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THE ROLE OF THE DROSOPHILA LEARNING AND MEMORY GENE
DUNCE (DNC)
IN SYNAPTIC TRANSMISSION

Alexander Joseph Shayan

A thesis submitted in conformity with the requirements
for the Degree of Master of Science
in the Graduate Department of Physiology
University of Toronto

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Department of Physiology, University of Toronto.

The *dunce* (dnc) gene of *Drosophila* encodes a cAMP-specific phosphodiesterase. *Drosophila dnc* null mutants, which exhibit elevated cAMP levels, have an enhanced number of synapse-bearing nerve ending varicosities at the larval neuromuscular junction (NMJ), coupled with augmented neurotransmission.

I investigated the effects of localized *dnc* overexpression on nerve terminal morphology and neurotransmission using transgenic lines of *Drosophila*. *dnc* overexpression in muscle cells alone, or in both muscle cells and motor neurons, results in a reduction of the number of varicosities and depresses whole-cell synaptic strength.

These effects are opposite to those in *dnc* null mutants and suggest that altered levels of *dnc* expression (and hence cAMP levels) influence the morphology of synaptic contacts and the strength of synaptic transmission. Consequently, an optimal level of cAMP is probably necessary for the normal formation of synaptic contacts and normal synaptic function.
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List of Abbreviations

CNS................................................................. central nervous system
EJC............................................................... excitatory junction current
EJP............................................................... excitatory junction potential
FITC............................................................ fluorescein-5-isothiocyanate
HRP............................................................. horseradish peroxidase
mEJC............................................................ miniature excitatory junction current
mEJP............................................................ miniature excitatory junction potential
NCAM......................................................... neuronal cell adhesion molecule
NMJ............................................................ neuromuscular junction

Nomenclature

Genes are *italicized* (e.g. *dunce*).

Proteins are capitalized in normal font (e.g. FasII).
Chapter One: Introduction

Using the genetically-tractable organism Drosophila melanogaster, this study examines properties of synaptic transmission in genetically-modified cells in the nervous system. I will first outline general features of neurotransmission and then discuss the advantages of utilizing Drosophila as a model to study the mechanisms that underlie synaptic transmission.

General features of neurotransmission

The nervous system is comprised of specialized cells called neurons and other associated supporting cells called glial cells. Neurons communicate with each other (in the central nervous system) and other types of target cells (such as muscle cells via the peripheral nervous system) by the release of chemical substances known as neurotransmitters. A neuron communicates with its target at specialized regions called synapses where neurotransmitter is released and received.

In general, neurons consist of several compartments, namely a cell body, dendritic regions, and axons. The dendrites are regions through which a neuron receives input from other cells at synaptic connections. The cell body contains the nucleus where gene transcription occurs. Products of gene transcription are transported to the cytoplasm where they are translated into protein products. Gene products synthesized in the cell cytoplasm can be transported to the axonal terminals by axonal transport. Axonal terminals (or nerve endings) have variable morphology but in general consist of the presynaptic cell processes, the sites at which a nerve forms synaptic contacts with its
target cell (the postsynaptic cell). The pre- and postsynaptic cells are separated by a synaptic cleft. Chemical neurotransmitter-containing synaptic vesicles are selectively localized to synaptic regions of the presynaptic cell. Upon nerve stimulation and the subsequent calcium entry into the presynaptic cell (through voltage-gated calcium channels), synaptic vesicles can fuse with the plasma membrane and release their transmitter content into the synaptic cleft. The process of vesicular release of transmitter is known as exocytosis. Once a synaptic vesicle has released its contents, its membrane is retrieved from the plasma membrane of the nerve terminal and recycled by an internalization process referred to as endocytosis. Released neurotransmitter diffuses across the cleft and binds to specific receptors on the postsynaptic target cell. Binding of a transmitter molecule to its appropriate receptor on the target cell can have differential physiological effects depending on the target cell. If the receptor is directly coupled to a specific ion channel, the binding of the transmitter to the receptor will allow the flow of specific ions into or out of the postsynaptic cell. Some receptors are coupled to G proteins and set off a second messenger cascade once they bind their transmitter. The effects of postsynaptic receptor activation on the target cell can be either excitatory or inhibitory in nature and will depend on the type of receptor itself and the specific ionotropic or metabotropic cellular machinery to which the receptor is coupled.

In recent years, there has been an enormous amount of effort invested in pharmacological, electrophysiological, and light and electron microscopical techniques in an attempt to understand the mechanisms that underlie synaptic transmission, using a variety of invertebrate and vertebrate animal models, each having its own particular
advantages and disadvantages. In this work, there has been a vast increase in the employment of molecular and neurogenetic approaches to genetically dissect the molecular mechanisms of synaptic transmission. Just in the process of exocytosis alone, a great number of proteins have been identified which closely interact with one another and synaptic vesicles in a highly complex chain of events which gives rise to exocytosis and release of transmitter (for reviews see Sudhof, 1995; Scheller, 1995).

Increasingly, neuroscientists have sought to identify molecules that give rise to the formation and modulation of synaptic contacts at the morphological and physiological levels. In the area of neurotransmission, the collective efforts and aspirations of many neuroscientists today have a common goal as their core: to understand the mechanisms by which synaptic transmission occurs. It is thought that by identifying and dissecting the mechanisms of action of key molecules involved in neuronal communication using model systems, one should be able to gain better insight into the way neurons interact with one another. Potentially, such knowledge will be relevant to the human nervous system, and could better enable us to deal with neurodegenerative diseases that afflict the human nervous system.

**Genetic advantages of Drosophila as a model**

A great deal of what is currently known about the function of pertinent molecules involved in synaptic transmission has been obtained by using *Drosophila* as a model system. This is because there are various advantages associated with the usage of
Drosophila as a model to carry out genetic dissections of the mechanisms underlying synaptic transmission.

The Drosophila genome is smaller than its vertebrate counterparts. The comparative ease of genetic manipulations of the Drosophila genome provides for the employment of genetic approaches to alter the molecules of interest, as a means of studying their functions.

There are numerous powerful mutagenesis techniques that can be employed to generate mutations in Drosophila. A traditional method of mutagenesis in Drosophila has involved rearing animals in an environment that contains chemical mutagens and looking for any interesting phenotypes that result (for review see Rubin, 1988). Another method of creating genetic mutations in Drosophila has involved the use of P-elements. This method has gained widespread application and will be discussed further, since genetically-modified Drosophila produced by this method are used in the present study.

Genetically-modified Drosophila

The Drosophila genome contains transposable elements, some of which have been studied in detail (Berg and Howe, 1989). In particular, the “P” family of transposable elements has been the subject of much research interest, since they can serve as invaluable tools for genomic manipulations in Drosophila (for review see Engels, 1989).

P-elements are endogenous sequences of DNA in Drosophila which have unique features. Part of their sequence encodes for an enzyme named transposase and sequences that are called inverted terminal repeats. Normally, P-elements can migrate since the
transposase provides a means for their mobility in the genome (Spradling and Rubin, 1982). The sequences of the P-element which encode for inverted terminal repeats allow for the insertion or extraction of the P-element within the genome. Removal of the transposase sequences of P-elements allowed creation of P-element insertions that are immobile (Rubin and Spradling, 1983). As such, P-element insertions into the genome can be used as a mutagenesis technique (Cooley et al., 1988). If the P-element is inserted into coding regions of a particular gene, that may alter the normal DNA sequence of that gene and hence result in its altered expression. However, once the insertion is complete, one can again mobilize the P-element by providing transposase (the mobilizing agent) by genetic crosses. Mobilizing an inserted P-element in this way can result in imprecise excisions of that P-element. Thus, DNA sequences flanking the P-elements may be deleted as part of the imprecise P-element excision. The DNA sequences flanking a P-element may be important coding regions of genes, hence their removal can lead to altered gene expression.

Perhaps one of the most important uses of P-elements is in generating transgenic lines of *Drosophila* (Spradling and Rubin, 1982; Rubin and Spradling, 1982). This involves placing a gene of interest into a P-element. The P-element can contain other genes that encode for identifiable markers such as eye-color genes, as a means of tracking the P-element. The P-element is injected into embryos in the presence of transposase. The transposase allows the P-element to transfer to a random genomic site. This brief overview of aspects involved in creating transgenic flies using the P-element insertion method is more fully detailed in Ashburner (1989) and Spradling (1986).
More recently, a powerful genetic approach called the GAL4-UAS (Upstream Activating Sequence) expression system has been designed that makes use of P-element insertions in conjunction with regulation by a foreign transcription factor (GAL4). The GAL4-UAS expression system allows for targeted expression of a gene in a specific subset of cells. Since this technique is used in this thesis to drive the expression of the dunce (dnc) gene in a subset of specific cells, I will discuss it further (see Fig. 1. for an overview).

The GAL4-UAS expression system was developed in Drosophila by Brand and Perrimon (1993). It allows for targeted expression or overexpression of a gene of interest in a subset of desired cells. The technique involves generating P-element constructs that contain a gene of interest under the transcriptional control of the UAS. UAS is the binding site for the yeast transcriptional activator GAL4. In Drosophila, GAL4 has no endogenous function. Thus, the gene of interest (part of the P-element construct) can only be expressed if GAL4 is present. Note that lines of flies specific for particular GAL4 expression patterns also need to be generated and selected. These lines of flies can then be used in driving the expression of the gene of interest in a subset of specific cells.

In summary, to achieve overexpression of a particular gene in a subset of cells using the UAS-GAL4 expression system, one needs to engineer transgenic animals that contain the construct containing that gene under the control of a UAS. Further, animals with a specific GAL4 expression pattern are needed since it is that pattern of GAL4 expression that dictates the expression pattern of the gene of interest. By crossing transgenic animals containing the (UAS-gene of interest) construct to animals specific for
Figure 1. Schematic diagram depicting the UAS-GAL4 expression system in *Drosophila* developed by Brand and Perrimon (1993). Flies from fly stock (a) represent transgenically-engineered flies that are specific for a particular GAL4 expression pattern. Stock (b) represents transgenic animals containing the gene of interest subcloned behind the UAS (Upstream Activating Sequence) which is the GAL4 binding site. The gene of interest is expressed as the result of crossing flies from stock (a) to flies of stock (b). In the progeny, the expression pattern of the gene of interest will be dictated by the pattern of GAL4 expression in stock a). Diagram modified from Brand and Perrimon (1993).
Tissue-specific expression of GAL4

Transcriptional activation of the gene of interest
a particular GAL4 expression pattern, one can drive the expression of the desired gene in a pattern governed by the GAL4 expression pattern.

Thus, taking advantage of the powerful UAS-GAL4 expression system, one can ectopically overexpress a protein of interest in a subset of cells and study the effects of such perturbation on synaptic transmission. Sweeney and colleagues (1995) used the technique to drive the expression of tetanus toxin light chain (which cleaves the vesicular protein synaptobrevin involved in the process of exocytosis) in motor neurons in order to study the effects of this perturbation on synaptic transmission. Since then, more studies have taken advantage of the technique to study the role of a neuronal cell adhesion molecule (NCAM) fasciclin II (FasII; Schuster et al., 1996a,b), postsynaptic glutamate receptor DGlurIIA (Petersen et al., 1997), and postsynaptic PKA (Davis et al., 1998) on morphological and physiological modulation of synaptic contacts at the *Drosophila* neuromuscular junction (NMJ).

