Calcium regulation and transmitter release at drosophila larval neuromuscular junction

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Abstract

Calcium ions act as the most versatile 2\textsuperscript{nd} messenger and are involved in an incredibly broad spectrum of biological processes. Proper cellular Ca\textsuperscript{2+} homeostasis is paramount in maintaining normal cell growth, functions, survival and programmed death. Hence, understanding Ca\textsuperscript{2+} regulation has been a goal of and a hot topic in cutting-edge biological research for decades. Recently, the advent of a new generation of Ca\textsuperscript{2+} indicators has made a major contribution to advances in understanding Ca\textsuperscript{2+} regulation (Gryniewicz et al., 1985). Likewise, the application of *Drosophila melanogaster* as an animal model has provided tantalizing clues and fueled the progress of research towards this goal.

This dissertation is composed of studies that are dedicated to a better understanding of Ca\textsuperscript{2+} regulation in *Drosophila* larval neuromuscular junction, which is a powerful model system for studying synaptic development and function (Keshishian et al., 1996). In chapter I, the Ca\textsuperscript{2+} regulation in the two most commonly used motor terminals, Ib and Is, was studied and the mechanisms responsible for the regulation were explored and discussed. Chapter II investigated the endogenous Ca\textsuperscript{2+} buffering system that helps shape the Ca\textsuperscript{2+} signals in Ib terminals. Chapter III examined the effects of ectopically expressing a slow Ca\textsuperscript{2+} buffering protein, parvalbumin (*PV*), on short-term synaptic enhancement, synaptic morphology and Ca\textsuperscript{2+} regulation. Lastly, chapter IV studied Ca\textsuperscript{2+} regulation under a pathological condition, i.e. chronic lead (Pb\textsuperscript{2+}) toxicity, and examined the corresponding changes in synaptic facilitation.
# Table of Contents

ACKNOWLEDGMENTS........................................................................................................i

ABSTRACT......................................................................................................................ii

TABLE OF CONTENTS.....................................................................................................iii

LIST OF FIGURES...........................................................................................................v

INTRODUCTION...............................................................................................................1

CHAPTER I DIFFERENCES IN CA\(^{2+}\) REGULATION FOR HIGH-OUTPUT IS AND LOW-OUTPUT IB MOTOR TERMINALS IN DROSOPHILA LARVAE..............................................................................................23

ABSTRACT......................................................................................................................23

INTRODUCTION...............................................................................................................25

MATERIALS AND METHODS...........................................................................................28

RESULTS.........................................................................................................................34

DISCUSSION....................................................................................................................46

CHAPTER II CA\(^{2+}\) BUFFERING AT A DROSOPHILA LARVAL SYNAPTIC TERMINAL.................................................................................................................................52

ABSTRACT......................................................................................................................52

INTRODUCTION...............................................................................................................53

MATERIALS AND METHODS...........................................................................................55

RESULTS.........................................................................................................................58

DISCUSSION....................................................................................................................64

CHAPTER III EFFECTS OF PRESYNAPTIC PARVALBUMIN EXPRESSION ON SYNAPTIC PHYSIOLOGY, MORPHOLOGY AND CA\(^{2+}\) REGULATION.................................................................................................................69

ABSTRACT......................................................................................................................69

INTRODUCTION...............................................................................................................71

MATERIALS AND METHODS...........................................................................................74
List of Figures

Figure                                           Page
1-1 Two separate Ib terminals on MF4…………………………………………35
1-2 Ca$^{2+}$ transients measured in the Is and Ib terminals innervating
MF6/7 and 4……………………………………………………………………36
1-3 The amplitude of Ca$^{2+}$ transients seen at Is and Ib boutons on
MFs 6/7 and 4……………………………………………………………………37
1-4 The Ca$^{2+}$ $\tau_{\text{decay}}$ after single APs and AP trains for Is and Ib
boutons was determined from the decay of $\Delta F/F$…………………………38
1-5 Comparison of single-AP Ca$^{2+}$ transients in Is and Ib terminals
with similar OGB-1 concentrations……………………………………………40

Table1-1 Comparison of Ca$^{2+}$ transients produced by single APs
and trains of APs on MF6/7 for [OGB-1]-matched Is and Ib terminals……40

1-6 Measurements of single-AP Ca$^{2+}$ transients using a photomultiplier tube.…41
1-7 The effect of bouton position on single-AP Ca$^{2+}$ transients
for Is terminals from MFs 4 and 6/7………………………………………………42
1-8 Staining of active zones along Is and Ib terminals on MF6/7………………44

Table1-2 Comparing structural parameters between most distal
boutons and more proximal ones for Is and Ib terminals on MF6/7………44
1-9 Comparison of the number of active zones along Is and Ib
terminals on MF6/7………………………………………………………………45
2-1 The $\Delta F/F$ recorded from Ib boutons during AP trains…………………58
2-2 Increases in $[\text{Ca}^{2+}]_i$ produced by single APs and AP trains…………60
2-3 The effect of [OGB-1] on single-AP Ca$^{2+}$ transients and
determination of $\kappa_S$…………………………………………………………62
3-1 Anti-Myc immunostaining revealed the expression of $PV$ at
the motor terminal innervating MF5 at NMJs (arrow)…………………………78
3-2 Expression of $PV$ produced a dramatic reduction in PPF at
Drosophila NMJs………………………………………………………………….81
3-3 $PV$-expressing terminals showed less synaptic facilitation
during a train of APs…………………………………………………………82
3-4 $PV$-expressing terminals showed greater single-AP transmitter
release than the controls.................................................................84
3-5 Expression of PV caused hypomorphic development of the
motor terminals................................................................................86
3-6 The amplitude and decay of Ca\(^{2+}\) transients seen at the
PV-expressing terminals and control terminals.................................89
4-1 Ca\(^{2+}\) transients recorded from synaptic boutons in control and
Pb\(^{2+}\)-exposed animals....................................................................109
4-2 The Ca\(^{2+}\) transient amplitude in synaptic boutons from control
larvae and those exposed to 100 or 250µM Pb\(^{2+}\).................................111
4-3 The Ca\(^{2+}\) transient decay time constant in synaptic boutons
from control and Pb\(^{2+}\)-exposed animals...........................................112
4-4 Ca\(^{2+}\) transient decay after 20Hz AP train normalized to the
final response at the end of the AP train.............................................112
4-5 Comparison of the resting fura-2 ratio (360/380) in control and Pb\(^{2+}\)-exposed
Ib terminals on muscle fiber 6/7 before and after TPEN application.....114
4-6 EPSPs recorded from muscle fibers in control and Pb\(^{2+}\)-exposed
larvae during 20Hz stimulation.........................................................116
4-7 Mean EPSP amplitudes and synaptic facilitation in control larvae
and those exposed to 250µM Pb\(^{2+}\)......................................................117
Introduction

The nervous system contains numerous neurons and an even greater number of synapses. In order for the organism to respond to the external and internal environment, these neurons need to be able to communicate with one another, and synapses make this happen. There are two classes of synapses: electrical synapse and chemical synapse, with the latter being the majority and most studied. Chemical synapses allow neuron-to-neuron or neuron-to-target communication by secreting neurotransmitter. This event is triggered by the propagation of action potentials along the axon to the presynaptic terminal, which then opens the voltage-gated calcium channels (VGCCs) on the presynaptic membrane. The great concentration gradient of Ca$^{2+}$ across the membrane (external: ~1mM; internal: ~100nM; about $10^4$-fold difference) will cause a rapid influx of Ca$^{2+}$ into the presynaptic terminal upon the opening of Ca$^{2+}$ channels, allowing synaptic vesicles, the presynaptic organelles that contain neurotransmitter, to fuse with the presynaptic membrane and release transmitter molecules into the synaptic cleft. These molecules bind to the postsynaptic receptors, producing postsynaptic potentials.

Ca$^{2+}$ is well known and proven to play a pivotal and essential role in synaptic transmission. Katz and Miledi showed for the first time that transmitter release depends on the presence of extracellular Ca$^{2+}$ rather than Na$^+$; they successfully elicited EPSPs while blocking the Na$^+$ channels on the presynaptic membrane with tetrodotoxin in the presence of extracellular Ca$^{2+}$ (Katz and Miledi, 1967). The role of presynaptic Ca$^{2+}$ in transmitter release has also been directly demonstrated by injecting Ca$^{2+}$ into the presynaptic terminals, which was found to trigger transmitter release without any presynaptic action potential (Miledi, 1971). When presynaptic Ca$^{2+}$ was chelated by
injecting the Ca\textsuperscript{2+} chelator BAPTA into the presynaptic terminals, it was found that action potentials were not able to trigger transmitter release (Adler et al., 1991). Llinas et al., using the voltage clamp technique at the squid giant synapse, demonstrated that Ca\textsuperscript{2+} influx occurs through voltage-gated calcium channels (VGCCs) in the presynaptic membrane; blockade of these VGCCs pharmacologically could inhibit the transmitter release (Llinas et al., 1981a; Llinas et al., 1981b). The presynaptic Ca\textsuperscript{2+} rise following the Ca\textsuperscript{2+} influx was confirmed by the fluorescence change reported from the Ca\textsuperscript{2+}-indicator, fura-2, injected into the giant presynaptic terminals (Smith et al., 1993). All this evidence shows the importance of presynaptic Ca\textsuperscript{2+} in the process of transmitter release.

Besides its role in transmitter release, presynaptic intracellular Ca\textsuperscript{2+} is also highly involved in many other neuronal functions and processes, such as gene expression (Bito et al., 1997), neuronal development (Aamodt and Constantine-Paton, 1999; Moody and Bosma, 2005), neuronal differentiation (Spitzer et al., 2004), neuronal migration (Komuro and Rakic, 1998), synaptogenesis (Cline, 2001), neuronal survival (Vicencio et al., 2010), and various forms of synaptic plasticity (Zucker and Regehr, 2002).

Meanwhile, postsynaptic intracellular Ca\textsuperscript{2+} is also of importance in the induction of long-term potentiation (LTP) and long-term depression (LTD) (Yang et al., 1999), dendrite growth or retraction (Wong and Ghosh, 2002), just to name a few.

The involvement of Ca\textsuperscript{2+} in these essential functions necessitates the precise regulation of intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}). In general, Ca\textsuperscript{2+} regulation is executed by several “functional units” that include Ca\textsuperscript{2+} influx, Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release, endogenous Ca\textsuperscript{2+} buffering, internal Ca\textsuperscript{2+} uptake/sequestration and Ca\textsuperscript{2+} extrusion system.

**Ca\textsuperscript{2+} regulation**
Ca\textsuperscript{2+} influx

At presynaptic terminals, Ca\textsuperscript{2+} entry through voltage-gated Ca\textsuperscript{2+} channels (VGCCs) produces local and global increases in [Ca\textsuperscript{2+}]. These local large increases in [Ca\textsuperscript{2+}], appear to be particularly important for regulating cellular processes: this has been most clearly demonstrated for transmitter release. Upon membrane depolarization, VGCCs open and a transient high concentration of Ca\textsuperscript{2+}, usually on the order of hundreds of µM in magnitude (Llinas et al., 1992b), will form near the cytoplasmic mouth of VGCCs. This high concentration of local presynaptic Ca\textsuperscript{2+} is usually termed “microdomain Ca\textsuperscript{2+} signal” by convention (Llinas et al., 1992b), and can be visualized using low-affinity fluorescent Ca\textsuperscript{2+} indicators with imaging technique (Tucker and Fettiplace, 1995; Llinas et al., 1995; DiGregorio and Vergara, 1997). The microdomain Ca\textsuperscript{2+} signal usually lasts very briefly, e.g. for about 1 msec in the squid giant synapse (Llinas et al., 1995); the magnitude of this Ca\textsuperscript{2+} is produced by the Ca\textsuperscript{2+} influx through VGCCs (see above) and has to rise to tens of µM in order to trigger neurotransmission, e.g. 50 µM in goldfish retinal bipolar neurons (Heidelberger et al., 1994) and about 75 µM at the crayfish NMJs (Lando and Zucker, 1994). The VGCCs are located immediately adjacent to and have direct interaction with the secretory machinery for transmitter release, i.e. the complex of SNARE proteins (Catterall, 2000; Catterall and Few, 2008). This co-localization enables fast transmitter release upon action potential invasion on the nerve terminals; it also makes VGCC a possible regulatory checkpoint for the Ca\textsuperscript{2+} signal. Ca\textsuperscript{2+} influx can also occur through receptor-operated channels such as the NMDA receptors; since these receptors are located primarily at postsynaptic sites, they are not discussed here.
VGCCs are composed of 4 or 5 distinct subunits (Catterall, 2000); the single membrane–spanning pore-forming subunit regulates Ca\(^{2+}\) influx upon membrane depolarization. The Ca\(^{2+}\) currents in different cell types have different gating kinetics, physiological roles and pharmacological profiles; an alphabetical nomenclature has been developed for the different classes of Ca\(^{2+}\) currents, such as N-type, P/Q-type and R-type Ca\(^{2+}\) currents (Tsien et al., 1988; Tsien et al., 1991). More recently, a new nomenclature based on sequence similarity in genes coding for the \(\alpha_1\) subunit of the channel was proposed (Ertel et al., 2000); it divides the Ca\(^{2+}\) channels into 3 subfamilies, i.e. Cav1, Cav2 and Cav3. In the Cav2 subfamily there are 3 members, Cav2.1, Cav2.2 and Cav2.3. Ca\(^{2+}\) responsible for synaptic transmission at fast synapses enters nerve terminals primarily through Cav2.1 and Cav2.2 channels (Dunlap et al., 1995); Cav2.1 channels conduct the P/Q-type Ca\(^{2+}\) transients and are responsible for the Ca\(^{2+}\) entry that triggers synaptic transmission at the majority of synapses in the mammalian nervous system (Day et al., 1997; Llinas et al., 1992a), whereas Cav2.2 channels conduct N-type Ca\(^{2+}\) currents and appear to play a role in mediating vesicle replenishment during stimulation at very high frequency (e.g. 50Hz) rather than in evoked transmitter release during low frequency of stimulation (e.g. 5Hz) (Perissinotti et al., 2008).

Ca\(^{2+}\)-dependent regulation of VGCCs has been observed in some types of cells and synapses. Generally speaking, for repetitive stimulation, Ca\(^{2+}\) currents become increasingly greater during the first few shocks due to current facilitation and then gradually decrease in size due to current inactivation for the subsequent pulses (Cuttle et al., 1998; Lee et al., 2000a). Current facilitation appears to have more rapid kinetics than current inactivation, since chelating Ca\(^{2+}\) with a fast buffer such as BAPTA has a great
effect on facilitation (Cuttle et al., 1998), whereas a high concentration of slow Ca\(^{2+}\) buffer such as EGTA can prevent current inactivation but not facilitation (Lee et al., 2000a). For current inactivation, it appears that the global rise in Ca\(^{2+}\) concentration mediated by multiple closely-located Ca\(^{2+}\) channels is required, since the Ca\(^{2+}\) currents do not show current inactivation in neuronal cell bodies where the channel density is low (Chaudhuri et al., 2005; Mochida et al., 2008), whereas in non-neuronal cells transfected with high density of Ca\(_V\)2.1 channels such Ca\(^{2+}\)-dependent inactivation was observed (Lee et al., 1999; Lee et al., 2000a). In summary, these two forms of Ca\(^{2+}\)-dependent regulation allow for activity-dependent fine-tuning of the Ca\(^{2+}\) signal; during short bursts of impulses current facilitation enhances the Ca\(^{2+}\) transients and increases release probability and thus transmitter release is ensured, whereas during long trains, current inactivation gradually decreases Ca\(^{2+}\) transients and thus helps somewhat prevent Ca\(^{2+}\) overload (Berridge et al., 2000). Note that, however, these forms of VGCC regulation were mostly described in transfected non-neuronal cells (Lee et al., 1999; Lee et al., 2000a; Liang et al., 2003; DeMaria et al., 2001; Kreiner and Lee, 2006) and occasionally in CNS synapses such as the calyx of Held (Forsythe et al., 1998) and the acutely isolated thalamocortical relay neurons of the dorsal lateral geniculate nucleus (Meuth et al., 2005); it has never been shown in the presynaptic terminals at NMJs. Moreover, this regulation does not exist in some CNS neurons (Chaudhuri et al., 2005; Mochida et al., 2008). Thus, the regulation of VGCCs through this mechanism does not appear to be universal.

\[ \text{Ca}^{2+}\text{-induced Ca}^{2+}\text{ release (CICR)} \]
CICR can contribute to the \([\text{Ca}^{2+}]_i\) by the release of \(\text{Ca}^{2+}\) from the \(\text{Ca}^{2+}\) store primarily held within the membrane systems of the endoplasmic reticulum (ER) in neuronal cells and synaptic terminals (Berridge et al., 2000). The \(\text{Ca}^{2+}\) release is operated by two main classes of receptors on ER (Henzi and MacDermott, 1992; Simpson et al., 1995): the inositol-1, 4, 5-trisphosphate receptors (IP3Rs), which are distributed ubiquitously throughout the brain with particularly high levels being reported in cerebellar Purkinje cells (Martone et al., 1997; Furuichi et al., 1994), and the ryanodine receptors (RYRs), which are primarily located in the soma of neurons but also exist in other regions (Kuwajima et al., 1992).

These two receptors are sensitive to a variety of regulators, the most important of which is \(\text{Ca}^{2+}\) itself (Berridge et al., 2000). \(\text{Ca}^{2+}\) can act on these receptors from either the lumenal or the cytoplasmic side of the ER. Within the lumen, increased \(\text{Ca}^{2+}\) levels caused by the ER uptake after neural activity can increase the sensitivity of both receptors to their ligands, i.e. IP3 and cyclic ADP ribose. On the cytosolic side, the action of \(\text{Ca}^{2+}\) is more complicated for IP3R (Simpson et al., 1995; Berridge et al., 2000). In the presence of low concentrations of IP3, \(\text{Ca}^{2+}\) action is “bell-shaped”: low concentrations of \(\text{Ca}^{2+}\) (100-300nM) are stimulatory, but above 300nM the action is inhibitory. In the presence of high concentration of IP3, \(\text{Ca}^{2+}\) action is sigmoidal, i.e. high cytosolic \(\text{Ca}^{2+}\) is stimulatory instead of inhibitory (Bootman and Lipp, 1999). For RYRs, the general action of cytosolic \(\text{Ca}^{2+}\) is to increase the receptor sensitivity, similar to that of the lumenal \(\text{Ca}^{2+}\) (Kano et al., 1995).

The most effective activation of these two types of receptors requires the presence of both \(\text{Ca}^{2+}\) and their ligands (i.e. IP3 and cyclic ADP ribose) or inducers (such as
caffeine for RYRs) (Berridge, 1998). This implies that the Ca\(^{2+}\) entering from outside serves as the trigger to promote the Ca\(^{2+}\) release from the internal stores (Li and Hatton, 1997; Shmigol et al., 1995), which in turn serves as the amplification of the Ca\(^{2+}\) influx. This internal amplification process has been found to operate in many cell types, such as dorsal root ganglion cells (Shmigol et al., 1995), rat cerebellar Purkinje neurons (Kano et al., 1995) and hippocampal neurons (Alford et al., 1993).

**Internal Ca\(^{2+}\) uptake/sequestration**

Mitochondria are an active and major player in Ca\(^{2+}\) internal sequestration. The Ca\(^{2+}\) uptake is achieved by the action of the mitochondrial Ca\(^{2+}\) uniporter which is allosterically activated by Ca\(^{2+}\) itself (Bernardi, 1999; Gunter et al., 2004) and the Ca\(^{2+}\)/H\(^+\) antiporter (Jiang et al., 2009; Sparagna et al., 1995). Mitochondria are considered as a low-affinity, high-capacity “Ca\(^{2+}\) sink” (Toescu, 2000), because the uniporter has a low sensitivity to Ca\(^{2+}\) (half-maximal activation around 15\(\mu\)M) (Rizzuto et al., 1993). For Ca\(^{2+}\)/H\(^+\) antiporter, the proton gradient that is necessary for ATP production is also utilized for Ca\(^{2+}\) uptake by the antiporter (Duchen, 1999). This mitochondrial Ca\(^{2+}\) uptake can produce significant effects on the global intracellular Ca\(^{2+}\) signals. For instance, it can cause a reduction in the amplitude of the global Ca\(^{2+}\) transient (Herrington et al., 1996); pharmacological blockade of mitochondrial uptake produced higher presynaptic Ca\(^{2+}\) levels (Suzuki et al., 2002; Bennett et al., 2007). This form of uptake can operate at intracellular Ca\(^{2+}\) concentrations as low as 150-300 nM (Pitter et al., 2002); it is also effective in sequestrating Ca\(^{2+}\) on the order of hundreds of \(\mu\)M during fast Ca\(^{2+}\) transients present in the vicinity of VGCCs or CICR sites of ER (Rizzuto et al., 2009). Notably, the
high-capacity of mitochondria for loading Ca\(^{2+}\) prevents the intracellular Ca\(^{2+}\) from rising too high; once the Ca\(^{2+}\) level returns to baseline, a mitochondrial Na\(^+/\)Ca\(^{2+}\) exchanger pumps the load of Ca\(^{2+}\) back to the cytoplasm for either ER uptake or extrusion (Szabadkai and Duchen, 2008); meanwhile, the slow release of the accumulated mitochondrial Ca\(^{2+}\) resulting from tetanic stimulation appears to be related to the production of posttetanic potentiation at the crayfish NMJs (Tang and Zucker, 1997).

Besides being a major site for CICR, ER is also considered to be a Ca\(^{2+}\) sink; it relies on its membrane Ca\(^{2+}\) pumps, i.e. SERCA, for the action of Ca\(^{2+}\) uptake. SERCA is an ATP-driven Ca\(^{2+}\) pump that was first discovered in skeletal muscle cells (Hasselbach and Makinose, 1961; Ebashi and Ebashi, 1962); it was found to transport two Ca\(^{2+}\) per ATP hydrolyzed into the ER lumen and meanwhile counter-transport H\(^+\) in exchange for Ca\(^{2+}\) (Yu et al., 1993). With this efficient Ca\(^{2+}\) transport system, ER serves as an effective buffering system in many cell types. For instance, blocking the SERCA pumps with thapsigargin resulted in a much slower clearance of the Ca\(^{2+}\) signal arising from the single action potential-evoked depolarization in rat neocortical layer V pyramidal neurons (Markram et al., 1995). This appears to be true in the *Drosophila* larval neuromuscular junction (NMJ): applying thapsigargin lowered the Ca\(^{2+}\) clearance rate after single APs as well as trains of APs, although this effect on Ca\(^{2+}\) clearance was less dramatic than that from inhibiting the plasmic membrane Ca\(^{2+}\) ATPase (Lnenicka et al., 2006a). As mentioned above, increased level of Ca\(^{2+}\) in the ER lumen resulting from the SERCA uptake can increase the sensitivity of the IP\(_3\)R and RYRs and thus the chance of triggering CICR by neural activity, i.e. it makes the cells more excitable to subsequent stimulations; this implies that ER might serve as a “memory” of neuronal activity: Ca\(^{2+}\)
influx resulting from brief pulses would be buffered, whereas the Ca\textsuperscript{2+} resulting from prolonged stimulation could charge up the ER enough to transmit regenerative signals to the nucleus for initiating gene transcription (Berridge, 1998).

\textit{Cytosolic Ca\textsuperscript{2+} binding proteins (CBPs)}

The most common physiologically significant CBPs include calbindin D\textsubscript{28K} (CB-D28K), calretinin (CR) and parvalbumin (PV). They belong to the EF-hand family, in which the EF-hand is a Ca\textsuperscript{2+}-coordinating helix-loop-helix structure that binds Ca\textsuperscript{2+} (Kretsinger and Nockolds, 1973). In the resting condition, these proteins remain largely in the Ca\textsuperscript{2+}-free state, since their dissociation constants for Ca\textsuperscript{2+} are about one order of magnitude larger than the resting Ca\textsuperscript{2+} level in the cytoplasm (~100nM) (Schwaller, 2010). Their chemodynamic features, including their intracellular concentration, Ca\textsuperscript{2+}-binding affinities, and mobility, will determine their impacts on the Ca\textsuperscript{2+} signal. For instance, PV shows a slow Ca\textsuperscript{2+} binding rate (\(K_{on} = 10.7\times10^7 \text{ M}^{-1}\text{S}^{-1}\)) (Schmidt et al., 2003b); this feature indicates that under physiological conditions, PV hardly has any effects on the Ca\textsuperscript{2+} transient amplitude, which is considered as a dynamically fast process, but it could dramatically spend up the initial decay of Ca\textsuperscript{2+} transients and prolong the late phase of Ca\textsuperscript{2+} decay due to Ca\textsuperscript{2+} unbinding from PV (Lee et al., 2000c). In other words, PV can convert the monoexponential Ca\textsuperscript{2+} decay into a biexponential one (Collin et al., 2005; Lee et al., 2000c). Another example is CB-D28K. It has a fast Ca\textsuperscript{2+} binding site (\(K_{on} =5.5\times10^6 \text{ M}^{-1}\text{S}^{-1}\)) and a medium Ca\textsuperscript{2+} binding site (\(K_{on} =4.35\times10^6 \text{ M}^{-1}\text{S}^{-1}\)) (Schmidt et al., 2003b). The fast Ca\textsuperscript{2+} binding site is fast enough to compete for the Ca\textsuperscript{2+} that triggers transmitter release, i.e. the Ca\textsuperscript{2+} transient peak will be significantly decreased
(Airaksinen et al., 1997); the Ca\(^{2+}\) unbinding will prolong the Ca\(^{2+}\) decay (Schmidt et al., 2003b).

Different CBPs are found in different subsets of cells. For instance, cells containing high concentrations of PV include most GABA-ergic neurons (Klausberger, 2009), fast-twitch muscle fibers (Berchtold et al., 2000) and epithelial cells in the early distal convoluted tubule in nephrons and collecting ducts of the kidney (Zacchia and Capasso, 2008). This selective distribution is believed to be physiologically significant. For example, PV-containing neurons are known to fire repetitively at very high frequency (Kawaguchi and Kubota, 1993; Baimbridge et al., 1992); this unique feature is thought to be contributed to by the ability of PV to bring down the presynaptic Ca\(^{2+}\) quickly, preventing the Ca\(^{2+}\) concentration from reaching the critically detrimental level, as well as the ability of PV to prevent cumulative facilitation so that the strength of synapse remains constant (Schwaller, 2010).

\textit{Ca}^{2+} \textit{extrusion}

In general, Ca\(^{2+}\) extrusion is accomplished by the action of Na\(^{+}/\text{Ca}^{2+}\) exchanger (NCX) and/or plasma membrane Ca\(^{2+}\)-ATPase (PMCA). NCX was first discovered in heart muscle (Reuter and Seitz, 1968) and squid axon (Blaustein and Hodgkin, 1969) and is a high capacity, low affinity Ca\(^{2+}\) transporter. Until now, five isoforms of NCX have been identified (Matsuoka, 2004); different isoforms have different tissue distributions (Blaustein and Lederer, 1999). In general, NCX relies on the net electrochemical driving force to move Ca\(^{2+}\) into or out of cells; the net Ca\(^{2+}\) movement mediated by NCX may change direction according to the different stages of a cell’s activity, e.g. when the
membrane potential changes or when the intracellular Na$^+$ or Ca$^{2+}$ concentration changes. In nervous system, NCX has very high expression levels, especially in regions where Ca$^{2+}$ activity across the membrane is very high, such as at synapses (Luther et al., 1992; Reuter and Porzig, 1995). In line with this, NCX isoforms found in nervous system are activated by the intracellular Ca$^{2+}$ (Blaustein and Lederer, 1999). It is found to play a major role in Ca$^{2+}$ extrusion in the growth cones of regenerating crayfish motor axon (Lnenicka et al., 1998) as well as in removing cytosolic free Ca$^{2+}$ from crayfish motor terminals, working together with mitochondrial Ca$^{2+}$ uptake (Mulkey and Zucker, 1992; Tang and Zucker, 1997). However, the isoform found in Drosophila (CalX) appears unique: it is inhibited by intracellular Ca$^{2+}$ (Hryshko et al., 1996). CalX is believed to play a less important role in Ca$^{2+}$ clearance at Drosophila larval NMJs, since inhibiting its activity did not affect the amplitude of the Ca$^{2+}$ transient during trains of action potentials and only slightly prolonged the decay of Ca$^{2+}$ (Lnenicka et al., 2006a). This Drosophila isoform is critical for the survival of photoreceptor cells, but its function is not known in the Drosophila nervous system (Wang et al., 2005).

