noticeable difference between this experiment and the other 9 experiments is that the size of the EPP during the train (EPP\textsubscript{t}) was typically greater than EPP\textsubscript{0}. This suggests F2, A and P may be detected as long as depression is not too great.
Figure 22. Observed data and fitted data curves for EPPs evoked in 2.0 mM Ca$^{2+}$ by stimuli given at a rate of 2.5 Hz (interstimulus interval of 400 ms) with a dropped or added impulse given every 20th stimulus; values for the components used in the model are shown separately. (A) The observed EPP (in black) for 400 stimuli is overlaid with values fitted by the model (in red).

(B) The responses in shown in (A) for stimuli numbers 80 – 130. (C) Magnitude of the underlying component $F1^*$ predicted by the model for the stimulation train used to collect the observed data. (D) The magnitude of $F1^*$ shown in (C) for stimuli number 80 - 130. (E) The amount of depletion predicted by the model for the RRP during the stimulation train, given an initial value of 10000. (F) The amount of depletion predicted by the model for the RRP shown in (E) for stimuli number 80 – 130. (G) Predicted size and rate of depletion of the DP. The values used in the model were: $f_{1}^* = 1.51$, $\tau_{RRP} = 5.67$ s, $DP_0 = 174336$, $\tau_{DP} = 133.9$ s, with $EPP_0 = 283$ vesicles.
Figure 23. Observed data and fitted data curves for EPPs evoked in 2.0 mM Ca²⁺ by 20 stimuli given at a rate of 6.6 Hz (interstimulus interval of 150 ms) followed by 10 stimuli at a rate of 3.3 Hz (interstimulus interval of 300 ms); with values for the components used in the model are shown separately. (A) The observed EPP (in black) for 400 stimuli is overlaid with values fitted by the model (in red). (B) The responses in shown in (A) for stimuli numbers 80 – 130. (C) Magnitude of the underlying component F₁* fit by the model for the stimulation train used to collect the observed data. (D) The magnitude of F₁* shown in (C) for stimuli number 80 - 130. (E) The amount of depletion predicted by the model for the RRP during the stimulation train, given an initial value of 10000. (F) The amount of depletion predicted by the model for the RRP shown in (E) for stimuli number 80 – 130. (G) Predicted size and rate of depletion of the DP. The values used in the model were:

\[ f_1^* = 1.07, \tau_{RRP} = 6.77 \text{ s}, \text{DP}_0 = 94839, \tau_{DP} = 104.4 \text{ s}, \text{with EPP}_0 = 212 \text{ vesicles}. \]
Figure 24. Observed data and fitted curves for EPPs evoked in 2.0 mM Ca$^{2+}$ by stimuli given at a rate of 33 Hz with impulses dropped or added impulse every 20th stimulus; values for the components used in the model are shown separately. (A) The observed EPP (in black) for 400 stimuli is overlaid with values fitted by the model (in red). (B) The amount of depletion fit by the model for the RRP during the stimulation train, given an initial value of 10000.

(C) Magnitude of the underlying component $F_1^*$ fit by the model for the stimulation train used to collect the observed data. (D) Model fitted size and rate of depletion of the DP. Model fitted magnitude of additional components included in the model for the stimulation train used to collect the observed data are shown in (E) $F_2^*$, (F) potentiation, $P^*$ and (G) augmentation, $A^*$.

The values used in the model were: $f^*_{1} = 1.31$, $\tau_{RRP} = 1.03$ s, $DP_0 = 169191$, $\tau_{DP} = 30$ s, with $EPP_0 = 107$ vesicles.
Results for stimulation trains in 1.0 mM extracellular Ca\textsuperscript{2+}

To examine whether apparent loss of F2, A and P is related to depression, the number of vesicles released during stimulation was reduced by using 1 mM instead of 2 mM extracellular Ca\textsuperscript{2+}. Figure 25 shows the observed and predicted response from two experiments (left (a) and right (b) panels) with different levels of depletion from the RRP. In both examples, four components of enhancement and a depleting RRP and DP were determined during the fitting.