**Advantages of the *Drosophila* larval neuromuscular junction as a model to study mechanisms underlying synaptic transmission**

There are numerous advantages that render the *Drosophila* larval neuromuscular junction (NMJ) a good candidate model for studying mechanisms underlying synaptic transmission. The larval musculature and motor innervation is composed of segmentally-repeated identifiable muscle cells and motor neurons that are organized in a highly
stereotypic fashion. Each muscle cell is innervated by one to four readily-identified motor neurons (Johansen et al., 1989). Thus, specific identifiable muscle cells and motor neurons in the Drosophila larval preparation are highly-accessible using a variety of experimental manipulations. In particular, living nerve terminals can be visualized with Nomarski optics. The Drosophila larval NMJ, which can be manipulated by genetic methods, serves as an ideal model for investigation with electrophysiological methods in combination with light and electron microscopical techniques.

Some of the basic physiological properties of the Drosophila larval NMJ were first described by Jan and Jan (1976a) who discovered that it is predominantly glutamatergic (Jan and Jan, 1976b), a feature it shares with central excitatory synapses in the vertebrate CNS. Furthermore, larval muscle fibers were found to be innervated by different motor neurons that are morphologically and physiologically distinct (Johansen et al., 1989; Atwood et al., 1993; Kurdyak et al., 1994). Synaptic contacts at the Drosophila NMJ have been shown to exhibit dynamic morphological and physiological plasticity through the use of genetic manipulations that perturb various molecules that play important roles in normal synaptic function. Some of these molecules include protein kinases (Wang et al., 1994), neuronal cell adhesion molecules (Stewart et al., 1996; Zhong and Shanley, 1995), potassium channels (Budnik et al., 1990), and second messenger components (Zhong and Wu, 1991).

As noted earlier, the general goal of these studies has been to gain insight into the molecular mechanisms of synapse formation and transmission. Synaptic contacts are not rigid. They have been shown to be flexible structures that can modify their
and physiological plasticity of synaptic contacts also play a role in general processes underlying learning and memory. In support of this notion, there has been a growing body of evidence in the literature suggesting that mutations of genes that reduce the capacity for physiological plasticity result in altered learning and memory in vertebrates.

While vertebrate models have been useful in assessing links between physiological plasticity and learning and memory, it has been more difficult to study morphological dynamics of synaptic contacts within the context of synaptic plasticity in these models. This is due to the highly inaccessible nature of synaptic contacts in the vertebrate CNS combined with their huge number and small size; these features make it difficult to examine specific synaptic regions of known neurons using light and electron microscopical techniques. Ideally, using a genetic approach, one could study behaviour in combination with morphological and physiological assessment of synaptic contacts, all in the same model. All of the above-mentioned techniques can be applied in Drosophila (Davis, 1996).

**Early findings implicating the importance of cAMP in physiological synaptic plasticity**

CAMP has been demonstrated to be involved in various forms of synaptic enhancement, such as short-term facilitation (STF; Castellucci et al., 1980; Brunelli et al., 1976) and long-term facilitation (LTF; Schacher et al., 1988) in Aplysia central synapses. CAMP has also been shown to play a role in LTF at the crustacean NMJ (Dixon and Atwood, 1989). Further, CAMP has been shown to initiate long-term
structural and functional synaptic changes, processes that require new protein synthesis 
(Bailey et al., 1992; Schacher et al., 1988; Montorolo et al., 1986).

It has been shown that cAMP may initiate new transcription of proteins by the 
phosphorylation and hence activation of the cAMP response element binding protein 
(CREB) by protein kinase A (PKA; Hunter and Karin, 1992). Altered CREB activity has 
been shown to result in learning deficits in *Drosophila* and rodents (Yin et al., 1995; 
Bourtchuladze et al., 1994 respectively). Cumulatively, these studies suggest that cAMP 
and components of its signalling pathway play a prominent role in the formation and 
function of synaptic contacts.

**Recent studies focusing on components of the cAMP signalling system and their role 
in morphological and physiological synaptic plasticity at the *Drosophila* larval NMJ.**

Null mutations in the *Drosophila dunce* (*dnc*) gene serve as excellent models to 
study synaptic plasticity and learning since all of these parameters have been shown to be 
altered due to mutations in the single gene. The *dnc* gene in *Drosophila* encodes a form 
II phosphodiesterase and null mutations in this locus result in elevated levels of cAMP 
and have been shown to result in olfactory learning deficits in the adult fly (Dudai et al., 
1976). Mutations in the same gene have been shown to give rise to morphological and 
physiological plasticity at the larval NMJ (Zhong et al., 1992; Zhong and Wu, 1991; see 
Table 1. for summary). Recent evidence suggests that in addition to exhibiting enhanced 
whole-cell neurotransmission (Zhong and Wu, 1991), release of neurotransmitter from 
individual nerve ending varicosities displays more variability in
Table 1. A summary of findings from recent studies focusing on the role of components of the cAMP signalling system on synaptic morphology and physiology at the *Drosophila* larval neuromuscular junction.
Table 1: Components of the cAMP signalling system: previous findings at the *Drosophila* neuromuscular junction.

<table>
<thead>
<tr>
<th>molecule of interest</th>
<th>nature of altered expression</th>
<th>morphological alterations</th>
<th>physiological perturbations</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>dnc</em> (PDE)</td>
<td>null mutation</td>
<td>- more nerve ending varicosities</td>
<td></td>
</tr>
<tr>
<td>Zhong and Wu (1991); Renger et al., (1997; 1998)</td>
<td>- more docked vesicles</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- poor synaptic definition by EM</td>
<td>- whole cell EJCs larger</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- whole cell mEJCs unaltered</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- extracellular recording reveals prolonged decay of mEJCs and EJCs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- facilitation replaced by depression</td>
</tr>
<tr>
<td><em>dnc</em> (PDE)</td>
<td>overexpression in motor neurons</td>
<td>less nerve ending varicosities</td>
<td>- whole cell mEJPs larger</td>
</tr>
<tr>
<td>(Cheung et al., 1997)</td>
<td></td>
<td></td>
<td>- other effects currently not known.</td>
</tr>
<tr>
<td>CREB (Davis et al., 1996)</td>
<td>transgenically ↑ CREB activity</td>
<td>none</td>
<td>- whole cell mEJCs unaltered</td>
</tr>
<tr>
<td>PKA (Davis et al., 1998)</td>
<td>transgenically ↑ PKA activity in muscle</td>
<td>none</td>
<td>- whole cell mEJPs smaller, decay faster</td>
</tr>
<tr>
<td></td>
<td>transgenically ↓ PKA activity in muscle</td>
<td>none</td>
<td>- whole cell mEJCs smaller,</td>
</tr>
<tr>
<td></td>
<td>transgenically ↓ PKA activity in muscle in the <em>dnc</em> null background (that already exhibits elevated cAMP levels)</td>
<td>none</td>
<td>- size of evoked EJCs unchanged.</td>
</tr>
<tr>
<td></td>
<td>pharmacologically ↑ PKA activity in muscle</td>
<td>none</td>
<td>- quantal content ↑</td>
</tr>
<tr>
<td></td>
<td>- results in suppression of the increase in mEJC size seen due to ↓ PKA activity in muscle in normal background (as stated above).</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- whole cell mEJCs become smaller in dose-dependent manner.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- with repeated iontophoresis of glu, get more rundown of the response as the result of repeated puffs (compared to normal rundown due to repeated puffs in absence of analog).</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- effect of PKA activity on mEJC/mEJP size is dependent on DGluRIIA subunit (i.e. in DGluRIIA null mutants, don't see PKA's effects on quantal size.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- suggests ↑ PKA activity in muscle in Drosophila has a desensitizing effect on the DGluRIIA Rc subunit (in contrast to vertebrate findings of PKA action by Wang et al., 1993).</td>
<td></td>
<td></td>
</tr>
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</table>
$dnc$ null mutants (Renger et al., 1997, 1998). Further, ultrastructural analysis as viewed by electron microscopy, reveals that synaptic regions display poorly-defined presynaptic membranes in $dnc$ null mutants (Renger et al., 1998; see Table 1.). The findings are significant since for the first time, learning and memory deficits occur in a model that also exhibits altered morphology and physiological function of synaptic contacts, all as the result of mutation in a single gene.

Also at the $Drosophila$ larval NMJ, there has been increasing evidence implicating the importance of other components of the cAMP signalling system (such as CREB and PKA) as important modulators of synaptic physiology. Increased CREB activity and enhanced postsynaptic PKA activity have been shown to enhance presynaptic neurotransmitter release at the $Drosophila$ larval NMJ (Davis et al., 1996; Davis et al., 1998 respectively; see Table 1.).

Recent findings from the $Drosophila$ larval NMJ in conjunction with earlier discoveries in other animal models implicate the importance of the cAMP signalling system in neurotransmission. Cumulatively, these studies warrant the further study of this system in an attempt to decipher the molecular mechanisms by which cAMP exerts its actions on synaptic transmission.

**Project goals**

Since initial work on the effects of $dnc$ null mutations on synaptic morphology and physiology at the $Drosophila$ larval NMJ, additional efforts have been underway to genetically dissect the role of other components of the cAMP signalling system in
synaptic transmission using the *Drosophila* NMJ as a model (for summary see Table 1). In this thesis I will build on previous work to further assess the role of *dnc* in synaptic transmission at the *Drosophila* larval NMJ. There are currently attempts underway to study the effects of *dnc* overexpression in motor neurons on synaptic strength taking advantage of the UAS-GAL4 expression system (Cheung et al., 1997). This work employs GAL4 lines of flies specific for motor neuron expression, and transgenic animals containing the UAS-*dnc* construct. Using the latter transgenic animals and flies that are specific for GAL4 expression in muscle alone, or in muscle and motor neurons in combination, I have investigated the effects of *dnc* overexpression (in the patterns specified above) on synaptic transmission.

In my thesis I address the following topics:

1) Previous studies have shown that null mutations in *dnc* result in an enhanced number of nerve terminal varicosities at the larval NMJ (Zhong et al., 1992; Davis et al., 1996). Thus, altered levels of cAMP affect nerve terminal morphology. I have investigated whether *dnc* overexpression in muscle alone, or in muscle and motor neurons in combination, affects the morphology of the larval NMJ as might be expected from previous findings in the *dnc* null mutants.

2) *dnc* null mutations have previously been shown to enhance whole-cell synaptic transmission at the larval NMJ (Zhong and Wu, 1991). As well, it has been shown that transmission at individual release sites (using the focal extracellular recording approach...
developed by Dudel, 1981 and previously employed in *Drosophila* by Mallart et al., 1991; Stewart et al., 1996) exhibits larger decay time constants (Renger et al., 1998).

Using the UAS-GAL4 expression system as described earlier, I have assayed the effects of *dnc* overexpression in muscle alone, or in muscle and motor neurons in combination, on synaptic strength. In particular, I have used the intracellular recording technique to measure spontaneously-occurring miniature excitatory junction potentials (mEJPs) and nerve-evoked excitatory junction potentials (EJPs) at the whole-cell level (from the entire muscle fiber as a whole). The former are attributed to the spontaneous release of neurotransmitter from the presynaptic cell, while the latter are due to neurotransmitter released by nerve stimulation. Measuring these events provides information on overall strength of synaptic transmission (as revealed by the amplitude of these events) at the whole-cell level and on properties of spontaneous release of quantal units of transmitter.