PMCA was first discovered in 1966 as an ATP-driven Ca$^{2+}$ transport system that expelled Ca$^{2+}$ from erythrocytes (Schatzmann, 1966); it is now known that PMCA pump exists ubiquitously in most cell types and has four isoforms with many splice variants (Strehler and Zacharias, 2001). This pump has a high Ca$^{2+}$ affinity ($K_d$ in the range of 0.2-0.5µM) when stimulated but a low transport capacity compared to NCX (Carafoli and Brini, 2000; Strehler and Zacharias, 2001; Furukawa et al., 1988) and operates with a 1:1 Ca$^{2+}$/ATP stoichiometry (Brini and Carafoli, 2009). Generally speaking, unstimulated PMCA pump has poor affinity for Ca$^{2+}$ ($K_m > 10$ µM) and therefore is essentially inactive.
under the resting condition when the Ca$^{2+}$ concentration is less than 100nM (Brini and Carafoli, 2009); its $K_d$ can be changed through binding of multiple activators. For instance, calmodulin (CaM) is an extensively studied activator of PMCA (Brini and Carafoli, 2009); it interacts with the CaM-binding-domain (CaM-BD) located in the C-terminal cytosolic tail of PMCA (Strehler and Zacharias, 2001) and releases the pump from its auto-inhibition state, leading to a decrease of $K_d$ for Ca$^{2+}$ to hundreds of nM (Enyedi et al., 1987; Falchetto et al., 1991). PMCA pumps are also activated by the acidic phospholipids and polyunsaturated fatty acids; this activation is even more powerful than CaM, since it decreases $K_d$ to about 100 nM (Enyedi et al., 1987), but its role in vivo is less clear than that of CaM (Brini and Carafoli, 2009). Besides the extrusion of cytosolic Ca$^{2+}$ from the cells and terminals, one important function of PMCA is to maintain the resting Ca$^{2+}$ level in the cells. For instance, the basal [Ca$^{2+}$]$_i$ in cultured DRG neurons was significantly changed by inhibiting PMCA (Benham et al., 1992). At Drosophila larval NMJs, PMCA appears to be paramount in presynaptic Ca$^{2+}$ clearance, since inhibiting PMCA doubled the decay time constant of Ca$^{2+}$ transients for single APs and increased it by fivefold for AP trains, whereas inhibiting other clearance mechanisms showed much less dramatic effects; meanwhile, inhibiting PMCA doubled the Ca$^{2+}$ transient plateau during 10Hz trains of APs, indicating that this pump was important for establishing the plateau as well (Lnenicka et al., 2006a).

$\text{Ca}^{2+}$ and exocytotic transmitter release

Communication between neurons or neurons and their targets is accomplished through exocytotic neurotransmitter release; this takes place at highly specialized sites:
the synapses. At fast synapses, the exocytotic transmitter release requires the presence of Ca²⁺ microdomains at the active zones near where the readily releasable vesicles are docked. The microscopic Ca²⁺ signal is believed to have a high cooperativity in inducing vesicle fusion at synapses, since peak rate of the depolarization-induced transmitter release is at least about 10⁵-10⁶ fold higher than the rate of spontaneous release, whereas the magnitude of the microdomain Ca²⁺ signal (about 50 µM) responsible for the evoked release is only about 10³ higher than that of the resting Ca²⁺ concentration (about 50nM) thought to be responsible for the spontaneous release (Schneggenburger and Neher, 2005). Studies showed that the microdomain Ca²⁺ signals at the active zones corresponds well to the sites of vesicle fusion, which give rise to transmitter release (Zenisek et al., 2003; Becherer et al., 2003); this configuration is believed to prevent the synapse from starting spontaneous transmitter release in a large scale, i.e. a release magnitude similar to that of AP-evoked transmitter release, since at least one Ca²⁺ channel per active zones have to be activated to trigger exocytotic transmitter release (Llinas et al., 1992b; Simon and Llinas, 1985).

**Ca²⁺ and activity-dependent synaptic enhancement**

The strength of chemical synapses can be dynamically modified to reflect the history of use. This change of synaptic strength, which is usually termed synaptic plasticity, is believed to play a fundamental role in information processing and learning and memory (Markram et al., 1998). Various forms of synaptic enhancement have been described, based on their distinct lifespan: (1) The most transient form is facilitation. At some synapses it can be clearly subdivided into F₁ and F₂ components; the F₁ component
lasts tens of milliseconds while the F₂ component lasts hundreds of milliseconds. They can be measured by paired-pulse stimulation or very brief AP trains (Mallart and Martin, 1967). (2) Another form of synaptic enhancement with a decay time constant of 5-10 seconds (Magleby and Zengel, 1976a; Zengel et al., 1980) is augmentation. It is sufficient to induce it by a train of action potentials delivered at a medium frequency. (3) The third form with a longer time course of up to 30 seconds to several minutes is post-tetanic potentiation (PTP) (Rosenthal, 1969; Magleby and Zengel, 1975b). It is often induced by delivering a relative long tetanus of action potentials. (4) The even longer-lasting forms of synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD), which have time courses of up to several hours, have also been described at CNS synapses (Nelson and Turrigiano, 2008), but these are not the focus of the current study and will not be discussed.

**F₁ and F₂ components of facilitation**

Mallart and Martin (Mallart and Martin, 1967) laid the foundation for the description of the two components of facilitation (F₁ and F₂) and provided the first evidence for F₁ and F₂ at the frog neuromuscular junction. In this research, the authors used the protocol of paired-pulse stimulation to elicit the facilitation; the quantification of the facilitation was achieved by measuring the fractional increase in the amplitude of the testing end-plate potential over the first end-plate potential. By this, they found that the decay of F₁ could be reasonably fit to a single exponential described as \( f = f₁ \exp(-bt) \), where \( f₁ \) was the zero intercept at Y-axis and \( 1/b \) was the time constant of decay. Based on this finding, they developed a model that allowed them to derive the \( f₁ \) and \( 1/b \) to
predict the decay of the facilitation elicited by the other protocol, such as 5-pulse conditioning train, and found that the experimental decay of the facilitation elicited by 5-pulse conditioning train fit to the prediction curve very well within the first 50 msec after the conditioning stimuli, which was obviously the F₁ component; beyond that time scale, the experimental points deviated from the prediction curve. By subtracting the predicted curve from the experimental curve, they derived the other component of facilitation, namely F₂, which had an onset time of 60-80 msec after the conditioning stimuli, peaked at about 120 msec with a decay time constant of several hundreds of msec. They came to the conclusions that the F₂ component was late in onset and that F₁ and F₂ were additive. There appeared to be neither potentiation nor fatigue of whatever processes were responsible for the two components during the short lifespan of them. Magleby (Magleby, 1973a) also used the above linear summation model and successfully predicted the buildup and decay of facilitation for the first few hundred msec after the conditioning shock until he saw additional synaptic enhancement after that time scale, which he called potentiation. Interestingly in the studies of the frog neuromuscular junction (Mallart and Martin, 1967), squid synapses (Charlton and Bittner, 1978), and crayfish neuromuscular junction (Bittner and Sewell, 1976), F₂ was obviously preceded by a “hump”, i.e. a slight additional facilitation, or a temporary plateau phase, whereas in other studies of the frog neuromuscular junction (Magleby, 1973a), mouse neuromuscular junction (Gage and Murphy, 1981), and crayfish neuromuscular junction (Linder, 1974), the facilitation was found to be a smooth, two-phase decay curve. It is not known why this occurs.

Pharmacologically, the addition of Sr²⁺ to the medium selectively increased the magnitude of F₂ but not F₁ (Zengel et al., 1980), indicating that they might be derived
from two distinct processes. But also note that the effects of Sr$^{2+}$ may be due to its buffering action or its interference in presynaptic Ca$^{2+}$ removal during F2 facilitation phase, supporting the conclusion drawn from previous research mentioned above (Xu-Friedman and Regehr, 2000).

**Augmentation**

Magleby et al. (Magleby and Zengel, 1976b) gave the first detailed description of augmentation as a process that acts to increase transmitter release and has a time course intermediate in duration between those of facilitation and post-tetanic potentiation (PTP). In the study, the authors used 20Hz trains for 15 sec to condition the nerve and then applied testing stimuli at 1.5-2 sec intervals immediately after the trains to examine the decay of augmentation and found that the decay time constant was about 7 sec. The important points they drew from the observations were that augmentation was usually present following repetitive stimulation and that the magnitude of augmentation was often sufficiently great to become a major factor in increasing EPSP amplitudes following repetitive stimulation. Augmentation proves to be a different process from facilitation and PTP, since pharmacological experiments showed that its magnitude could be selectively enhanced by Ba$^{2+}$ but neither facilitation nor PTP; however, its time constant of decay was not affected by Ba$^{2+}$ (Zengel et al., 1980). It should be noted that, it is difficult to measure augmentation separately in the absence of PTP, since the regular protocols used to elicit augmentation also could produce a certain degree of PTP. Thus, augmentation could only be estimated in terms of certain models that define the relationship between these two forms of enhancement. It was found that using a different
model could only affect the magnitude of augmentation, but its time constant of decay remained the same (Zengel and Magleby, 1980).

**Post-tetanic potentiation (PTP)**

PTP could be measured readily without much contamination of other forms of synaptic enhancement, since $F_1$, $F_2$ and augmentation decay to insignificant levels in ~200 msec, ~2 sec and ~20 sec, respectively (Zengel and Magleby, 1980). PTP typically decays with a time constant of about 20 sec following a few conditioning pulses, and decays slower with a time constant of several minutes following hundreds to thousands of conditioning pulses (Magleby and Zengel, 1976b).

**Proposed mechanisms of facilitation**

Katz and Miledi first proposed that “residual $\text{Ca}^{2+}$” was responsible for facilitation (Katz and Miledi, 1968). Although the mechanisms underlying synaptic enhancement are not understood, all of the components appear to be dependent upon residual $\text{Ca}^{2+}$ (Zucker and Regehr, 2002). After $\text{Ca}^{2+}$ enters to evoke transmitter release, the residual $\text{Ca}^{2+}$ equilibrates in the terminal. Whereas most of the $\text{Ca}^{2+}$ binds to buffering proteins, some may be compartmentalized by intracellular organelles, and all the $\text{Ca}^{2+}$ is ultimately extruded. Both $\text{Ca}^{2+}$ buffers and $\text{Ca}^{2+}$ extrusion mechanisms play an important role in shaping the residual $\text{Ca}^{2+}$ transients at synaptic terminals.

The “multiple-site hypothesis” proposes that synaptic enhancement is induced by $\text{Ca}^{2+}$ binding to high-affinity site(s) that are separate from the low-affinity sensor for exocytosis (Zucker and Regehr, 2002). This high-affinity site(s) acts cooperatively with
the Ca\(^{2+}\) sensor for exocytosis. There could be separate high-affinity binding sites for each phase of synaptic enhancement or only a single high-affinity site and the accumulation/decay of Ca\(^{2+}\) could occur in multiple phases leading to the multiple phases of synaptic enhancement (Zucker and Regehr, 2002). The latter hypothesis is favored by modeling studies of facilitation using a single high-affinity site (Atluri and Regehr, 1996; Bennett et al., 2004; Bennett et al., 2007). \(F_1\) and \(F_2\) facilitation can be modeled with the low-affinity exocytosis binding site 25 nm above the Ca\(^{2+}\) channels and the high-affinity facilitation site 100 nm above it (Bennett et al., 2004; Bennett et al., 2007). In this scenario, \(F_1\) facilitation results from a high local Ca\(^{2+}\) concentration at the high-affinity site immediately after Ca\(^{2+}\) influx; \(F_1\) decays rapidly as the Ca\(^{2+}\) gradient dissipates and Ca\(^{2+}\) equilibrates in the terminal. The slower decay of \(F_2\) facilitation results from the clearance of the equilibrated Ca\(^{2+}\) from the terminal. The unbinding of Ca\(^{2+}\) from the high affinity site may be slow since facilitation has been found to decay more slowly than residual Ca\(^{2+}\) (Atluri and Regehr, 1996; Matveev et al., 2004). The decay of augmentation and post-tetanic potentiation could result from multiple phases of Ca\(^{2+}\) clearance during an AP train resulting from Ca\(^{2+}\) uptake and extrusion mechanisms (Zucker and Regehr, 2002).

A second model proposes that facilitation results from the (partial) saturation of fast Ca\(^{2+}\) buffers (Rozov et al., 2001). Normally, fast buffers bind a portion of entering Ca\(^{2+}\) and prevent it from acting at the Ca\(^{2+}\) sensor. According to this buffer saturation model, prior synaptic activity leaves buffers partially saturated and subsequent Ca\(^{2+}\) influx results in a greater increase in the Ca\(^{2+}\) concentration and transmitter release. This mechanism appears to operate at a couple of synapses in the mouse CNS, which contain
the fast Ca$^{2+}$ buffer Calbindin (Blatow et al., 2003). It may be that this mechanism only operates where there is an optimal concentration of a fast mobile buffer, such as Calbindin (Matveev et al., 2004). It appears that this model cannot account for facilitation seen at many synapses since it is inconsistent with some of the experimental findings. For example, a moderate concentration of EGTA usually reduces facilitation and a decrease in external Ca$^{2+}$ often increases facilitation; this is consistent with the residual Ca$^{2+}$ model, but not the buffer saturation model (Blatow et al., 2003; Matveev et al., 2004). Also, buffer saturation cannot account for the magnitude or long duration of F2 facilitation at the amphibian neuromuscular junction (Bennett et al., 2004).

**Synaptic enhancement at the Drosophila larval neuromuscular junction**

Synaptic enhancement can be studied at the *Drosophila* larval NMJs, which is one of the preferred systems due to its identifiability and accessibility to electrophysiological studies. In prior studies, the combined EPSP produced by the Is and Ib synaptic terminals innervating muscle fiber 6/7 was used to study paired-pulse facilitation (PPF) and frequency facilitation during AP trains (Rohrbough et al., 1999; Zhong and Wu, 1991). This preparation was not ideal since the Is and Ib terminals show differences in synaptic facilitation (Lnenicka and Keshishian, 2000). In this study, facilitation was studied at a single *Drosophila* motor terminal on muscle fiber 5, which should be a better preparation since muscle fiber 5 is innervated by only a single Ib terminal (Hoang and Chiba, 2001). It seems likely that facilitation at this synapse follows the multiple-site hypothesis due to the clear presence of an F1 and F2 component (see chapter 3).
Measurements of \([\text{Ca}^{2+}]_i\)

All forms of short-term synaptic enhancement, including facilitation, augmentation and PTP, are believed to be closely related to the change in \([\text{Ca}^{2+}]_i\) (Zucker and Regehr, 2002). It is thus important to measure the change in \([\text{Ca}^{2+}]_i\) using \([\text{Ca}^{2+}]_i\) imaging techniques. The technique used in this study was first described by Macleod et al. (Macleod et al., 2002), using the long-wavelength calcium indicator Oregon Green 488 BAPTA-1 (OGB-1) conjugated to dextran (see chapter 1 Materials and methods for technical details). The major advantage of this indicator over those conventional ones such as fura-2 is that upon binding \([\text{Ca}^{2+}]_i\), OGB-1 increases its fluorescence emission intensity with very little shift in wavelength, introducing great convenience by indicating the \([\text{Ca}^{2+}]_i\) change without the need of doing double-wavelength ratiometric measurements; the indicator conjugated to dextran is also effective in preventing dye leakage and compartmentalization over time. This technique allows for producing \([\text{Ca}^{2+}]_i\) signals with excellent signal-to-noise ratio, good temporal resolution and stable indicator concentration in cells.

This thesis is trying to achieve the following goals and address the following questions: (1) Characterization of the \([\text{Ca}^{2+}]_i\) regulation at the *Drosophila* larval motor terminals by examining the terminal-specific differences in \([\text{Ca}^{2+}]_i\) regulation and determining the \([\text{Ca}^{2+}]_i\) buffering capacity at Ib terminals followed by a quantitative analysis of \([\text{Ca}^{2+}]_i\) regulation; (2) What are the effects of expressing a \([\text{Ca}^{2+}]_i\) buffering protein (*parvalbumin*) on \([\text{Ca}^{2+}]_i\) regulation at these terminals? (3) What are the effects of chronic lead (\([\text{Pb}^{2+}]_i\)) exposure on \([\text{Ca}^{2+}]_i\) regulation at these terminals?
The major findings presented in this thesis are as follows. (1) Differences in Ca\(^{2+}\) regulation in two classes of larval motor terminals—Is and Ib terminals: for single APs, Is boutons were found to have greater Ca\(^{2+}\) transients than Ib boutons due to greater Ca\(^{2+}\) influx per bouton volume; for AP trains, Is boutons showed larger Ca\(^{2+}\) transients and longer decay of the transients, most likely due to a lower PMCA activity in Is terminals (see chapter I). (2) The Ca\(^{2+}\)-binding ratio (\(\kappa_S\)), defined as the ratio of buffer-bound Ca\(^{2+}\)/free Ca\(^{2+}\), was found to be 77, indicating nearly 99% of entering Ca\(^{2+}\) will be immediately bound by endogenous fast Ca\(^{2+}\) buffers. Quantitative analysis yielded an increase in [Ca\(^{2+}\)]\(_i\) of 196 nM, a decay time constant of 46 msec and a Ca\(^{2+}\)-removal rate constant of 1641 s\(^{-1}\) for single APs in the absence of the exogenous Ca\(^{2+}\) indicator; for AP trains, the increase in [Ca\(^{2+}\)]\(_i\) was 185 nM/10 Hz and the Ca\(^{2+}\) extrusion rate constant was 827 s\(^{-1}\) (see chapter II). (3) The expression of a slow Ca\(^{2+}\) buffer protein, parvalbumin (PV), in the Drosophila motor terminals reduced paired-pulse facilitation and facilitation during trains of APs; as a homeostatic mechanism, single-AP transmitter release in the examined terminals was found to be increased to compensate partially for the reduced facilitation. Meanwhile, the expression caused a hypomorphic development of the synaptic terminals, presumably due to the perturbed cAMP signaling pathway resulting from the depressed intracellular Ca\(^{2+}\) signals. This was supported by Ca\(^{2+}\) imaging, which showed an alteration of Ca\(^{2+}\) regulation in PV-expressing terminals (see chapter III). (4) Under certain pathological conditions such as chronic lead (Pb\(^{2+}\)) exposure, the Ca\(^{2+}\) regulation in the terminals was shown to be altered: although the resting [Ca\(^{2+}\)]\(_i\), and the Ca\(^{2+}\) transients produced by single APs were not changed, the plateau of the Ca\(^{2+}\) transients produced by trains of APs was found to be significantly increased in the Pb\(^{2+}\)-
exposed terminals in a dose-dependent pattern, and the decay of these transients was also prolonged. These findings appear to be due to the slower PMCA activity in Pb\(^{2+}\)-exposed animals, apparently resulting from the inhibition of PMCA by Pb\(^{2+}\). This alteration of Ca\(^{2+}\) regulation increased synaptic facilitation during trains of APs, a finding that is in accordance to that seen in chapter III (see chapter IV). This serial of studies, together with previous publications (Lnenicka and Keshishian, 2000; Lnenicka et al., 2006a; Lnenicka et al., 2003), contributes to a better understanding of Ca\(^{2+}\) regulation in presynaptic terminals at the *Drosophila* larval NMJs.
Chapter I: Differences in Ca\textsuperscript{2+} Regulation for High-output Is and Low-output Ib Motor Terminals in Drosophila Larvae

Abstract

This study determined whether two classes of Drosophila larval motor terminals with known differences in structure and transmitter release also showed differences in Ca\textsuperscript{2+} regulation. Larval motor neurons can be separated into those producing large synaptic boutons (Ib) and those with small boutons (Is). Ib terminals release less transmitter during single action potentials (APs) than Is terminals, but show greater facilitation during high-frequency stimulation. The current study measured the Ca\textsuperscript{2+} transients produced by single APs and AP trains after loading the terminals with the dextran-conjugated Ca\textsuperscript{2+} indicator Oregon Green 488 BAPTA-1 (OGB-1). The two pairs of Is and Ib terminals innervating muscle fiber 4 and fibers 6 and 7 were examined. The OGB-1 concentrations were measured in order to compare measurements from terminals with similar OGB-1 loading. For single APs, the change in OGB-1 fluorescence (\(\Delta F/F\)) in Is boutons was significantly larger than in Ib boutons due to greater Ca\textsuperscript{2+} influx per bouton volume. The Is boutons had greater surface area and active zone number per bouton volume than Ib boutons; this could account for the differences in Ca\textsuperscript{2+} influx and argues for similar Ca\textsuperscript{2+} influx at Is and Ib active zone. As previously reported for the Ib boutons, the distal Is boutons had larger single-AP Ca\textsuperscript{2+} transients than proximal ones on muscle fibers 6 and 7, but not on fiber 4. This difference was not due to proximal-distal differences in surface area or active zones per bouton volume and may be due to greater Ca\textsuperscript{2+} influx at distal active zones. During AP trains, the Is Ca\textsuperscript{2+} transients were larger in amplitude and had longer decay time constants than Ib ones. This can be explained by a slower rate of Ca\textsuperscript{2+}
extrusion from the Is boutons apparently due to lower plasma membrane Ca^{2+} ATPase activity at Is boutons compared to Ib boutons.
**Introduction**

Calcium plays an important role in neuronal function especially at the synapse. Ca$^{2+}$ entering through voltage-gated Ca$^{2+}$ channels to trigger the release of transmitter and subsequently equilibrates in the terminal (Zucker, 1996). This “residual Ca$^{2+}$” likely serves multiple functions, one of which is the facilitation of subsequent transmitter release (Zucker and Regehr, 2002). Differences in Ca$^{2+}$ regulation at synaptic terminals are likely to play an important role in specifying transmitter release and facilitation. The “volume-averaged” residual Ca$^{2+}$ can be measured using fluorescent Ca$^{2+}$ indicators, and its amplitude is often used as an indirect measurement of Ca$^{2+}$ influx, since the Ca$^{2+}$ current cannot be directly measured at most terminals. However, other factors influence the amplitude and duration of these Ca$^{2+}$ transients including: Ca$^{2+}$ chelation by endogenous Ca$^{2+}$ buffers (Neher, 1995); sequestration by intracellular organelles (Scotti et al., 1999); extrusion across the plasma membrane (Reuter and Porzig, 1995; Lnenicka et al., 2006a); and synaptic terminal geometry (Jackson and Redman, 2003; Lnenicka et al., 2006a).

*Drosophila* larval motor terminals have become an important model system for the study of synaptic function, development and plasticity (Keshishian et al., 1996). Powerful genetic approaches have provided valuable insights to molecular mechanisms of synaptic function (Schwarz, 2006); however, Ca$^{2+}$ regulation at these terminals has not been thoroughly characterized. Future progress using this preparation will require a more complete understanding of the mechanisms regulating Ca$^{2+}$ and the neuron-specific differences.
In this study, differences in Ca$^{2+}$ regulation in two types of motor terminals with known differences in structure and function in *Drosophila* larvae were examined. Larval motor terminals have been classified as Is (s stands for small) or Ib (b stands for big) based on the differences in the size of the synaptic boutons (Atwood et al., 1993). These two classes of motor terminals originate from separate motoneurons and show differences in synaptic physiology. For single action potentials (APs), Is terminals generate larger excitatory postsynaptic potentials (EPSPs), and Ib terminals appear to release more transmitter per synapse than Ib terminals; stimulation at moderate frequencies results in depression of Is synapses and facilitation at Ib synapses (Atwood et al., 1993; Lnenicka and Keshishian, 2000; Kurdyak et al., 1994).

Some of the factors that shape Ca$^{2+}$ transients at the larval motor terminals have been described. For a given terminal, the amplitude and duration of the single-AP Ca$^{2+}$ transient are related to bouton size, and the activity of the plasma membrane Ca$^{2+}$ ATPase (PMCA) also influences the amplitude and duration of Ca$^{2+}$ signals produced by AP trains (Lnenicka et al., 2006a). In addition, the amplitude of the Ca$^{2+}$ transients is related to bouton position for the Ib terminals on MF6/7 (Guerrero et al., 2005; Lnenicka et al., 2006a).

In this study, it was found that Is terminals had greater single-AP-induced Ca$^{2+}$ transients than Ib terminals; this can be explained by their greater surface area/bouton volume ratio and it appears unlikely that there are differences in Ca$^{2+}$ influx per synapses or active zone. The single-AP-induced Ca$^{2+}$ transients were greater in distal boutons than more proximal ones for both Is and Ib terminals on MF6/7, but not for the Is and Ib terminals on MF4. The Ca$^{2+}$ transients produced by AP trains in Is boutons were larger
and decayed more slowly than those in Ib boutons, presumably due to a slower rate of \( \text{Ca}^{2+} \) extrusion by PMCA.
Materials and methods

Dissection

Experiments were performed on Drosophila Canton S wandering 3rd-instar, female larvae raised on Jazz-Mix Drosophila Food (Fischer Scientific) at 25°C and 70-80% relative humidity. Terminals innervating MF6/7 and MF4 in segment 3 on the right side were chosen for Ca$^{2+}$ imaging study. For dissection (Jan and Jan, 1976), the larva was placed in chilled Schneider’s insect medium (Sigma, St. Louis MO) in the dissection dish and was held in a prone position using forceps; its head and tail were then pinned down for immobilization. The larva was cut open along the dorsal midline using a pair of spring micro-dissection scissors (straight, 6mm blades; Fine Science Tools). The skin edges on both sides of the cut were flipped over and pinned down; all the internal organs were then removed in order to expose the internal ventral wall for experiments.

Loading the terminals with Ca$^{2+}$ indicator

All experiments were done at room temperature. To prepare for the loading, a specialized glass tube (Kimax 1.5-1.8 × 100mm; Kimble Glass Inc., NJ) was melted and pulled in a Narishige puller (PP-83; Scientific Instrument Lab, Japan) to make pipettes. The tip of the pipette was then etched and broken so that the outside diameter of the tip was between 120-140µm and the inside diameter was about 100µm. A suction electrode was made by fire polishing the tip of the pipette so that the inside diameter was between 10-12µm. The single wavelength Ca$^{2+}$ indicator, Oregon Green 488 BAPTA-1 (OGB-1) coupled to 10,000 MW dextrans with a dissociation constant ($K_d$) of 1180nM (Invitrogen, Carlsbad, CA, USA), was dissolved in extra-pure distilled water with a concentration of
5mM and was used in this study. After exposing the internal ventral wall as described above, the segmental nerve innervating the right third segment was cut, using a pair of spring micro-dissection scissors (straight, 8 mm blades; Fine Science Tools), close to the ventral ganglion. Within 5 min of making the cut, the cut end of the nerve was then sucked up by the suction electrode, and a small volume of the OGB-1 solution was introduced into the tip of the suction electrode for the incubation of the nerve cut end for 40 minutes at room temperature in a dark room. After this, the nerve was blown out of the suction electrode, and the Schneider’s was changed several times to make sure the OGB-1 solution that was blown out together with the nerve did not remain on the larval body unfavorably raising the background fluorescence and thus decreasing the signal-to-noise ratio. The preparation was then left for another hour to ensure that the indicator molecules were substantially transported to the terminals. This technique for loading the indicator was developed by Macleod et al. (Macleod et al., 2002). After the loading, the Ca\textsuperscript{2+} measurements were performed in HL3 saline (Stewart et al., 1994) containing 1mM Ca\textsuperscript{2+}. 10 µM philanthotoxin-433 or 7 mM glutamate (Sigma-Aldrich, St. Louis, MO, USA) were added to the saline to prevent muscle contraction (Macleod et al., 2004; Frank et al., 2006).

**Measurement of OGB-1 \( \Delta F/F \) (Fluorescence-resting fluorescence/resting fluorescence)**

The terminals were imaged using an upright, fixed-stage BH2 microscope (Olympus Optical, Tokyo, Japan) equipped with epifluorescence, DIC, a water-immersion 40× Zeiss (Carl Zeiss, Thornwood, NY, USA) lens (NA 0.75) and a cooled-DDT camera (CoolSNAP HQ, Photometrics, Tucson, AZ, USA). Excitation illumination
from a 75W xenon arc lamp was passed through a Lambda-10 Optical Filter Changer (Sutter Instruments, Novato, CA) containing a 480 ± 15nm bandpass excitation filter (Chroma Technology, Brattleboro, VT). Excitation and emission wavelengths were separated with a 500 nm dichroic mirror, and emitted light traveled through a high-pass 515 nm filter. During the imaging, the nerve was stimulated with a suction electrode connected to an S11 stimulator (Grass Technologies, West Warwick, RI) and MetaFluor 6.1 software (Molecular Devices, Palo Alto, CA, USA) was used for image acquisition and to measure fluorescence intensity along the terminal and background fluorescence from the muscle. Images were streamed at 50 Hz for single APs and 20 Hz for AP trains. MetaMorph 6.1 software (Molecular Devices, Palo Alto, CA, USA) was used to produce pseudo-color maps of the percent of ∆F/F and measure bouton widths. The Is and Ib terminals could usually be distinguished by the differences in their bouton size, especially at the end of the terminals. The frequency for firing single APs was 0.25 Hz; usually 20 APs were obtained for each pre-determined region on every terminal. For AP trains, stimulations lasted 5 sec for all frequencies.