![Graphs](image.png)

**Figure 25.** Observed data and fitted curves in 2 different preparations labeled (a) and (b) for EPPs evoked in 1.0 mM Ca\textsuperscript{2+} by stimuli given at a rate of 33 Hz with impulses dropped or added impulse every 20\textsuperscript{th} stimulus; values for the RRP and DP are shown separately. (1) The observed EPP (in black) for 400 stimuli is overlaid with values fit by the model (in red). (2) The amount of depletion predicted by the model for the RRP during the stimulation train, given an initial value of 10000. (3) Predicted size and rate of depletion of the DP. The values used in the model were for (a) $f_1^*=0.82$, $f_2^*=0.10$, $A^*=0.01$, $P^*=0.0001$, $\tau_{\text{RRP}}=1.0$ s, $\text{DP}=195013$, $\text{EPP}=100$; and for (b) $f_1^*=1.16$, $f_2^*=0.03$, $A^*=0.008$, $P^*=0.0001$, $\tau_{\text{RRP}}=1.0$ s, $\text{DP}=92020$, $\text{EPP}=58$. 
Table 1 compares the size of the incremental enhancement for four components added by each impulse in the two experiments shown in Fig. 25. As shown, the magnitude of components for both A and P are comparable.

<table>
<thead>
<tr>
<th>Enhancement</th>
<th>$f_1^*$</th>
<th>$f_2^*$</th>
<th>Power (n)</th>
<th>$a_0^*$</th>
<th>$p_0^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 23 A</td>
<td>0.8209</td>
<td>0.0984</td>
<td>1.0</td>
<td>0.01</td>
<td>0.0001</td>
</tr>
<tr>
<td>Figure 23 B</td>
<td>1.157</td>
<td>0.0303</td>
<td>1.7</td>
<td>0.01</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

$f_1^*$, $f_2^*$, $A^*$ and $P^*$ are defined by Eqns. 10-17, n is the power relationship between ($f_1^* + f_2^* + 1$) and transmitter release, and $Z_{ST} = 1.0$.

Four components of enhancement were needed to fit the data, although the magnitude of depletion of vesicles from the RRP was variable: In Figure 23A, 60 % of the RRP was depleted and Figure 23 B, 80 % of the RRP was depleted. The larger amount of F1 enhancement seen in Figure 23B ($F_1 \sim 1.157, n = 1.7$) vs Figure 23A ($F_1 \sim 0.821, n = 1$) correlated with depletion of the RRP. Previously shown experiments in 2.0 mM Ca$^{2+}$ typically had a loss of $> 70$ % of the RRP and F2, A and P were not needed to fit the data. In all experiments, however, when the $EPP_t / EPP_0$ remained $> 1$ during the train, four components of enhancement were needed to fit the data.

Not all stimulus trains in 1.0 mM extracellular Ca$^{2+}$ produced depression during the train. An example, using the same pattern of stimulation (drop/add impulse during 33 Hz stimulation) is shown in Figure 26. Four components of enhancement were detected in the data. This data set, compared with the previous sets in 1.0 extracellular Ca$^{2+}$,
depletion fitted by the model for the RRP during the stimulation train, given an initial value of 10000. (C) Magnitude of the underlying component $F1^*$ fit by the model for the stimulation train used to collect the observed data. (D) Fitted size and rate of depletion of the DP. Fitted magnitude of additional components included in the model for the stimulation train used to collect the observed data are shown in (E) $F2^*$, (F) potentiation, $P^*$ and (G) augmentation, $A^*$. The values used in the model were: $f_1^* = 0.45, f_2^* = 0.07, A^* = 0.009, P^* = 0.002, \tau_{RRP} = 1.0 \text{ s}, DP_0 = 495592, \tau_{DP} = 30 \text{ s},$ with $EPP_0 = 37$ vesicles.

**Figure 26.** Observed data and fitted curves for EPPs evoked in 1.0 mM Ca$^{2+}$ by stimuli given at a rate of 33 Hz with impulses dropped or added impulse every 20th stimulus; values for the components used in the model are shown separately. (A) The observed EPP (in black) for 400 stimuli is overlaid with values fit by the model (in red). (B) The amount of
had comparable magnitudes for F2, A and P components but the component for F1 was considerably smaller (F1 ~ 0.449) and fewer vesicles were depleted from the RRP.