3) I have used the focal loose-patch extracellular recording technique (Dudel, 1981; Mallart et al., 1991; Stewart et al., 1996) to measure spontaneous and evoked excitatory junction currents (mEJCs and EJCs respectively) from individual nerve ending varicosities. Extracellular recordings provide:

(a) information about synaptic strength at individual release sites through estimations of quantal content as an index of presynaptic transmitter release.
(b) information about possible alterations in the kinetics of the recorded responses for comparison with previous reports of such alterations due to null mutations in \textit{dnc} (Renger et al., 1997, 1998) and altered PKA activity (Davis et al., 1998).

Cumulatively, the results obtained in this study can be used to assess the effects of \textit{dnc} overexpression, which lowers cAMP concentration in the targeted cells, on synaptic morphology and physiology at the \textit{Drosophila} larval NMJ. The observations build on previous findings on the effect of null mutations in \textit{dnc} and thus provide additional insight into the role of \textit{dnc} as a component of the cAMP signalling system in synaptic transmission at the larval NMJ of \textit{Drosophila}. 
Chapter Two: Materials and Methods

Control animals

Specimens of the Canton-S strain of *Drosophila melanogaster* were used as wild-type controls in this study.

Experimental animals

The *dnc* gene was subcloned into a pUAST vector. P-element plasmids containing the P-UAS-*dnc* construct were made and transgenic flies containing the construct were generated and balanced on the X chromosome by Ms. Una Cheung (Cheung et al., 1997).

The mhc\textsuperscript{82}-GAL4 line contains the myosin heavy chain promoter fused to GAL4 and is a homozygous viable insert on the 3d chromosome (Meg Winberg, personal communication). It is used to drive gene expression selectively in muscle cells.

The e62-GAL4 line was generated from an enhancer trap screen described originally by Lin and Goodman (1994) and later in Schuster et al., (1996a,b). It drives GAL4 expression in many muscles and motor neurons, including muscles 6 and 7 and their innervating motor neurons (Meg Winberg, personal communication). Further, this line drives expression in muscle 3, motor neuron 3a, but not motor neuron 3b (Schuster et al., 1996a, b).

In this study, *dnc* was overexpressed in muscle alone by crossing virgin females from the UAS-*dnc* line to males from the mhc\textsuperscript{82}-GAL4 line. The "mhc" notation on all graphs/figures in this thesis indicates specimens where *dnc* was overexpressed in muscle fibers alone using the mhc\textsuperscript{82}-GAL4 line.
Overexpression of \( dnc \) conjointly in muscle and motor neurons was achieved by crossing males from the e62-GAL4 line to virgin females of the UAS-\( dnc \) line. The “e62” designation on all graphs/figures in this thesis indicates specimens where \( dnc \) was overexpressed conjointly in muscle cells and motor neurons.

All animals were reared on cornmeal medium at 25°C. Wandering third instar larvae were used in all experiments.

**Immunostaining of nerve endings**

Typically, a wandering third instar larva was selected and pinned down at its anterior and posterior ends. Two drops of saline was placed on top of the larval specimen at this point. The larval cuticle was then punctured along the centre of the dorsal midline using a sharp insect pin. Using fine-tipped scissors, the dorsal midline was cut towards the anterior and posterior from the central incision made earlier. The larval viscera was removed using fine-tipped forceps and the dissected larva was unrolled and flattened into a fillet using the dissecting pins of the dissection chamber.

Dissected larvae were fixed using Bouin’s fixative (Grimstone, 1972) for 30 minutes to prepare them for further manipulations. The fixed larval specimens were washed in 0.1 M phosphate buffered saline (PBS) containing 0.2% Triton X-100 (hence referred to as 0.1M PBT) every twenty minutes for one hour.

The preparations were incubated overnight at room temperature in fluorescein isothiocyanate (FITC)- conjugated anti-horseradish peroxidase (HRP) antibody. It has previously been shown that this antibody labels an HRP-like molecule in insect nervous
systems. As such, it can serve as a powerful tool for labeling and visualizing nerve endings in *Drosophila* larvae (Jan and Jan, 1982). This antibody was used at a dilution of 1:100.

Following overnight incubation, the specimens were once more rinsed in 0.1 M PBT for one hour at twenty-minute intervals. The specimens were then mounted on a glass slide in a drop of Permafluor mounting medium (Immuron) under a coverslip.

Mounted preparations were imaged using a BioRad MRC 600 confocal imaging apparatus (and COMOS software) coupled to a Nikon microscope for fluorescence microscopy. Barrier and excitation filters suitable for the FITC fluorochrome were used in order to visualize the labelled larval neuromuscular junctions. The specimen was imaged along the z-plane by obtaining optical sections that were 1μm in thickness. Optical slices obtained in 3-D were then projected to obtain a 2-D image of the NMJ. Moderate gain and black-level settings were used to minimize bleaching of the fluorochrome throughout imaging.

**Improving the design of the traditional recording chamber**

The design of the traditional recording chamber used to carry out electrophysiological assays was modified to overcome several disadvantages associated with this design.

The traditional chamber is made by gluing a magnetic strip (from which a central hole has been cut out; Fig. 2A) onto a large glass slide (Corning). Insect pins are glued onto small metal tabs that stick to the magnetic strip. The insect pins are used to
Figure 2. Schematic diagram depicting the improvement of the *Drosophila* larval recording chamber. (A) traditional prototype; (B) newly-improved design. The new chamber is taller and has a more generous basin for containing the physiological saline. As such, it reduces the chance of saline leakage due to capillary flow along the dissecting pins and electrodes that are lowered into the dish from either side. Scale bar: 1.5 cm.
maneuver and dissect the larval specimen in the chamber and serve functions that are analogous to surgical clamps (although on a much more minute scale).

Once the dissection is complete, in order to carry out physiological recordings from the larval specimens, two electrodes must be lowered towards the specimen (one from each side of the chamber). Also, visualization of the neuromuscular junction (NMJ) during the experiment is done using a water-immersion lens. For this, adequate amounts of physiological solution (bath) must be present in the chamber to provide a respectable distance between the lens and the electrodes. However, since there is very little depth associated with the chamber of traditional design, the bath can slowly seep out of the dish by capillary action along the dissecting pins and with the electrodes.

To correct for these factors, I designed a new chamber which has a roomier basin (Fig. 2B.), allowing for comfortable positioning of the electrodes and the simultaneous usage of a water-immersion lens without potential problems of fluid escaping due to capillary action.

Solutions

HL3 physiological solution (Stewart et al., 1994) was used in all experiments in this study. This solution contains (in mM): NaCl, 70; KCl, 5; MgCl₂, 20; NaHCO₃, 10; trehalose, 5; sucrose, 115; N,N-bis(2-Hydroxyethyl)-2-aminoethanesulfonate (B.E.S.), 5. Calcium was added to the extracellular physiological solution (at the concentrations indicated) as a chloride salt.
Electrophysiology

(a) Whole-cell intracellular recording

All measurements were made from ventral longitudinal muscle fiber 6 of abdominal segment 4. Resting membrane potentials, spontaneously-occurring miniature excitatory junction potentials (mEJPs), and evoked excitatory junction potentials (EJPs) were recorded using sharp voltage-recording glass microelectrodes that were backfilled with 3M KCl, and had resistances of ~ 40-60 MΩ. mEJPs and EJPs provide a profile of spontaneous and nerve-evoked release at the whole-cell level.

Evoked EJPs were elicited by stimulating the segmental nerve at 1Hz through a Kimax glass suction electrode of 10 µm inside diameter (see Fig. 3). Stimulating pulses were generated by a Grass stimulator coupled to a Grass stimulation isolation unit.

Electrophysiological data were collected on a Power Macintosh computer coupled to a Maclab Version 4/S system (AD Instruments, Australia). The amplitudes, rise times, and time constants of decay of mEJPs and EJPs were analyzed using the Scope and Chart softwares for Macintosh.
Figure 3. Schematic diagram representing the arrangement for electrophysiological recordings from the larval NMJ. Whole-cell voltage measurements were made using sharp intracellular electrodes (A). Dimple in (A) denotes impalement of the muscle fiber by a sharp intracellular electrode. Extracellular recordings from individual Type Ib varicosities were done by placing focal macropatch electrodes over selected varicosities (B). Evoked responses were elicited by stimulating the nerve through a suction electrode as depicted. All recordings were made from muscle fiber 6.
The frequency of mEJPs was determined by counting the number of events during a period of one minute. For each individual experiment involving mEJPs, the frequency of mEJPs was measured three times over different one-minute intervals. The average of the three measures was taken to represent the mEJP frequency for that particular experiment.

The measured amplitudes of evoked EJPs were corrected for non-linear summation using a correction formula described in MacLachlin and Martin (1981) and utilized by Stewart et al. (1996) at the Drosophila NMJ. The correction formula used is as follows:

$$v' = v / \left[1 - f \left(\frac{v}{E}\right)\right]$$

where $v'$ is the corrected amplitude, $v$ is the measured amplitude, $E$ is the driving force [$E = (\text{membrane potential}) - (\text{reversal potential})$]. Membrane potential of -60 mV and reversal potential of -10 mV was used for corrections. $f$ depends on the duration of neurotransmitter action ($\Delta t$) and the time constant of the membrane $\tau$ (where $f = \Delta t / \tau$). Based on findings from Stewart et al. (1996), where ($\Delta t$) is thought to be ~10 ms judged from focal recordings, and the time constant of the membrane is estimated at ~40 ms, $f = 0.25$ for Drosophila larval muscles. I used this value for $f$, as did Stewart et al. (1996).

(b) Focal extracellular recording

Focal synaptic currents were recorded from Type Ib varicosities (see Fig. 3) using focal loose-patch microelectrodes (Dudel, 1981; Mallart et al., 1991; Stewart et al., 1996). The focal extracellular recording technique allows one to assess synaptic transmission
from individual nerve ending varicosities. Extracellular electrodes were made using 1.10 mm Kimax glass tubing. Since previously it has been shown that Type Ib varicosities are up to ~5μm in diameter (Atwood et al., 1993), the focal electrodes were made with an inside diameter of 5μm. The tips of the electrodes were bent ~45° using a heated filament. The bend allows for the positioning of the patch electrode on top of an identified varicosity under a 40x water immersion lens.

Recordings were made from Type Ib varicosities identified by Nomarski optics on muscle fiber 6 of abdominal segment 4. A typical experimental paradigm involved recording spontaneously-occurring miniature excitatory junction currents (mEJCs) during a five-minute period, followed by recording 900 nerve-evoked EJCs elicited by stimulating the nerve at 1 Hz. Signals were low-pass filtered at 5 kHz and collected at a sampling rate of 40 kHz. EJCs and mEJCs in this study are reported as voltages recorded as a result of current flow at the tip of the recording macro-patch electrode. Amplitudes reported are comparative rather than absolute, since the seal resistance at the electrode tip is relatively low (typically ~1 MΩ). Electrophysiological data were collected on a PowerMacintosh via a Maclab System Version 4/S (AD Instruments, Australia). Amplitudes, rise times, and time constants of decay of synaptic currents were analyzed using the Igore and Scope softwares for Macintosh.