For single APs, images were streamed at 50 Hz; this would likely underestimate the real peak of the ∆F/F measurement. Thus, in a few experiments, single-AP measurements were made at higher sampling rates. This was accomplished by replacing the cooled-CCD camera with a photomultiplier tube (Photosensor module H5784-06, Hamamatsu Corp., Bridgewater, NJ, USA). To selectively excite OGB-1 in one specific terminal, the aperture of the fluorescence illuminator was modified so that it only illuminated a region of 10-15 µm in diameter. The output of the photomultiplier tube was
digitized at 10 kHz using a Digidata 1200A and pCLAMP 9.2 software (Molecular Devices) and the signals were subsequently filtered at 1 kHz.

For measurement, region of interest (ROI) was drawn for individual boutons and their immediately adjacent areas as background. These data were then imported to Sigmaplot 8.0 (SPSS Inc., Plover, WI, USA) and were subsequently transformed and analyzed. The background fluorescence was subtracted from the terminal fluorescence in order to calculate the change of fluorescence $\Delta F/F$ by $100 \times (\text{fluorescence-resting fluorescence})/\text{resting fluorescence}$. Single exponentials were fit to the $\Delta F/F$ decay for the decay time constant ($\tau_{\text{decay}}$). To eliminate noisy data, only the $\tau_{\text{decay}}$ for which the fit to a single exponential gave $r^2 > 0.9$ was used for the study; greater than 90% if the measurements met this criterion. The increase in $[\text{Ca}^{2+}]_i$ during trains of APs was measured at the plateau by averaging the measurements of the last second of the trains.

For indicator concentration calibration, the [OGB-1] in the terminals was estimated by determining the $\text{in vitro}$ fluorescence for an array of [OGB-1] in capillary tubes with an inside diameter of 18-23 µm. To do this: (1) capillary tubes with an inside diameter of 50 µm (VitroCom, Mountain Lakes, NJ, USA) were pulled on a Narishige PN-30 electrode puller (Narishige International USA, Inc., East Meadow, NY, USA) using “Heat max: 102; Magnet sub: 20” as well as blocking the photodiode switch with a piece of aluminium foil to prevent a second pull. The tube was held with a rubber band and a 4mm-pull was allowed in order to make a satisfactory capillary tube with an inside diameter around 20 µm. (2) these tubes were then filled with various [OGB-1] in 100 nM-Ca$^{2+}$ intracellular solution (Djamgoz, 1987; Dawson and Djamgoz, 1988; Djamgoz and Dawson, 1988), and images were captured using the setup described above. Data
were analyzed and a standardized plot of [OGB-1] versus fluorescence was created (graph not shown). A plot of \([\text{Ca}^{2+}]\) versus fluorescence in the presence of 10 µM [OGB-1] was created using a similar protocol for the adjustment of resting \([\text{Ca}^{2+}]\). It was assumed that these tubes were thin enough to capture all the emitted fluorescent light; and it appeared to be true since after standardizing the fluorescent values to a 20 µm tube, the plot of [OGB-1] versus fluorescence was linear. The average resting fluorescence was measured in a terminal region of uniform width (apparent diameter), and fluorescence intensity was adjusted by \((\text{fluorescence intensity}) \times (20 \, \mu\text{m})/\text{terminal width}\). This value could then be converted to [OGB-1] with the assumption of resting \([\text{Ca}^{2+}]\) being 40 nM in the motor terminals (Klose et al., 2008) using the above plots.

**Measuring active zone density at Is and Ib terminals**

For immunocytochemistry, the larvae were dissected in HL3 saline (0 Ca²⁺) and fixed in 4% paraformaldehyde for an hour at room temperature. Active zones were stained using the primary monoclonal antibody nc82 (1:100; Developmental Studies Hybridoma Bank, Iowa City, IA, USA), which recognizes the active zone component Bruchpilot (Wagh et al., 2006), for the overnight incubation at 4°C. In addition, goat anti-houseradish peroxidase (HRP) (1:200) was used to label the motor terminals (Jan and Jan, 1982). The secondary antibodies Alexa Fluor 488 donkey anti-mouse IgG (1:200) and Alexa Fluor 546 rabbit anti-goat IgG (1:200; Molecular Probe, Invitrogen, Carlsbad, CA, USA) were used for 5-hour incubation at room temperature. The preparations were then washed several times and were mounted in SlowFade gold antifade reagent (Invitrogen) and viewed under a Zeiss LSM 510 confocal microscope with a Zeiss 63×, 1.4 NA objective.
objective (Carl Zeiss). Using multitracking, Alexa Fluor 488 was imaged using a 488 laser with a BP 505-530 emission filter and Alexa Fluor 546 was viewed with a 543 laser and a LP560 emission filter. Stacks of slices (0.3µm thickness) were acquired from MF6/7 in segments 3 and 4. These images were then analyzed using MetaMorph 6.1 software (Molecular Devices). For each slice, the number of spots recognized by nc82 per bouton was counted assuming that each spot represented one active zone. When an active zone spanned more than one slice it was only counted once. The bouton volume and surface area were estimated using the equations for a prolate spheroid (Beyer WH. *Standard mathematical tables and formulae. 1990; Boston: CRC Press*): volume=$\frac{4}{3}\pi ab^2$ and surface area=$2\pi b\left( b + \left( \frac{e}{a} \right) \sin^{-1}\frac{e}{a} \right)$; where $e=(a^2-b^2)^{1/2}/a$, $a=$major radius and $b=$minor radius. Statistical analyses were performed using $t$ tests. All $n$-values represent (# of boutons, # of animals) unless otherwise specified.
**Results**

*Is and Ib terminals show differences in Ca\(^{2+}\) transients*

To measure Ca\(^{2+}\) transients, the terminals were loaded with a dextran conjugate of OGB-1. The OGB-1 reported changes in cytosolic [Ca\(^{2+}\)] since the dextran prevented compartmentation of OGB-1. Ca\(^{2+}\) transients produced by single APs and 10 Hz trains of APs in the two pairs of Is and Ib terminals innervating muscle fibers (MF) 4 and 6/7 for MF 6/7 were measured. These 4 terminals are all generated by different axons (Lnenicka and Keshishian, 2000; Hoang and Chiba, 2001). It was found that MF4 occasionally has two Ib terminals. In this study, only measurements were made where there was clearly one Is terminal and one Ib terminal. However, the previous observation raised the possibility that two different motoneurons could supply Ib terminals on MF4 and that the Ib measurements in the study were not always from the same motor neuron. Alternatively, it may be that the same motor neuron always supplies Ib terminals, but sometimes two branches arising from the same axon form separate terminals. To test this, a preparation with two separate Ib terminals along with an Is terminal on MF4 was examined (Fig. 1) by slowly increasing and decreasing the stimulation voltage and monitoring the Ca\(^{2+}\) signals in these two terminals. It was found that the Ca\(^{2+}\) signals always occurred simultaneously in the two terminals and thus, they likely originated from the same axon.
Figure 1. Two separate Ib terminals on MF 4. To determine whether these two Ib terminals originate from the same axon, stimulation voltage was adjusted while monitoring the Ca\(^{2+}\) signals in the two Ib motor terminals. In the above case, the voltage was set near threshold so the axon only fired intermittently during 10 Hz stimulation. It is clear that the two terminals always fired together: there were two Ca\(^{2+}\) signals (*) with a short interval at the beginning of the 10 Hz train and a single Ca\(^{2+}\) signal towards the end. The traces represent the \(\Delta F/F\) and images were streamed at 20 Hz.

The Is and Ib terminals innervating MF6/7 run together and often can only be clearly distinguished at their ends (Fig. 2). The amplitude of the Ca\(^{2+}\) transients recorded from the Is boutons was larger than that seen in the Ib boutons for both single APs and AP trains (Fig. 2: top). Similar results were observed for the Is and Ib boutons on MF4: the Ca\(^{2+}\) transients were larger for the Is boutons compared to the Ib boutons (Fig. 2: bottom). The peak of the \(\Delta F/F\) was measured for single APs ((fluorescence-resting fluorescence)/resting fluorescence during a single action potential, \(\Delta F/F_{AP}\)) and the \(\Delta F/F\) plateau during 10 Hz AP trains ((fluorescence-resting fluorescence)/resting fluorescence during an AP train, \(\Delta F/F_{train}\)), which was determined by averaging the \(\Delta F/F\) during the final 1 sec of the train. For MF 6/7, combined measurements from a number of terminals (Fig. 3: top) showed that the \(\Delta F/F_{AP}\) peak was 63% greater in boutons of Is terminals compared to Ib terminals and the \(\Delta F/F_{train}\) plateau for Is boutons was 54% greater than for Ib boutons. For MF4, the \(\Delta F/F_{AP}\) peak was 26% greater for Is boutons compared to Ib
boutons and the $\Delta F/F_{\text{train}}$ plateau was 64% greater for Is boutons compared to Ib boutons (Fig. 3: bottom).

Figure 2. $\text{Ca}^{2+}$ transients measured in the Is and Ib terminals innervating MF 6/7 and 4. For both muscle fibers, the $\Delta F/F$ is shown for the most distal Is and Ib boutons during single APs and 10 Hz AP trains. Top: For MF 6/7, the $\Delta F/F$ was greater for the Is bouton compared to the Ib bouton for both single APs and trains of APs. Bottom: The $\text{Ca}^{2+}$ transients produced during single APs and trains of APs were also greater in Is boutons than Ib boutons on MF 4. Calibration: single APs- 30 % and 1 sec.; AP trains- 30 % and 4 sec.
Figure 3. The amplitude of Ca\(^{2+}\) transients seen at Is and Ib boutons on MFs 6/7 and 4. The relative increase in [Ca\(^{2+}\)]\(_i\) was determined from the ΔF/F measured at the peak for single APs or the plateau for AP trains. Top: For MF 6/7, the ΔF/F\(_{AP}\) peak and ΔF/F\(_{train}\) plateau was significantly greater for Is boutons compared to Ib boutons. Bottom: The same was true for the Is and Ib boutons found on MF 4. Is values were compared to corresponding Ib values; * p < 0.001. n represents bouton # and animal #.
It was also found that Is boutons and Ib boutons showed differences in the decay rate of the Ca$^{2+}$ transients. For MF6/7, the $\Delta F/F_{\text{train}}$ $\tau_{\text{decay}}$ for Is boutons was 20% greater than that for Ib boutons; however, no significant difference was seen in the $\Delta F/F_{\text{AP}}$ $\tau_{\text{decay}}$ for Is boutons and Ib boutons (Fig. 4: top). For MF4, the $\Delta F/F_{\text{AP}}$ $\tau_{\text{decay}}$ for Is boutons was 37% greater than that for Ib boutons; and the $\Delta F/F_{\text{train}}$ $\tau_{\text{decay}}$ for Is boutons was 51% greater than that for Ib boutons (Fig. 4: bottom).

**Figure 4.** The Ca$^{2+}$ $\tau_{\text{decay}}$ after single APs and AP trains for Is and Ib boutons was determined from the decay of $\Delta F/F$. Top: For MF 6/7, the $\tau_{\text{decay}}$ was significantly greater for Is boutons compared to Ib boutons after an AP train, but not after single APs. Bottom: For both single APs and AP trains, the $\tau_{\text{decay}}$ was significantly greater for Is boutons compared to Ib boutons on MF 4. Is values were compared to corresponding Ib values; * $p<.01$, ** $p < 0.001$. 
**Effect of OGB-1 concentration**

Ca$^{2+}$ buffering by the Ca$^{2+}$ indicator can decrease the amplitude and increase the $\tau_{\text{decay}}$ of the Ca$^{2+}$ transients (Neher, 1995). The OGB-1 concentration for some of the above measurements performed on MF6/7 was measured. In these experiments, the OGB-1 concentration was higher in Ib terminals ($53.8 \pm 6.5$ $\mu$M, $n=22$ terminals) compared to Is terminals ($43.3 \pm 7.8$ $\mu$M, $n=13$ terminals). To examine the effect of differences in OGB-1 loading, the Is and Ib boutons were divided into groups with similar OGB-1 concentration (Fig.5). This showed that increasing OGB-1 concentration decreased the $\Delta F/F_{\text{AP}}$ peak and increased the $\Delta F/F_{\text{AP}} \tau_{\text{decay}}$. However, for all groupings the $\Delta F/F_{\text{AP}}$ peak was significantly greater for Is boutons and the $\Delta F/F_{\text{AP}} \tau_{\text{decay}}$ was not significantly different for Is and Ib boutons. To match OGB-1 concentration for the entire population of Is and Ib terminals, four Ib terminals with the highest OGB-1 concentration were eliminated; this resulted in mean OGB-1 concentrations of $43$ $\mu$M for both Is and Ib terminals. For these populations, the $\Delta F/F_{\text{AP}}$ peak for Is boutons was $37\%$ greater than for Ib boutons and the $\Delta F/F_{\text{AP}} \tau_{\text{decay}}$ for Ib boutons was not significantly different from Is (Table. 1). For many of these experiments, the Ca$^{2+}$ transients produced by 10 Hz stimulation were also measured. After eliminating the two Ib terminals with the highest OGB-1 concentrations, both Is and Ib terminals had a mean OGB-1 concentration of $48$ $\mu$M. In these OGB-1-matched populations, the $\Delta F/F_{\text{train}}$ plateau for Is boutons was $48\%$ greater than for Ib boutons and the $\Delta F/F_{\text{train}} \tau_{\text{decay}}$ for Is boutons was $27\%$ greater than for Ib boutons (Table. 1).
Figure 5. Comparison of single-AP Ca\(^{2+}\) transients in Is and Ib terminals with similar OGB-1 concentrations. The terminals were grouped according to their OGB-1 concentration. An increase in OGB-1 loading results in a decrease in the peak and an increase in the τ\(_{\text{decay}}\) of the Ca\(^{2+}\) transient. For all the groupings, the Is ΔF/F peak was significantly than the Ib peak. The ΔF/F\(_{\text{AP}}\) τ\(_{\text{decay}}\) was not significantly different for Is and Ib boutons. * p<0.0001.

<table>
<thead>
<tr>
<th></th>
<th>ΔF/F(_{\text{AP}}) peak</th>
<th>ΔF/F(_{\text{train}}) peak</th>
<th>ΔF/F(<em>{\text{AP}}) τ(</em>{\text{decay}})</th>
<th>ΔF/F(<em>{\text{train}}) τ(</em>{\text{decay}})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Is</strong></td>
<td>22.5 ± 0.7%*</td>
<td>34.0 ± 1.2%*</td>
<td>76 ± 2 msec</td>
<td>180 ± 7 msec*</td>
</tr>
<tr>
<td></td>
<td>(N = 90, 13)</td>
<td>(N = 55, 9)</td>
<td>(N = 90, 13)</td>
<td>(N = 54, 9)</td>
</tr>
<tr>
<td><strong>Ib</strong></td>
<td>16.4 ± 0.5%</td>
<td>23.0 ± 1.0%</td>
<td>74 ± 3 msec</td>
<td>142 ± 8 msec</td>
</tr>
<tr>
<td></td>
<td>(N = 93, 18)</td>
<td>(N = 48, 10)</td>
<td>(N = 89, 18)</td>
<td>(N = 45, 10)</td>
</tr>
</tbody>
</table>

Table 1. Comparison of Ca\(^{2+}\) transients produced by single APs and trains of APs on MF6/7 for [OGB-1]-matched Is and Ib terminals.

n=number of boutons, number of animals;
*: p<0.001.
Comparing single-AP Ca\(^{2+}\) transients using high sampling rates

The above measurements of \(\Delta F/F_{AP}\) were performed by capturing images at 50 Hz. This sampling rate likely results in an underestimation of the actual peak. To determine the extent of this underestimation, \(\Delta F/F_{AP}\) was measured at higher sampling rates using a photomultiplier tube. In these experiments, the aperture of the fluorescent illuminator was reduced to excite the boutons at the end of terminals (Fig. 6). The output of the photomultiplier tube was digitized at 10 kHz and subsequently filtered at 1 kHz. It was found that the \(\Delta F/F_{AP}\) peak for Is boutons was still greater than that for Ib boutons (31.4% versus 20.2%), similar to the above finding when images were streamed at 50 Hz. For the 4 Ib terminals and 2 Is terminals examined using both photomultiplier and camera, it was found that the underestimation of the peak was about 26%; however, this underestimation was similar for Is and Ib terminals. Thus the relative measurements using the camera in the above experiments should be accurate.

Figure 6. Measurements of single-AP Ca\(^{2+}\) transients using a photomultiplier tube. The aperture of the fluorescent illuminator was reduced (approximately 15 \(\mu\)m diameter) to selectively excite isolated regions of the Is and Ib terminal on MF 6/7. The \(\Delta F/F_{AP}\) was measured for the circled regions of the Is (top) and Ib (bottom) terminals located on the same muscle fiber. The arrowheads point to the adjacent Ib (top) and Is (bottom) terminals. The corresponding \(\Delta F/F_{AP}\) is shown for each region. The \(\Delta F/F_{AP}\) for the Is terminal (top) was 30.1 % and the Ib terminal (bottom) had a peak of 20.2 %. Calibration: 400 msec, 20% \(\Delta F/F\).
**Effect of Is bouton position on Ca\textsuperscript{2+} transient**

A previous study showed that bouton position could affect the peak of the single-AP Ca\textsuperscript{2+} transient (Lnenicka et al., 2006a). For the MF6/7 Ib terminals, the distal boutons had a larger ∆F/\textit{F}_\text{AP} peak than more proximal ones. In that study, there was no apparent effect of bouton position for the MF4 Ib terminals (Lnenicka et al., 2006a). To determine whether bouton position influenced the Ca\textsuperscript{2+} transients in the Is terminals, the ∆F/\textit{F}_\text{AP} in the most distal three boutons from Is terminals were compared. For the MF6/7 Is terminal, the end bouton (bouton 1) had a significantly greater ∆F/\textit{F}_\text{AP} peak than the 3\textsuperscript{rd} bouton from the end (bouton 3); however, the ∆F/\textit{F}_\text{AP} peak was not significantly different for bouton 1 and bouton 3 along the MF4 Is terminals (Fig. 7). For the Is terminals on MF6/7, the ∆F/\textit{F}_\text{AP} τ\text{decay} was not significantly different for bouton 1 (79.3 ± 5.0 msec) and the bouton 3 (79.0 ± 4.0 msec; \(p>0.10\)).

**Figure 7.** The effect of bouton position on single-AP Ca\textsuperscript{2+} transients for Is terminals from MFs 4 and 6/7. The ∆F/\textit{F}_\text{AP} peak was compared for the 3 most distal boutons to determine whether it was influenced by bouton position. The ∆F/\textit{F}_\text{AP} peak was significantly greater for distal boutons than proximal ones on MF 6/7, but not on MF 4. Mean values are from 9 MF 6/7 and 7 MF 4 terminals. The end bouton (bouton 1) was compared to the 3rd bouton from the end (bouton 3) using a paired t-test. *\(p<0.01\).
**Active zones at Is and Ib terminals**

The $\Delta F/F_{AP}$ peak was greater at Is terminals than Ib terminals on both MF6/7 and MF4 indicating that there was great Ca$^{2+}$ influx per bouton volume. In addition, it was shown here and in a previous study (Lnenicka et al., 2006a) that the $\Delta F/F_{AP}$ peak was greater in the distal boutons compared to the more proximal boutons for both the Is and Ib terminals on MF6/7. Assuming that the amount of Ca$^{2+}$ entering per membrane area is constant, differences in Ca$^{2+}$ influx per terminal volume could result from difference in the bouton surface area/volume. Alternatively, if Ca$^{2+}$ influx occurs predominantly at active zones, then the number of active zones per bouton volume could be the determining factor. A previous electron-microscopy study reported that the Ib terminal had more synapses and dense bodies (putative active zones) per length of terminal than the Is terminal (Atwood et al., 1993). Also, staining active zones at the Ib terminal (Guerrero et al., 2005). However, these did not examine the number of synapses or active zones per bouton volume.

The surface area and number of active zones per bouton volume for MF6/7 were estimated by staining the active zones with an anti-Bruchpilot (Wagh et al., 2006) and the terminal membrane with anti-HRP (Fig. 8). All measurements were performed on the most distal three boutons from 21 pairs of Is and Ib terminals in 15 larvae. The surface area/bouton volume ($\mu m^2$) was not significantly different for bouton 1 compared to bouton 3 for the Ib terminal or the Is terminal. Also, the number of active zones/bouton volume ($\mu m^3$) was not significantly different for bouton 1 compared to bouton 3 for the Ib terminal or the Is terminal. Results are shown in Table 2. Thus, it does not appear that the differences in $\Delta F/F_{AP}$ peak can be accounted for by the differences in bouton structure.
Figure 8. Staining of active zones along Is and Ib terminals on MF 6/7. The active zones were stained with an antibody to the Bruchpilot protein (green) and motor terminal membranes were stained with anti-HRP (red). This confocal image passes through the central region of the terminals at their distal ends.

<table>
<thead>
<tr>
<th></th>
<th>Surface area/bouton volume ($\mu$m$^{-1}$)</th>
<th>Number of active zones/bouton volume ($\mu$m$^{-3}$)</th>
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</thead>
<tbody>
<tr>
<td>Ib</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bouton 1</td>
<td>1.1 ± 0.1</td>
<td>1.12 ± 0.10</td>
</tr>
<tr>
<td>Bouton 3</td>
<td>1.4 ± 0.2</td>
<td>1.35 ± 0.16</td>
</tr>
<tr>
<td>Is</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bouton 1</td>
<td>2.3 ± 0.3</td>
<td>2.27 ± 0.25</td>
</tr>
<tr>
<td>Bouton 3</td>
<td>1.9 ± 0.1</td>
<td>1.95 ± 0.11</td>
</tr>
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Table 2. Comparing structural parameters between most distal boutons and more proximal ones for Is and Ib terminals on MF 6/7. No significant difference was seen for the two selected parameters ($p > 0.10$).

To compare Is and Ib boutons, measurements taken from the three most distal boutons were combined (Fig. 9). The Ib boutons had twice as many active zones as the Is boutons consistent with the previous electron microscopy study (Atwood et al., 1993). However, the number of active zones/bouton volume was 63% greater for Is boutons than...
for Ib boutons (Fig. 9). This difference in active zones per bouton volume was mainly due to the greater bouton surface area/bouton volume for Is boutons. The surface area/bouton volume was 48% greater for Is boutons (3.7 ± 0.1; 63, 15) than that for Ib boutons (2.5 ± 0.1; 63, 15; p<0.001). Thus, the difference in the ΔF/FAP peak between Is terminals and Ib terminals could be accounted for by the differences in active zones/bouton volume and bouton surface area/volume.

**Figure 9.** Comparison of the number of active zones along Is and Ib terminals on MF 6/7. The number of active zones was counted for the three most distal bouton along Is and Ib terminals. The number of active zones per bouton was greater for the Ib terminals; however, the number of active zones per bouton volume was greater for the Is terminals. Measurements were performed on 63 Is and Ib boutons from 15 animals. Is and Ib values were compared. * p<0.001.

**Discussion**

*Is-Ib differences in Ca<sup>2+</sup> transients*
ΔF/F was measured by capturing images of the terminals during nerve stimulation and it was found that the Ca\(^{2+}\) transients were larger for Is than Ib boutons. The ΔF/F\(_{\text{AP}}\) peak and ΔF/F\(_{\text{train plateau}}\) were greater at Is boutons than at Ib boutons for both MFs 4 and 6/7. Measurements of ΔF/F also showed differences in the τ\(_{\text{decay}}\) for Is and Ib boutons. The ΔF/F\(_{\text{AP}}\) τ\(_{\text{decay}}\) was longer for Is boutons than for Ib boutons on MF 4, but not on MF 6/7. For AP trains, the τ\(_{\text{decay}}\) was longer for Is boutons than for Ib boutons for both MFs 4 and 6/7. These Is-Ib differences in Ca\(^{2+}\) transients were not due to differences in OGB-1 loading. When Is and Ib boutons with similar OGB-1 concentrations were compared, the same significant differences in the Ca\(^{2+}\) transients were still seen. These findings conflict with a previous study of MF 6/7 that reported Ib boutons had similar or slightly larger Ca\(^{2+}\) transients for AP trains compared to Is boutons; however, their findings were based upon a relatively small sample (Karunanithi et al., 1997).

Variation in the Ca\(^{2+}\) signal for different populations of synaptic terminals has been reported. For example, the Δ[Ca\(^{2+}\)] for single APs in boutons formed by dentate gyrus granule cells (Jackson and Redman, 2003) is approximately twice that seen at the calyx of Held (Helmchen et al., 1997) or boutons produced by pyramidal cells in the neocortex (Koester and Sakmann, 2000). The postsynaptic target can influence the presynaptic Ca\(^{2+}\) signal; the amplitude of the single-AP Ca\(^{2+}\) transient in terminals of pyramids in the rat somatosensory cortex was dependent on the type of postsynaptic cell (Koester and Johnston, 2005). However, the synaptic terminals in our study show differences in Ca\(^{2+}\) transients even though they innervate the same postsynaptic cell.
The Is-Ib differences for single AP Ca\(^{2+}\) transients appear to be due to differences in bouton structure rather than differences in Ca\(^{2+}\) influx per membrane area

The $\Delta F/F_{AP}$ peak was 63% greater for Is boutons than for Ib boutons for a large sample of boutons on MF 6/7 and 37% greater for a smaller number of boutons where the OGB-1 concentrations were matched. This indicates that Is terminals have greater Ca\(^{2+}\) influx per bouton volume than Ib terminals. The greater Ca\(^{2+}\) influx per bouton volume can be explained by the 48% greater surface area/ bouton volume for Is boutons compared to Ib boutons assuming that the Ca\(^{2+}\) influx per membrane area was similar at Is and Ib boutons. Since much of the Ca\(^{2+}\) influx might be expected to occur at active zones, the number of active zones/ bouton volume was also estimated; the number of active zones/ bouton volume was 63% greater for Is boutons compared to Ib boutons.

This represented a similar Is-Ib difference in active zone area and synaptic area/ bouton volume since dense body length, the number of dense bars/ synapse and synapse size are all similar in Is and Ib terminals (Atwood et al., 1993). Thus, the Is-Ib differences in $\Delta F/F_{AP}$ peak can be accounted for by differences in membrane area, active zones and/or synapses per bouton volume that result from differences in bouton diameter. A similar effect of synaptic bouton size on $\Delta F/F_{AP}$ amplitude was reported when comparing boutons along a single larval motor terminal (Lnenicka et al., 2006a) or a population of boutons in the mammalian CNS (Jackson and Redman, 2003).

Although the Is-Ib difference in $\Delta F/F_{AP}$ peak can be explained by the differences in bouton structure, this could also result from a greater concentration of fast endogenous Ca\(^{2+}\) buffers for Ib boutons compared to Is boutons (Neher, 1995). However, this appears unlikely since more fast Ca\(^{2+}\) buffering should increase the $\Delta F/F_{AP} \tau_{decay}$ as seen when the
OGB-1 concentration was increased (Fig. 5), and the $\Delta F/F_{AP}\tau_{\text{decay}}$ was similar for Ib and Is boutons.