**Results for stimulation trains in low (0.14 - 0.20 mM) extracellular Ca\(^{2+}\)**

To compare the previous data with data obtained in low extracellular Ca\(^{2+}\), two different stimulation patterns were used (N=8). In Figure 27, a drop/add impulse pattern during a 33 Hz train identical to the stimulation pattern used in Figures 20, 24, 25, 26 was used to obtain the data. For Figure 28, trains of 40 stimuli were delivered at a rate of 1/25 ms (40 Hz), a pattern identical to the train used to obtain the data in Figure 21. In low extracellular Ca\(^{2+}\), the underlying loss of vesicles from the RRP was not seen visually as depression in the observed data (see Figures 27 and 28), but could be quantified by solving for the components necessary to fit the data with the model described by Equation 9. The amount of depletion was less in low Ca\(^{2+}\) because, as Ca\(^{2+}\) is reduced, a smaller number of vesicles are released per impulse (Dodge and Rahamimoff, 1967). A reduced EEP\(_{0}\) is indicated as a reduction in the size of the EEP\(_{0}\). The calculated number of vesicles released at EPP\(_{0}\) indicated a 94% reduction in low Ca\(^{2+}\) (an average of 13 vesicles, vs. 231 vesicles in 2.0 mM Ca\(^{2+}\); see Table 5). In low extracellular Ca\(^{2+}\), both stimulation patterns produced data that required all four components of enhancement to predict the responses during the train, consistent with previous work using low extracellular Ca\(^{2+}\) (Zengel and Magleby, 1982). When [Ca\(^{2+}\)]\(_{0}\) was low, estimates of the DP were usually very large. However, fixing the DP at any value > 75000 had no effect on the quality of the fitting, indicating that the DP is poorly defined in low Ca\(^{2+}\) and that the estimated values for the DP are probably not accurate in low Ca\(^{2+}\).
Figure 27. Observed data and fitted curves for EPPs evoked in 0.14 mM Ca^{2+} by stimuli given at a rate of 33 Hz with impulses dropped or added impulse every 20th stimulus; values for the components used in the model are shown separately. (A) The observed EPP (in black) for 400 stimuli is overlaid with values fit by the model (in red). (B) The amount of depletion predicted by the model for the RRP during the stimulation train, given an initial value of 10000. (C) Magnitude of the underlying component $F1^*$ predicted by the model for the stimulation train used to collect the observed data. (D) Predicted size and rate of depletion of the DP. Predicted magnitude of additional components included in the model for the stimulation train used to collect the observed data are shown in (E) $F2^*$, (F) potentiation, $P^*$ and (G) augmentation, $A^*$. The values used in the model were: $f_1^* = 0.18$, $f_2^* = 0.10$, $A^* = 0.0003$, $P^* = 0.005$, $\tau_{RRP} = 1.9$ s, $DP_0 = 500000$, $\tau_{DP} = 30$ s, with EPP_0 = 2 vesicles.
Figure 28. Observed data and fitted curves for EPPs evoked in 0.14 mM Ca\(^{2+}\) by stimulation trains of 40 stimuli delivered at a rate of 40 Hz (interstimulus interval of 25 ms) with 2 s intervals between each train; values for the components used in the model are shown separately. (A) The observed EPP (in black) for 400 stimuli is overlaid with values fit by the model (in red). (B) The amount of depletion predicted by the model for the RRP during the stimulation train, given an initial value of 10000. (C) Magnitude of the underlying component \(F1^*\) predicted by the model for the stimulation train used to collect the observed data. (D) Predicted size and rate of depletion of the DP. Predicted magnitude of additional components included in the model for the stimulation train used to collect the observed data are shown in (E) \(F2^*\), (F) potentiation, \(P^*\) and (G) augmentation, \(A^*\). The values used in the model were: \(f_1^* = 0.12, f_2^* = 0.07, A^* = 0.002, P^* = 0.02, \tau_{RRP} = 5.8 \text{ s}, DP_0 = 500000, \tau_{DP} = 30 \text{ s}, \) with \(\text{EPP}_0 = 1 \text{ vesicle.}\)
Comparison of the components obtained in low (0.14 – 0.2 mM), medium (1.0 mM) and physiological (2.0 mM) Ca\(^{2+}\)