**Statistical Analysis**

Data are presented as mean±s.e.m. throughout the text. The Student’s t-test was used for all comparisons of means between experimental and control groups with p<0.05
chosen as the level of significance. Otherwise, the level of significance is specified. If data were such that the assumptions for normality and equal variance for the t-test were violated, the Mann-Whitney non-parametric equivalent U-test was used. Other statistical tests are specified in the text. The SigmaStat statistical software Version 1.01 (Jandel Scientific) was used to carry out all statistical analysis.
Chapter Three: Results

I. Morphology

A. Muscle overexpression of dnc gives rise to fewer synapse-bearing varicosities at the NMJ

*Drosophila* larval muscles are innervated by two types of motor neuron classified as Type I and Type II (Keshishian et al., 1993) based on their morphological features. The commonly studied muscle fibers 6 and 7 are innervated by Type I motor neurons that have been further subdivided into Type Ib and Type Ia motor neurons (Atwood et al., 1993). At the NMJ, Type Ib motor neurons give rise to bigger varicosities, while Type Ia motor neurons exhibit smaller varicosities.

Assessing the number of varicosities at the NMJ is important when one is considering the effects of mutations that alter synaptic efficacy. This is because the majority of individual synapses at the larval NMJ are known to occur on the varicosities (Atwood et al., 1993). Thus, as a first measure, any gross change in the number of these synapse-bearing varicosities (as revealed by light microscopy) could indicate morphological perturbations that might affect synaptic transmission.

Previously, it has been reported that null mutations in *dnc* result in an enhanced number of nerve ending varicosities at the larval NMJ (Zhong et al., 1992). In order to assess whether overexpression of *dnc* also results in altered morphology of the NMJ, FITC-conjugated anti-horseradish peroxidase (HRP) antibody was used as a visible marker to label the NMJ of larval specimens. This fluorochrome-labelled antibody has previously been shown to label the *Drosophila* larval nervous system (Jan and Jan, 1982)
and has been used extensively as a means to visualize the NMJ in order to assess morphological parameters of axonal terminal varicosities (Zhong et al., 1992; Atwood et al., 1993; Kurdyak et al., 1994; Stewart et al., 1996).

To check for morphological effects, I accounted for the number of varicosities in experimental and control animals. I found that dnc overexpression in muscle alone, or in muscle and motorneurons in combination, leads to ~ 35% fewer Type Ib and Type Is varicosities at the NMJ of muscle fibers 6 and 7 of abdominal segment 4 (Fig 4A-C, n=43 control, 32 muscle-overexpressed (mhc), and 39 muscle and motorneuron combination-overexpressed (e62) specimens). The number of Type Ib varicosities in specimens where dnc was overexpressed in muscle alone, or in muscle and motor neurons in combination, were 19.1±0.9 and 20.1±0.8 respectively (mean± s.e.m.) compared to 31.0±0.7 in controls (Fig. 5A.). The numbers of Type Is varicosities in the above-mentioned groups were 15.7±0.9 and 16.6±1.2 respectively (mean± s.e.m.) compared to 24.6±1.2 in controls (Fig 5A.). The number of axonal terminal branches was also correspondingly altered.

The number of Type Ib axonal branches in muscle-overexpressed specimens or in specimens where dnc was overexpressed in both muscle and motorneurons, were 3.7±0.2 and 3.3±0.2 respectively (mean± s.e.m.) compared to 5.2±0.19 in controls (Fig 5B.). The number of Type Is axonal branches in the above-mentioned groups were 2.8±0.2 and 3.1±0.2 respectively (mean± s.e.m.) compared to 4.3±0.3 in controls (Fig 5B.). The differences in varicosity and axonal branch numbers between experimental and control groups are significant (p<0.001).
Figure 4. Light micrographs of neuromuscular junctions of muscle fibers 6 and 7 labelled with anti-HRP antibody. (A) control specimen; (B) specimen in which dnc was overexpressed in muscle alone (mhc); and (C) a preparation in which dnc was overexpressed in muscle and motor neurons in combination (e62). Arrowheads indicate Type Ib, whereas small arrows indicate Type Ia varicosities. Scale bars =10μm.
Figure 5. *dnc* overexpression results in altered morphology of nerve endings at the NMJ. (A) Summary of varicosity counts; and (B) axonal branches from 43 control, 32 muscle-overexpressed (mhc), and 38 muscle- and motorneuron combination-overexpressed (e62) specimens indicates that there are fewer nerve ending varicosities and branches at the NMJ as the result of *dnc* overexpression. The error bars represent the standard error of the mean (s.e.m.) in this and subsequent figures. The designations (mhc) and (e62) denote the described experimental animals in all subsequent figures. Asterisks represent significant differences in this and all subsequent bar graphs.
II. Physiology

A. Overexpression of dnc results in altered whole-cell synaptic transmission

The number of synapse-bearing contacts between a presynaptic nerve ending and its postsynaptic target is thought to be a significant factor in determining the strength of synaptic transmission. It has previously been shown that the nerve terminal varicosities at the larval NMJ possess most of the synapses in this preparation (Atwood et al., 1993). Since the collective participation of all the varicosities at the NMJ underlies whole-cell EJPs, I investigated whether dnc overexpression, which results in a reduction of synaptic contacts at the NMJ, also affects the amplitude of whole-cell responses in a parallel manner. To test this, I measured whole-cell intracellular recordings of EJPs.

A-I: Intracellular recording of whole-cell evoked excitatory junction potentials (EJPs)

Previously it has been shown that Type Ib and Type Is varicosities give rise to physiologically-distinct evoked responses. Type Ib motor axons are often recruited at lower stimulation voltages and give rise to evoked excitatory junction potentials (EJPs) that are smaller in amplitude. Type Is motor axons give rise to larger evoked EJPs and are often recruited at higher stimulation voltages (Kurdyak et al., 1994). In the present study, whole-cell evoked synaptic strength was determined by recording the compound EJPs (at 1 Hz frequency of stimulation; see Fig. 6.) that are elicited by stimulation voltages set to recruit both axons.
Figure 6. Whole-cell recording of evoked EJPs. Sample raw traces (top panel) and average of 300 whole-cell EJPs (bottom panel) recorded from muscle fiber 6 in 1.0 mM \([\text{Ca}^{2+}]_o\) in typical experiments are shown.
It was found that dnc overexpression leads to whole-cell evoked responses that are smaller in amplitude than those of control animals (Fig 7A.). The amplitude of the compound EJPs, in 1.0 mM [Ca\(^{2+}\)]_o in muscle-overexpressed specimens (mhc) or in preparations where dnc was overexpressed in muscle and motor neurons in combination (e62), was 31.8±0.6 mV, and 33.0±0.5 mV respectively compared to 44.7±1.1 mV in controls (n=5 cells in each group). These differences are significant (p<0.001, Kolmogorov-Smirnov two-sample test; Siegel, 1956). The raw values of measured amplitudes were corrected for non-linear summation using a correction factor described by MacLachlin and Martin (1981) and employed previously by Stewart et al. (1996), as outlined above in Methods. In this study, all values for evoked EJP amplitudes reported are corrected values.

In addition to the reduction in amplitude of whole-cell compound EJPs already discussed, these responses exhibited faster rise times and smaller time constants of decay (Fig 7B-C). Rise time for a given EJP was measured from baseline to peak of that response while the time constant of decay was determined as a single exponential curve fit of the falling phase of the EJP. The rise times and time constants of decay of EJPs in the muscle-overexpressed group (mhc) were 13.9±0.5 and 45.2±0.7 ms, respectively. EJPs recorded from muscle- and motoneuron-overexpressed specimens (e62) exhibited rise times and time constants of decay that were 12.0±0.2 and 42.8±0.7 ms respectively. Rise times and time constants of decay of EJPs in control specimens were 16.6±0.5 and 54.5±1.5 ms respectively (Fig. 7B-C.). The differences between control and both experimental groups are significant (p<0.001; n=5 cells in each group).
Figure 7. Whole-cell EJPs are altered due to *dnc* overexpression. (A) EJPs are smaller in amplitude in mhc and e62 experimental lines. The kinetics of EJPs are also altered as they exhibit faster rise times (B) and smaller time constants of decay (C). Results shown are a summary of EJPs measured in 1.0 mM [Ca$^{2+}$], (n=100 evoked EJPs from 5 muscle fibers in each group). Asterisks denote significant differences (p<0.001) between both experimental groups and controls.
A-II: Intracellular recording of whole-cell spontaneously-occurring miniature excitatory junction potentials (mEJPs)

Miniature EJPs (mEJPs) are miniature postsynaptic potentials caused by the spontaneous presynaptic release of neurotransmitter. An mEJP is thought to represent the effects of a single quantum of released neurotransmitter. Fatt and Katz in the early 1950's (1951) first discovered the occurrence of spontaneously occurring potentials at the frog NMJ. They concluded that because they much resembled nerve-evoked responses, only on a much smaller scale, many of the spontaneously-occurring potentials must underlie a nerve-evoked potential. They postulated that in response to a presynaptic action potential, many “packets” of neurotransmitter were released which they named “quanta” (with a single quantum representing an individual “packet” of neurotransmitter which when released would give rise to an mEJP). These initial discoveries by Fatt and Katz paved the way for quantal analysis of synaptic transmission as a measure of presynaptic neurotransmitter release which is commonly employed today.

Thus, to further assess whether dnc overexpression perturbed whole-cell synaptic transmission, spontaneously-occurring mEJPs were recorded (Fig. 8.) and analyzed for their amplitudes, rise times, and time constants of decay. It was found that the amplitude of the mEJPs was significantly larger (p<0.001, Kolmogorov-Smirnov two-sample test) in specimens where dnc was overexpressed in muscle alone (mhc; Fig. 9A.), suggesting possible postsynaptic alterations. In the mhc group, the amplitude of mEJPs was 2.3±0.07 mV while the amplitude of mEJPs in the muscle- and motor neuron-overexpressed specimens (e62) was 1.2±0.03 mV. Control mEJPs were 1.3±0.04 mV in
Figure 8. Whole-cell recording of spontaneously-occurring mEJPs. Sample traces of mEJPs recorded from muscle fiber 6 in experimental and control groups as specified. Calibration bar: 2 mV, 1.5 seconds.
Figure 9. (A) Cumulative distribution of mEJP amplitudes shows a significant shift (p<0.001) towards larger values as the result of dnc overexpression in muscle alone (n=400 mEJPs from 10 fibers in each group). Summary of mEJP rise times and time constants of decay shown in (B) and (C) respectively (n=200 mEJPs from 5 muscle fibers in each group) indicates significant differences (p<0.05) in the kinetics of mEJPs due to overexpression of dnc.
amplitude (mean±s.e.m. of 400 mEJPs recorded from 10 cells are reported in each group). Amplitudes of mEJPs in e62 specimens were not significantly different from controls (p>0.05).

Miniature EJPs in both experimental groups also exhibited altered rise times and time constants of decay (Fig 9B-C). The rise times and time constants of decay of mEJPs in muscle-overexpressed (mhc) specimens were 17.2±0.4 and 38.3±0.9 ms respectively. The rise times and time constants of decay of mEJPs in e62 specimens, were 13.2±0.3 and 39.7±1.1 ms respectively. The duration of the rise times and time constants of decay for control mEJPs were 15.9±0.3 and 35.0±1.0 ms respectively (Fig. 9B-C). The differences in mEJP rise times and time constants of decay between both experimental groups and controls are significant (p<0.05; mean±s.e.m. of 200 mEJPs are reported in each group).