**Effect of bouton position on Ca$^{2+}$ transients**

The more distal Ib boutons on MF6/7 release more transmitter and have larger Ca$^{2+}$ transients produced by single APs and AP trains than more proximal ones (Guerrero et al., 2005; Lnenicka et al., 2006a); however, this was not the case for all Ib terminals since the Ib terminals on MF4 did not show this position effect (Lnenicka et al., 2006a). A similar result was obtained for the Is boutons; the distal boutons had a larger $\Delta F/F_{AP}$ peak than proximal ones for MF 6/7, but not for MF4. The difference in $\Delta F/F_{AP}$ peak must be due to differences in Ca$^{2+}$ influx per bouton volume since there were no differences in the $\Delta F/F_{AP}\tau_{\text{decay}}$ indicating that the fast Ca$^{2+}$ buffering was similar at proximal and distal boutons. However, this difference in Ca$^{2+}$ influx was not due to structural differences since surface area or active zones per bouton volume were similar in distal and proximal boutons for both Is or Ib terminals. This finding supports greater Ca$^{2+}$ influx per active zone at distal boutons, which could be responsible for the greater transmitter release from distal boutons as previously proposed (Guerrero et al., 2005). The function of this proximal-distal difference in Ca$^{2+}$ influx and transmitter release is not known. Also, it is not clear why the differences are seen on MF 6/7, but not on MF 4. However, it could be related to the differences in terminal length since the Is terminals on MF 6/7 are more than twice as long as those on MF 4 (Lnenicka and Keshishian, 2000; Lnenicka et al., 2006b).
Ib boutons show more rapid Ca\textsuperscript{2+} transient decay than Is boutons due to greater Ca\textsuperscript{2+} extrusion by the PMCA

The decay of $\Delta F/F_{\text{train}}$ was slower for Is boutons than for Ib boutons on both MF 4 and 6/7. In a previous study of larval motor terminals, it was found that Ca\textsuperscript{2+} clearance during 10 Hz AP trains was primarily due to Ca\textsuperscript{2+} extrusion by the PMCA: inhibiting the PMCA had a large effect on $\Delta F/F_{\text{train}} \tau_{\text{decay}}$, and immunocytochemistry showed that the PMCA was localized at the neuromuscular junction (Lnenicka et al., 2006a). Based upon the differences in $\tau_{\text{decay}}$, the Ca\textsuperscript{2+} extrusion rate per bouton volume should be 27% greater for Ib boutons compared to Is boutons. The differences in PMCA activity per membrane area would be even larger due to the smaller surface area/ volume for Ib boutons. In the crayfish, axons with higher impulse activity develop stronger Ca\textsuperscript{2+} clearance mechanisms (Lnenicka et al., 1998; Fengler and Lnenicka, 2002). This could also be true in *Drosophila* since Ib terminals appear to have greater impulse activity than Is terminals (Kurdyak et al., 1994).

For single APs, there was no significant difference in the $\Delta F/F_{\text{AP}} \tau_{\text{decay}}$ when we compared Is and Ib boutons with matched OGB-1 concentrations. This may reflect a reduced role of the PMCA in determining the $\tau_{\text{decay}}$ after single APs. This is supported by the previous finding that inhibiting the PMCA had a greater effect on $\Delta F/F \tau_{\text{decay}}$ after an AP train than after a single AP (Lnenicka et al., 2006a). Other factors, such as slow Ca\textsuperscript{2+} buffers, could also play a role in the $\Delta F/F_{\text{AP}}$ decay (Lin et al., 2005).

The lower rate of Ca\textsuperscript{2+} extrusion for Is boutons likely contributes to the higher $\Delta F/F_{\text{train}}$ plateau seen at Isboutons compared to Ib boutons. During an AP train, the increase in [Ca\textsuperscript{2+}]\textsubscript{i} results in greater Ca\textsuperscript{2+} extrusion until Ca\textsuperscript{2+} influx and efflux per unit
time are equal and the plateau is reached (Tank et al., 1995). Lower PMCA activity should result in a higher $\Delta F/F_{\text{train}}$ plateau; in fact, inhibiting the PMCA was shown to produce a large increase in the $\Delta F/F_{\text{train}}$ plateau for Ib boutons (Lnenicka et al., 2006a).

The Is-Ib difference in surface area/volume should have less effect on the average $\Delta F/F_{\text{train}}$ during the plateau than on the $\Delta F/F_{\text{AP}}$ peak since an increase in surface area/volume would increase both Ca$^{2+}$ influx and efflux per bouton volume; i.e., a greater Ca$^{2+}$ transient for each AP would be offset by a decrease in $\tau_{\text{decay}}$. This is supported by the previous study which failed to find a correlation between the averaged $\Delta F/F_{\text{train}}$ during the plateau and bouton width for the Ib terminal (Lnenicka et al., 2006a).

**Relationship of Is-Ib differences in Ca$^{2+}$ regulation to differences in transmitter release**

It was estimated that the Is synapses on MF 6/7 release 4 times more transmitter than the Ib synapses for single APs; this difference was proposed to result from greater Ca$^{2+}$ sensitivity of transmitter release rather than greater Ca$^{2+}$ influx per synapse (Atwood et al., 1997). Results in this study provide further support for this hypothesis since evidence of differences in Ca$^{2+}$ influx for Is and Ib synapses was not found. In addition, the Ib terminals on MF 6/7 show synaptic facilitation, whereas the Is terminals show depression during 10 Hz stimulation (Lnenicka and Keshishian, 2000). Clearly differences in residual Ca$^{2+}$ do not play a role in this synaptic differentiation since Is boutons show a greater increase in [Ca$^{2+}$], during 10 Hz trains than Ib boutons.

It was previously shown that the Is-Ib differences in motor terminal structure and transmitter release resemble the phasic-tonic differences seen for crustacean motor terminals (Atwood and Wojtowicz, 1986; Atwood et al., 1993; Kurdyak et al., 1994;
Lnenicka and Keshishian, 2000); this includes greater transmitter release per synapse/active zone for phasic terminals compared to tonic ones (Msghina et al., 1998). It appears that the Is-Ib differences in Ca\(^{2+}\) regulation are also similar to those seen in phasic and tonic terminals. In the crayfish, the amplitude of the [Ca\(^{2+}\)] plateau seen during AP trains was greater for phasic terminals compared to tonic terminals and [Ca\(^{2+}\)], decayed more slowly in phasic terminals than in tonic ones (Msghina et al., 1999). Based upon the differences in the [Ca\(^{2+}\)] plateau and decay \(\tau\), it was concluded that phasic terminals had greater Ca\(^{2+}\) influx per bouton volume compared to tonic terminals. Similar to the Is and Ib terminals, this difference was attributed to more active zones per bouton volume in phase terminals and not differences in Ca\(^{2+}\) influx per active zone (Msghina et al., 1999). Subsequently, it was shown that the Ca\(^{2+}\) sensitivity of transmitter release differed at phasic and tonic synapses (Millar et al., 2005).

A number of mutations in *Drosophila* affect transmitter release from larval motor terminals. The techniques and baseline data presented here should prove useful in examining the role of altered Ca\(^{2+}\) regulation in these synaptic changes.

**Chapter II: Ca\(^{2+}\) Buffering at a *Drosophila* Larval Synaptic Terminal**

**Abstract**

A quantitative analysis of Ca\(^{2+}\) dynamics requires knowledge of the Ca\(^{2+}\)-binding ratio (\(\kappa_S\)); this has not been measured at *Drosophila* synaptic terminals or any invertebrate
synaptic terminal. We measured $\kappa_S$ at a Ib motor terminal in Drosophila larvae comparing single-AP Ca\(^{2+}\) transients in synaptic terminals that contained varying concentrations of the Ca\(^{2+}\) indicator, Oregon Green 488 BAPTA-1 (OGB-1). Using a linear single-compartment model, $\kappa_S$ was calculated based upon the effect of [OGB-1] on the time constant ($\tau_{\text{decay}}$) for the decay of intracellular free Ca\(^{2+}\) concentration ($[\text{Ca}^{2+}]_i$). This gave a $\kappa_S$ of 77 indicating that nearly 99% of entering Ca\(^{2+}\) is immediately bound by endogenous fast Ca\(^{2+}\) buffers. Extrapolation to zero [OGB-1] gave a $\tau_{\text{decay}}$ of 46 msec and a Ca\(^{2+}\)-removal rate constant of 1641 s\(^{-1}\) for single APs. We calculated that a single AP produced an increase in $[\text{Ca}^{2+}]_i$ of 196 nM and an increase in the total intracellular [Ca\(^{2+}\)] (free + bound) of 15.3 µM. The increase in $[\text{Ca}^{2+}]_i$ for AP trans was 185 nM/10 Hz; this gave a Ca\(^{2+}\) extrusion rate constant of 827 s\(^{-1}\), which likely reflects the activity of the plasma membrane Ca\(^{2+}\) ATPase. Experiments were performed to examine the effect of altering external Ca\(^{2+}\) and Mg\(^{2+}\) on Ca\(^{2+}\) influx at these terminals.

**Introduction**

The *Drosophila* larval neuromuscular junction is a popular model system to apply genetic approaches to the study of synaptic function, development and plasticity (Keshishian et al., 1996). These studies often require an analysis of intracellular Ca\(^{2+}\) regulation since the intracellular free Ca\(^{2+}\) concentration ($[\text{Ca}^{2+}]_i$) plays an important role in transmitter release and synaptic plasticity (Atluri and Regehr, 1996; Delaney et al.,
intracellular Ca\(^{2+}\) regulation at these terminals. Some of the factors that shape Ca\(^{2+}\) transients at larval motor terminals have been described. For example, Ca\(^{2+}\) extrusion by the plasma membrane Ca\(^{2+}\) ATPase (PMCA) and Ca\(^{2+}\) uptake by endoplasmic reticulum (ER) play a role in Ca\(^{2+}\) clearance at these motor terminals (Lnenicka et al., 2006a; Sanyal et al., 2005); whereas, mitochondria do not appear to play an important role in Ca\(^{2+}\) clearance during physiological firing rates (Chouhan et al., 2010). The amplitude and duration of the single-AP Ca\(^{2+}\) signal is related to bouton size (He et al., 2009; Lnenicka et al., 2006a) and for some terminals, distal boutons produce larger Ca\(^{2+}\) transients than proximal ones (Guerrero et al., 2005; Lnenicka et al., 2006a).

A quantitative analysis of Ca\(^{2+}\) dynamics requires the measurement of fast Ca\(^{2+}\) buffering. Typically less than 5% of the Ca\(^{2+}\) entering during a single AP remains free; the rest is rapidly bound by endogenous fast Ca\(^{2+}\) buffers (Neher, 1995). The endogenous Ca\(^{2+}\)-binding ratio (\(\kappa_S\)), which is also referred to as Ca\(^{2+}\)-buffering capacity (Sabatini et al., 2002), is the ratio: amount of Ca\(^{2+}\) bound/ amount of Ca\(^{2+}\) remaining free (Neher, 1995). The \(\kappa_S\) influences the amplitude and duration of the Ca\(^{2+}\) transients and it is necessary to know \(\kappa_S\) in order to determine the total increase in intracellular Ca\(^{2+}\) concentration and calculate the rates of Ca\(^{2+}\) influx and extrusion. The \(\kappa_S\) has not been measured at the *Drosophila* larval motor terminals; in fact, it has not been measured at any invertebrate synaptic terminal.

The endogenous Ca\(^{2+}\) buffering in the terminals can be estimated using a linear single-compartment linear model (Neher, 1998; Neher and Augustine, 1992), which has been applied to a variety of dendrites and synaptic terminals (Helmchen et al., 1996;
Helmchen et al., 1997; Jackson and Redman, 2003; Koester and Sakmann, 2000; Sabatini et al., 2002). The addition of a high-affinity Ca\textsuperscript{2+} indicator will contribute to the total Ca\textsuperscript{2+} buffering reducing the amplitude and increasing the duration of Ca\textsuperscript{2+} transients produced by single APs. The $\kappa_S$ can be determined by comparing the Ca\textsuperscript{2+} signal amplitude or decay in terminals filled with a range of Ca\textsuperscript{2+} indicator concentrations; this has yielded values ranging from 20 to 140 for synaptic terminals in the mammalian CNS (Jackson and Redman, 2003; Koester and Sakmann, 2000). We determined $\kappa_S$ for the Ib terminals on muscle fibers 6 and 7 in Drosophila larvae by loading the terminals with a range of [OGB-1] and examining the single-AP Ca\textsuperscript{2+} transients.

**Materials and Methods**

**Loading the terminals with OGB-1**

Procedures were largely the same as those in chapter I for loading the terminals. All experiments were performed on the Ib terminals innervating MF6/7 in segment 3 of Drosophila (CS) wandering 3\textsuperscript{rd}-instar larvae.
**Determination of [Ca\(^{2+}\)]\(_i\) using OGB-1**

OGB-1 loaded terminals were imaged using the same setting as in chapter I. The change of OGB-1 fluorescence (\(\Delta F/F\)) was calculated: (fluorescence – resting fluorescence)/ resting fluorescence.

In order to calculate the actual [Ca\(^{2+}\)]\(_i\) change, the measured OGB-1 \(\Delta F/F\) needs to be calibrated. A previously established technique for a single-wavelength Ca\(^{2+}\) indicator was used (Maravall et al., 2000; Sabatini et al., 2002). It gave the following equations for the [Ca\(^{2+}\)]\(_i\) at rest ([Ca\(^{2+}\)]\(_{\text{rest}}\)) and the change in [Ca\(^{2+}\)]\(_i\) (\(\Delta [\text{Ca}^{2+}]_i\)):

\[
[\text{Ca}^{2+}]_{\text{rest}} = K_D \left( (1 - R_f^{-1})/(\Delta F/F)_{\text{max}} - R_f^{-1} \right) \quad 1
\]

\[
\Delta [\text{Ca}^{2+}]_i = (K_D)((\Delta F/F)_{\text{max}}+1)(1 - R_f^{-1}) (\Delta F/F)/(((\Delta F/F)_{\text{max}} - \Delta F/F)(\Delta F/F)_{\text{max}}) \quad 2
\]

The dissociation constant (\(K_D\)) and dynamic range (\(R_f\)) were determined for OGB-1 and it was necessary to measure (\(\Delta F/F\))\(_{\text{max}}\) for each synaptic terminal: \(R_f\) equals fluorescence at saturating Ca\(^{2+}\) (\(f_{\text{max}}\)) / fluorescence at 0 Ca\(^{2+}\) (\(f_{\text{min}}\)) and (\(\Delta F/F\))\(_{\text{max}}\) is equal to (\(f_{\text{max}} – \text{resting fluorescence}\))/ resting fluorescence.

The \(f_{\text{max}}, f_{\text{min}}\) and \(K_D\) for the OGB-1 dextran needed for the calibration were determined in vitro as follows: (1) prepare the same type of capillary tubes with an inside diameter of 20 \(\mu\)m as in Materials and Methods in chapter 1; (2) prepare Drosophila intracellular solution with 160 mM KCl, 13 mM NaCl, 10 mM KHEPES, pH 7.2 according to the literature (Dawson and Djamgoz, 1988; Djamgoz, 1987). OGB-1 and Na-EGTA were added to the solution so that the final [OGB-1] was 10 \(\mu\)M and the final [EGTA] was 10 mM, respectively. (3) Ca\(^{2+}\) calibration solutions were made by adding
increasing amounts of CaCl₂ to the solution in step 2; the final [Ca²⁺] was determined using the MaxChelator program (Bers et al., 1994): http://www.stanford.edu/~cpatton/maxc.html. (4) Capillary tubes were loaded with different Ca²⁺ calibration solutions for imaging and further measurement and calculation. \( f_{\text{max}} \) was the fluorescence when free Ca²⁺ concentration in the calibration solution was very high (45 µM); \( f_{\text{min}} \) was the fluorescence when free Ca²⁺ concentration in the calibration solution was 0. This gave an \( R_f \) of 2.5. (5) For \( K_D \), a fluorescence spectrofluorometer (FluoroLog-3, Horiba Jobin Yvon Inc. Edison NJ) was used through a similar procedure, which gave a \( K_D \) of 640.

For the measurement of \((\Delta F/F)_{\text{max in vivo}}\), it was accomplished by applying AP trains of increasing frequencies (Fig. 1: Bottom); \((\Delta F/F)_{\text{max}}\) was extrapolated from the relationship between stimulation frequency and \( \Delta F/F \) as OGB-1 began to saturate (Maravall et al., 2000).

\[
(\Delta F/F)_{\text{max}} = \Delta F_{V_2} (1-V_1/V_2)/(1-(\Delta F_{V_2}V_1/\Delta F_{V_1}V_2))
\]

where \( V_1 \) is the lower train frequency; \( V_2 \) is the higher train frequency; \( \Delta F_{V_2} \) equals the plateau \( \Delta F/F \) for \( V_2 \); and \( \Delta F_{V_1} \) is the plateau \( \Delta F/F \) for \( V_1 \). Typically, 40 Hz was used for \( V_1 \) and 60 Hz for \( V_2 \) (Fig. 1B) and a single \((\Delta F/F)_{\text{max}}\) was determined for each terminal. This assumed that the \( \Delta [\text{Ca}^{2+}]_i \) was linearly related to stimulation frequency, which was shown to be the case for these motor terminals (Hendel et al., 2008).

To measure \( R_f \) \textit{in vivo}, the above determined \((\Delta F/F)_{\text{max}}\) was used and then measured \( f_{\text{min}} \) by reducing intracellular Ca²⁺ through the addition of HL3 containing 0 Ca²⁺, 10 mM EGTA and the Ca²⁺ ionophore 4-Br-A23187 (5 µM) (SigmaAldrich). This gave an \( R_f \) of 2.2; the value was lower than that obtained \textit{in vitro}, which was consistent
with previous studies (Harkins et al., 1993; Maravall et al., 2000; O'Malley et al., 1999) and was used in the study. As described in chapter 1, OGB-1 concentration in the synaptic terminals were estimated by comparing resting terminal fluorescence with that measured in capillary tubes filled with intracellular solutions containing known [OGB-1] and [Ca\(^{2+}\)] with the calculated [Ca\(^{2+}\)]\_{\text{rest}} of 51 nM.

For the OGB-1 dextran, the K_D was much higher and the R_f much lower than previously reported values for the unconjugated OGB-1 (Maravall et al., 2000). Also, the values for OGB-1 dextran appear to be lot specific: the K_D for OGB-1 dextran has ranged from 454 nM to 1040 nM for different lots (Invitrogen). The same lot of OGB-1 dextran was used for all experiments in this study.

Comparison of means was performed using t-tests. All n values represent number of terminals, which is also the number of animals, since only one terminal was examined per animal. Single exponential were fit to the [Ca\(^{2+}\)]_i decay to determine the decay time constant. Bouton widths were measured from fluorescence images using MetaMorph 6.1 software (Molecular Devices).

Results

Ca\(^{2+}\) dynamics in the Ib terminals that innervate muscle fibers 6 and 7 (MF6/7) were examined. MF6/7 are innervated by two separate motor neurons; one supplies the Ib terminal and the other generates the Is terminal (Atwood et al., 1993; Hoang and Chiba, 2001; Lnenicka and Keshishian, 2000). These are the most commonly used terminals for
synaptic studies in *Drosophila* larvae. Results from the two terminals could not be combined since the Ib and Is terminals have differences in Ca\(^{2+}\) regulation (He et al., 2009). Thus, only the Ib terminals were studied; different terminals could be distinguished based upon the differences in bouton size, particularly at the end of them (Fig. 1: *Top*).

**Figure 1.** The ΔF/F recorded from Ib boutons during AP trains. *Top:* The Is and Ib terminals were viewed on muscle fiber 6/7 after loading with OGB-1. The terminals can be distinguished by the differences in the size of their boutons. Three boutons along the Ib terminal are identified with arrows. *Bottom:* The ΔF/F was measured at synaptic boutons. The nerve was stimulated at increasing frequencies (5 sec duration) and OGB-1 began to saturate as the frequency was increased from 40 to 60 Hz. These frequencies were used to calculate the \((\Delta F/F)_{\text{max}}\) using equation 3 and gave a value of 1.03 for this terminal.

**The increase in \([Ca^{2+}]_i\) produced by single APs and AP trains**

In this study, OGB-1 was used since it allowed the direct measurement of the Ca\(^{2+}\) transients produced by single APs as in previous studies (Lnenicka et al., 2006a; Hendel et al., 2008). 7 mM glutamate was added to the saline in order to prevent muscle contraction during stimulations at high frequencies. It was previously shown that the
presence of glutamate did not affect the amplitude of the Ca\textsuperscript{2+} transients produced by 10 Hz AP trains (Macleod et al., 2004). To verify that glutamate did not affect the Ca\textsuperscript{2+} transients produced by single APs, measurements made in the presence of glutamate were compared with experiments in which glutamate was absent. There was no significant difference in peak ΔF/F in the presence (14.7±0.9%, n = 23) and absence (15.5 ± 1.2%, n = 26; p>0.10) of glutamate.

To estimate [Ca\textsuperscript{2+}]_{rest} and Δ[Ca\textsuperscript{2+}], a previously described technique was used to calibrate a single-wavelength Ca\textsuperscript{2+} indicator (Maravall et al., 2000). For this calibration, it was necessary to determine the increase in fluorescence at OGB-1 saturation, i.e. (ΔF/F)\textsubscript{max}, for each terminal (see Methods). A mean [Ca\textsuperscript{2+}]_{rest} of 51 ± 12 nM (n=29) was obtained; this was similar to previous values of [Ca\textsuperscript{2+}]_{rest} (31, 40, 65 nM) reported for larval motor terminals (Hendel et al., 2008; Klose et al., 2008; Macleod et al., 2004). The Δ[Ca\textsuperscript{2+}], produced by single APs and AP trains was then determined (Fig. 2: Top). The Ib boutons had a peak Δ[Ca\textsuperscript{2+}], for single APs (Δ[Ca\textsuperscript{2+}]\textsubscript{AP}) of 121 ± 9 nM, a time constant of Δ[Ca\textsuperscript{2+}]\textsubscript{i} decay (τ\textsubscript{decay}) of 79 ± 1 msec and the bouton width was 2.5 ± 0.1 µm (n=29). The Δ[Ca\textsuperscript{2+}]\textsubscript{AP} was an underestimation and the τ\textsubscript{decay} was an overestimation since these measurements will be influenced by Ca\textsuperscript{2+} buffering by OGB-1 itself (see below). For AP trains, the Δ[Ca\textsuperscript{2+}], measured at the plateau (Δ[Ca\textsuperscript{2+}]\textsubscript{train}) increased with increasing AP frequency in a linear fashion (Fig. 2: Bottom) and the slope of the linear regression was 185 nM/10 Hz. For the AP frequencies (10, 20, 30, 40 and 60 Hz), the τ\textsubscript{decay} at the end of the train was 150 ± 10 msec, 188 ± 13 msec, 225 ± 35 msec, 202 ± 20 msec and 229 ± 19 msec, respectively.
Figure 2. Increases in \([\text{Ca}^{2+}]_i\) produced by single APs and AP trains. Top: The \(\text{Ca}^{2+}\) transients produced by single APs and AP trains. The traces represent the averaged response from 10 boutons at a synaptic terminal. Calibration: 40 nM and 0.2 sec for single APs; 200 nM and 2 sec for AP trains. Bottom: The dependence of the \(\Delta[\text{Ca}^{2+}]_{\text{train}}\) on AP frequency. AP trains (5 sec duration) were evoked in the Ib boutons at increasing AP frequencies and the \(\Delta[\text{Ca}^{2+}]_{\text{train}}\) was measured. The linear regression \((r = .89, p< .001)\) gave a slope of 185 nM/ 10 Hz \((n = 12\text{ terminals})\).

**Endogenous \(\text{Ca}^{2+}\) buffering**

The \(\text{Ca}^{2+}\) binding ratio \((\kappa_S)\) for the terminals can be determined by examining the effect of fast \(\text{Ca}^{2+}\) buffering by OGB-1 on the single-AP \(\tau_{\text{decay}}\). According to the linear single-compartment model, the \(\tau_{\text{decay}}\) is influenced by both \(\kappa_S\) and the incremental \(\text{Ca}^{2+}\)-binding ratio \((\kappa'_B)\) for OGB-1 according to the equation (Helmchen et al., 1996; Neher and Augustine, 1992):

\[
\tau_{\text{decay}} = \frac{(1 + \kappa_S + \kappa'_B)}{\gamma}
\]
where $\gamma$ is the rate constant for Ca$^{2+}$ removal from the cytoplasm and $\tau_{\text{decay}}$ plotted against $\kappa'_B$ should follow a straight line which intercepts the horizontal axis at $-(1+\kappa_S)$. $\kappa'_B$ is given by the equation (Helmchen et al., 1996; Neher and Augustine, 1992):

$$
\kappa'_B = \frac{\text{[OGB-1]} K_D}{((K_D + [\text{Ca}^{2+}]_{\text{rest}})(K_D + [\text{Ca}^{2+}]_{\text{rest}} + \Delta[\text{Ca}^{2+}]_{\text{AP}}))}
$$

A range of $\kappa'_B$ values can be obtained by comparing preparations that are loaded with different concentrations of the Ca$^{2+}$ indicator. Ib terminals on MF6/7 were examined in which the range of [OGB-1] was 15-fold and it was found that this produced a dramatic effect on $\Delta[\text{Ca}^{2+}]_{\text{AP}}$ and $\tau_{\text{decay}}$. Terminals with a higher [OGB-1] and $\kappa'_B$ showed a smaller $\Delta[\text{Ca}^{2+}]_{\text{AP}}$ and greater $\tau_{\text{decay}}$ (Fig. 3: Top). The $\tau_{\text{decay}}$ was determined for each bouton and these values were averaged to give a single value for each terminal; these values were positively correlated with $\kappa'_B$ (Fig. 3: Bottom). The data were fit by a linear regression ($n = 26$, $r = 0.78$, $p < 0.001$), which was then extrapolated to the x-axis. The negative x-axis intercept is equal to $\kappa_S + 1$ (Neher and Augustine, 1992) and gave a value of 77 for $\kappa_S$. The y-intercept of this plot was 46 msec, which is the $\tau_{\text{decay}}$ in the absence of OGB-1.

**Figure 3.** The effect of [OGB-1] on single-AP Ca$^{2+}$ transients and
determination of $\kappa_S$. Top: Changes in $[\text{Ca}^{2+}]_i$, produced by a single AP when the terminal was loaded with a low [OGB-1] (left) and a high [OGB-1] (right). Both [OGB-1] and $\kappa'_B$ are given. An increase in [OGB-1] resulted in a higher $\kappa'_B$, which reduced the amplitude and increased the duration of the Ca$^{2+}$ transient: left- $\Delta[\text{Ca}^{2+}]_{\text{AP}}$ 142 nM, $\tau_{\text{decay}}$ 42 msec; right- $\Delta[\text{Ca}^{2+}]_{\text{AP}}$ 70 nM, $\tau_{\text{decay}}$ 145 msec. The trace on the left is the mean response from 6 boutons and the right trace is the mean from 8 boutons. Bottom: The dependence of the single-AP $\tau_{\text{decay}}$ on $\kappa'_B$. The $\tau_{\text{decay}}$ was positively correlated with $\kappa'_B$. The linear regression was extrapolated to the x-axis intercept (arrow) to give a value of -78.