A comparison of the contribution of the components for F1 and F2, to enhanced responses, is shown on Table 2. Mean values for F1 can be compared with previous values of ~ 0.8. The amount of enhancement from the F2 component decreased as Ca\(^{2+}\) increased, from ~ 0.1 in low Ca\(^{2+}\) to 0.01 in normal Ca\(^{2+}\) (and only one data set used any appreciable amount of F2 at normal Ca\(^{2+}\) when EPP\(_t\)/EPP\(_0\) was typically >1). The combined \((f_1^* + f_2^* + 1)\) to the nth power gives values of 0.4, 0.8 and 0.7 for low, medium, and normal Ca\(^{2+}\), respectively, indicating that facilitation increased as Ca\(^{2+}\) increased. That the power n decreased as Ca\(^{2+}\) was increased is not surprising as the power relationship between Ca\(^{2+}\) and release is best observed at very low levels of Ca\(^{2+}\), with possible saturation at higher levels of Ca\(^{2+}\).

Table 2. A comparison between different Ca\(^{2+}\) concentrations and the average incremental enhancement produced by \(F1^*\) and \(F2^*\) predicted by the model for different trains of patterned stimulation using 500 stimuli.

<table>
<thead>
<tr>
<th>Ca(^{2+}) (mM)</th>
<th>N</th>
<th>(f_1^*) ± SEM</th>
<th>(f_2^*) ± SEM</th>
<th>Power (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1-0.2</td>
<td>8</td>
<td>0.5064 ± 0.159</td>
<td>0.0993 ± 0.030</td>
<td>2.0</td>
</tr>
<tr>
<td>1.0</td>
<td>3</td>
<td>0.8090 ± 0.203</td>
<td>0.0672 ± 0.020</td>
<td>1.5</td>
</tr>
<tr>
<td>2.0</td>
<td>10</td>
<td>0.7771 ± 0.147</td>
<td>0.0123 ± 0.012</td>
<td>1.6</td>
</tr>
</tbody>
</table>

N is the number of experiments at a given concentration of Ca\(^{2+}\), \(f_1^*\) and \(f_2^*\) are defined by Equation 7 and n is the power relationship between \((f_1^* + f_2^* + 1)\) and transmitter release.

Table 3 compares the increment of augmentation added by the first impulse for different concentrations of Ca\(^{2+}\). Augmentation \((a_0^*)\) increased from 0.005 in low Ca\(^{2+}\) to 0.009 in medium Ca\(^{2+}\). In comparison, previous studies show an increment of of ~ 0.01 at
low Ca\(^{2+}\) (Magleby and Zengel, 1982). At normal Ca\(^{2+}\) concentrations, the component A was negligible and was detected in only one experiment where \(\text{EPP}_t / \text{EPP}_0 > 1\) for all but two dropped points at the end of the train of stimulation.

The Z factor, a constant that determines the rate of increase in augmentation added by each impulse (Eqn. 13), ranged from 1.000 to 1.0013 and was typically less than previous values of 1.003 to 1.005 (Magleby and Zengel, 1982), although the total time for the stimulation patterns was comparable or longer than in previous studies.

**Table 3.** A comparison between different Ca\(^{2+}\) concentrations and the average incremental enhancement produced by \(a_0^*\), predicted by the model for different trains of patterned stimulation using 500 stimuli, and \(Z\), the constant that determines the incremental increase in \(a_0^*\) with each impulse.

<table>
<thead>
<tr>
<th>Ca(^{2+}) (mM)</th>
<th>(a_0^*) ± SEM</th>
<th>(Z) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1-0.2</td>
<td>0.0051 ± 0.0014</td>
<td>1.0013 ± 0.0005</td>
</tr>
<tr>
<td>1.0</td>
<td>0.0091 ± 0.0004</td>
<td>1.0000*</td>
</tr>
<tr>
<td>2.0</td>
<td>&lt; 0.00004</td>
<td>1.0009 ± 0.001</td>
</tr>
</tbody>
</table>

* \(a_0^*\) and \(Z\) are defined by Equation 13.
* There was no variation between experiments.