Further, the frequency of occurrence of mEJPs was altered as the result of dnc overexpression (Fig. 10.). The frequency of mEJPs in muscle-overexpressed specimens was 2.7±0.2 Hz. mEJPs in specimens where dnc was overexpressed in muscle and motor neurons in combination, occurred at a frequency of 1.7±0.2 Hz. Control mEJPs occurred at a frequency of 3.9±0.2 Hz (Fig. 10.). The differences in mEJP frequency between control and both experimental groups are significant (p<0.001; n=10 cells in each group).
Figure 10. Whole-cell mEJP s occur less frequently due to dnc overexpression. Summary of the frequency of occurrence of mEJPs indicates that dnc overexpression in muscle (mhc) or conjoint overexpression in muscle and motor neurons (e62) leads to reduced frequency of mEJP occurrence. Sample size is 10 muscle fibers in each group.
B. Neurotransmitter release from individual varicosities

As shown above, evoked whole-cell synaptic transmission was reduced when *dnc* was overexpressed in muscle alone, or in muscle and motor neurons in combination (Fig. 7A.). I wanted to assess whether this was solely due to a reduction in the number of nerve endings at the NMJ or whether synaptic transmission at individual nerve ending varicosities was also reduced to give rise to the whole-cell effect.

To test this, I assessed synaptic efficacy at individual release sites (varicosities) at the NMJ. Individual varicosities were identified by Nomarski optics and focal loose-patch extracellular electrodes (Dudel, 1981; Mallart et al., 1991; Stewart et al., 1996) were used to record extracellular synaptic events from an identified varicosity. Recordings were made from Type Ib varicosities on muscle 6.

A typical experimental paradigm involved extracellularly recording spontaneously-occurring miniature excitatory junction currents (mEJCs) from an identified varicosity. Further, 900 evoked excitatory junction currents (EJCs) were recorded at low frequency of stimulation (1Hz) in 1.0 mM [Ca$^{2+}$]$_o$. Following this period, the bath solution containing 1mM [Ca$^{2+}$]$_o$ was removed and the preparation was washed in 0 Ca$^{2+}$ physiological solution for about 5 minutes to allow for a period of rest and removal of the previous experimental solution. After that, solution containing 1.5 mM [Ca$^{2+}$]$_o$ replaced that containing 0 Ca$^{2+}$, and after ~ 5 minutes of rest in the new solution, 900 additional evoked EJCs were recorded to obtain a profile of release at 1.5 mM [Ca$^{2+}$]$_o$. This was done in order to get a comparison of release at individual varicosities at the commonly used 1.0 mM [Ca$^{2+}$]$_o$, and at 1.5 mM [Ca$^{2+}$]$_o$ which is thought to be a more
physiologically-relevant calcium concentration. It is currently thought that the calcium concentration in the animal’s own haemolymph to be \( \sim 1.5 \) mM (Stewart et al., 1994).

mEJCs recorded in \( \sim 5 \) minutes of spontaneous activity monitoring (see Fig. 11 for examples of mEJCs), and 300 EJCs (from 900 collected) recorded at 1.0 mM \([\text{Ca}^{2+}]_o\) (see Fig. 12 for examples of EJCs) were analyzed for their amplitudes, rise times and time constants of decay in the experimental and control groups.

**B-I: Spontaneous miniature excitatory junction potentials (mEJCs) from individual release sites**

In specimens where \( dnc \) was overexpressed in muscle and motorneurons in combination, there was a significant reduction in the amplitude of mEJCs (\( p<0.001; \) Kolmogorov-Smirnov two-sample test) compared to controls (Fig. 13A.). The mEJCs in the former group were \( 0.21 \pm 0.03 \) mV (n=7 release sites; mean\( \pm \)s.e.m.) while in controls they were \( 0.29 \pm 0.04 \) mV in amplitude (n=8 release sites). mEJCs in specimens where \( dnc \) was overexpressed in the muscle alone, were \( 0.25 \pm 0.02 \) mV in amplitude (n=8 release sites) and were not significantly different from controls (\( p>0.05 \)).

A significantly faster (\( p<0.001 \)) rise time was detected only in mEJCs of specimens where \( dnc \) was overexpressed in muscle and motor neurons in combination (\( e62; \) Fig. 13B). Time constants of decay of mEJCs were significantly smaller in both experimental groups compared to controls (\( p<0.001 \)). Rise times and time constants of decay of mEJCs in muscle-overexpressed specimens (mhc) were \( 1.3 \pm 0.05 \) and \( 5.9 \pm 0.02 \) 0.7 ms respectively (Fig. 13. B-C.). The rise times and time constants of decay of mEJCs in specimens where \( dnc \) was overexpressed in both muscle and motorneuron in
Figure 11. Spontaneously-occurring mEJCs from individual varicosities. Averaged traces of spontaneously-occurring mEJCs in ~ 5 minutes of recording from individual Type Ib release sites on muscle fiber 6 are shown using focal macropatch electrodes.
Figure 12. Nerve-evoked EJCs from individual release sites. Raw traces (top panel) and average of 900 evoked EJCs (bottom panel) recorded from individual Type Ib release sites on muscle fiber 6 at 1.0 mM $[Ca^{2+}]_o$ are shown using focal macropatch electrodes.
Figure 13.  Spontaneous mEJCs are altered due to dnc overexpression. (A) dnc overexpression pre- and postsynaptically (e62) leads to mEJCs that are smaller in amplitude. Conjoint overexpression of dnc (e62) or exclusive muscle-overexpression of dnc (mhc) results in mEJCs that are faster to rise (B) and exhibit smaller time constants of decay (C). n= 8 release sites in each group.
A

mEJC amplitude (mV)

control
mhc
e62

*
combination (e62), were 1.0±0.07 and 4.8±0.6 ms respectively. The rise times and time constants of decay of control mEJCs were 1.5±0.09 and 7.1±0.9 ms respectively (Fig. 13. B-C.).

Further, the frequency of occurrence of mEJCs was not different in experimental animals compared to controls. mEJCs recorded from ‘mhc’ varicosities occurred at a frequency of 0.09±0.01 Hz, while control mEJCs occurred at a frequency of 0.07±0.01 Hz. ‘e62’ mEJCs occurred at 0.1±0.02 Hz (see Fig. 14.; n=15 control varicosities and 8 release sites in each experimental group).

**B-II: Evoked excitatory junction potentials (EJCs) from individual release sites**

There was not a significant difference (p>0.05) between experimental and control groups in the amplitude of evoked EJCs (Fig. 15A.). Evoked EJCs recorded from individual release sites in specimens where dnc was overexpressed in muscle alone had a mean amplitude of 0.8±0.1 mV (mean±s.e.m; n=8 release sites). EJCs in specimens where dnc was overexpressed in muscle and motor neurons in combination, averaged 0.7±0.1 mV (n=7 release sites). Control EJCs averaged 0.7±0.1 mV (n=8 release sites).

Focally-recorded EJCs in both experimental groups exhibited significantly faster rise times and smaller time constants of decay compared to controls (p<0.001; Fig. 15B-C.). Rise times and time constants of decay of EJCs in specimens where dnc was overexpressed in muscle were 1.6±0.05 ms and 4.1±0.1 ms respectively (mean±s.e.m; n=8 release sites). EJCs in specimens where dnc was overexpressed in muscle and motor neurons in combination, exhibited rise times and time constants of decay of 1.4±0.05 ms.
Figure 14. mEJC frequency is not altered at single varicosities due to \textit{dnc} overexpression. There are no detectable differences in the frequency of occurrence of spontaneous mEJCs as the result of \textit{dnc} overexpression in either experimental group. n=15 control varicosities and 8 varicosities in each experimental group.
Figure 15. Evoked EJCs from individual varicosities are altered in their kinetics, but not in their amplitude due to dnc overexpression. (A) Summary of EJC amplitudes indicates a large variability in the amplitude of these responses. There are no detectable differences in EJC amplitudes between experimental and control groups. However, EJCs are faster to rise (B) and exhibit smaller time constants of decay (C) in experimental animals compared to controls (300 evoked EJCs from each release site were analyzed for their amplitudes, rise times, and time constants of decay. Sample size is 8 varicosities in each experimental and control group). Asterisks denote significant differences (p<0.001) between experimental groups and controls.
and 3.8±0.3 ms, respectively (n=7 release sites). Rise times and time constants of decay for control EJCs were 2.2±0.1 and 5.5±0.3 ms respectively (n=8 release sites).

B-III: Quantal content from individual varicosities as an index of presynaptic transmitter release

Miniature EJCs (mEJCs) and EJCs have previously been reported to exhibit a large variability in their amplitudes (Stewart et al., 1996; present observations). The direct method of quantal content estimation (Cooper et al., 1995) involves dividing the mean amplitude of evoked EJCs by the mean amplitude of spontaneous mEJCs for a given release site (see Fig. 16.). This estimation would provide an approximate indication of how many spontaneously-occurring mEJCs (quanta) underlie an evoked response. Estimations of quantal content at individual release sites (as an index of presynaptic transmitter release) using this direct method have proven difficult for definitive comparisons between experimental and control groups (Stewart et al., 1996). Despite the problems posed by the large variability in response amplitudes within a single, and between different release sites, the direct method of quantal content estimation was employed as a first approximation of quantal content. This was done at individual release sites in both 1.0 and 1.5 mM [Ca^{2+}]_o. Examples of quantal content estimates for control specimens are listed in Table 2. to indicate the variability in estimates from one varicosity to another.

There was no significant difference in direct quantal content estimates among experimental and control groups (p>0.05). Quantal content estimations for
Figure 16. Direct estimations of quantal content. Averaged traces of spontaneously-occurring mEJCs (A; top panel), evoked EJCs at 1.0 mM [Ca$^{2+}$]$_o$ (B; top panel), and evoked EJCs at 1.5 mM [Ca$^{2+}$]$_o$ (C; top panel) for typical experiments from Type Ib varicosities on muscle fiber 6 using focal extracellular electrodes are shown. Bottom traces in A, B, C represent examples of corresponding traces of individual trials. Direct estimates of quantal content are made by dividing the mean EJC amplitude by the mean mEJC amplitude. Estimates were done for two different [Ca$^{2+}$]$_o$ concentrations, as specified.
A  
spontaneous mEJC

B  
evoked EJC (1.0 mM $[Ca^{2+}]_o$)

C  
evoked EJC (1.5 mM $[Ca^{2+}]_o$)
Table 2. Examples of direct quantal content estimates at the commonly-used 1.0 mM [Ca^{2+}]_o, and the more physiologically-relevant 1.5 mM [Ca^{2+}]_o for identified Type Ib varicosities from control specimens.
Table 2. Examples of quantal content estimates for identified varicosities

<table>
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<tr>
<th></th>
<th>Quantal content (1.0 mM[Ca^{2+}])</th>
<th>Quantal content (1.5 mM[Ca^{2+}])</th>
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<tbody>
<tr>
<td>exp1</td>
<td>2.07</td>
<td>5.53</td>
</tr>
<tr>
<td>exp2</td>
<td>2.89</td>
<td>7.0</td>
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<tr>
<td>exp3</td>
<td>1.4</td>
<td>2.76</td>
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<tr>
<td>exp4</td>
<td>1.55</td>
<td>2.0</td>
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<tr>
<td>exp5</td>
<td>1.75</td>
<td>3.47</td>
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<tr>
<td>exp6</td>
<td>3.04</td>
<td>---</td>
</tr>
<tr>
<td>exp7</td>
<td>---</td>
<td>4.73</td>
</tr>
<tr>
<td>exp8</td>
<td>---</td>
<td>2.13</td>
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<tr>
<td>exp9</td>
<td>3.5</td>
<td>5.54</td>
</tr>
<tr>
<td>exp10</td>
<td>---</td>
<td>7.37</td>
</tr>
<tr>
<td>mean±s.e.m.</td>
<td>2.31±0.3</td>
<td>4.50±0.7</td>
</tr>
</tbody>
</table>
individual Type Ib release sites, at 1.0 mM [Ca$^{2+}$]$_o$, in specimens where dnc was overexpressed in muscle alone, or in muscle and motor neurons in combination, was 2.5±0.4 (mean±s.e.m.; n=11 release sites) and 2.7±0.6 (n=6 release sites) respectively. Quantal content estimates for control release sites were 2.3±0.3 (n=8 release sites; Fig. 17A).