More data were added to the above plot by including some measurements in which the $\Delta F/F_{\text{max}}$ was not determined. For these experiments, the $\Delta F/F$ values were calibrated by assuming that $[\text{Ca}^{2+}]_{\text{rest}}$ was 51 nM, which would give a ($\Delta F/F_{\text{max}}$) of 1.02 (equation 1). When this larger data set was plotted, the obtained values were similar to those reported above. The linear regression ($n = 34$, $r=0.74$, $p<0.001$) gave a value of 82 for $\kappa_S$ and 46 msec for $\tau_{\text{decay}}$. 
Effect of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} concentrations on Ca\textsuperscript{2+} influx

The recent salines used for physiological studies of the larval NMJs have contained a variety of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} concentrations, which likely influenced Ca\textsuperscript{2+} influx through the voltage-gated Ca\textsuperscript{2+} channels. The ionic composition of salines HL3 and HL3.1 are identical (both contain 1.5 mM Ca\textsuperscript{2+}) except that HL3 has 20 mM Mg\textsuperscript{2+} and HL3.1 contains 4 mM Mg\textsuperscript{2+} (Feng et al., 2004; Stewart et al., 1994). Another saline, HL6, contains 0.5 mM Ca\textsuperscript{2+} and 15 mM Mg\textsuperscript{2+} (Macleod et al., 2002). In this study, HL3 with 1.0 mM Ca\textsuperscript{2+} was used. The effect of reducing external Ca\textsuperscript{2+} from 1.5 to 1.0 mM or reducing Mg\textsuperscript{2+} from 20 to 4 mM on Ca\textsuperscript{2+} influx was examined. Single-AP Ca\textsuperscript{2+} transients in HL3 saline where the Ca\textsuperscript{2+} concentration was changed from 1.0 mM to 1.5 mM were first measured; this was followed by the measurements in which Mg\textsuperscript{2+} concentration was reduced from 20 mM to 4 mM as was in HL3.1 saline. For analysis, the peak $\Delta F/F$ of the Ca\textsuperscript{2+} transients was normalized to that measured in HL3 with 1.5 mM Ca\textsuperscript{2+}. As expected, there was a reduction in Ca\textsuperscript{2+} influx when external Ca\textsuperscript{2+} was reduced or external Mg\textsuperscript{2+} was increased. The Ca\textsuperscript{2+} transients in HL3 with 1.0 mM Ca\textsuperscript{2+} (79.1 ± 1.8%; n=139 boutons, 8 animals) were smaller than those in 1.5 mM Ca\textsuperscript{2+} (100%). The Ca\textsuperscript{2+} transients in HL3.1 (203.8 ± 3.8%) were approximately twice as large as those in HL3 with 1.5 mM Ca\textsuperscript{2+}.
Discussion

Endogenous Ca\(^{2+}\) buffering at Ib terminals

In this study, it was found that the endogenous Ca\(^{2+}\) buffering (κ\(_S\)) for the Ib terminals on MF 6/7 was 77 and thus, nearly 99% of the Ca\(^{2+}\) entering the terminals upon a single AP will be bound by the endogenous Ca\(^{2+}\) buffers. This κ\(_S\) falls within the range of values previously reported for synaptic terminals. The κ\(_S\) was 20 for dentate granule cell boutons (Jackson and Redman, 2003), 20 and 27 for CA1 pyramidal neuron spines and dendrites, respectively (Sabatini et al., 2002; Rozsa et al., 2004), 40 for the calyx of Held (Helmchen et al., 1997), 110 for layer 2/3 neocortical pyramidal neuron dendrites (Koester and Sakmann, 2000) and 120 for layer 5 neocortical pyramidal neuron dendrites. The identity of these endogenous proteins is unclear. Calbindin and calretinin are fast Ca\(^{2+}\)-binding proteins (Edmonds et al., 2000; Lee et al., 2000b; Nagerl et al., 2000; Schwaller et al., 2002); however, they are mobile (subject to wash out) and in many cases, the fast Ca\(^{2+}\) buffering did not wash out during cell dialysis (Helmchen et al., 1997; Neher and Augustine, 1992; Stuenkel, 1994; Zhou and Neher, 1993). In addition, not all neurons express calbindin or calretinin, and these proteins have a low \(K_d\) (hundreds of nanomolar) (Baimbridge et al., 1992; Schwaller et al., 2002), resulting in greater saturation of the buffer than normally seen (Neher and Augustine, 1992; Zhou and Neher, 1993). *Drosophila* expresses a homologue of vertebrate calbindin, i.e. calbindin-32 (Buchner et al., 1988; Reifegerste et al., 1993); however, it does not appear to be expressed at this Ib motor terminals. A calbindin-32 antibody demonstrated little or no calbindin-32 expression at the NMJs (Dr. Erich Buchner, personal communication) and
in our lab it has been confirmed that the MF 6/7 Ib terminal does not show immunostaining with this antibody (generous gift of Dr. Erich Buchner).

In the study of chapter 1, it was found that Is terminals produced larger single-AP Ca\(^{2+}\) transients than those produced by Ib terminals; this could result from more Ca\(^{2+}\) influx per bouton volume or less endogenous Ca\(^{2+}\) buffering (He et al., 2009). Previous evidence indicated that this was due to more Ca\(^{2+}\) influx per bouton volume and the preliminary data from Is terminals supports this conclusion: the Is terminals do not appear to have a lower \(\kappa_S\) than that for Ib terminals.

**Ca\(^{2+}\) influx produced by single APs**

It is necessary to determine the \(\Delta[Ca^{2+}]_{AP}\) in order to estimate the Ca\(^{2+}\) influx during a single AP. The sampling rate in this study (50 Hz) resulted in an underestimation of the actual \(\Delta[Ca^{2+}]_{AP}\), however, this underestimation would be less at terminals containing a high [OGB-1] and having a long \(\tau_{decay}\). For instance, in our lab Ca\(^{2+}\) dynamics was modeled for single APs using a compartment model and Virtual cell software (Schaff et al., 1997; Slepchenko et al., 2003). These simulations were resampled using 20 msec integration times as in this study. For an [OGB-1] of 120 \(\mu\)M, the peak was underestimated by 7-10% when it fell within the first 5 msec of the 20 msec interval as in the current experiments. However, the underestimation may be less than this since the rise of the Ca\(^{2+}\) transients is likely slower than in the simulations; the simulations use a compartment model, which does not consider the time required for entering Ca\(^{2+}\) to reach diffusional equilibrium. Note that the measurements of \(\tau_{decay}\) used to determine \(\kappa_S\)
were not affected by sampling error since it was found that the sampling rate used in this study would overestimate the time constant by less than 1% at high and low [OGB-1].

For the current analysis, two terminals with the highest [OGB-1] (117 µM and 107 µM) were chosen; they had mean $\Delta[\text{Ca}^{2+}]_{\text{AP}}$ of 70.1 nM and 73.1 nM, respectively. According to the linear single-compartment model (Helmchen et al., 1996; Neher and Augustine, 1992), equation 6 predicts that the total increase in intracellular $[\text{Ca}^{2+}]$ ($\Delta[\text{Ca}^{2+}]_{\text{total}}$) was 15.3 µM and 15.2 µM, respectively; the $\Delta[\text{Ca}^{2+}]_{\text{AP}}$ would be approximately 196 nM in the absence of indicator.

$$
\Delta[\text{Ca}^{2+}]_{\text{AP}} = \frac{\Delta[\text{Ca}^{2+}]_{\text{total}}}{1 + \kappa_S + \kappa_B}
$$

The averaged surface/volume of the MF6/7 Ib boutons was 2.5 based upon images from confocal microscopy and the assumption that the boutons were prolate spheroid (He et al., 2009). Based upon these measurements, it was calculated that during a single AP $3.68 \times 10^3$ Ca$^{2+}$ ions entered per µm$^2$ surface membrane.

These values could be larger under physiological conditions since they were obtained in HL3 with 1.0 mM Ca$^{2+}$ and larval hemolymph has been reported to contain 1.5 mM Ca$^{2+}$ (Stewart et al., 1994). It was found in this study that the single-AP Ca$^{2+}$ transient measured in 1.0 mM Ca$^{2+}$ was 79% of that measured in 1.5 mM Ca$^{2+}$. These values are similar to those of the calyx of Held where measurements of single-AP Ca$^{2+}$ transients showed that Ca$^{2+}$ influx for 1.0 mM Ca$^{2+}$ was approximately 75% of that measured in 1.5 mM Ca$^{2+}$ (Helmchen et al., 1997). The *Drosophila* saline HL3.1 contains 4 mM Mg$^{2+}$ rather than the 20 mM Mg$^{2+}$ found in HL3; it was found that this reduction in Mg$^{2+}$ gave physiological phenotypes for channel mutants that were more consistent with
the behavioral phenotypes (Feng et al., 2004). HL3.1 produced a 104% increase in the Ca\(^{2+}\) transient amplitude compared to HL3 presumably due to the less blocking of Ca\(^{2+}\) channels by external Mg\(^{2+}\) (Lansman et al., 1986). If the physiological levels of Ca\(^{2+}\) and Mg\(^{2+}\) are 1.5 and 4.0 mM, respectively, then the \(\Delta [\text{Ca}^{2+}]_{\text{AP}}\) would be expected to be 506 nM. In the mammalian CNS, the \(\Delta [\text{Ca}^{2+}]_{\text{AP}}\) for synaptic terminals range from 400 nM for the calyx of Held to 500 nM for pyramidal cells in the neocortex and 1000 nM for dentate gyrus granule cells (Helmchen et al., 1997; Jackson and Redman, 2003; Koester and Sakmann, 2000).

**Ca\(^{2+}\) clearance and efflux**

The rate constant for Ca\(^{2+}\) removal after a single AP was 1696 s\(^{-1}\) based upon the \(\tau_{\text{decay}}\) (46 msec) with no added buffer and equation 4. This removal rate likely involves Ca\(^{2+}\) extrusion by the PMCA and Ca\(^{2+}\) uptake by ER based upon pharmacological studies (Lnenicka et al., 2006a). However, it cannot be ruled out that the involvement of other mechanisms such as slow Ca\(^{2+}\) buffering, which appears to play a role in \([\text{Ca}^{2+}]_{i}\) decay at crayfish motor terminals (Lin et al., 2005).

The Ca\(^{2+}\) extrusion rate is likely lower than this removal rate and this was supported by determining the extrusion rate constant during AP trains. The \(\Delta [\text{Ca}^{2+}]_{\text{train}}\) represents a steady-state where the rate of Ca\(^{2+}\) influx is equal to the rate of Ca\(^{2+}\) extrusion (Tank et al., 1995): (AP frequency)( \(\Delta [\text{Ca}^{2+}]_{\text{total}}/\text{AP}\))=( \(\Delta [\text{Ca}^{2+}]_{\text{train}}\))(Ca\(^{2+}\) extrusion rate constant). Using a \(\Delta [\text{Ca}^{2+}]_{\text{train}}\) of 185 nM/ 10 Hz (Fig. 2) and a \(\Delta [\text{Ca}^{2+}]_{\text{total}}\) of 15.3 \(\mu\)M gave a Ca\(^{2+}\) extrusion rate constant of 827 s\(^{-1}\), which was approximately half the rate constant for Ca\(^{2+}\) removal after a single AP. These values generally fall within
the range of values seen at other synaptic terminals. The $\tau_{\text{decay}}$ measured in boutons of dentate gyrus granule cells (43 msec), the calyx of Held (47 msec) and neocortical pyramidal cells (56 msec) were similar to that in $Drosophila$ boutons (Helmchen et al., 1997; Jackson and Redman, 2003; Koester and Sakmann, 2000). For the calyx of Held and neocortical pyramidal cells, the Ca$^{2+}$ removal constants were 900 and 2600 s$^{-1}$, respectively.
Chapter III: Effects of Presynaptic Parvalbumin Expression on Synaptic Physiology, Morphology and Ca\textsuperscript{2+} Regulation

Abstract

Synapses show a diversity of transmitter releasing properties that influence the processing of information by neural circuits. Although the molecular mechanisms responsible for this diversity remain largely unknown, differences in Ca\textsuperscript{2+} regulation could play an important role. Current study has focused on the effects of altered presynaptic Ca\textsuperscript{2+}-buffering on synaptic facilitation, morphology and Ca\textsuperscript{2+} regulation in genetically-modified larvae expressing parvalbumin (PV), which is a slow Ca\textsuperscript{2+}-buffering protein and is expressed in specific subsets of neurons in the mammalian brain but not in Drosophila. PV expression in the nervous system in Drosophila was achieved by crossing the transgenic UAS-PV fly line with the elav-GAL4 driver line and experiments were performed at larval neuromuscular junctions of muscle fiber 5, which is innervated only by one Ib terminal and is thus a preferred animal model. It was found that PV-expressing terminals showed dramatic reduction in the F\textsubscript{1} component of paired-pulse facilitation and to a lesser extent, a decreased F\textsubscript{2} component; the reduction in F\textsubscript{1} component was not due to the difference in the initial EPSP amplitude, since the reduced F\textsubscript{1} was still apparent when PV-expressing terminals and control terminals with the same initial EPSP amplitude were compared. The facilitation during an AP train was also found to be significantly decreased in PV-expressing terminals; meanwhile, an increased single-AP transmitter release was seen in these terminals, presumably as a homeostatic change to partially compensate for the reduction in facilitation. The reduced facilitation
indicates a decreased impulse activity in PV-expressing terminals; this may be related to
the hypomorphic development of the motor terminals found in the study, presumably
through depressing Ca\(^{2+}\) signals. This was supported by the findings from Ca\(^{2+}\) imaging,
which showed an alteration of Ca\(^{2+}\) regulation in the PV-expressing terminals.
**Introduction**

Ca\(^{2+}\) buffers can shape Ca\(^{2+}\) signals in neurons and thus, potentially influence many aspects of neuronal development and function. The three proteins *calbindin*, *calretinin* and *parvalbumin* have been classified as Ca-buffering proteins since they have multiple Ca\(^{2+}\) binding sites and no apparent direct role in cell signaling (Baimbridge et al., 1992; Schwaller et al., 2002). These Ca\(^{2+}\) buffers all have a high affinity for Ca\(^{2+}\) (K\(_d\) in the nanomolar range), but differ in their Ca\(^{2+}\) binding rates; *calbindin* and *calretinin* are considered fast buffers and *parvalbumin* a slow buffer (Lee et al., 2000c; Nagerl et al., 2000). The expression of Ca-buffering proteins in the nervous system is broad, distinctive and dependent on the developmental stage; they are localized to well-defined subpopulations of neurons either alone or in combination (Rogers, 1989; Jandet al., 1981; Andressen et al., 1993; Hof et al., 1999; DeFelipe, 1997; Bastianelli, 2003).

Although their physiological function in these neurons is largely unknown, there is evidence that both *calbindin* and *parvalbumin* can influence synaptic facilitation (Blatow et al., 2003). Their influence is determined by their Ca\(^{2+}\)-binding affinities and their kinetics of Ca\(^{2+}\) handling. In this regard, *parvalbumin* has attracted much attention because of its unique chemodynamic features. Under resting condition, it is 60-90% bound by Mg\(^{2+}\) ions *in vivo* (Hou et al., 1991); Mg\(^{2+}\) ions must dissociate first before *parvalbumin* can bind Ca\(^{2+}\) ions. The slow dissociation process of Mg\(^{2+}\) makes *parvalbumin* a slow Ca\(^{2+}\) buffer: its K\(_{on}\) is about 20-fold greater than that in the fast Ca\(^{2+}\) buffer, *calbindin*, even though *parvalbumin* has a much higher Ca\(^{2+}\) affinity than *calbindin* (Schmidt et al., 2003b). *Parvalbumin* appears to act by mainly influencing the decay of residual Ca\(^{2+}\). After Ca\(^{2+}\) enters to evoke transmitter release, the residual Ca\(^{2+}\)
equilibrates in the terminal and studies of central and peripheral synapses in vertebrates and invertebrates have shown that the magnitude of PTP, augmentation and F₂ facilitation were correlated with residual Ca²⁺ (Zucker and Regehr, 2002). The effect of parvalbumin on residual Ca²⁺ and synaptic facilitation has been studied at two different populations of synapses in the mammalian CNS; in both cases, parvalbumin knockout mice were used to eliminate parvalbumin expression. The major effect of eliminating parvalbumin was to slow the decay of residual Ca²⁺ produced by a single AP or short train of APs (Collin et al., 2005; Muller et al., 2007) and influence the magnitude and decay of paired-pulse facilitation (Caillard et al., 2000; Muller et al., 2007). However, parvalbumin’s effect on different components of PPF, i.e. F₁ facilitation and F₂ facilitation was not studied; moreover, the buildup and decay of facilitation during AP trains was not examined.

In addition to its direct effects on synaptic function, the presence of a Ca²⁺ buffer could have effects on synaptic development. For example, it was proposed that an increase in Ca²⁺ can promote terminal growth by increasing cAMP (Zhong et al., 1992) and knocking out parvalbumin and calbindin in mice alters the size of spines on Purkinje cell dendrites (Vecellio et al., 2000). Also, a reduction in synaptic facilitation produced by parvalbumin could trigger a homeostatic response restoring the effective synaptic strength. Invertebrate motor terminals have shown homeostatic changes in transmitter release in response to experimental manipulations that alter synapse efficacy including changes in muscle fiber growth, membrane resistance and sensitivity to glutamate (Lnenicka and Mellon, Jr., 1983; Petersen et al., 1997; Paradis et al., 2001).

The synaptic changes produced by parvalbumin can be detailed by examining identified synapses at the Drosophila larval NMJ. In a previous study parvalbumin was
expressed at the larval motor terminals and the changes in Ca$^{2+}$ transients produced by single APs and AP trains were demonstrated (Harrisingh et al., 2007). In the current study, the synaptic physiology, morphology and Ca$^{2+}$ regulation at the motor terminal innervating muscle fiber 5 were examined. It was found that parvalbumin expression dramatically reduced PPF (especially the F$_1$ component) and reduced the buildup of facilitation during AP trains; meanwhile, an increase in single-AP transmitter release was found, presumably as a homeostatic change to partially compensate for the reduced facilitation. In addition, PV expression also caused a hypomorphic development in the motor terminals, presumably resulting from the depressed Ca$^{2+}$ signals in the terminals; this was confirmed by Ca$^{2+}$ imaging which showed the alteration of Ca$^{2+}$ regulation in the PV-expressing terminals.
Materials and methods

Drosophila stocks and crosses

All experiments were performed at 20°C. The Parvalbumin (PV) expressing transgenic fly strain (x/x(y); PVPV/Cyo; PVPV/TM3, Ser, or TM6C, Sb, and/or TM6B, Hu; +/+) was produced in Dr. Michael Nitabach’s lab (Harrisingh et al., 2007) and called UAS-PV in this study. Virgin females were selected to mate with the pan-neuronal elav driver elavC155 (flybase). The 3rd-instar female larvae from the F1 generation of the cross were used for experiments.

Immunohistochemistry

A Myc epitope tag was fused to the UAS-PV transgene and the expression of the PV protein was detected using an anti-Myc antibody (mouse monoclonal, 1:50, Santa Cruz). Goat anti-horseradish peroxidase (HRP; 1:200; Cappel, OH) was used to label the terminals in order to calculate the number and the size of boutons and the monoclonal antibody nc82 (1:100; Developmental Studies Hybridoma Bank, Iowa City, IA, UAS), which recognizes an active zone component Bruchpilot (Wagh et al., 2006), was used to label the active zones. Procedures for immunostaining were previously described (He et al., 2009). In brief, 3rd-instar larvae were dissected and fixed in 4% formaldehyde for an hour. The fixed larvae were incubated overnight (4°C) in primary antibody (anti-Myc, anti-HRP or nc82) followed by corresponding secondary antibody (Alexa Flour 488 goat anti-mouse IgG for anti-Myc; Alexa Flour 546 rabbit anti-goat IgG for HRP and Alexa Flour goat anti-mouse IgG for nc82; all 1:200; Invitrogen) for 5 hours at room temperature. Preparations were mounted in SlowFade gold antifade reagent (Invitrogen).
and viewed under a Zeiss LSM 510 confocal microscope with a Zeiss 63x, 1.4 NA objective (Carl Zeiss). Alexa Flour 488 was imaged using a 488 laser with a BP 505-530 emission filter and Alexa Fluor 546 with a 543 laser and a LP 560 emission filter.

For the morphological measurements, stacks of slices (0.3 µm thickness) were acquired from MF5 in segments 3 and 4 using a confocal microscope (LSM 510 Meta with Argon, HeNe1 and HeNe2 lasers, Carl Zeiss) in order to count the bouton number and determine the bouton size using anti-HRP, and the number of active zones using nc82. These images were then analyzed using Zeiss LSM Image Browser (Carl Zeiss). After measuring the bouton width and length, bouton volume and surface area were estimated using equations for a prolate spheroid (Beyer, 1990): volume = \( \frac{4}{3} \pi a b^2 \) and surface area = \( 2\pi b \left( b + \frac{a^2}{e} \right) \sin^{-1} \left( \frac{e}{a} \right) \); where \( e = \frac{\left( \frac{a^2}{e} - b^2 \right)^{1/2}}{a} \), \( a \) = major radius and \( b \) = minor radius. For the number of active zones, the number of spots recognized by nc82 per bouton was counted, assuming that each spot represented one active zone; when an active zone spanned more than one slice it was counted only once. For the length of terminals, the measurements began at the first bifurcation where the terminals left the axon. The number of branches was determined by counting all segments arising from each branch point. Data were analyzed using Sigmaplot 10.0 (SPSS Inc., Plover, WI, USA) and statistical analyses were performed using \( t \)-test or a one-way ANOVA.

**Electrophysiology**

All experiments were performed in HL3 saline with 1.0 mM Ca\(^{2+}\). Synaptic responses were evoked in segments 3 or 4 by sucking up the cut end of the segmental
nerve with a suction electrode (approximately 10 µm inside diameter) and stimulating the
nerve. EPSPs from muscle fiber 5 were recorded using sharp microelectrodes (10-20 MΩ
filled with 3 M KCl) connected to a preamplifier (Axoclamp 2A; Axon Instruments, Inc.,
Foster City, CA). Data were acquired (sampling rate 5 KHz) and analyzed using a
Digidata 1322A (Axon Instruments Inc.) interface and pCLAMP 9.2 (Axon Instruments
Inc.) software. There were two stimulation protocols used in the study: (1) paired-pulse
facilitation (PPF) protocol. Interstimulus intervals (ISIs) were varied to obtain the PPF
decay. For very short ISI such as 10 ms, only the experiments where the two EPSPs did
not heavily overlap and the peaks were discernible were selected, and the amplitudes of
these peaks were measured from the “projected tail” of the proceeding conditioning
EPSPs. Paired-pulse ratio (PPR) was calculated as $\text{PPR} = (\text{EPSP}_1 - \text{EPSP}_2)/\text{EPSP}_1$, where
$\text{EPSP}_1$ is the response produced by the proceeding conditioning action potential and
$\text{EPSP}_2$ is the response produced by the subsequent testing action potential. To determine
the magnitude for $F_1$ facilitation, PPRs for ISIs of 10, 15, 20, 30, 40 and 50 msec were
plotted semilogarithmically against time and the best fit line was created using linear
regression to determine the magnitude and decay time constant. Likewise for $F_2$
facilitation, PPRs for ISIs greater than 200 msec were plotted for the best fit line, and the
value predicted by the line at the ISI of 150 msec was taken as the magnitude of $F_2$
facilitation, based on the previous studies that showed $F_2$ usually reached a maximum
about 120-150 msec after the conditioning pulse (Bittner and Sewell, 1976; Mallart and
Martin, 1967). (2) Facilitation buildup protocol. Stimulations at 0.5 Hz for 40 seconds
were used to establish the baseline EPSP level, followed by 10-second 20 Hz tetanus and
then another 40-second 0.5 Hz. The averaged EPSP level during the last second of the 20
Hz tetanic stimulation was taken as the plateau of the tetanus, and the facilitation after the train was calculated as $100 \times \frac{\text{plateau of the tetanus} - \text{averaged pre-tetanus EPSPs}}{\text{averaged pre-tetanus EPSPs}}$. Magnitude of post-tetanic potentiation (PTP) was also determined by fitting the points 10 sec after the end of the 20 Hz stimulation to create a best fit line and extrapolating the line to the time when the 20 Hz train ended. For miniature EPSP recording, microelectrodes with resistance of 5 MΩ were used.

$Ca^{2+}$ imaging

All experiments were performed on terminals innervating MF5 in HL3 saline with 1 mM Ca$^{2+}$ and 7 mM glutamate. Procedures of loading the Ca$^{2+}$ indicator, Oregon Green 488 BAPTA-1 (OGB-1), and the imaging were described previously (Lnenicka et al., 2006a). Fluorescence change was calculated as $(\text{fluorescence} - \text{resting fluorescence})/\text{resting fluorescence}$. 
**Results**

To examine the effect of the Ca^{2+} buffer protein parvalbumin (PV) on the development and function of synapses, larvae in which PV was expressed pan-neuronally in the otherwise PV-negative *Drosophila* nervous system were studied. The PV transgenes were fused to a Myc epitope tag for the detection of PV expression, as well as a mammalian nuclear export signal to ensure accumulation in the cytoplasm (Harrisingh et al., 2007). Larvae expressing PV were generated using an elav (embryonic lethal, abnormal vision, *Drosophila*)-GAL4 transgene driving the UAS-PV transgene by standard genetic crosses.

To investigate the effects of ectopic expression of PV on synaptic development and physiology, muscle fiber 5 (MF5) was used since it is innervated by only a single motor neuron (Hoang and Chiba, 2001). The synaptic properties of this motor neuron was examined previously and it was found that the properties were sexually differentiated (Lnenicka et al., 2006b) so only females were used in this study. Immunohistochemistry revealed expression of PV in motor terminals at neuromuscular junctions (Fig 1) as well as in the brain (not shown) of elav>PV females but not in the UAS-PV females or CS females that served as controls.

*Fig. 1: Anti-Myc immunostaining revealed the expression of PV at the motor terminals innervating MF5 at NMJs (arrow). This single-plane confocal image passes through the central region of the terminal at the end.*
PV expression reduces paired-pulse facilitation and the build-up of facilitation during AP trains

The effect of PV expression on short-term synaptic enhancement was investigated. First paired-pulse facilitation (PPF) was compared between the PV-positive (elav>PV) and PV-negative larvae (UAS-PV, CS). In the initial studies of PPF at the frog neuromuscular junctions (Mallart and Martin, 1967), it was demonstrated that PPF could be separated temporally into two distinct components: an early component with a rapid decay rate (termed F1 facilitation) and a subsequent component with a slower decay rate (termed F2 facilitation). These F1 and F2 components of PPF have not been demonstrated at the larval NMJ.

The time course of PPF for this Ib terminal was determined by varying the interstimulus intervals (ISIs) from 10 to 1000 msec (Fig. 2). The control terminals (UAS-PV, CS) clearly showed the two phases of the PPF (Fig. 2: A and B). The PV-positive terminals showed reduced PPF; this is particularly true for F1 facilitation and to a lesser extent F2 (Fig. 2: C). The effects on F1 and F2 facilitation can be compared by examining PPF at a 10 msec ISI where F1 predominates and a 200 msec ISI where only F2 facilitation is present. For the ISI of 10 ms, the averaged paired-pulse ratios (PPRs) for controls were 0.94 ± 0.23 (n= 18) and 0.90 ± 0.09 (n= 14) for CS and UAS-PV, respectively; both were greater than that for elav>PV (0.19 ± 0.04, n= 36; p<0.001, one-way ANOVA) (Fig. 2: D). For ISI of 200 msec, the averaged PPRs were: 0.25 ± 0.04 (n= 16) for CS, 0.30 ± 0.06 (n= 11) for UAS-PV, and were also greater than that for elav>PV (0.17 ± 0.02, n= 34; p<0.05, one-way ANOVA) but to a lesser extent (Fig. 2: D). For F1 component, the difference in PPRs between PV-positive and PV-negative groups did not
appear to result from the difference in the initial EPSP amplitude produced by the conditioned pulse, since the difference in PPRs still existed when comparing these two types of terminals with the same initial EPSP amplitude (Fig. 2: E). To determine the magnitude and time constants of decay for each component, all the PPRs were plotted semilogarithmically against time and the best fit lines were created to probe the magnitude as well as the decay time constant of F1 and F2 facilitation: for F1, the (magnitude, decay time constant) for CS and UAS-PV was (1.35, 25 msec) and (1.17, 31 msec), respectively. These F1 parameters were not obtainable in elav>PV. For F2, the (magnitude, decay time constant) for CS, UAS-PV and elav>PV was (0.24, 845 msec), (0.26, 747 msec) and (0.18, 671 msec), respectively.

The effects of PV on the facilitation during AP trains were also investigated. First the baseline EPSP amplitude was determined by stimulating the nerve at 0.5 Hz for 40 sec, and then the facilitation of EPSP amplitude during 20 Hz for 10 sec was examined and lastly the synaptic enhancement during 0.5 Hz stimulation for 40 sec. Only experiments without any failure were selected and analyzed. It was found that during 20 Hz stimulation, the averaged EPSP amplitude during the last second of the tetanus for the PV-expressing terminals (elav>PV: 6.6 ± 0.3 mV; n= 10) was significantly less than that of controls (CS: 8.3 ± 0.4 mV, n= 10; UAS-PV: 8.9 ± 0.3 mV, n= 13; p<0.01, one-way ANOVA). On average, EPSPs in elav>PV showed 75.8 ± 15.7% increase in amplitude during the last second of the 20 Hz tetanus compared to the baseline (measured by averaging EPSPs from the pre-tetanus 0.5 Hz stimulation), while for the CS and UAS-PV the increases were 145.5 ± 12.4% and 222.3 ± 19.8%, respectively (p<0.01, one-way ANOVA; Fig. 3: B). No significant difference was found for post-tetanic
Fig. 2: Expression of PV produced a dramatic reduction in PPF at Drosophila NMJs. PPF was studied by measuring the facilitation at various inter-stimulus intervals (ISIs) and determining the paired-pulse ratio (PPR) calculated by (EPSP2-EPSP1)/EPSP1. Decay of PPF was described by semi-log plot of PPRs versus ISIs; this was used to determine the amplitude and decay time constant of the PPF components. For the F1 component, ISIs of 10, 15, 20, 30, 40 and 50 msec were used and its amplitude was determined by extrapolating the line to zero time. For F2 component, ISIs >200 msec were used and the amplitude was determined at an ISI of 150 msec (see Materials and Methods for details). In controls (A and B), a two-phase decay of PPF was clearly seen, whereas in PV-expressing terminals (C), only the F2 component was apparent. Insets are representative traces; the first EPSP is followed by superimposed second EPSPs for various ISIs. (D) At an ISI of 10 msec where F1 predominates, PPR in PV-expressing terminals was significantly smaller than that of controls; the difference was also seen for ISI of 200 msec where F2 predominates, although less dramatic (*p<0.01; **p<0.001; one-way ANOVA). For ISI of 10 msec, n (# of animals) in three groups was 18, 14 and 36 for CS, UAS-PV and elav>PV, respectively; for ISI of 200 msec, it was 16, 11 and 34, respectively. (E) The decreased PPF for ISI of 10 msec in PV-expressing terminals (elav>PV) was still apparent when comparing PV and control terminals with the same initial EPSP amplitude.
potentiation (PTP) between $elav>PV$ and the controls ($UAS-PV$ and $CS$) (for the enhanced transmission accounted for by PTP after the 20Hz stimulation: $elav>PV$: $0.56 \pm 0.17$; $UAS-PV$: $0.69 \pm 0.09$; CS: $0.60 \pm 0.08$; $p>0.05$, one-way ANOVA).