Potentiation was not present in data sets collected in normal quantal release conditions (2.0 mM Ca\(^{2+}\)), and the component was smaller for lower release conditions (Table 4, \(p^* < 0.005\)) when compared with previous studies (~ 0.01) (Magleby and Zengel, 1975, 1976b, 1976c, 1982). The measure of the increase in the potentiation component time constant, \(B\), during repetitive stimulation, increased as the level of extracellular Ca\(^{2+}\) increased, as expected. \(G\), a possible measure of saturation for
potentiation, decreased as extracellular Ca\textsuperscript{2+} increased, as expected, although the value of G was larger than previously reported (2.0 – 3.0).

<table>
<thead>
<tr>
<th>Ca\textsuperscript{2+} (mM)</th>
<th>p* ± SEM</th>
<th>B ± SEM</th>
<th>G ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1-0.2</td>
<td>0.0047 ± 0.0023</td>
<td>4.10 ± 1.43</td>
<td>14.03 ± 2.49</td>
</tr>
<tr>
<td>1.0</td>
<td>0.0008 ± 0.0007</td>
<td>7.37 ± 2.60</td>
<td>13.54 ± 6.27</td>
</tr>
<tr>
<td>2.0</td>
<td>&lt; 0.0001</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

p* is defined by Equation 16, B is defined by Equation 15, and G is defined by Equation 17.

Comparison of the time constants for refilling the RRP in experiments conducted under different levels of extracellular Ca\textsuperscript{2+} are shown in Table 5. Differences between the low and normal Ca\textsuperscript{2+} groups were insignificant (p > 0.3). The median τ\textsubscript{RRP} was between 1.0 – 1.4 s for all groups (not shown). The spread within groups was greater: low Ca\textsuperscript{2+}: 1.0 - 5.7 s; 1.0 mM Ca\textsuperscript{2+}: 1.0 - 1.5 s; 2.0 Ca\textsuperscript{2+}: 1.0 - 6.8 s.

The calculated size of the EPP\textsubscript{0} increased as the Ca\textsuperscript{2+} concentration in the extracellular bath increased. EPPs ranged from a low of 13 vesicles in low Ca\textsuperscript{2+} to 231 vesicles in physiological Ca\textsuperscript{2+}, and were within the expected values for these groups (Dodge and Rahamimoff, 1967; Zengel and Magleby, 1981; Zengel et al., 1994).

The average least squares error per EPP is also presented, and indicates an average error of ~ 3% in the fitting of EPP amplitudes by the model.
Table 5. A comparison between different Ca\(^{2+}\) concentrations, the average time constant for the loss of vesicles from the RRP, and the average size of the EPP at time \(t_0\), predicted by the model for different trains of patterned stimulation using 500 stimuli, for N data sets. LSE/pt is the least squares error per point (stimulus number).

<table>
<thead>
<tr>
<th>Ca(^{2+}) (mM)</th>
<th>τRRP (ms)</th>
<th>EPP</th>
<th>N</th>
<th>LSE/pt</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1-0.2</td>
<td>2118 ± 582</td>
<td>13</td>
<td>8</td>
<td>0.0006</td>
</tr>
<tr>
<td>1.0</td>
<td>1177 ± 177</td>
<td>65</td>
<td>3</td>
<td>0.0012</td>
</tr>
<tr>
<td>2.0</td>
<td>2464 ± 688</td>
<td>231</td>
<td>10</td>
<td>0.0012</td>
</tr>
</tbody>
</table>

The RRPl (ms) is the time constant for the recovery of the RRP given by Equation 9. The size of the EPP is derived from the equations for the model. N is the number of data sets from different preparations.
CHAPTER 3. DISCUSSION

The purpose of this study was to examine the interaction between four components of enhancement (F1, F2, A and P) and two components of depression during stimulation under physiological conditions. A model was developed to delineate these components under a wide range of Ca\textsuperscript{2+} concentrations, using equations constructed from four previously discovered components of enhancement and two components of depression. The depression components were modeled by the depletion of vesicles from a readily releasable pool (RRP) replenished by a depot pool (DP) of vesicles. Ca\textsuperscript{2+} concentration was not factored into the model. Rather, the initial size of the RRP was set at 10000, consistent with vesicle recycling studies in the frog (Rizzoli and Betz, 2005), and the model solved for quantal content. When depression was included in the model data obtained over 10-fold variations in both Ca\textsuperscript{2+} concentration and stimulation frequency were described. Under the stimulation patterns used in these experiments, the contributions of the A and P components of enhancement were small, so that additional observations using conditioning and testing patterns of longer duration would be necessary to assess these factors more completely.