At the more physiologically-relevant [Ca$^{2+}$]$_o$ of 1.5 mM, it was found that quantal content estimates were generally higher, indicating more presynaptic release of neurotransmitter due to higher extracellular [Ca$^{2+}$]. However, there was also no significant difference (p>0.05) among experimental and control groups in quantal content estimates in 1.5 mM [Ca$^{2+}$]$_o$. Quantal content estimations for individual release sites, in 1.5 mM [Ca$^{2+}$]$_o$, in specimens where dnc was overexpressed in muscle alone (mhc), or in muscle and motor neurons in combination (e62), were 3.5±0.6 (mean±s.e.m.; n=12 release sites) and 3.9±0.8 (n=9 release sites) respectively. Quantal content estimates for control release sites were 4.2±0.7 (n=12 release sites; Fig. 17B.).

B-IV: Frequency of failures of evoked transmission in low [Ca$^{2+}$]$_o$ as an alternate measure of synaptic strength from individual varicosities

While direct estimations of quantal content provide an approximate profile of presynaptic transmitter release at individual release sites, the large variability typically seen in estimates between release sites makes it difficult for conclusive comparisons to be made among groups without very large samples. As an alternate measure, failure of neurotransmitter release can be used as an index of synaptic strength. del Castillo and
Figure 17. Quanta1 content of individual varicosities is not altered due to \textit{dnc} overexpression. (A) Direct estimates of quanta1 content for individual Type Ib varicosities in 1.0 mM $[\text{Ca}^{2+}]_0$, and (B) in 1.5 mM $[\text{Ca}^{2+}]_0$ do not reveal alterations in quanta1 content of presynaptic neurotransmitter release as the result of \textit{dnc} overexpression in either experimental group compared to controls. Number of sampled varicosities in 1.0 mM $[\text{Ca}^{2+}]_0$ were 11 mhc, 6 e62, and 8 control varicosities. Sample sizes in 1.5 mM $[\text{Ca}^{2+}]_0$ were 12 mhc, 9 e62, and 12 control varicosities.
Katz (1954) counted failures of release as a method of estimating quantal content. Their approach assumes a Poisson distribution for the release of “packets” of neurotransmitter (or quanta) per presynaptic action potential. Assessing the frequency of failures of transmitter release in low \([\text{Ca}^{2+}]_o\) may be a more accurate means of estimating synaptic strength at individual release sites at the *Drosophila* NMJ, rather than attempting to extrapolate quantal content based on counting the number of failures in a given number of trials. This is because assumptions underlying a Poisson distribution may not be adequately fulfilled at the *Drosophila* larval NMJ (Stewart et al., 1996).

In physiologically-relevant calcium concentrations, failures of release seldom occur (Cooper et al., 1995; present observations). Thus, to assess synaptic strength at individual varicosities using an alternate measure to direct quantal content estimates, I chose to study the frequency of failures of neurotransmitter release from individual release sites. To do this, I used the low external \([\text{Ca}^{2+}]_o\) of 0.35 mM. At this concentration of \([\text{Ca}^{2+}]_o\), there are many failures of neurotransmitter release from individual release sites (Stewart et al., 1996). I employed methods of assessing failure of transmitter release similar to those of Stewart and colleagues (1996) in order to address the strength of synaptic transmission at individual release sites (Fig. 18.).

At the low \([\text{Ca}^{2+}]_o\) of 0.35 mM, a large proportion of stimuli give rise to failures of synaptic transmission (Stewart et al., 1996). I found that there was no significant difference in the frequency of failures between experimental and control groups (p>0.05) in 0.35 mM \([\text{Ca}^{2+}]_o\). For individual Type I\(b\) release sites in specimens in which *dnc* was overexpressed in muscle alone, or in muscle and motorneurons in
Figure 18. Failure analysis of evoked release at individual varicosities. (A) Three sample traces where nerve stimulation resulted in failures of transmitter release and (B), three examples where stimulation gave rise to an evoked response, from a control specimen. Asterisks in (B) denote an evoked response. Experiments were carried out in 0.35 mM $[Ca^{2+}]_o$. 100 stimuli from each release site were graded as failures or evoked responses.
combination, the percentage of stimuli that failed to evoke neurotransmitter release were 70.1±3.7% and 75.2±2.6% respectively (mean±s.e.m.; n=14 release sites in each experimental group). In control release sites, 76.1±2.6% of the stimulation trials exhibited failures of neurotransmitter release (n=14 release sites; Fig. 19.). Thus, synaptic strength at the individual release sites, assessed by percentage of failures and direct quantal content estimates, does not seem to be altered in the experimental groups compared to controls.

C: Summary of alterations in the amplitude and kinetics of postsynaptic responses due to *dnc* overexpression (see Table 3.)

In Table 3, a summary of the electrophysiological findings is provided, indicating the changes in amplitude and kinetics due to *dnc* overexpression.

The features that differ from control values in both experimental groups are:

a) amplitude of whole-cell EJPs (significantly lower)

b) rise times of EJPs (significantly faster)

c) rise times of mEJCs and EJCs (significantly faster)

d) time constants of decay of EJPs, mEJCs, and EJCs (significantly smaller)

Overall, there is a clear reduction in the amplitude of the EJPs and on the kinetics of the mEJCs and EJCs in both experimental groups, and these differences probably represent the major effects of *dnc* overexpression in muscle cells.
Figure 19. Failures of transmitter release are not altered due to \textit{dnc} overexpression. Summary of the mean percentage of failures of release reveals no differences between experimental and control groups in the frequency of failures of evoked neurotransmission from individual varicosities. (n=14 release sites in each group; 100 stimuli from each release site were graded as failures or evoked responses).
Table 3. Summary of amplitudes, rise times, and time constants of decay of mEJPs, EJPs, mEJC, and EJC in specimens in which dnc was overexpressed in muscle alone (mhc), or in muscle and motor neurons in combination (e62).
Table 3. Summary of amplitudes, rise times, and time constants of decay for mEJPs, EJPs, mEJCs, and EJCs.

<table>
<thead>
<tr>
<th>Amplitude (mV)</th>
<th>whole-cell EJP</th>
<th>whole-cell mEJP</th>
<th>spontaneous mEJC</th>
<th>evoked EJC</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>mean 44.7 % change</td>
<td>mean 1.31 % change</td>
<td>mean 0.29 % change</td>
<td>mean 0.69 % change</td>
</tr>
<tr>
<td>mhc (muscle only)</td>
<td>31.8 ↓ 29%</td>
<td>2.29 ↑ 74%</td>
<td>0.25 none</td>
<td>0.75 none</td>
</tr>
<tr>
<td>e62 (muscle + motor neurons)</td>
<td>33.0 ↓ 27%</td>
<td>1.24 none</td>
<td>0.21 ↓ 16%</td>
<td>0.67 none</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Rise time (ms)</th>
<th>whole-cell EJP</th>
<th>whole-cell mEJP</th>
<th>spontaneous mEJC</th>
<th>evoked EJC</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>mean 16.6 % change</td>
<td>mean 15.9 % change</td>
<td>mean 1.5 % change</td>
<td>mean 2.2 % change</td>
</tr>
<tr>
<td>mhc (muscle only)</td>
<td>13.9 ↓ 16%</td>
<td>17.2 ↑ 8.2%</td>
<td>1.3 ↓ 13%</td>
<td>1.6 ↓ 27%</td>
</tr>
<tr>
<td>e62 (muscle + motor neurons)</td>
<td>12.0 ↓ 28%</td>
<td>13.2 ↓ 17%</td>
<td>1.0 ↓ 33%</td>
<td>1.4 ↓ 36%</td>
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</table>

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<thead>
<tr>
<th>Time constant of decay (ms)</th>
<th>whole-cell EJP</th>
<th>whole-cell mEJP</th>
<th>spontaneous mEJC</th>
<th>evoked EJC</th>
</tr>
</thead>
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<tr>
<td>control</td>
<td>mean 54.5 % change</td>
<td>mean 35.0 % change</td>
<td>mean 7.1 % change</td>
<td>mean 5.5 % change</td>
</tr>
<tr>
<td>mhc (muscle only)</td>
<td>45.2 ↓ 17%</td>
<td>38.3 ↑ 9.4%</td>
<td>5.9 ↓ 17%</td>
<td>4.1 ↓ 25%</td>
</tr>
<tr>
<td>e62 (muscle + motor neurons)</td>
<td>42.8 ↓ 21%</td>
<td>39.7 ↑ 13%</td>
<td>4.8 ↓ 32%</td>
<td>3.8 ↓ 31%</td>
</tr>
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Chapter Four: Discussion

In this study, I have presented evidence that overexpression of the dnc gene postsynaptically, or both pre- and postsynaptically, (and hence reduction of cAMP in targeted cells), results in a reduction of the number of axonal branches and varicosities at the Drosophila larval NMJ. I also found that dnc overexpression results in a reduction of whole-cell synaptic strength, but that synaptic transmission at the individual varicosities is not greatly affected. These observations suggest that physiological compensation for morphological changes does not occur in the presynaptic varicosities; that is, synaptic transmission does not increase at individual varicosities to compensate for their reduced number.

These results complement previous studies of dnc null mutants which have elevated levels of cAMP (Byers et al., 1981). These mutants exhibit an increased number of nerve terminal varicosities and axonal arborization (Zhong et al., 1992; Davis et al., 1996) and enhanced whole-cell synaptic transmission in low Ca^{2+} solutions (Zhong and Wu, 1991). More recently, it has been shown that the strength of synaptic transmission at individual varicosities is not greatly altered in the dnc null mutants (Renger et al., 1997, 1998). It can be postulated that the enhancement of whole-cell synaptic strength as the result of null mutations in dnc (Zhong and Wu, 1991) or the reduction of whole-cell synaptic transmission due to ectopic dnc overexpression (present observations), could be explained by a corresponding alteration in the number of varicosities and synaptic contacts.
Effect of \textit{dnc} overexpression on spontaneous release of neurotransmitter: overall effects

Postsynaptic overexpression of \textit{dnc} resulted in an increased amplitude and reduced frequency of occurrence of whole-cell mEJPs, whereas only a reduction in the frequency of mEJP occurrence was observed when \textit{dnc} was overexpressed both pre- and postsynaptically.