Fig. 3: PV-expressing terminals showed less synaptic facilitation during a train of APs. Nerves were stimulated at 0.5 Hz for 40 sec, followed by 10 sec of a 20 Hz train of APs and then 0.5 Hz for 40 sec. (A) Averaged representative EPSPs for each group. Pre-tetanic and post tetanic EPSPs were made by averaging all EPSPs that preceded and followed the 20 Hz stimulation. 20 Hz plateau was made by averaging the EPSPs during the last second of the 10-sec 20 Hz stimulation. Sample numbers were 10, 13 and 10 animals for CS, UAS-PV and $elav>PV$, respectively. (B) Facilitation during a train of APs in PV-expressing terminals ($elav>PV$) was significantly smaller than that in controls (CS and UAS-PV) (*$p<0.01$; one-way ANOVA). Facilitation was calculated as $100\%$ (averaged amplitude of EPSPs during the last second of the train-averaged amplitude of pre-tetanic EPSPs)/averaged amplitude of pre-tetanic EPSPs. (C) Averaged EPSP amplitude was plotted against time to show the facilitation during a train. The initial EPSP was the averaged amplitude of pre-tetanic EPSPs; the following 20 points were for the 20 Hz stimulation, with one point representing the averaged EPSP amplitude during 0.5 sec; the rest were the points for the averaged amplitude of post-tetanic EPSPs. Sample numbers for creating the plot were the same as in (A). (D) The data from C were normalized to the initial EPSP.

82
Parvalbumin expression results in an increase in transmitter release per AP that partially compensates for the reduced facilitation

A noticeable finding from the above results was that the baseline EPSP amplitude (combined measurements from the EPSP1 in PPF experiments and the pre-tetanic EPSPs in train facilitation experiment, at stimulation frequencies of 0.1 Hz and 0.5 Hz, respectively) was significantly greater in *PV*-expressing terminals (*elav>*PV: 4.1 ± 0.3 mV, n= 26) than those in the controls (CS: 3.2 ± 0.2 mV, n= 27; *UAS-PV*: 2.9 ± 0.2 mV, n= 22; *p*<0.05, one-way ANOVA) (Fig. 4: B).

To determine whether the increased EPSP amplitude was due to an increase in transmitter release, the amplitude and the frequency of the miniature EPSPs (mEPSPs) were compared. Each muscle fiber was recorded for about 10 min for mEPSP. It was found that there was no significant difference in mEPSP amplitudes between the *PV*-expressing terminals (*elav>*PV: 0.40 ± 0.02 mV, n= 7) and controls (CS: 0.37 ± 0.02 mV, n= 7; *UAS-PV*: 0.37 ± 0.02 mV, n= 6; *p*>0.5, one-way ANOVA) (Fig. 4: C); thus, single APs released more transmitter from *PV*-expressing terminals compared to controls. It was estimated that upon single impulse the *elav>*PV terminals released on average 10.3 quanta compared to 8.7 and 7.8 for CS and *UAS-PV*, respectively. The greater transmitter release in the *PV*-expressing terminals was supported by the greater frequency of mEPSPs in these terminals (*elav>*PV: 0.37 ± 0.05 Hz, n= 7) than in controls (CS: 0.12 ± 0.02 Hz, n= 7; *UAS-PV*: 0.13 ± 0.01 Hz, n= 6; *p*<0.01) (Fig. 4: E). This increased single-AP transmitter release may be a homeostatic change that was developed to compensate for the reduced facilitation after a train of APs (see discussion) (Fig. 3: C and D).
Fig. 4: PV-expressing terminals showed greater single-AP transmitter release than the controls. (A) Representative EPSPs for each group; traces were averaged from 10 animals each group. (B) Amplitude of EPSP level in PV-expressing terminals (elav>PV) was significantly greater than that in controls (CS and UAS-PV) (*p<0.05; one-way ANOVA). Samples were taken from all EPSP1 in PPF experiments in Fig 2 and the pre-tetanic responses in train facilitation experiments in Fig 3. n (# of animals) values were 27, 22 and 26 for CS, UAS-PV and elav>PV, respectively. (C) Representative traces of mEPSP for each group. (D) No difference was seen in mEPSP amplitude between PV-expressing terminals (elav>PV) and controls (CS and UAS-PV) (p>0.5; one-way ANOVA). n (# of animals) values were 7, 6 and 7 for CS, UAS-PV and elav>PV, respectively. (E) mEPSP frequency in elav>PV was significantly greater than that in controls (*p<0.01; one-way ANOVA).
**Effect of parvalbumin expression on motor terminal morphology**

The effect of expressing PV on the morphology of the motor terminals was examined. It has been shown that the expression of calcium-binding proteins can affect the development of neuronal morphology (Vecellio et al., 2000); however synaptic terminals have not been examined. By examining this small identified synaptic terminal, it should be feasible to detect relatively subtle changes in terminal morphology. Since the PV-expressing terminals (elav>PV) showed an increase in single-AP transmitter release, the number of active zones per bouton for PV-expressing and the control terminals was also examined. The terminals were stained with an antibody to HRP and the active zones were stained with an antibody to Bruchtpilot.

Using confocal microscopy, the length of terminal contacting the muscle fiber was first measured and then the number of boutons along the terminal and the bouton widths were examined. The terminal lengths for the PV-expressing terminals were about 16% shorter than for the control terminals (elav>PV: 73.7 ± 3.5 µm, n= 16; CS: 88.5 ± 4.7 µm, n= 8; UAS-PV: 86.5 ± 2.8 µm, n= 16; p<0.05, one-way ANOVA) (Fig. 5: B), although the branching number of the terminals among the groups was not significantly different (data not shown) The number of boutons in the PV-expressing terminals was about 25% less than for controls (elav>PV: 9.1 ± 0.3, n= 23; CS: 11.9 ± 0.5, n= 14; UAS-PV: 11.8 ± 0.4, n= 16; p<0.05, one-way ANOVA). In addition, boutons along the PV-expressing terminals were smaller than for control terminals; the width of PV-positive boutons was approximately 17% less than for control boutons (elav>PV: 2.4 ± 0.1 µm, n= 23; CS: 2.8 ± 0.1 µm, n= 8; UAS-PV: 3.0 ± 0.1 µm, n= 16; p<0.001, one-way ANOVA). Calculations of bouton volume and surface area estimated that the bouton
volume was approximately 34% less for $PV$-expressing boutons and the surface area was approximately 24% less. In spite of the smaller bouton size for $PV$-positive terminals, the number of active zones per bouton was similar for experimental and control terminals ($elav>PV$: 11.4 ± 0.5, n=11; CS: 11.6 ± 0.4, n=12; UAS-$PV$: 11.7 ± 0.6, n=6; $p>0.5$, one-way ANOVA).

Fig. 5: Expression of $PV$ caused hypomorphic development of the motor terminals. (A) Representative images of the motor terminals (left two images) and active zones (right two images). Each image is a montage of about 50-70 optical sections (slice width, ~50 µm) through MF terminals in segments 3 and 4. The morphology of boutons and the presence of active zones were clearly shown using anti-HRP and anti-Bruchpilot staining, respectively. Morphological features of the terminals including terminal length (B), number of boutons (C), bouton width (D) and number of active zones per bouton (E) were compared between $PV$-expressing terminals ($elav>PV$) and controls (CS and UAS-$PV$). Significant differences were seen in the terminal length (B; *$p<0.05$; one-way ANOVA), number of boutons (C; **$p<0.001$; one-way ANOVA) and bouton width (D; **$p<0.001$; one-way ANOVA), but not in the number of active zones per bouton (E; $p>0.5$; one-way ANOVA). n (# of animals) values for CS, UAS-$PV$ and $elav>PV$ in each comparison were: B- 8, 16, 16; C- 14, 16, 23; D- 8, 16, 23; E- 12, 18, 11.
Thus, $PV$ expression had an effect on the development of synaptic boutons resulting in fewer and smaller boutons. Based upon the number of boutons and the number of active zones per bouton, the $PV$-expressing terminals had about 25% fewer active zones than the controls. Therefore, the greater transmitter release from $PV$-expressing terminals was not due to a greater number of active zones compared to controls.

**PV expression affects presynaptic $Ca^{2+}$ transients**

The effect of $PV$ expressing on synaptic morphology and physiology is presumably due to changes in $Ca^{2+}$ buffering and the $Ca^{2+}$ signal (Vecellio et al., 2000). It was previously showed that there was an effect of $PV$ expression on $Ca^{2+}$ transients in Ib terminals on muscle fiber 4 (Harrisingh et al., 2007). In the current study the $Ca^{2+}$ transients in the Ib terminals on MFs 5 were also found to be affected by $PV$ expression. The transients were measured during single APs and AP trains. Since the concentration of OGB-1 in these terminals has a large effect on the $Ca^{2+}$ transients (He and Lnenicka, 2011), the study matched these factors in the experimental and control terminals. This was accomplished by selecting terminals that had similar ratios of resting OGB-1 fluorescence/bouton size ($elav>PV$: 15.1, n= 9; $UAS>PV$: 16.2, n= 6; $p>0.5$; $t$-test); one animal in $elav>PV$ and five animals in $UAS-PV$ with extreme values were removed.

It was found that $PV$ expression significantly reduced the amplitude and duration of the $Ca^{2+}$ signal produced by single APs (Fig. 6): the $\Delta F/F_{AP}$ peak was 29% less ($elav>PV$: 9.9 ± 0.8%, n= 48 boutons, 9 animals; $UAS-PV$: 14.4 ± 1.5%, n= 45, 6; $p<0.05$; $t$-test) and the decay time constant ($\Delta F/F_{AP} \tau_{\text{decay}}$) was 26% less for $PV$-expressing
terminals compared to controls (elav>PV: 44.4 ± 4.5 msec, n=46,9; UAS-PV: 59.6 ± 5.2 msec, n=45,6; p<0.05; t-test) (Fig. 6: B). The decrease in \( \tau \text{decay} \) is expected for a slow Ca\textsuperscript{2+} buffer (Lee et al., 2000c; Collin et al., 2005), whereas the decrease in \( \Delta F/F_{AP} \) peak could occur only with high concentration of the slow buffer (Atluri and Regehr, 1996; Chard et al., 1993). For 5 sec trains of APs delivered at 10 and 20 Hz, the amplitude of the Ca\textsuperscript{2+} signal at the plateau and the decay of Ca\textsuperscript{2+} transients at the end of the trains were measured (Fig. 6: B). For both 10 and 20 Hz stimulation, the amplitude of the Ca\textsuperscript{2+} signal was not significantly different between PV-expressing terminals and control terminals (10Hz: elav>PV: 13.1 ± 1.4%, n=42, 8; UAS-PV: 13.5 ± 1.0%, n=35, 6; p>0.05; 20Hz: elav>PV: 26.8 ± 3.0%, n=42, 8; UAS-PV: 29.8 ± 1.3%, n=29, 5; p>0.05; t-test); however, the \( \tau \text{decay} \) was significantly greater for the PV-expressing terminals compared to control (10Hz: elav>PV: 166.6 ± 18.8 msec, n= 46, 9; UAS-PV: 89.9 ± 14.0 msec, n= 26, 5; p<0.05; 20Hz: elav>PV: 237.6 ± 20.0 msec, n= 41, 8; UAS-PV: 169.6 ± 21.5 msec, n= 29, 5; p<0.05; t-test). For AP trains, the buildup of Ca\textsuperscript{2+} is slower in the PV-expressing terminals than in controls (Fig. 6: C). Thus for AP trains, at least during the first second of the AP trains the amplitude of the Ca\textsuperscript{2+} signal will be less in PV-expressing terminals than in control ones. The results are likely the direct effect of adding a slow Ca\textsuperscript{2+} buffer such as PV to the terminals, since PV was shown to cause a rapid initial decay of Ca\textsuperscript{2+} transients and thus slow down the Ca\textsuperscript{2+} buildup during trains of APs (Lee et al., 2000c).
Fig. 6: The amplitude and decay of Ca$^{2+}$ transients seen at the PV-expressing terminals and control terminals. The relative increase in [Ca$^{2+}$], was determined from the ΔF/F measured at the peak for single APs or the plateau for AP trains. (A) Representative traces for PV-expressing terminals (elav>PV) and control terminals (UAS-PV) averaged from all the available measurements. (B) Top: ΔF/F$_{AP}$ peak in elav>PV was significantly smaller than that in UAS-PV, whereas there was no difference in ΔF/F$_{train}$ plateau between these two groups. Bottom: ΔF/F$_{AP}$ τ$_{decay}$ in elav>PV was smaller than that in UAS-PV, whereas the ΔF/F$_{train}$ τ$_{decay}$ in elav>PV was consistently greater than that in UAS-PV. n represents (# of boutons, # of animals). *p<0.01; t-test. (C) Comparison of ΔF/F amplitude during the first second of the 20 Hz train. Each column was the average of two Ca$^{2+}$ transient amplitudes. During the first second of the train, the increase in [Ca$^{2+}$], for elav>PV was always smaller than that in UAS-PV.
For Ib boutons, a decrease in bouton size was shown to increase the Ca$^{2+}$ transient amplitude for single APs and decrease the decay time constant (Lnenicka et al., 2006a). It was previously shown that the PV-expressing boutons were smaller than the control ones. For the boutons used for Ca$^{2+}$ measurements, it was also found that bouton width of in PV-expressing terminals (2.7 ± 0.1 μm, n= 9) was significantly smaller than that for control terminals (3.2 ± 0.2 μm, n= 6; p< 0.05; t-test). Thus, the decrease in bouton size for PV-expressing terminals should have increased the amplitude of the single AP Ca$^{2+}$ transient and decreased its time constant of decay. This would have offset some of the effect of Ca$^{2+}$ buffering by PV on the single AP Ca$^{2+}$ transient amplitude and the Ca$^{2+}$ decay after a train, but added to the effect of Ca$^{2+}$ buffer on the Ca$^{2+}$ decay after a single AP.
Discussion

Effects of parvalbumin expression on facilitation

In this study the effect of PV expression on short-term synaptic enhancement at Drosophila NMJ was examined using paired-pulse facilitation (PPF) protocol and 20 Hz trains of APs. It was found that PV-expressing terminals showed a dramatic decrease in F1 facilitation and to a lesser extent, a decrease in F2 facilitation; the decreased F1 was not due to the differences in the initial EPSP, since the decrease in facilitation was still apparent when comparing the two types of terminals with the same initial EPSP amplitude. The buildup of facilitation was also found to be significantly decreased during trains of APs in PV-expressing terminals. These results are in good agreement with previous studies. For example, Caillard et al. showed that one population of CNS synapses that normally show paired-pulse depression were found to produce PPF in PV-knockout mice (Caillard et al., 2000), indicating that PV produces a decrease in the peak amplitude of PPF. In another group of mammalian CNS synapses, PPF decayed more slowly but the peak amplitude of PPF was not affected by knocking out PV (Muller et al., 2007). However, these terminals contained calbindin, which may have also affected facilitation (Felmy et al., 2003). Furthermore, the effect of PV on F1 and F2 facilitation was not compared since neither synapse showed a clear F1 and F2 component. In the current study, PV’s effects on both components of facilitation were clearly shown; and these effects were not contaminated by those from calbindin, since there was little or no expression of calbindin at Drosophila NMJs (He and Lnenicka, 2011).

Previous studies showed that EGTA, an inorganic Ca\(^{2+}\) buffer that shows similar Ca\(^{2+}\)-binding kinetics as PV, affects F1 facilitation in a dose-dependent manner: high
buffer concentrations reduce the $F_1$ magnitude whereas low ones do not. This effect is more significant when the concentration of EGTA is greater than 1 mM (Atluri and Regehr, 1996; Caillard et al., 2000; Muller et al., 2007), which is expected to be as effective as 500 µM of $PV$, since $PV$ binds $Ca^{2+}$ with a stoichiometry of two (Haiech et al., 1979). In this study, the $PV$ concentration in presynaptic terminals is likely to be high, since $F_1$ facilitation was found to be affected profoundly. The current preferred model to explain synaptic facilitation is the “multiple-site hypothesis”, which proposes that synaptic facilitation is due to $Ca^{2+}$ acting at high-affinity, facilitating site ($Y$-site), separate from the low-affinity site ($X$-site) responsible for exocytotic transmitter release (Zucker and Regehr, 2002). These two sites act cooperatively to enhance transmitter release. $F_1$ facilitation is thought to be produced by local high $Ca^{2+}$ concentrations at the $Y$-site; the $Ca^{2+}$ microdomain formed near the $Ca^{2+}$ entry site is a candidate potentially responsible for producing $F_1$ (Llinas et al., 1992b; DiGregorio et al., 1999); computational simulations also support this idea (Naraghi and Neher, 1997; Bennett et al., 2007). This $Y$-site is believed to be in close proximity to the $X$-site, considering that fast buffers which reduce exocytotic transmitter release can also strongly affect $F_1$ facilitation, regardless of their concentrations (Suzuki et al., 2000; Ogawa et al., 2000). On the other hand, although low concentration of slow buffers does not usually affect exocytosis, moderate to high concentration of them does reduce it as well as $F_1$ facilitation (Borst and Sakmann, 1996; Atluri and Regehr, 1996; Muller et al., 2007); this appears counter-intuitive, since the action of slow buffers is too slow to affect the fast processes. The finding can be explained by simple calculations proposed by Roberts: (1) $\tau_C=(K_{on}B_0)^{-1}$ and (2) $R=B_dyD_{Bu}$, where $\tau_C$ is the mean time before the buffer captures a free calcium ion,
$K_{on}$ is the association rate constant of the buffer, $B_0$ is the concentration of the buffer, $R$ is the replenishment factor and $D_{Bu}$ is the buffer’s diffusional mobility (Roberts, 1994). These calculations indicate that the effects of a slow buffer on the Ca\(^{2+}\) microdomain can mimic those of a fast buffer, provided that its concentration is high enough and diffusion mobility is great enough; this is supported by a modeling study which showed that very high concentration of a slow buffer could affect the local Ca\(^{2+}\) concentration near the membrane in a way similar to that from a fast buffer (Nowycky and Pinter, 1993). It is tempting to speculate PV affects the Ca\(^{2+}\) microdomain signal through this mechanism, since its concentration is likely very high in the terminals and it is considered a highly mobile buffer and can greatly facilitate Ca\(^{2+}\) dissipation (Gabso et al., 1997; Schmidt et al., 2003a).

According to the “multiple-site hypothesis”, F\(_2\) facilitation is believed to be in close relationship with residual Ca\(^{2+}\) after Ca\(^{2+}\) influx and its subsequent equilibration in the terminal. This residual Ca\(^{2+}\) can be measured using the Ca\(^{2+}\) imaging technique described in this study. The time required for Ca\(^{2+}\) to equilibrate after it enters the boutons is given by $(\text{radius})^2/6D$ (Tank et al., 1995), where $D$ is the effective diffusion coefficient for Ca\(^{2+}\) and the best estimate for $D$ is $0.2 \times (10^{-6})$ cm\(^2\)/sec (Zhou and Neher, 1993). The apparent diameters for elav> PV and UAS-PV boutons in this study were 2.7 $\mu$m and 3.2 $\mu$m, respectively; based upon the above equation, the equilibration time for these two types of boutons were about 15.2 msec and 21.3 msec, respectively. Images were acquired at 50 Hz in the Ca\(^{2+}\) imaging (i.e. 20 msec per image) for single APs; thus the measured Ca\(^{2+}\) peaks for single APs could be plausibly considered as the residual Ca\(^{2+}\). It was found that the residual Ca\(^{2+}\) in elav> PV terminals was around 76% of that in
UAS-PV terminals for single APs; meanwhile, PPF with ISI of 200 msec to 1000 msec (mainly F₂ facilitation) in elav>PV terminals was found to be about 60-70% of that in UAS-PV terminals, supporting the idea that residual Ca²⁺ is at least in part responsible for producing F₂ facilitation (Zucker and Regehr, 2002). Also, the decay time course of F₂ facilitation appears to be determined in part by the slow kinetics of Ca²⁺ unbinding from the Y-site (Atluri and Regehr, 1996). Presumably this would produce the observation that F₂ facilitation outlasts residual Ca²⁺ (Atluri and Regehr, 1996; Matveev et al., 2004). This was the case in this study: residual Ca²⁺ returned to baseline 250 msec after Ca²⁺ influx at the latest, whereas F₂ facilitation was still evident 1000 msec after the conditioning impulse.

The current study showed that the facilitation during 10 sec of the 20 Hz tetanic stimulation in PV-expressing terminals was dramatically reduced. At this frequency (50 msec between two adjacent APs), the loss of F₁ is not expected to contribute much to the reduced facilitation seen upon the train of APs, since its τdecay was found to be about 30 msec and in 50 msec, F₁ should fall to a negligible level. Therefore, the reduction of the later forms of short-term synaptic enhancement (STE), i.e. F₂ facilitation and augmentation, are likely to contribute to this finding; significant reduction of PTP was not seen in the study. It was previously showed that augmentation was usually present following repetitive stimulation and its magnitude was often great enough to become a major player in the buildup of facilitation (Magleby and Zengel, 1976b). In one study where the stimulation frequency was so low (5 Hz) that the impact of F₁ could be neglected, the application of high concentration of a slow buffer (25 mM EGTA) was also shown to reduce the magnitude of facilitation after a train (Feller et al., 1996). Even
when the stimulation frequency was high enough (50 Hz) but the concentration of the slow buffer was too low (0.15 mM EGTA) to affect F₁, the magnitude of facilitation after a 50 Hz-train was also reduced, presumably due to the reduction of the later forms of STE (Ogawa et al., 2000). These forms of STE are thought to be closely related to residual Ca²⁺ (Zucker and Regehr, 2002); results from Ca²⁺ imaging support this idea: at least for the first second of the 20 Hz stimulation, PV-expressing terminals showed lower level of residual Ca²⁺ than the control terminals (Fig. 6: C). Note that, however, the difference in facilitation during an AP train toward the end of the stimulation (a time when the [Ca²⁺]ᵢ in these two types of terminals showed no difference (Fig. 6:B)) was still dramatic (Fig. 3: D); this is unexpected according to the “multiple-site hypothesis” (Zucker and Regehr, 2002) and the reason is unknown.

**Effect of PV expression on Ca²⁺ regulation**

The results in the current study showed ΔF/F_Ap peak was decreased in PV-expressing terminals; similar modulatory effect from PV were seen in some other studies (Chard et al., 1993; Dreessen et al., 1996). Meanwhile, the ΔF/F_Ap τ_decay was also decreased; this is expected since PV is well known to facilitate the decay of Ca²⁺ transient (Lee et al., 2000c; Muller et al., 2007). For the ΔF/F_train plateau (both 10 Hz and 20 Hz), no significant difference was found between PV-expressing terminals and the control; a computational simulation also showed a 4-second train of 10 Hz produced the same magnitude of Ca²⁺ transients in the absence and presence of 200 µM PV in bovine chromaffin cells (Lee et al., 2000c). However, for the terminals innervating MF4, a 10 Hz train produced a greater plateau in the PV-positive animals and it was proposed that this
resulted from a compensatory decrease in the activity of the Ca$^{2+}$ pump (Harrisingh et al., 2007); it appears that there are neuron-specific differences in the response to PV expression. For the $\Delta F/F_{\text{train}} \tau_{\text{decay}}$, the $PV$-expressing terminals showed consistent prolonged decay of Ca$^{2+}$ transients, in agreement with the previous findings (Harrisingh et al., 2007) and presumably results from the dissociation of Ca$^{2+}$ from PV as the Ca$^{2+}$ levels decrease.

**Effects of PV expression on terminal morphology and transmitter release**

In the study it was found that in the $PV$-expressing terminals, boutons were smaller and there were fewer boutons along the terminals; meanwhile, these terminals were also found to be shorter compared to control. Previous studies have shown that the level of impulse activity can influence the development of the number and size of synaptic boutons. For example, terminals of hyperactive, K$^+$-channel mutant *eag Sh* were shown to bear more synaptic boutons, whereas when hypo-excitability mutation *napts* was combined with *eag Sh* to lower its activity level, the morphological change in *eag Sh* mutants was completely reversed (Budnik et al., 1990). Another study also showed that reduced impulse activity resulted in a decrease in bouton size (Lnenicka et al., 2003). The effect of increased impulse activity may be mediated by an increased level of intracellular Ca$^{2+}$, which would increase levels of cAMP by activating the adenyl cyclases (Dunn and Feller, 2008; Zaccolo and Pozzan, 2003; Davis et al., 1996); the increase in cAMP would ultimately leads to the change in synaptic structure (Davis et al., 1996). In this study, PV has been shown to decrease the global Ca$^{2+}$ concentration, especially during single APs and the first few APs of a train; this could reduce the activation of the adenyl cyclase,
resulting in smaller and fewer boutons along the terminals. However, it cannot be ruled out that PV directly reduced the impulse activity in the motoneurons and thus reduced the bouton size and number along the terminals; or the morphological changes seen in the study could be the combination of these two effects.

An increase in single-AP transmitter release was also found in the PV-expression terminals; this could result from a homeostatic mechanism that helps to compensates for the reduced facilitation after a train. This facilitation plays a major role in synaptic efficacy. By itself, EPSP produced by single APs is unlikely to be an accurate measurement of synaptic effectiveness since these Drosophila terminals fire trains of impulses, not single APs, under the physiological conditions (Budnik et al., 1990); thus a reduction in facilitation after a train seen in the PV-expressing terminals would reduce the effective synaptic strength and there would be attempts for the organism to correct this defect by the accompanied homeostatic changes.