All data sets were fit multiple times using different starting parameters to increase the chances of obtaining the best fits. Averages from best fit data sets were chosen to fix the time constants for enhancement. The fixed values for the time constants of decay for F1, F2, A and P were 46, 220, 1600, and 20000 ms, respectively, and were faster than those previously reported previously for low Ca\textsuperscript{2+}: 50 ms, 300 ms, 6 s and seconds to minutes, respectively (Magleby and Zengel, 1982). In particular, the time constant of decay for augmentation (A) was three times faster with a τ of 1600 ms compared to 6 s.
The use of physiological Mg\(^{2+}\) (1.0 mM) vs 5.0 mM (typically used in other studies of this type) may have played a role in reducing the \(\tau\) in the present study. Additional experiments are needed to verify this observation, although few visually obvious differences were seen when compared with data obtained from a drop/add train of stimulation in 5 mM Mg\(^{2+}\).

Under conditions of low Ca\(^{2+}\), the model predicted four components of enhancement, a slightly depleted RRP, a barely perceptible decline in the DP and a low number of vesicles released upon the first stimulus \((m = 13)\) (see Fig. 27 and Tables 5). As Ca\(^{2+}\) was increased, the number of vesicles released by the first stimulus increased \((at 2 \text{ mM Ca}^{2+} m = 231)\), and the RRP and DP declined more rapidly \(\) (see Fig. 20 and Table 5).

The major findings regarding depression are as follows: 1) Depression during a train of stimuli is accounted for by a model where vesicle release depletes a RRP of vesicles that is replenished at a mobilization rate slower than the rate of depletion. 2) The RRP recovers with three time constants, \(\tau_s\), ~185 ms, 3.5 s and 10.5 minutes. 3) When the RRP is depleted, the mobilization per impulse is inversely related to the stimulus frequency. That the mobilization per unit time does not change with stimulation frequency suggests that the mobilization rate during repetitive stimulation at rates between 50 and 200 Hz is independent of the changes in \([\text{Ca}^{2+}]_i\), assuming \([\text{Ca}^{2+}]_i\) changes with different stimulation rates. Other studies have suggested a \([\text{Ca}^{2+}]_i\) dependent mobilization rate (Dittman and Regehr, 1998; Wang and Kaczmarek, 1998), but Dittman and Regehr did not include an RRP in their model and Wang and Kaczmarek...
did not account for underlying components of enhancement (see Stevens and Wesseling, 1999).

**The RRP may be comprised of two types of vesicles**

The results regarding depression are consistent with the proposal (Richards et al., 2003) that two types of vesicles in the RRP are released, one type that is endocytosed slowly and another type that is rapidly recycled. Richards et al. (2003) found two time constants for vesicle recovery after depletion: a recycling pool that was depleted in 6 s and recycled in 1 min, and a reserve pool that was depleted in 40 s and refilled slowly (half-time of about 8 min). Interestingly, in these studies, low frequency (2-5 Hz) stimulation-induced release was maintained solely by the recycling pool whereas high frequency stimulation evoked release from the reserve pool. The data obtained presently indicate the RRP may be composed of vesicles from both of these pools for the following two reasons: 1) The depression of quantal release is described by two exponentials, with fast (ms) and slow (s) time constants, and 2) Release continues after depletion of the RRP. If the recycling pool (which responds to low frequency) comprises part of the RRP and is continually active but at a low rate of release, and the DP responds to high frequency stimulation but the DP vesicles in the RRP are depleted quickly, then this could explain continued release (from the recycling pool) after depletion of the pool of vesicles that respond to high frequency (the reserve pool). If this were the case both the DP and the RRP would be depleted of vesicles, as is shown in Figure 23. At rates of stimulation > 40 Hz, depression was frequency dependent (see Figures 2, 3), which may reflect the loss of vesicles from the reserve pool. Recycled vesicles could maintain mobilization and subsequent release after depletion of the RRP. Time would be required
to re-release these vesicles, which may be reflected in the present experiments by the
time course to reach a steady state of release after a step change in frequency in the
presence of depletion (Figure 9).