Interestingly, the frequency of spontaneous release of transmitter from individual varicosities does not seem to be greatly altered as the result of \textit{dnc} overexpression. Hence the reduction in the frequency of mEJPs at the whole-cell level could reflect the reduced number of nerve ending varicosities seen with \textit{dnc} overexpression. Since whole-cell mEJPs are due to the collective spontaneous release of transmitter from all of the varicosities at the NMJ, a reduction in the number of varicosites could lead to a reduced frequency of spontaneously-occurring mEJPs at the whole-cell level, assuming that the frequency of spontaneous release of quanta per synapse remains unaltered due to \textit{dnc} overexpression.
The effects of \( dnc \) overexpression on the kinetics of mEJPs/mEJCs: implications

In this study, \( dnc \) overexpression postsynaptically, or pre- and postsynaptically, has been shown to result in mEJPs that had larger time constants of decay. While mEJPs in the former exhibited slower rise times, mEJPs in the latter were faster-rising compared to controls.

Although the amplitudes of spontaneously-occurring mEJCs from individual nerve ending varicosities were not greatly altered, mEJCs exhibited faster rise times and smaller time constants of decay due to \( dnc \) overexpression postsynaptically, or due to pre- and postsynaptic overexpression of \( dnc \).

The differential alteration of the kinetics of mEJPs as opposed to mEJCs is paradoxical in that one would expect similar alterations in kinetics to be detected both in mEJPs and in mEJCs. Alterations in the kinetics of mEJCs as described may be due to alterations in the kinetics of postsynaptic receptors (Davis et al., 1998). Further, possible alterations in the membrane properties of the muscle fibers may be contributing to the differential alterations of mEJP kinetics as opposed to mEJC kinetics since the former, but not the latter, are affected by membrane properties (membrane resistance and time constant) of the muscle fiber.

To address whether possible alterations in the muscle fiber properties could be a contributing factor to altered properties of mEJPs, I made some preliminary measurements of input resistances \( (R_{in}) \) of the muscle fibres. \( R_{in} \) reflects the specific surface resistance of the muscle fiber membrane \( (R_m) \) provided cell size is constant. The relationship between \( R_{in} \) and \( R_m \) is given by:
\[ R_{\text{in}} = \frac{1}{\pi \sqrt{R_m R_i / d^3}}, \]

where \( R_{\text{in}} \) is the input resistance between inside and and outside of the muscle fiber, \( R_m \) is the specific membrane resistance of the muscle fiber, and \( R_i \) is the specific intracellular resistance, and \( d \) is the muscle fiber diameter (Katz and Thesleff, 1957). Further, the time constant of the membrane (\( \tau_m \)) is determined by the product of its specific membrane resistance (\( R_m \)) and membrane capacitance (\( C_m \)). Increasing \( R_{\text{in}} \) (assuming other factors remain the same) would give rise to a greater voltage excursion for a given injected current. Furthermore, if increased \( R_{\text{in}} \) is mainly due to increased \( R_m \), the muscle cell would have a longer \( \tau_m \), and and mEJPs would decay more slowly. Preliminary evidence presented in Appendix 1 suggests that postsynaptic \( dnc \) overexpression results in muscle fibers with larger input resistances. Enhanced membrane resistance of the muscle fibers could explain the increase in amplitude and prolongation of the time constants of decay of mEJPs that have been observed. At present, it is not clear how overexpression of \( dnc \) as specified can lead to the alterations in kinetics of receptors suggested by the altered time course of mEJCIs, or to possible modifications of muscle fiber properties. What the preliminary evidence suggests, however, is that perturbations in the properties of the muscle fibers may influence the amplitude and time constants of decay of mEJPs. Thus, possible alterations in the membrane properties of the muscle fibers need to be considered in parallel with other factors when trying to assess the mechanisms underlying alterations of the mEJP properties.
Appendix 1. Preliminary measurements of muscle fiber electrical properties (input resistance, \( R_{\text{in}} \)). \( dnc \) overexpression in the muscle fibers, or in combination with \( dnc \) overexpression in motor neurons, results in muscle fibers that exhibit higher \( R_{\text{in}} \). \( (n=7 \) control fibers and 8 muscle fibers in each experimental group).
Muscle fiber input resistance (mega Ohms)

- Control
- MHC
- e62

* indicates statistically significant difference.
Effects of *dnc* overexpression on properties of evoked whole-cell neurotransmission

One would expect that alterations in the membrane properties of the muscle fibers would affect the amplitudes and decay time constants of whole-cell mEJPs and EJPs in an analogous fashion. In other words, since both whole-cell mEJPs and EJPs are affected by passive properties of the muscle fiber, any alteration in the latter should affect whole-cell mEJPs and EJPs in similar ways. This, however, is not the observation as specimens in which *dnc* was overexpressed displayed whole-cell EJPs that exhibit reduced amplitudes and smaller time constants of decay, directly in contrast to our findings from mEJPs.

With respect to the amplitude of EJPs, increased input resistance ($R_{in}$), likely resulting from increased specific resistance of the muscle membrane ($R_{m}$), would theoretically be expected to give rise to increased EJP amplitudes, if other cell properties remain the same, as discussed above for mEJPs. However, the ~ 35% reduction in the number of varicosities that would normally participate in a whole-cell evoked response, coupled with unaltered quantal emission per varicosity, would lead to reduced EJP amplitudes, counteracting the increases in EJP amplitude that possibly could have resulted from increase in $R_{m}$ of the muscle fiber. Thus, since there are fewer nerve ending varicosities at the NMJ, fewer quanta are released onto the whole cell per nerve stimulus, giving rise to whole-cell EJPs that are smaller in amplitude. This explanation requires additional quantitative verification through further more detailed measurements of muscle cell electrical properties.

The smaller time constants of decay of EJPs in *dnc* overexpressors cannot be readily explained at the present time. It could reflect more synchronous release of fewer
quanta or possible differences in membrane conductances of the muscle membrane turned on by voltage. These possibilities should be considered in the future in addition to solidifying the examination of possible alterations in muscle membrane properties as the result of \textit{dnc} overexpression. Further, such factors are equally important to consider in the \textit{dnc} null mutant and in specimens in which \textit{dnc} is overexpressed exclusively in motor neurons (Cheung et al., 1997). These are some of the missing links from earlier studies (Zhong and Wu, 1991; Cheung et al., 1997) that need to be addressed in the future.

**Effects of \textit{dnc} overexpression on properties of evoked neurotransmission from individual varicosities**

Although the amplitude of evoked EJCs from individual varicosities was found not to be altered due to \textit{dnc} overexpression postsynaptically alone, or pre- and postsynaptically in conjunction, these responses exhibited altered kinetics, namely faster rise times and smaller time constants of decay compared to controls. The alterations in the kinetics observed for EJCs from individual release sites were analogous to those observed for mEJCs from individual varicosities. Since mEJCs and EJCs are a measure of inward flow of current into the postsynaptic cell, such measurements are not affected by the passive membrane properties of the muscle fibers. Thus, the altered kinetics of mEJCs and EJCs should more closely reflect possible alterations in the kinetics of the synaptic glutamate receptors. Altered kinetics of EJCs could also reflect differences in synchrony of release of quantal units in response to evoked stimuli. While the precise mechanism by which \textit{dnc} overexpression leads to altered kinetics of mEJCs and EJCs
remains largely unknown, it should be pointed out that results from this study are consistent with findings in the *dnc* null mutants, in which Renger et al. (1998) observed mEJCs and EJCs with lengthened time constants of decay from individual varicosities. Thus, these studies taken together suggest that increased intracellular cAMP leads to larger time constants of decay of mEJCs and EJCs, while reduced intracellular cAMP leads to smaller time constants of decay of these postsynaptic responses.

**PKA-dependent modulation of properties of postsynaptic responses at the *Drosophila* NMJ - critical review of recent work (Davis et al., 1998)**

There have been other recent attempts to address the effects of altered expression of pertinent postsynaptic molecules on synaptic transmission at the *Drosophila* larval NMJ (see Table 1). Increasing the postsynaptic PKA activity by transgenic postsynaptic overexpression of a PKA catalytic subunit results in whole-cell mEJPs that are smaller in amplitude and display smaller time constants of decay (Davis et al., 1998). Similar effects have been shown for whole-cell mEJCs as they are also smaller in amplitude, although assessment of the time constants of decay for these responses was not presented in this report. Further, these authors show that the amplitude of whole-cell EJCs are unaltered with enhanced postsynaptic PKA activity, although they do not present data on the time constant of decay of these responses. Pharmacological enhancement of PKA activity by bath-application of a cAMP analog also results in reduced mEJC amplitudes. As well, repeated iontophoresis of glutamate in combination with pharmacological enhancement of PKA activity leads to a more rapid desensitization of the response to
iontophoresed glutamate compared to fibers with normal PKA activity, which exhibit a more gradual desensitization in their response to repeated glutamate iontophoresis. These authors provide additional evidence that the PKA-mediated effects on mEJC/mEJP size are dependent on presence of the endogenous *Drosophila* glutamate receptor subunit DGluRIIA. In DGluRIIA null mutants (lacking this receptor subunit), PKA-mediated reduction of mEJC/mEJP size is not observed. Thus, the authors conclude that enhanced PKA activity in the muscle results in: (a) mEJPs that are smaller and exhibit smaller time constants of decay; and (b) responses to iontophoresed glutamate that exhibit more rapid desensitization with repeated puffs of glutamate. The authors conclude that their observations reflect the desensitization of the DGluRIIA receptor subunit, and attribute this to phosphorylation of the DGluRIIA receptor subunit by PKA. They further point out that these findings are in contrast to findings from the vertebrate CNS where increased PKA activity has been shown to result in the enhancement of postsynaptic responses (Wang et al., 1993).

Attempts by Davis and colleagues (1998) as described above serve as first attempts in elucidating the role of PKA modulation of glutamate receptors at the *Drosophila* larval NMJ. However, these authors do not address numerous pertinent questions which leaves the implications of their findings in some doubt. For example, although they stress that increased postsynaptic PKA activity results in alterations of the amplitude and time constant of decay of whole-cell mEJPs, they do not provide definitive assessments of the kinetics of whole-cell EJPs, or of single-varicosity mEJC and EJCs. Further, as already discussed, muscle fiber membrane electrical properties can play a role
in determining the kinetics of postsynaptic potentials, and these properties were not examined.

In order to further illustrate the importance of assessing the properties of muscle fibers as role-players in determining synaptic strength, a review of certain caveats in previous studies that failed to address such factors can shed additional insight. Petersen et al. (1997) reported that whole-cell mEJPs were smaller in amplitude as the result of null mutations in the Drosophila glutamate receptor subunit DGluRIIA. Davis et al. (1998) further reported that in addition to reduced amplitudes, mEJPs exhibited smaller time constants of decay. These authors also showed that whole-cell mEJCIs in the DGluRIIIA null mutants were unaltered in their amplitudes and they did not report any alterations in the time constant of decay of mEJCIs. It would be difficult to ascertain why there would be a preferential effect on the properties of whole-cell mEJPs and not whole-cell mEJCIs as the result of the postsynaptic perturbations that null mutations in DGluRIIA represent unless alterations in the muscle fiber membrane properties also occur and play a role in the alterations of whole-cell mEJPs. This example illustrates that accounting for the properties of the muscle fibers can be important and should be considered. This becomes particularly important in studies just discussed (Petersen et al., 1997; Davis et al., 1998) that specifically create perturbations in the postsynaptic cell itself (which includes the muscle fibers, receptors, channels and any pertinent molecules that the muscle houses) and try to assess the effects of these alterations on synaptic strength and kinetics of electrophysiological responses.
Thus, in order to attribute any alterations in the kinetics of whole-cell postsynaptic potentials (spontaneously-occurring mEJPs or evoked EJPs) to exclusive modulations of postsynaptic receptors, possible effects of muscle fiber membrane and electrical properties need to be ruled out. Addressing such effects would be a crucial step in attempts to elucidate the role of PKA on receptor kinetics.