Synaptic homeostasis is a well-established phenomenon. Early studies focused on the maintenance of synaptic strength during growth at crustacean NMJs. For example, it was found that the EPSP amplitude was maintained during the dramatic muscle growth due to an increase in transmitter release from the motor terminals and an increase in the miniature excitatory postsynaptic current (mEPSC) (DeRosa and Govind, 1978); experimentally reducing the muscle growth delayed the increase in transmitter release (Lnenicka and Mellon, Jr., 1983). Major advances in the study of synaptic homeostasis have come from using the powerful genetic tools in Drosophila along with the well-defined larval neuromuscular system. For instance, the fasciclin II mutants, which have abnormally small motor terminals, still had a normal-sized EPSP (Stewart et al., 1996);
this synaptic homeostasis resulted mainly from increased transmitter release, apparently due to an increase in the density of active zones. Transgenic larvae were also shown to compensate for a reduced sensitivity to glutamate at the NMJ by increasing transmitter release from the motor terminals (DiAntonio et al., 1999; Petersen et al., 1997; Davis et al., 1998). These studies all focused on the maintenance of the EPSP amplitude produced by single APs; however, synaptic homeostasis could also act to maintain a constant EPSP amplitude during AP trains as is proposed here.
Chapter IV: Chronic lead exposure alters presynaptic calcium regulation and synaptic facilitation in *Drosophila* larvae

**Abstract**

Chronic exposure to inorganic lead (Pb²⁺) has been shown to influence activity-dependent synaptic plasticity in the mammalian brain, possibly by altering the regulation of intracellular Ca²⁺ concentration ([Ca²⁺]). To explore this possibility, the effect of Pb²⁺ exposure on [Ca²⁺], regulation and synaptic facilitation was studied at the neuromuscular junction of larval *Drosophila*. Wild-type *Drosophila* (CS) larvae were raised in media containing either 0, 100 µM or 250 µM Pb²⁺ and identified motor terminals were examined in wandering third-instar larvae. To compare resting [Ca²⁺], and the changes in [Ca²⁺], produced by impulse activity, the motor terminals were loaded with a Ca²⁺ indicator dye, either Oregon Green 488 BAPTA-1 (OGB-1) or fura-2 conjugated to a dextran. It was found that rearing in Pb²⁺ did not significantly change the resting [Ca²⁺], nor the Ca²⁺ transient produced in synaptic boutons by single action potentials (APs); however, the Ca²⁺ transients produced by 10 and 20 Hz AP trains were larger in Pb²⁺-exposed boutons and decayed more slowly. For larvae raised in 250 µM Pb²⁺, the increase in [Ca²⁺], during an AP train (20 Hz) was 29% greater than in control larvae and the [Ca²⁺], decay τ was 69% greater. These differences appear to result from reduced activity of the plasma membrane Ca²⁺ ATPase (PMCA), which extrudes Ca²⁺ from these synaptic terminals. These findings are consistent with studies in mammals showing a Pb²⁺-dependent reduction in PMCA activity. It was also observed that a Pb²⁺-dependent enhancement of synaptic facilitation was present at these larval neuromuscular synapses. Facilitation of EPSP amplitude during AP trains (20 Hz) was 55% greater in Pb²⁺-reared
larvae than in controls. These results showed that Pb$^{2+}$ exposure produced changes in the regulation of [Ca$^{2+}$], during impulse activity that could affect various aspects of nervous system development. At the mature synapse, this altered [Ca$^{2+}$]$_i$ regulation produces changes in synaptic facilitation that are likely to influence the function of neural networks.
Chronic Pb\(^{2+}\) exposure can produce changes in the structure and function of the mammalian brain (Costa et al., 2004; Toscano and Guilarte, 2005). This is particularly true for synapses where chronic Pb\(^{2+}\) exposure influences synaptic development (Kiraly and Jones, 1982; Petit and LeBoutillier, 1979) and plasticity (Altmann et al., 1993; Gilbert et al., 1996; Gilbert and Mack, 1998; Ruan et al., 1998). The effects of Pb\(^{2+}\) exposure on synaptic development and plasticity could result from alteration in \([Ca^{2+}]_i\) regulation. Intracellular Ca\(^{2+}\) can influence multiple steps in synaptic development; e.g., growth cone guidance (Jin et al., 2005), synapse formation (Xu et al., 2009) and synapse elimination and stabilization (Lohmann and Bonhoeffer, 2008; Pratt et al., 2003). At the mature synapse, altered \([Ca^{2+}]_i\) regulation can influence long and short-term forms of synaptic plasticity (MacDonald et al., 2006; Zucker and Regehr, 2002).

The effects of Pb\(^{2+}\) on Ca\(^{2+}\) influx and efflux are well documented. Acute exposure to Pb\(^{2+}\) blocks Ca\(^{2+}\) influx through both invertebrate (Audesirk and Audesirk, 1989; Busselberg et al., 1990) and mammalian voltage-dependent Ca\(^{2+}\) channels (Audesirk and Audesirk, 1991; Audesirk and Audesirk, 1993; Busselberg et al., 1993; Evans et al., 1991; Peng et al., 2002). Also, micromolar concentrations of Pb\(^{2+}\) can inhibit the extrusion of Ca\(^{2+}\) by the plasma membrane Ca\(^{2+}\) ATPase (PMCA) in humans and rats (Bettaiya et al., 1996; Mas-Oliva, 1989; Sandhir and Gill, 1994) although the PMCA is stimulated by lower (nanomolar) Pb\(^{2+}\) concentrations (Ferguson et al., 2000; Mas-Oliva, 1989). However, it is not known whether the effects of Pb\(^{2+}\) exposure on synaptic development and plasticity are due to altered regulation of \([Ca^{2+}]_i\). In fact, the effect of Pb\(^{2+}\) exposure on the regulation of \([Ca^{2+}]_i\) has not been well characterized. For example, there are no studies examining the effect of Pb\(^{2+}\) exposure on the changes in \([Ca^{2+}]_i\)
produced by impulse activity. It is difficult to examine the acute effects of Pb$^{2+}$ on [Ca$^{2+}$], regulation since Pb$^{2+}$ can enter through voltage-dependent Ca$^{2+}$ channels (Simons and Pocock, 1987; Tomsig and Suszkiw, 1991) and Ca$^{2+}$ indicators can respond to both Ca$^{2+}$ and Pb$^{2+}$ (Kerper and Hinkle, 1997; Tomsig and Suszkiw, 1990). However, the long-term effects of chronic Pb$^{2+}$ exposure on [Ca$^{2+}$], can be measured in Pb$^{2+}$-free saline. In fact, fura-2 has been used to measure resting [Ca$^{2+}$], in synaptosomes isolated from rats after chronic Pb$^{2+}$ exposure (Sandhir and Gill, 1994).

First, the changes in [Ca$^{2+}$], produced in synaptic terminals by impulse activity were measured in control and Pb$^{2+}$-exposed larvae. Second, after defining the changes in [Ca$^{2+}$], regulation resulting from Pb$^{2+}$ exposure, whether there were correlated changes in synaptic plasticity was determined. In these experiments Drosophila larval NMJ was used; this is one of the preferred systems for studies of the molecular basis for synaptic function, development and plasticity (Keshishian et al., 1996). In addition to its advantage for genetic analyses, the larval motor terminals are identifiable and accessible for electrophysiological and optical studies. Previously, Pb$^{2+}$ exposure was shown to affect the development of behavior in Drosophila (Hirsch et al., 2003; Hirsch et al., 2009) and synaptic development at the larval NMJ (Morley et al., 2003).

It was found that chronic Pb$^{2+}$ exposure did not produce a significant effect on the size of the Ca$^{2+}$ signal produced by a single AP suggesting that Ca$^{2+}$ influx is not affected. However, Pb$^{2+}$-exposed synaptic boutons show a greater increase in [Ca$^{2+}$], during 10 and 20 Hz AP trains and a slower decay of [Ca$^{2+}$], at the end of these trains. This is apparently due to a decrease in the activity of the PMCA. Prior exposure to Pb$^{2+}$ did not affect the amplitude of the EPSP produced by single APs; however, these terminals showed a
greater buildup of synaptic facilitation during an AP train. Presumably the greater buildup of residual Ca$^{2+}$ results in greater synaptic facilitation and both responses are ultimately due to reduced PMCA activity.

*Materials and Methods*
To produce larvae, 20 female and male adult flies (wild type, Canton S) were added to 25 mm vials containing Instant Drosophila Medium Formula 4-24 (Carolina Biological Supply Company, Burlington, NC) mixed with either dH₂O (nominal 0 Pb²⁺), 100 µM Pb acetate in dH₂O or 250 µM Pb acetate in dH₂O. Female wandering 3ʳᵈ instar larvae were collected for Ca²⁺ measurements and synaptic physiology.

**Loading the Ca²⁺ indicator**

To measure [Ca²⁺]ᵢ, the dual-wavelength Ca²⁺ indicator fura-2 (Kₐ 594 nM) or the single-wavelength indicator, Oregon Green 488 BAPTA-1 (OGB-1; Kₐ 1180 nM) were used. In both cases, they were coupled to 10,000 MW dextrans (Invitrogen, Carlsbad, CA). As in previous studies (He et al., 2009; Lnenicka et al., 2006a), the indicator was loaded into the motor terminals using the technique developed by (Macleod et al., 2002). Briefly, larvae were dissected in Schneider’s media (Sigma, St. Louis, MO) and the nerve supplying segment 3 was cut. The cut end of the nerve was immediately sucked up into a snug-fitting pipette and then a small volume of indicator (5 mM) was introduced into the end of the pipette tip using very thin tubing. After 40 min, the nerve was removed from the pipette and the preparation was left at room temperature for another hour. The Schneider’s solution was then replaced with HL3 saline (Stewart et al., 1994) containing 1 mM Ca²⁺ for physiological measurements. The membrane-permeant, heavy-metal chelator N,N,N’,N’-tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN; Invitrogen) was dissolved in ethanol (200 mM) and added to the saline for a final concentration of 100 µM for some experiments.

**Measurement of [Ca²⁺]ᵢ changes**
The terminals were imaged using an upright, fixed-stage BH2 microscope (Olympus) equipped with epifluorescence, DIC, a water-immersion 40x Zeiss lens (NA 0.75) and a digital cooled-CCD camera (CoolSNAP HQ, Photometrics, Tucson, AZ). Excitation illumination from a 75W xenon arc lamp was passed through a Lambda-10 Optical Filter Changer (Sutter Instrument Co., Novato, CA)

Fura-2 was excited using 360 ± 5 and 380 ± 5 nm bandpass filters (Chroma Technology Corp., Brattleboro, VT); excitation and emission wavelengths were separated with a 410 nm dichroic mirror and the emitted light was passed through a barrier filter of 510 ± 10 nm. For OGB-1, a 480 ± 15nm excitation filter was used, as well as a 500 nm dichroic mirror and a high-pass 515 nm barrier filter. Images were captured using 100 msec exposures for fura-2. For OGB-1, images were streamed using 20 msec exposures (image size- 50 x 350 pixels) for single APs and 50 msec (image size- 100 x 350 pixels) exposures for AP trains. Metafluor 6.1 software (Molecular Devices, Downingtown, PA) was used for image acquisition and to measure fluorescent intensity at synaptic boutons and background fluorescence from the muscle. Metamorph 6.1 software (Molecular Devices) was used to measure bouton widths.

As in a previous study, the [OGB-1] was estimated in the synaptic terminals by measuring the fluorescence for a range of [OGB-1] in capillary tubes with an inside diameter of approximately 20 µm (He et al., 2009). The fluorescent values were normalized to a 20 µm diameter giving a linear plot of [OGB-1] versus fluorescent intensity. For each terminal, the average resting fluorescence was measured at a region of uniform terminal width (apparent diameter) and the fluorescence intensity was normalized to a 20 µm diameter. These capillary tubes contained 40 nM Ca^{2+}; we
assumed a resting [Ca\(^{2+}\)], of 40 nM in the motor terminals (Klose et al., 2008). This fluorescent value was then converted to [OGB-1] using the above plot. Capillary tubes with a 20 µm inside diameter were produced from 50 µm capillary tubes (VitroCom, Mountain Lakes, NJ) using a Narishige PN-30 electrode puller (Narishige International USA Inc., East Meadow, NY).

**Synaptic physiology**

The nerve was stimulated with a suction electrode connected to a S11 stimulator (Grass-Telefactor, West Warwick, RI) and EPSPs were recorded from muscle fibers with sharp electrodes using an Axoclamp 2A amplifier (Axon Instruments Inc., Foster City, CA). pCLAMP 9.2 software and a Digidata 1200A digitizer (Axon Instruments Inc., Foster City, CA) were used to acquire and measure EPSPs.

**Data analysis**

Sigmaplot 10.0 and SPSS 16.0 (SPSS Inc. Plover, WI) were used for data transformation and statistical analysis. The background fluorescence was subtracted from the bouton fluorescence for each image. For fura-2, the ratio of fluorescence produced by 360 and 380 nm excitation (360/380) was calculated. For OGB-1, the percentage change in fluorescence (ΔF/F) was calculated by 100 x (fluorescence – initial fluorescence)/initial fluorescence. Single exponentials were fit to the ΔF/F decay to determine the [Ca\(^{2+}\)], decay time constant (τ). Only [Ca\(^{2+}\)], decay τ for which the fit to a single exponential gave r\(^2\) > 0.9 was reported in order to eliminate noisy measurements. The increase in [Ca\(^{2+}\)], during trains of impulses was measured after the [Ca\(^{2+}\)], increase
plateaued by averaging the last 20 measurements of the train. Statistical analyses were performed using t-tests with a Welch correction for unequal variances or an ANOVA followed by a post-hoc analysis for which p values were adjusted using either a Bonferroni correction (variances not significantly different), or a Games-Howell correction (variances are significantly different). All n values were reported as (# boutons, # animals) unless otherwise noted.

Results
To examine the effect of chronic Pb\(^{2+}\) exposure on intracellular [Ca\(^{2+}\)], regulation at synaptic terminals, *Drosophila* larvae were raised from egg stages in control media (nominal 0 Pb\(^{2+}\) concentration) and in media containing 100 µM or 250 µM Pb\(^{2+}\). In a previous study, the Pb\(^{2+}\) burdens were measured in larvae raised in 100µM Pb\(^{2+}\) using inductively coupled mass spectrometry; this gave values of 12.37 ng Pb\(^{2+}\) per larva, which had an average wet weight of 1.6 mg (Morley et al., 2003). It was found that the water content of the *Drosophila* larvae was about 80% of wet weight consistent with a previous study (Folk et al., 2001). Probably about half of this water content is hemolymph since hemolymph is about 40% of wet body weight for insect larvae (Nation, 2001). If the Pb\(^{2+}\) were evenly distributed in the body fluids then the average Pb\(^{2+}\) concentration in hemolymph would be 48 µM. However, since the mid and hindgut contain about 73% of the Pb\(^{2+}\) (Wilson, DT. *The development of Drosophila as an animal model for studying the behavioral genetics of lead toxicology. Ph.D. dissertation, University at Albany; 2004*.), where it is likely they are sequestered (Vijver et al., 2004), and contribute little to total larval volume, the average effective Pb\(^{2+}\) concentration would be about 13 µM. This value could increase up to 2-fold if the hemolymph Pb\(^{2+}\) concentration were considerably higher than the intracellular Pb\(^{2+}\) concentration. In conclusion, it appears likely that the levels of total Pb\(^{2+}\) in the larval hemolymph are micromolar, which falls within the range of Pb\(^{2+}\) blood levels observed to influence nervous system development and synaptic plasticity in other organisms (Cline et al., 1996; Gilbert et al., 1996; Lorton and Anderson, 1986; Petit and LeBoutillier, 1979).

In wandering third instar larvae, [Ca\(^{2+}\)]\(_{i}\) in the Ib motor terminals innervating muscle fibers 6 and 7 (MF 6/7) was compared. MF 6/7 are innervated by Is and Ib motor
terminals supplied by two separate motor neurons (Hoang and Chiba, 2001; Lnenicka and Keshishian, 2000). The terminals can be distinguished by their different morphology: Ib terminals have large synaptic boutons and Is terminals have small boutons (Kurdyak et al., 1994).

**Increase in \([\text{Ca}^{2+}]_i\) produced by impulse activity**

The increase in \([\text{Ca}^{2+}]_i\), produced by single APs and trains of APs was examined in control and Pb\(^{2+}\)-reared larvae. The single-wavelength \(\text{Ca}^{2+}\) indicator OGB-1 was used; this allowed us to measure the rapid, “volume-averaged” changes in \([\text{Ca}^{2+}]_i\), produced by single APs (Lnenicka et al., 2006a). The change in OGB-1 fluorescence (\(\Delta F/F\)) produced by single APs (\(\Delta F/F_{\text{AP}}\)) and AP trains (\(\Delta F/F_{\text{train}}\)) was determined for individual synaptic boutons (Fig 1). In general, the Pb\(^{2+}\)-exposed synaptic boutons showed similar \(\Delta F/F_{\text{AP}}\) amplitudes but larger \(\Delta F/F_{\text{train}}\) amplitudes than boutons from larvae grown in 0 Pb\(^{2+}\).

![Figure 1. Ca\(^{2+}\) transients recorded from synaptic boutons in control and Pb\(^{2+}\)-exposed animals.](image)

**Figure 1.** \(\text{Ca}^{2+}\) transients recorded from synaptic boutons in control and Pb\(^{2+}\)-exposed animals. Left: Ib terminals on MF 6/7 filled with OGB-1 from control larvae and those exposed to Pb\(^{2+}\). The arrows point to two typical synaptic boutons where \(\text{Ca}^{2+}\) transients were measured. Right: Typical \(\text{Ca}^{2+}\) transients for single APs and AP trains seen at control and Pb\(^{2+}\)-exposed (250 µM) boutons. The \(\Delta F/F_{\text{AP}}\) was similar for control and Pb\(^{2+}\)-exposed synaptic boutons; however, the \(\Delta F/F_{\text{train}}\) was generally larger in Pb\(^{2+}\)-exposed boutons compared to controls. Calibration: Single AP- 20% \(\Delta F/F\), 0.4 sec; AP train- 20% \(\Delta F/F\), 2 sec.

The differences in \(\text{Ca}^{2+}\) transients for control and Pb\(^{2+}\)-exposed larvae were quantified by comparing a number of animals. In these experiments, it was necessary to
use a similar [OGB-1] in the terminals of control and experimental larvae; the Ca\textsuperscript{2+} indicator acts as a buffer and high concentrations will decrease the amplitude of the $\Delta F/F_{AP}$ and reduce the rate of $[\text{Ca}^{2+}]$, decay at the end of the Ca\textsuperscript{2+} transient (Neher, 1995). In two experiments, the [OGB-1] was very high (> 100 µM) and these experiments were not included. For the remaining experiments, the [OGB-1] was similar for terminals from control (43.3 ± 7.3 µM, 11 terminals), 100 µM Pb\textsuperscript{2+}-exposed (43.3 ± 8.0 µM, 7 terminals) and 250 µM Pb\textsuperscript{2+}-exposed larvae (49.2 ± 4.1 µM, 7 terminals). In addition, the Ca\textsuperscript{2+} transients can be influenced by bouton size so it was necessary to compare bouton populations that were of similar size (Lnenicka et al., 2006a); bouton width was similar for control (2.5 ± 0.1 µm; n=94, 11), 100 µM Pb\textsuperscript{2+} (2.6 ± 0.1 µm; n=59, 7) and 250 µM Pb\textsuperscript{2+} terminals (2.7 ± 0.1 µm; n=121, 7).

The $\Delta F/F_{AP}$ amplitude was not significantly different for Pb\textsuperscript{2+}-reared larvae compared to controls; however, Pb\textsuperscript{2+}-reared larvae showed a significantly larger $\Delta F/F_{train}$ amplitude than controls (Fig. 2). The $\Delta F/F_{train}$ amplitude increased with increasing concentrations of Pb\textsuperscript{2+}. For 20 Hz stimulation, the $\Delta F/F_{train}$ amplitude was 16% and 29% larger than controls for 100 µM and 250 µM Pb\textsuperscript{2+}, respectively. An increase in the $[\text{Ca}^{2+}]_{plateau}$ during an AP train can be produced by greater Ca\textsuperscript{2+} influx or a reduction in the rate of Ca\textsuperscript{2+} extrusion (Tank et al., 1995). Since the $\Delta F/F_{AP}$ amplitudes were similar for control and Pb\textsuperscript{2+}-exposed terminals, it is unlikely that the greater $\Delta F/F_{train}$ amplitude resulted from differences in Ca\textsuperscript{2+} influx. Rather, this difference in amplitude likely reflected differences in Ca\textsuperscript{2+} extrusion.

The decay of $[\text{Ca}^{2+}]$, after an AP train reflects the rate of Ca\textsuperscript{2+} extrusion: a higher extrusion rate will result in a smaller $[\text{Ca}^{2+}]$, decay time constant ($\tau_{\text{decay}}$). We compared
the [Ca$^{2+}$]$_{\text{i}}$ $\tau_{\text{decay}}$ for control and Pb$^{2+}$ exposed terminals after single APs and AP trains (Fig. 3). There was no clear effect of Pb$^{2+}$ exposure on the $\Delta F/F_{\text{AP}}$ $\tau_{\text{decay}}$ since the $\tau_{\text{decay}}$ was significantly greater for larvae exposed to 100 µM Pb$^{2+}$, but not for those exposed to 250 µM Pb$^{2+}$. For AP trains, Pb$^{2+}$ exposure produced an increase in the $\tau_{\text{decay}}$ in a dose-dependent manner (Fig. 4). For 20 Hz stimulation, the $\Delta F/F_{\text{train}}$ $\tau_{\text{decay}}$ was 27% and 69% greater than controls for animals exposed to 100 µM and 250 µM Pb$^{2+}$, respectively.

Figure 2. The Ca$^{2+}$ transient amplitude in synaptic boutons from control larvae and those exposed to 100 or 250 µM Pb$^{2+}$. The OGB-1 $\Delta F/F$ was measured at the peak of the Ca$^{2+}$ transient for single APs ($\Delta F/F_{\text{AP}}$ peak) and at the plateau for AP trains ($\Delta F/F_{\text{train}}$ plateau).

Top: The $\Delta F/F_{\text{AP}}$ peak was not significantly different for Pb$^{2+}$-exposed larvae compared to control larvae.

Middle: The $\Delta F/F_{\text{train}}$ plateau during 10 Hz stimulation was significantly greater for Pb$^{2+}$-exposed animals compared to control ones.

Bottom: The $\Delta F/F_{\text{train}}$ plateau during 20 Hz stimulation was significantly greater for the Pb$^{2+}$-exposed larvae compared to control larvae. Since data for the different stimulation rates are not independent, we used the ANOVA routines of SPSS to analyze amplitude separately for single APs, 10Hz and 20Hz. For a single AP there was no significant overall effect of Pb$^{2+}$-exposure ($F=2.70; df=1; p>0.05$), but there were significant linear effects of dose for both 10Hz ($F=54.21; df=1; p<0.001$) and 20Hz ($F=28.12; df=1; p<0.001$). Significantly greater than control: ***$p<0.001$; Post-hoc analysis with Games-Howell correction for multiple comparisons.
Figure 3. The Ca$^{2+}$ transient decay time constant in synaptic boutons from control and Pb$^{2+}$-exposed animals. The $\tau_{\text{decay}}$ of the OGB-1 $\Delta F/F$ was measured after a single AP ($\Delta F/F_{\text{AP}} \tau_{\text{decay}}$) or at the end of an AP train ($\Delta F/F_{\text{train}} \tau_{\text{decay}}$). Top: The $\Delta F/F_{\text{AP}} \tau_{\text{decay}}$ for larvae raised in 100 µM Pb$^{2+}$, but not 250 µM Pb$^{2+}$, was significantly greater than that from control animals. Middle: For 10 Hz stimulation, the $\Delta F/F_{\text{train}} \tau_{\text{decay}}$ increased with increasing Pb$^{2+}$ concentration; the $\Delta F/F_{\text{train}} \tau_{\text{decay}}$ was significantly larger than control for 250 µM Pb$^{2+}$, but not for 100 µM Pb$^{2+}$. Bottom: The $\Delta F/F_{\text{train}} \tau_{\text{decay}}$ increased with increasing Pb$^{2+}$ concentration for 20 Hz stimulation; the $\Delta F/F_{\text{train}} \tau_{\text{decay}}$ was significantly greater than control for both 100 and 250 µM Pb$^{2+}$. For single APs, the ANOVA gave no significant linear effect of dose ($F=0.264; d_f=1; p>0.05$) but there was a significant quadratic effect of dose ($F=8.23; d_f=1; p<0.01$). For 10Hz there was a significant linear effect of dose ($F=11.12; d_f=1; p<0.002$) as there was for 20 Hz ($F=57.55; d_f=1; p<0.001$); there were no significant higher-order effects for these two groups. Significantly greater than control: *$p<0.05$, **$p<0.01$, ***$p<0.001$; Post-hoc analysis with Bonferroni correction for multiple comparisons.

Figure 4. Ca$^{2+}$ transient decay after 20 Hz AP train normalized to the final response at the end of the AP train. The mean normalized $\Delta F/F_{\text{train}} \tau_{\text{decay}}$ was compared for animals exposed to 0 µM Pb$^{2+}$ (68, 10), 100 µM Pb$^{2+}$ (53, 7) and 250 µM Pb$^{2+}$ (74, 7). The $\Delta F/F_{\text{train}} \tau_{\text{decay}}$ decays more slowly in Pb$^{2+}$-exposed synaptic boutons in a dose-dependent manner.
It was possible that the Pb\textsuperscript{2+}-exposed terminals still contained free Pb\textsuperscript{2+} that bound to OGB-1 and compromised the \([\text{Ca}^{2+}]_i\) measurements. To test for this, 100 \(\mu\text{M}\) TPEN, a membrane-permeant, heavy-metal chelator, was added; TPEN has a much higher affinity for Pb\textsuperscript{2+} than Ca\textsuperscript{2+} and reduces intracellular [Pb\textsuperscript{2+}]\textsubscript{i}, but not resting [Ca\textsuperscript{2+}]\textsubscript{i} (Arslan et al., 1985). In 8 larvae raised in 250 \(\mu\text{M}\) Pb\textsuperscript{2+}, resting OGB-1 fluorescence and \(\Delta F/F\) were measured in the same boutons before and after applying TPEN. It was found that resting fluorescence (25.1 ± 1.4 AU) and \(\Delta F/F_{\text{AP}}\) (8.2 ± 0.5 %) did not significantly change after TPEN addition (26.4 ± 1.5 AU and 8.2 ± 0.6 %, respectively; \(p> 0.10\)). Note that the \(\Delta F/F_{\text{AP}}\) amplitude was small because we included terminals containing high concentrations of OGB-1. This confirms that OGB-1 can be used to accurately measure \([\text{Ca}^{2+}]_i\) changes in Pb\textsuperscript{2+}-exposed terminals.

\textit{Comparison of resting \([\text{Ca}^{2+}]_i\)}

Pb\textsuperscript{2+} exposure has been shown to produce both an increase and decrease in resting \([\text{Ca}^{2+}]_i\), in neurons (Ferguson et al., 2000; Sandhir and Gill, 1994). To compare resting \([\text{Ca}^{2+}]_i\) in synaptic terminals from control and Pb\textsuperscript{2+}-exposed larvae, ratiometric Ca\textsuperscript{2+} measurements were performed using fura-2. The fura-2 ratio for experimental and control terminals was also compared in the presence of TPEN in case there was any free Pb\textsuperscript{2+} remaining in the Pb\textsuperscript{2+}-exposed terminals; fura-2 binds Pb\textsuperscript{2+} with a high affinity and this can produce an increase in the fura-2 ratio (Tomsig and Suszkiw, 1990). The change in indo-1 or fura-2 fluorescence produced by an increase in \([\text{Pb}^{2+}]_i\), was shown to be reversed by the addition of TPEN (Kerper and Hinkle, 1997; Mazzolini et al., 2001).
The resting fura-2 ratio was not significantly different for larvae raised in 0 Pb\textsuperscript{2+} (0.35 ± 0.01) and those raised in 250 µM Pb\textsuperscript{2+} (0.33 ± 0.02; \(p>0.10\)) before adding TPEN (Fig. 5). After adding TPEN, the fura-2 ratio in Pb\textsuperscript{2+}-reared animals was not significantly different either for 0 Pb\textsuperscript{2+} (0.36 ± 0.01) and 250µM Pb\textsuperscript{2+} terminals (0.32 ± 0.02; \(p>0.10\)). Also, the fura-2 ratio in the Pb\textsuperscript{2+}-exposed terminals was not significantly reduced after adding TPEN (\(p>0.10\)). The data were from four control and four Pb\textsuperscript{2+}-exposed animals and an ANOVA was used for statistical comparisons.

Figure 5. Comparison of the resting fura-2 ratio (360/380) in control and Pb\textsuperscript{2+}-exposed Ib terminals on muscle fiber 6/7 before and after TPEN application. To determine whether Pb\textsuperscript{2+} exposure affected resting [Ca\textsuperscript{2+}], the fura-2 ratio was compared in terminals of larvae reared in nominal 0 Pb\textsuperscript{2+} and those reared in 250 µM Pb\textsuperscript{2+}. In addition, the fura-2 ratios were compared in the presence of the heavy-metal chelator TPEN. We found no significant difference in the fura-2 ratios for 0 Pb\textsuperscript{2+} and 250 µM Pb\textsuperscript{2+} terminals before adding TPEN (\(p > 0.10\)). After adding TPEN, there was also no significant difference in the fura-2 ratios (\(p > 0.10\)). Also, the fura-2 ratio in the Pb\textsuperscript{2+}-exposed terminals was not significantly reduced after adding TPEN (\(p > 0.10\)). The data are from 4 control and 4 Pb\textsuperscript{2+}-exposed animals and an ANOVA was used for statistical comparisons.
Synaptic facilitation

Synaptic facilitation is dependent on the “residual” Ca\(^{2+}\) that equilibrates in the terminal after entering through voltage-dependent Ca\(^{2+}\) channels (Zucker and Regehr 2002). Since we have found that an AP train results in a greater buildup of residual Ca\(^{2+}\) in Pb\(^{2+}\)-exposed terminals, these terminals might show greater synaptic facilitation compared to control terminals. To test this, EPSP amplitude during low-frequency stimulation and during trains of APs delivered at higher frequencies was measured. By performing these experiments on muscle fiber 5, which is innervated by a single axon, facilitation at a single synapse could be measured (Lnenicka et al., 2006b).