The major findings regarding enhancement are as follows: 1) The F1 component
of enhancement is independent of depression; 2) Four components of enhancement are
needed to predict data obtained during a stimulation train in low [Ca\textsuperscript{2+}]\textsubscript{o} but only F1 is
needed to predict data obtained during a train of stimuli under 2 mM [Ca\textsuperscript{2+}]\textsubscript{o} conditions; 3) The magnitudes of F2, A and P are larger in low [Ca\textsuperscript{2+}]\textsubscript{o} than in 1 mM [Ca\textsuperscript{2+}]\textsubscript{o}; 4) The lack of measurable F2, A and P during a train of stimuli under normal quantal release
conditions requires sufficient depression so that EPP\textsubscript{t} / EPP\textsubscript{0} < 1.

**The number of quanta available for release determines the number of detected
components of enhancement**

The most surprising result of the data is that the three components of
enhancement, F2, A and P, are not required to describe the data during repetitive
stimulation when depression is pronounced and mobilization is supplying synaptic
vesicles. It is possible that the newly mobilized vesicles are missing some factors
required for the expression of F2, A and P, or alternatively, high internal Ca\textsuperscript{2+}
accumulating during the trains could saturate possible receptors for these components.
The model in its present form does not allow saturation of the activation of the various
components. Consequently, the presence of the components in a masked form not
accounted for by the model cannot be excluded (Kalkstein and Magleby, 2004). Four
components of enhancement are present when [Ca\textsuperscript{2+}]\textsubscript{o} \leq 1 mM, consistent with the idea
that possible receptors may saturate at levels of Ca\textsuperscript{2+} that are associated with an increase
in depression. Support for this idea is shown by the decrease in the apparent power
relationship for Ca\(^{2+}\) as the external Ca\(^{2+}\) concentration is increased (Table 2), indicating that 2 mM [Ca\(^{2+}\)]\(_{o}\) is a concentration well beyond that needed for activation of mechanisms underlying F2, A and P.

Several lines of observation suggest the site of action for enhancement is near the release site (Rettig and Neher, 2002; Stevens and Wesseling 1998, 1999b; Sudhof, 2004; Voets, 2000; Voets et al., 2001). For example, augmentation is unrelated to the size of the RRP but due to an increased probability of release from the RRP (Stevens and Wesseling, 1999a; Kalkstein and Magleby, 2004). Enhancement produced by F2, A and P may be related to the activation of Ca\(^{2+}\) sensors that are coupled to vesicles at the release site. When vesicles are released, their sensors are de-coupled and no longer active. Re-coupling to new vesicles may take more time than available for actively recycled and re-released vesicles, so that F2, A and P disappear during heavy stimulation / recycling. If there are enough vesicles in the RRP, then there is time to couple vesicles to the sensor and this would explain why F2, A and P are needed to predict the data when quantal release is > EPP\(_0\) or after conditioning trains when there is time for recovery.

Several proteins acting in concert may activate release. For example, multiple Ca\(^{2+}\) sensors acting in synergy but different from the Ca\(^{2+}\) sensor that actually triggers fusion have been proposed to account for enhanced quantal release (Bennett et al 2004, 2007; Matveev et al, 2002, 2006; Tang et al., 2000). High affinity Ca\(^{2+}\) sensors such as NCS-1 activate dormant vesicles and increase the probability of release (Sippy et al., 2003). There are also a considerable number of Ca\(^{2+}\) binding proteins such as rabphillin, doc2, and scinderin that can alter the priming of mobilized vesicles or the re-priming of recycled vesicles (Calakos and Scheller, 1996; Zhang et al., 1996). The overexpression
of Munc13-1 was found to increase the size of the RRP ~ 3-fold in bovine chromaffin cells. It is not unlikely that additional sensors may also be involved in synaptic release at the frog NMJ but little is known about the properties of potential co-factors for release in this synapse and additional work is needed to shed light on possible mechanisms.