Synaptic physiology was compared in control larvae and those raised in 250 μM Pb\(^{2+}\) since this concentration produced a greater effect on [Ca\(^{2+}\)], regulation than 100 μM Pb\(^{2+}\).

To determine the EPSP amplitude for single APs, the axon was stimulated at 0.5 Hz, a stimulation frequency that did not produce facilitation. To compare synaptic facilitation, the axon was stimulated at 20 Hz; it was previously shown that Pb\(^{2+}\)-exposed and control terminals showed clear differences in the buildup of residual Ca\(^{2+}\) at this frequency. It was found that there was greater synaptic facilitation in Pb\(^{2+}\)-exposed animals during 20 Hz stimulation compared to control (Fig. 6). It was found that the amplitude of the EPSP produced by single APs was not significantly different in 0 Pb\(^{2+}\) and 250 μM Pb\(^{2+}\) larvae (Fig. 7). However, the EPSP amplitude during 20 Hz stimulation was significantly greater for Pb\(^{2+}\)-exposed larvae compared to control.
Figure 6. EPSPs recorded from muscle fibers in control and Pb²⁺-exposed larvae during 20 Hz stimulation. Top: Typical EPSPs recorded from muscle fiber 5 during 20 Hz stimulation. The EPSPs were larger in larvae raised in media containing 250 µM Pb²⁺ compared to EPSPs from larvae in 0 Pb²⁺. Bottom: Mean EPSP amplitudes recorded from muscle fiber 5 during 20 Hz stimulation. The data are from 24 muscle fibers in 7 larvae raised in control media (0 Pb²⁺) and 29 muscle fibers in 8 larvae in media containing 250 µM Pb²⁺. The initial response (0 sec) was the average of 20 EPSPs produced by 0.5 Hz stimulation. The subsequent points are the average of 10 successive EPSPs during 20 Hz stimulation. The final 2 points are individual EPSPs produce by 0.5 Hz stimulation. Twenty Hz stimulation resulted in greater facilitation of EPSP amplitude in Pb²⁺-exposed larvae compared to controls.

The differences in the EPSP amplitude seen during 20 Hz stimulation appear to be mainly due to differences in the buildup of synaptic facilitation. During a train of APs, the enhancement of EPSP amplitude can result from the buildup of facilitation plus the onset of the longer-lasting augmentation and post-tetanic potentiation (PTP). Synaptic facilitation is the shortest component, decaying with a time constant of tens to hundreds of milliseconds (Mallart and Martin, 1967; Zengel et al., 1980). Augmentation decays with a time constant of 5-10 sec. (Magleby and Zengel, 1976b; Zengel et al., 1980) and PTP has a decay time constant of 30 sec to several minutes (Magleby, 1973a; Magleby, 1973b; Magleby and Zengel, 1975b; Magleby and Zengel, 1975a; Rosenthal, 1969;
Zengel et al., 1980). The differences in EPSP amplitude for Pb$^{2+}$-exposed and control larvae were nearly gone 2 seconds after the end of the 20 Hz train (Fig. 6). At this time, facilitation would have decayed and the remaining synaptic enhancement likely reflects augmentation. Thus, the differences are seen only during the synaptic facilitation phase. During the 20 Hz train, synaptic facilitation was significantly greater in Pb$^{2+}$-exposed larvae than in control larvae (Fig. 7).

**Figure 7. Mean EPSP amplitudes and synaptic facilitation in control larvae and those exposed to 250 μM Pb$^{2+}$.** Left: The EPSP amplitude produced by single APs (0.5 Hz) was not significantly different in 0 Pb$^{2+}$ and 250 μM Pb$^{2+}$ larvae. During 20 Hz stimulation, the EPSP amplitude was significantly larger for Pb$^{2+}$-exposed larvae compared to control larvae. The mean value for 20 Hz was determined from a train of 200 stimuli and the 0.5 Hz value was the mean of 20 EPSPs. Right: Synaptic facilitation was calculated for each experiment and the Pb$^{2+}$-exposed larvae showed significantly greater facilitation than control larvae. Facilitation (%) = (20 Hz EPSP amplitude/ 0.5 Hz EPSP amplitude) x 100. The mean values were obtained from 29 muscle fibers in 8 Pb$^{2+}$-exposed larvae and 24 muscle fibers in 7 control larvae. * p < 0.05, ** p < 0.01; t-test with Welch correction.
**Discussion**

*Pb\(^{2+}\) exposure influences the \([Ca^{2+}]_i\) increase produced by APs*

In this study it was found that the Ca\(^{2+}\) transient produced by single APs was not influenced by chronic Pb\(^{2+}\) exposure; however, Pb\(^{2+}\) exposure resulted in a larger Ca\(^{2+}\) transient during AP trains. The \(\Delta F/F_{AP}\) amplitude was similar in control synaptic boutons and those exposed to 100 or 250 µM Pb\(^{2+}\). Since the amplitude of the single-AP Ca\(^{2+}\) transient is determined by the amount of Ca\(^{2+}\) influx and the concentration of fast Ca\(^{2+}\) buffers, it appears that Ca\(^{2+}\) influx was similar in control and Pb\(^{2+}\)-exposed terminals. Alternatively, there could have been Pb\(^{2+}\)-related changes in Ca\(^{2+}\) influx that were counterbalanced by changes in fast Ca\(^{2+}\) buffering. This seems unlikely since changes in Ca\(^{2+}\) buffering would result in a change in the \(\Delta F/F_{AP} \tau_{\text{decay}}\) (Neher, 1995) and a consistent effect of Pb\(^{2+}\) exposure on the \(\Delta F/F_{AP} \tau_{\text{decay}}\) was not seen in this study. The inhibition of Ca\(^{2+}\) influx by acute Pb\(^{2+}\) exposure (nanomolar to micromolar) has been demonstrated for a variety of voltage-dependent Ca\(^{2+}\) channels in both invertebrate (Audesirk and Audesirk, 1989; Busselberg et al., 1990) and mammalian voltage-dependent Ca\(^{2+}\) channels (Audesirk and Audesirk, 1991; Audesirk and Audesirk, 1993; Busselberg et al., 1993; Evans et al., 1991; Peng et al., 2002). In most cases, the inhibition of Ca\(^{2+}\) influx by Pb\(^{2+}\) was reversible (Audesirk, 1993) and it was assumed in the study that any inhibitory effects of Pb\(^{2+}\) were washed out before measurements were performed. Thus, the measurements do not rule out the possibility that Pb\(^{2+}\) in the hemolymph inhibited Ca\(^{2+}\) influx *in vivo*.

For AP trains, chronic Pb\(^{2+}\) exposure resulted in a linear dose-dependent increase in the \(\Delta F/F_{\text{train}}\) amplitude and \(\tau_{\text{decay}}\). The study assumes that both of these effects resulted
from a decrease in the rate of Ca\(^{2+}\) extrusion by the PMCA. During an AP train, the buildup of \([\text{Ca}^{2+}]\) results in greater Ca\(^{2+}\) extrusion until Ca\(^{2+}\) influx and efflux per unit time are equal and the plateau is reached (Tank et al., 1995); a reduction in the Ca\(^{2+}\) extrusion rate constant will result in a greater $\Delta F/F_{\text{train}}$ amplitude. At the end of the train, the \([\text{Ca}^{2+}]\), decay reflects the rate of Ca\(^{2+}\) extrusion and a lower extrusion rate will result in a greater $\Delta F/F_{\text{train}}$ $\tau_{\text{decay}}$. The PMCA is responsible for Ca\(^{2+}\) extrusion from these larval synaptic terminals; inhibition of the PMCA resulted in an increase in $\Delta F/F_{\text{train}}$ amplitude and the $\Delta F/F_{\text{train}}$ $\tau_{\text{decay}}$ and immunostaining showed localization of the PMCA at the NMJ (Lnenicka et al., 2006a). It appears unlikely that Pb\(^{2+}\) also acts on other Ca\(^{2+}\)-clearance mechanisms to produce the increase in $\Delta F/F_{\text{train}}$ amplitude since blocking Ca\(^{2+}\) extrusion by the Na\(^+\)/Ca\(^{2+}\) exchanger or Ca\(^{2+}\) uptake by mitochondria or ER did not result in an increase in $\Delta F/F_{\text{train}}$ amplitude in the previous study (Lnenicka et al., 2006a).

There was no clear and consistent effect of Pb\(^{2+}\) exposure on the $\Delta F/F_{\text{AP}}$ $\tau_{\text{decay}}$ (the $\Delta F/F_{\text{AP}}$ $\tau_{\text{decay}}$ increased significantly for the 100 $\mu$M Pb\(^{2+}\) group and then dropped for the 250 $\mu$M Pb\(^{2+}\) group). This may be because the PMCA plays a greater role in determining the $\Delta F/F_{\text{train}}$ $\tau_{\text{decay}}$ than the $\Delta F/F_{\text{AP}}$ $\tau_{\text{decay}}$. This is supported by the finding that blocking the PMCA had a greater effect on $\Delta F/F_{\text{train}}$ $\tau_{\text{decay}}$ than on $\Delta F/F_{\text{AP}}$ $\tau_{\text{decay}}$ (Lnenicka et al., 2006a). Factors other than the PMCA may play a role in the $\Delta F/F_{\text{AP}}$ $\tau_{\text{decay}}$, e.g., studies of crayfish motor terminals indicate that slow Ca\(^{2+}\) buffers play a role in the $\Delta F/F_{\text{AP}}$ $\tau_{\text{decay}}$ (Lin et al., 2005).

Pb\(^{2+}\) exposure has been shown to influence PMCA activity in other systems. Acute exposure to micromolar concentrations of Pb\(^{2+}\) reduced the activity of the PMCA in humans and rats (Bettaiya et al., 1996; Mas-Oliva, 1989; Sandhir and Gill, 1994). The
in vitro studies indicate that Pb\textsuperscript{2+} inhibits PMCA activity by binding directly to the PMCA (Mas-Oliva, 1989; Sandhir and Gill, 1994). Studies examining the effect of in vivo chronic Pb\textsuperscript{2+} application have shown a persistent inhibition of PMCA activity; i.e., PMCA activity was inhibited when measured in nominal Pb\textsuperscript{2+}-free solutions. For example, the PMCA activity in erythrocytes from mothers and newborns was negatively correlated with hair Pb\textsuperscript{2+} levels (Campagna et al., 2000). Rats were exposed to Pb\textsuperscript{2+} for 8 weeks and PMCA activity per total protein was reduced by 31\% in synaptic plasma membranes isolated from the brain (Sandhir and Gill, 1994). It was also found in the current study that a persistent effect of Pb\textsuperscript{2+} on PMCA activity and experiments using TPEN showed no evidence for free Pb\textsuperscript{2+} remaining in the synaptic terminals. The persistent effects of chronic Pb\textsuperscript{2+} exposure could be due to irreversible binding of Pb\textsuperscript{2+} to the PMCA. Alternatively, Pb\textsuperscript{2+} exposure could have reduced the expression of PMCA. The level of [Ca\textsuperscript{2+}]	extsubscript{i} is one factor regulating PMCA expression (Guerini et al., 1999) and it may be that Pb\textsuperscript{2+} exposure decreases PMCA expression by reducing Ca\textsuperscript{2+} influx through Ca\textsuperscript{2+} channels.

In mammals, there are 4 PMCA genes and over 20 possible splice variants (Strehler and Treiman, 2004; Strehler and Zacharias, 2001) expressed in a tissue- and development-specific manner; all cell types express at least one of the isoforms (Strehler and Treiman, 2004). Nervous tissue appears to show the greatest abundance and variety of PMCA expression: many of the isoforms have been demonstrated in the brain (Strehler and Treiman, 2004). Drosophila has fewer PMCA genes and isoforms than found in mammals. Based upon the annotation of the genome, and BLASTp analysis of a mouse PMCA (PMCA-4) against the Drosophila annotated protein sequences, Drosophila has
only a single highly homologous PMCA gene (CG42314) and 6 potential isoforms (E value = 0).

No effect of chronic Pb\textsuperscript{2+} exposure on resting [Ca\textsuperscript{2+}]\textsubscript{i} was found in the study and this appears unexpected since the PMCA plays an important role in maintaining resting [Ca\textsuperscript{2+}]\textsubscript{i}. However, previous in vitro studies have reported an increase in resting [Ca\textsuperscript{2+}]\textsubscript{i} during exposure to 5 µM Pb\textsuperscript{2+} (Schanne et al., 1989) and a decrease in resting [Ca\textsuperscript{2+}]\textsubscript{i} during exposure to 0.1 µM Pb\textsuperscript{2+} (Ferguson et al., 2000). This apparently occurs because Pb\textsuperscript{2+} can stimulate the PMCA activity as well as inhibit it. Normally, an increase in [Ca\textsuperscript{2+}]\textsubscript{i} activates the PMCA through the formation of Ca-calmodulin, which binds to the C-terminal tail of the PMCA releasing it from autoinhibition (Strehler and Treiman 2004). Pb\textsuperscript{2+} can substitute for Ca\textsuperscript{2+} in calmodulin activation (Goldstein and Ar, 1983; Habermann et al., 1983; Kern et al., 2000) and this can result in an increase in PMCA activity (Ferguson et al., 2000). Synaptosomes isolated from rats exposed to Pb\textsuperscript{2+} in vivo (50 mg/kg body weight) showed an increase in resting [Ca\textsuperscript{2+}]\textsubscript{i} due to reduced PMCA activity even though there was also an increase calmodulin activity (Sandhir and Gill, 1994). The balance of PMCA inhibition and activation likely determines the effect of Pb\textsuperscript{2+} on resting [Ca\textsuperscript{2+}]\textsubscript{i}; this likely depends on the Pb\textsuperscript{2+} concentration since activation of calmodulin occurs at a lower concentration than Pb\textsuperscript{2+} inhibition (Ferguson et al., 2000).

In this study, there may have been an equal balance of PMCA inhibition and excitation for resting [Ca\textsuperscript{2+}]\textsubscript{i}; however, the effects of PMCA inhibition may have predominated at higher [Ca\textsuperscript{2+}]\textsubscript{i}. For example assuming Michaelis-Menton kinetics and fewer functional PMCA (lower V\textsubscript{max}) in Pb\textsuperscript{2+}-exposed larvae, the effects of PMCA inhibition on the steady-state [Ca\textsuperscript{2+}]\textsubscript{i} would be accentuated at a higher [Ca\textsuperscript{2+}]\textsubscript{i}. As Ca\textsuperscript{2+} influx increased,
the steady-state $[\text{Ca}^{2+}]$ concentration would increase more rapidly in Pb$^{2+}$-exposed animals since the PMCA would approach saturation at a lower $[\text{Ca}^{2+}]$.

**Synaptic facilitation**

The current study tested whether the change in $[\text{Ca}^{2+}]$ regulation resulting from Pb$^{2+}$ exposure produced changes in synaptic plasticity. It was found that during a train of impulses there was a greater increase in EPSP amplitude for Pb$^{2+}$-exposed terminals than for control terminals. This activity-dependent synaptic enhancement is the most common form of synaptic plasticity and is found at most, if not all synapses (Zucker and Regehr, 2002). It results from an increase in transmitter release and can be divided into synaptic facilitation, augmentation and PTP based upon differences in time course. It appeared that differences in synaptic enhancement seen for control and Pb$^{2+}$-exposed terminals were due to differences in the buildup of synaptic facilitation.

The greater synaptic facilitation seen at the Pb$^{2+}$-exposed terminals is likely due to the greater increase in $[\text{Ca}^{2+}]$ seen at these terminals. Although the mechanisms underlying synaptic enhancement are not understood, studies of synapses in vertebrates and invertebrates have shown that facilitation, augmentation and PTP appear to be dependent upon residual Ca$^{2+}$ (Zucker and Regehr, 2002). For example, adding the Ca$^{2+}$ chelator ethylene glycol tetraacetic acid (EGTA) alters the buildup and decay of Ca$^{2+}$ and synaptic enhancement during a train at mammalian central synapses and the lobster NMJ (Ogawa et al., 2000; Regehr et al., 1994).

Previous studies examining the effects of *in vivo* Pb$^{2+}$ exposure on synaptic plasticity have mainly focused on long-term potentiation (LTP). Postnatal Pb$^{2+}$ exposure
increased the threshold for producing LTP, decreased its amplitude and reduced the
duration of LTP in the rat hippocampus, possibly due to the effect of Pb^{2+} on the NMDA
receptor (Gilbert et al., 1996; Gilbert and Mack, 1998; Lasley et al., 1993; Ruan et al.,
1998). However, the effect of Pb^{2+} exposure on short-term plasticity may differ since
exposure to levels of Pb^{2+} that impaired LTP resulted in an increase in the amplitude of
short-term potentiation (STP) in the rat hippocampus (Grover and Frye, 1996). Since STP
may involve presynaptic mechanisms (Lauri et al., 2007), the increase in STP could
involve an increase in residual Ca^{2+} as seen at the larval NMJs.

The changes that were observed in [Ca^{2+}], regulation and synaptic facilitation at
the neuromuscular synapses are likely to occur at synapses in the central nervous system
since the PMCA plays an important role in Ca^{2+} extrusion from synaptic terminals in the
central nervous (Juhaszova et al., 2000; Morgans et al., 1998) and synaptic facilitation at
central synapses is dependent on residual Ca^{2+} (Atluri and Regehr, 1996). In fact, a
reduced PMCA activity has been shown to enhance synaptic facilitation at central
synapses; these studies involved blocking PMCA activity pharmacologically or knocking
out expression of one of the PMCA isoforms (Empson et al., 2007; Jensen et al., 2007).
Conclusions

The *Drosophila* larval neuromuscular junction (NMJ) is an ideal model system for studying the cellular and molecular mechanisms of a broad spectrum of neural activities, such as neurotransmission, synaptogenesis, neuronal development, differentiation, migration, and survival, due to its easy identifiability and great accessibility to various techniques such as electrophysiology, patch clamping and back-fill Ca\(^{2+}\) imaging. Moreover, the abundance of loss-of-function and gain-of-function mutants of *Drosophila* also makes it possible to study these activities on a scale of one single molecule (Keshishian et al., 1996). It is well known that proper Ca\(^{2+}\) homeostasis plays essential roles in these activities. It is therefore important to understand Ca\(^{2+}\) regulation in these synapses in order to decode these events. The studies detailed in this dissertation investigated the different aspects of Ca\(^{2+}\) regulation in this model system.

Studies presented in the dissertation utilized a single-wavelength fluorescent Ca\(^{2+}\) indicator, Oregon Green BAPTA 488-1 (OGB-1), for Ca\(^{2+}\) imaging. Conventionally, two-wavelength ratiometric dyes such as fura-2 were used extensively in earlier studies (Tsien, 1989a; Tsien, 1989b). Ratiometric measurements can cancel out differences in optical path length, detector efficiency and biologic auto-fluorescence (Tsien, 1989b). However, these ratiometric dyes require slow experimental set-ups for switching excitation wavelength; this limits their use in imaging fast Ca\(^{2+}\) signals such as those produced by single APs. In addition, ratiometric dyes usually have short wavelength excitation, which will be absorbed by living tissues, producing light scattering and photodamage to the tissues (Koester et al., 1999). The use of single-wavelength Ca\(^{2+}\) dyes can avoid these problems, and expressing fluorescence changes for the single-wavelength dye as \(\Delta F/F\)
yields a measure that, as for ratiometric dyes, is also independent of dye concentrations, optical path length and detector efficiency (Maravall et al., 2000). In addition, the greatest advantage of the single-wavelength dye, as seen in the current studies, is its ability to achieve large signal-to-noise ratios, which are required for Ca\textsuperscript{2+} imaging in small structures such as synaptic boutons. However, note that the limitation of OGB-1 used in these studies is the distortion of the Ca\textsuperscript{2+} signals due to its high Ca\textsuperscript{2+} affinity, producing a slight overestimation of the Ca\textsuperscript{2+} signals (Neher, 2008).

Using this model system and Ca\textsuperscript{2+} imaging technique, I started my studies by examining whether there were terminal-specific differences in Ca\textsuperscript{2+} regulation between Is and Ib terminals (Chapter I). This question arose from previous research which showed differences in synaptic physiology between these two classes of motor terminals: for single APs, Is terminals produced greater EPSPs and appeared to release more transmitter per synapse than did Ib terminals. Is terminals also showed depression upon stimulation at moderate frequencies, in contrast to the synaptic facilitation that was seen in Ib terminals (Atwood et al., 1993; Atwood et al., 1997; Kurdyak et al., 1994; Lnenicka and Keshishian, 2000). The common perception is that these findings can be explained by a greater Ca\textsuperscript{2+} influx per active zone in Is terminals. The study in Chapter I was performed to test this idea. It was found that the peak of Ca\textsuperscript{2+} transients for single APs was greater in Is terminals than in Ib terminals. This was still the case after comparing terminal populations with similar OGB-1 concentrations, which obviously had effects on the Ca\textsuperscript{2+} signals. These results indicate the existence of a greater Ca\textsuperscript{2+} influx per bouton volume in Is terminals. This conclusion was further supported by the finding that in Is boutons, the number of active zones per bouton volume was greater than in Ib boutons, arguing
against the idea that there was greater Ca\(^{2+}\) influx per active zone in Is terminals. Differences in endogenous fast Ca\(^{2+}\) buffering between Is and Ib terminals does not appear likely either, since there was no difference in the decay of Ca\(^{2+}\) transients for single APs. Additionally, after 10Hz AP trains, the decay of Ca\(^{2+}\) transients in Is terminals was found to be slower than that in Ib terminals. This appeared to be due to the greater Ca\(^{2+}\) extrusion by the PMCA in Ib boutons, which also contributed to the finding that AP trains produced higher plateau of Ca\(^{2+}\) transients in Is terminals than in Ib terminals.

Another aspect of Ca\(^{2+}\) regulation in *Drosophila* larval motor terminals, the Ca\(^{2+}\)-binding ratio (\(\kappa_S\)) of the endogenous Ca\(^{2+}\) buffering system, was investigated in the second study. A quantitative analysis of Ca\(^{2+}\) dynamics had not been done at *Drosophila* motor terminals or any invertebrate synaptic terminals because of the lack of knowledge of the nature of the endogenous Ca\(^{2+}\) buffering at these terminals. In this study, the \(\kappa_S\), which is defined as the amount of Ca\(^{2+}\) bound/ amount of Ca\(^{2+}\) remaining free after it enters the terminals (Sabatini et al., 2002), was calculated at a Ib motor terminal in *Drosophila* larvae based on the effect of different concentrations of the Ca\(^{2+}\) indicator, OGB-1, on the decay time constant of Ca\(^{2+}\) transients produced by single APs. These measurements yielded a value of 77, which indicated that nearly 99% of entering Ca\(^{2+}\) was bound immediately by the endogenous fast Ca\(^{2+}\) buffers. With this knowledge, a quantitative analysis of the Ca\(^{2+}\) dynamics could then be performed at Ib terminals: with 1.0 mM external Ca\(^{2+}\), one single AP produced an increase in total intracellular Ca\(^{2+}\) of 15.3 \(\mu\)M, of which 196 nM remained free. In the absence of exogenous Ca\(^{2+}\) buffer (OGB-1), the Ca\(^{2+}\) transients produced by single APs decayed with a time constant of 46
msec and a Ca^{2+}-removal rate constant of 1641 s^{-1}. For AP trains, the increase in intracellular free Ca^{2+} was 185 nM/10 Hz with a Ca^{2+} extrusion rate constant of 827 s^{-1}, which is likely due to the activity of PMCA. Thus far, the combination of study 1 and 2 represents the descriptive analyses of the Ca^{2+} regulation at *Drosophila* larval motor terminals, providing the baseline data for future studies.

Ca^{2+} buffering proteins (CBPs) are likely to play important roles in regulating transmitter releasing properties by modifying Ca^{2+} signals in different cell types or different stages of development. One of the physiologic CBPs, *parvalbumin* (*PV*), has attracted much attention due to its unique Ca^{2+}-handling abilities (Lee et al., 2000c; Collin et al., 2005) and the selective expression mostly in a subset of GABA-ergic neurons (Baimbridge et al., 1992). Although its physiological role is largely unknown, *PV* has been shown to affect synaptic facilitation (Caillard et al., 2000; Muller et al., 2007) and alter Ca^{2+} regulation in mammalian CNS synapses (Collin et al., 2005; Muller et al., 2007). However, its effect on different components and forms of short-term synaptic enhancement has not been studied in detail. Neither has the direct effect of *PV* expression on synaptic development been studied. Thus in chapter III, I went on to explore these issues by ectopically expressing *PV* in our model system, which normally does not express *PV* in the motor terminals. It was found that *PV* expression in terminals dramatically reduced the early component of paired-pulse facilitation and slightly decreased the late component. The facilitation during AP trains was also found to be significantly decreased by *PV* expression. Meanwhile, an increased single-AP transmitter release was seen in the *PV*-expressing terminals, presumably as a homeostatic change to partially compensate for the reduced facilitation. The reduced facilitation indicates a
decreased impulse activity in \( PV \)-expressing terminals. This may be related to the hypomorphic development of the motor terminals seen in these terminals, presumably through altering \( Ca^{2+} \) regulation during development. This conclusion was supported by the findings from \( Ca^{2+} \) imaging, which showed depressed \( Ca^{2+} \) signals for single APs and at the beginning of AP trains. This study demonstrated the differences in transmitter release and \( Ca^{2+} \) regulation between the \( PV \)-positive and –negative terminals. These differences resemble those seen between the high-output Is terminals and low-output Ib terminals presented in chapter I, indicating that endogenous buffers such as \( PV \) might play a role in switching the types of synapses under physiological conditions.

In the last study, I tried to address the following unanswered questions: what was the effect of chronic lead (\( Pb^{2+} \)) exposure during development on the presynaptic \( Ca^{2+} \) regulation? If there were changes, were they correlated with changes in synaptic facilitation? The study showed no changes in \( Ca^{2+} \) signals produced by single APs, whereas there was a linear dose-dependent increase in the plateau and the decay time constants of \( Ca^{2+} \) transients produced by AP trains in the \( Pb^{2+} \)-exposed terminals. The latter finding indicates that the PMCA, the \( Ca^{2+} \) pump that plays a major role in extruding \( Ca^{2+} \) at these motor terminals, was inhibited by chronic \( Pb^{2+} \) exposure. A \( Pb^{2+} \)-dependent enhancement of synaptic facilitation was also seen in the \( Pb^{2+} \)-exposed terminals, presumably due to the inhibition of the PMCA that leads to excessive \( Ca^{2+} \) accumulation during AP trains. It was thus concluded that at the mature synapse, the altered \( Ca^{2+} \) regulation due to chronic \( Pb^{2+} \) exposure would produce changes in synaptic facilitation that are likely to influence the function of neural networks.
This series of studies, together with previous publications in our lab (Lnenicka and Keshishian, 2000; Lnenicka et al., 2003; Lnenicka et al., 2006a), contribute to a better understanding of the relationship between Ca\(^{2+}\) regulation and transmitter release in the *Drosophila* larval NMJs. However, a few more experiments still need to be done in the future to address the following questions in order to make the study more informative:

1. What is the Ca\(^{2+}\)-binding ratio for Is terminals? With this knowledge, a quantitative study for these terminals would then be possible. In addition, this set of experiments would help explain the differences in Ca\(^{2+}\) regulation between Is and Ib terminals.

2. What is the amount of *PV* that is expressed in the transgenic larvae, as in chapter III? This knowledge would help explain the effects of *PV* expression on the synaptic enhancement and Ca\(^{2+}\) regulation seen in chapter III. Meanwhile, it would also be interesting to see if the important Ca\(^{2+}\) pump, the PMCA, participates in the homeostatic regulation of Ca\(^{2+}\) signals resulting from the early ectopic expression of *PV* by quantitatively determining its expression between the transgenic flies and the controls.

3. Lastly, *PV* expression could be selectively activated in a certain stage of the larval development, rather than at the very beginning as in chapter III. This would presumably avoid the homeostatic changes resulting from the early expression of *PV*, and thus allows for direct examination of the effects of *PV* expression on synaptic physiology and Ca\(^{2+}\) regulation without involving other complicating factors. The studies presented in this dissertation have provided mature methodology to address these questions, as well as baseline data for comparison.


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