The first and second components of enhancement, facilitation (F1* and F2*), were additive indicating that two different pathways with relatively fast time constants that differ by a factor of 5 – 6 are involved in release. If there were additional Ca^{2+} sensors closely connected to the vesicle or the release sites, one with high affinity and one with low affinity, then in general when [Ca^{2+}]_i levels rise, the high affinity sensor (low K_D, high k_on rate) would saturate rapidly whereas the low affinity sensor would saturate slowly. This is in agreement with findings where intraterminal application of BAPTA affects F1 but not F2 (Delaney and Tank, 1994; Tanabe and Kijima, 1992 also see Suzuki et al., 2000; Winslow et al., 1994; Kamiya and Zucker, 1994). If F2 were related to a high affinity mechanism then it is possible that this component would be saturated during high rates of repetitive stimulation and would not be detected in higher levels of extracellular Ca^{2+}.

The model used presently does not predict either the amplitude of enhancement during the first few impulses in the trains (see Figure 20 and 21) or, as in the case of Figure 21, the loss of enhancement during the first few 2 s interstimulus intervals between the trains. However, the observed data may have been affected by the use of curare to reduce the size of the EPP and hence non-linear summation. Curare is known to increase both the amplitude of the EPP during the first few impulses of a stimulation train and the rundown of the EPP during the train (Magleby et al., 1981) so that the results
obtained for the first few impulses and the time constant for depletion of the RRP would be affected accordingly. The enhancement seen in responses to the first few stimuli could not be accounted for by adding either an additional pool of vesicles in series with the RRP and DP or multiple time constants of mobilization to the model.

F2, A or P may activate postulated additional Ca\(^{2+}\) sensors that are lost after the release of vesicles by the first few impulses. On this basis, during a train of stimulation, F2, A and P would not be needed to describe the majority of EPPs because the RRP is being depleted so quickly that there is insufficient time for sensors to couple to the newly replaced vesicles. F2, A and P arising from the activation of different sensors for Ca\(^{2+}\) would be consistent with their multiplicative relationship seen in low Ca\(^{2+}\) conditions if the Ca\(^{2+}\) sensors acted independently to increase the probability of release. If this were the case, then the resolution of F2 might be improved by including saturation terms in the model.

The loss of augmentation (A) during trains in physiological Ca\(^{2+}\) was unexpected, as Kalkstein and Magleby (2004) show enhancement of vesicle release probability with the same time constant of depression of the RRP in the frog NMJ (also see Klyachko and Stevens, 2006). But their experimental demonstration of augmentation was after, not during the train, and only appeared after a delay. In the present study, when additional trains were given after a depleting train of stimuli, they were not fit well by the model (not shown). The model gave too little enhancement of release when additional stimuli were given after a quiescent period (2 seconds) followed a depleting train of stimulation. Since the experimental enhancement was greater than predicted by the model, this agrees
CONCLUSIONS

At the neuromuscular junction of the frog sartorius muscle, short term synaptic plasticity during trains of high-frequency repetitive stimulation at physiological levels of external Ca\(^{2+}\) involves substantial depression and is described by a model in which (a) a single component of enhancement, F1, increases the probability of synaptic vesicle release and (b) depletion of a pool of vesicles readily available for release accounts for depression. Assuming that each presynaptic impulse normally releases 200 vesicles, findings indicate the readily releasable pool has a capacity of 6,000 to 10,000 vesicles and is replenished from a larger pool with a capacity of ~ 80,000 vesicles. When extracellular Ca\(^{2+}\) is reduced, the number of synaptic vesicles depleted from the RRP of the NMJ is less and four enhancement components, F1, F2, A, and P, are required to accurately describe release during stimulation trains. Future experiments include expanding the model to include physical representations of the enhancement components to complement the physical representations of the depression components in order to determine the structural and Ca\(^{2+}\) handling mechanisms that can account for the observed results, including the apparent disappearance of F2, A, and P during the stimulation trains.
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