**Comparisons of observations from this study to previous findings**

In the present study, I have provided evidence that postsynaptic overexpression of \textit{dnc} alone, or in combination with presynaptic \textit{dnc} overexpression, leads to alterations in the kinetics of mEJPs. Presuming that the effects of \textit{dnc} overexpression are to reduce cAMP levels (Cheung et al., 1997), one would expect PKA activity to be down-regulated as the result of the transgenic reduction of cAMP availability. Based on the model proposed by Davis et al. (1998), one would expect an increase in amplitude and a lengthening of the time constant of decay of mEJPs due to postsynaptic overexpression of \textit{dnc} and hence the reduction of cAMP (and presumed reduced PKA activity). I have shown this to be the case and these observations coincide with the findings of Davis and colleagues (1998) based on whole-cell mEJPs. However, I have also provided preliminary evidence (Appendix 1) that suggests alterations in the membrane properties of the muscle fibers may contribute to the alterations of mEJP properties.

Davis et al. (1998) provide evidence only for altered time constants of decay of mEJPs as the result of enhanced PKA activity (see Table 4). Further, since they do not address other factors such as possible alterations in the membrane properties of the muscle fibers, one must assume that their findings also apply to EJPs, mEJCs, and EJCs.
That is, based on evidence presented in their study, one must assume that increased PKA activity gives rise to faster-decaying EJPs, mEJCs, and EJCs. This would mean that reduced PKA activity should have a lengthening effect on the time constant of decay of postsynaptic responses based on the model presented by Davis and colleagues (1998). In the present study, my findings from spontaneously-occurring mEJPs indicate that dnc overexpression in the muscle and hence reduced cAMP levels alter the amplitude and the time constant of decay of mEJPs in a manner that is consistent with the model presented by Davis et al. (1998; see Table 4.).

However, I have also shown that whole-cell EJPs in addition to mEJCs and EJCs from individual varicosities, all exhibit smaller time constants of decay as the result of dnc overexpression entailing reduction in cAMP levels (which implies reduced PKA activity). These observations are in contrast to those predicted by the model presented by Davis et al. (1998). Further, my observations from mEJCs and EJCs from individual varicosities are in direct agreement with previous findings in the dnc null mutants (Renger et al., 1997, 1998) which have shown that mEJCs and EJCs from individual varicosities display larger time constants of decay as the result of elevated cAMP levels (and presumably, enhanced PKA activity). Observations by Renger et al. (1998) in the dnc null mutants, in conjunction with observations from this study, suggest that possible alterations of receptor kinetics may underlie the alterations of the kinetics of mEJCs and EJCs from individual varicosities, which would be inconsistent with the proposed model of PKA-dependent modulation of the DGluRIIA receptor subunit proposed by Davis et al. (1998).
Table 4. PKA-mediated modulation of mEJPs. Enhanced postsynaptic PKA activity by elevated cAMP levels results in mEJPs that exhibit reduced amplitudes and smaller time constants of decay (Davis et al., 1998). Consistently, postsynaptic dnc overexpression (with reduced cAMP levels and presumed reduced postsynaptic PKA activity) results in mEJPs that exhibit enhanced amplitudes and larger time constants of decay.
Table 4. Alterations of the properties of whole-cell mEJP's by altered levels of cAMP

<table>
<thead>
<tr>
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<th>cAMP level</th>
<th>PKA activity</th>
<th>mEJP amplitude</th>
<th>mEJP time constant of decay</th>
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<td>Davis et al.</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>smaller</td>
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<td>(1998)</td>
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<td>present study</td>
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Possible ways in which altered cAMP levels could modulate the kinetics of synaptic responses

There may be alternate ways by which altered dnc expression results in altered kinetics of synaptic responses. These effects may be independent of PKA and may not involve the DGluRIIA receptor subunit.

While the Drosophila larval NMJ has been shown to be predominantly glutamatergic (Jan and Jan, 1976; Johansen et al., 1989), very little is known about its glutamate receptor and channel subunit composition. Only two postsynaptic glutamate receptor subunits have been identified at the Drosophila NMJ. These two receptors are named DGluRIIA (Schuster et al., 1991) and DGluRIIB (Petersen et al., 1997) and cannot be classified as either AMPA or kainate types by sequence analysis. They resemble each other more closely than any receptor subunits identified in the mammalian CNS. These two receptors have been shown to be specifically present in somatic musculature and are not present in the nervous system (Currie et al., 1995; Petersen et al., 1997).

Progress in the identification and characterization of glutamate receptors and the role of subunit compositions in synaptic strength has been vigorously pursued in the vertebrate CNS. A wide variety of ionotropic and metabotropic glutamate receptors have been identified in the vertebrate CNS and their roles in the mechanisms underlying synaptic transmission have been the subject of much study (for review see Hollmann and Heinemann, 1994).

Undoubtedly, other currently-unidentified and uncharacterized receptor and channel subunits may exist at the Drosophila NMJ, each of which can possibly be
modulated in ways that differ from the mechanism of receptor modulation that has been shown to date at the *Drosophila* NMJ, implicating PKA and the DGlurIIIA subunit (Davis et al., 1998). In particular, if metabotropic glutamate receptors (mGluRs) or cyclic nucleotide-gated channels (CNGCs, see Finn et al., 1996 for review) exist at the larval NMJ, it is possible that they can be modulated by cAMP and components of its signalling system.

The common feature of mGluRs and CNGCs is that they are G-protein linked. G-protein-linked signal transduction involves the complex interaction of G-proteins with enzymatic effector molecules which through a cascade of complex interactions involving intermediary molecules can result in the modulation of the function of receptors and channels. Potentially, the components of such an intricate system could be modulated by alterations in the cAMP signalling pathway. Form II phosphodiesterase (*dnc*) may be important in the modulation of particular effector molecules and other components of G-protein-mediated signalling pathways of potential mGluRs or CNGCs that may exist at the NMJ. Thus, alterations in the expression of *dnc* could possibly be involved in alterations of intracellular signalling cascades giving rise to altered modulation of receptors and channels, and hence ultimately resulting in perturbed kinetics of postsynaptic responses. While no mGluRs or CNGCs have been identified at the *Drosophila* NMJ, that doesn’t preclude their existence. It is possible that such receptors could exist at the *Drosophila* larval NMJ and that alterations of cAMP levels may have a modulatory effect on these receptors/channels directly or via the modulation of the effector molecules that they are coupled to.
The finding that CNGCs are important for olfactory and visual signal transduction in vertebrates (for review, see Finn et al., 1996) and the observation that null mutations in \textit{dnc} result in olfactory learning deficits in adult \textit{Drosophila} may be related. It has been shown that CNGCs exist in the visual system of adult \textit{Drosophila} (Baumann et al., 1994). Possibly, multiple isoforms of CNGCs could exist at various regions of the CNS of adult \textit{Drosophila} including the olfactory learning centres. The observation that \textit{dnc} has been shown to be heavily expressed in the olfactory learning centres of adult \textit{Drosophila} may indicate that \textit{dnc} is an important modulator of cAMP levels in these regions. Thus, \textit{dnc} may play an important role in the modulation of receptors and channels (by regulating cAMP levels) important for olfactory signal transduction. It has already been shown that \textit{dnc} is present at the \textit{Drosophila} larval nervous system (Cheung et al., 1997). It remains to be shown whether CNGCs or mGluRs do exist at the \textit{Drosophila} NMJ, and whether the modulation of such receptors could be altered as the result of reduced cAMP levels due to \textit{dnc} overexpression.

Consequently, our very limited knowledge of the molecular receptor and channel composition of the \textit{Drosophila} NMJ make it difficult to interpret how \textit{dnc} overexpression and its presumed effects on cAMP level lead to altered kinetics of postsynaptic responses. In order to be able to dissect the molecular mechanisms by which cAMP and components of its signalling system can modulate key postsynaptic molecules to alter the postsynaptic responses to neurotransmitter release, a better understanding of the molecular composition of the postsynaptic target is necessary.
Homeostasis of synaptic transmission

Previous findings suggested that null mutations in dnc result in an enhanced number of nerve endings (Zhong et al., 1992; Davis et al., 1996) and an enhancement of synaptic transmission at the whole-cell level (Zhong and Wu, 1991). Leaving aside alterations in kinetics of mEJCS and EJCs from individual varicosities, the amplitude of these events as a measure of overall synaptic strength is not greatly altered as the result of null mutations in dnc (Renger et al., 1998). Conversely, results from the present study suggest that dnc overexpression results in a reduced number of nerve ending varicosities and in reduced whole-cell synaptic transmission, although synaptic strength at individual varicosities is unaltered. Cumulatively, these findings suggest that an optimal level of dnc expression (and hence an optimal level of cAMP) is needed for normal formation and function of synaptic contacts. This suggests that perturbations in dnc expression are such that they do not allow for compensatory mechanisms to maintain whole-cell synaptic transmission at normal levels.

The results discussed above are in contrast to those found with respect to alterations in the expression of the Drosophila neuronal cell adhesion molecule fasciclin II (FasII). It has been shown that hypomorphic alleles of fasII exhibiting < 10% (Stewart et al., 1996) and ~ 50% of normal FasII levels (Davis et al., 1996), have opposing effects on the formation and function of synaptic contacts.

Mutations in fasII that result in FasII levels that are < 10%, exhibit ~ 40% fewer varicosities at the NMJ (Stewart et al., 1996). Electron microscopical analysis of ultrastructure at these varicosities indicated compensatory mechanisms (in particular,
larger and more complex synapses) which allow for more robust synaptic strength at individual varicosities. Thus, maintenance of whole-cell synaptic transmission is sustained (Stewart et al., 1996).

In contrast, fasII alleles exhibiting ~ 50% of normal FasII levels display an enhanced number of varicosities at the NMJ. However, synaptic transmission at individual varicosities is weaker and displays enhanced degree of failures of synaptic transmission. Therefore, whole-cell synaptic transmission was shown to be maintained here as well (Davis et al., 1996).

The findings from mutations in fasII are in contrast to those found as the result of perturbations in dnc expression with respect to the normal maintenance of synaptic transmission at the whole-cell level. Cumulatively, these studies suggest that perturbations in the normal expression of particular molecules may have effects that do not allow for compensatory mechanisms to take effect in order to sustain whole-cell synaptic transmission. Further, these studies indicate that maintenance of whole-cell transmission, despite perturbations at the molecular level, would be specific to the particular molecule that is being altered, the degree of the perturbation, and the pivotal importance of that molecule to the morphological formation and physiological function of synaptic contacts.
Suggestions for future experiments

In order to better define the effects of dnc overexpression on synaptic strength at the *Drosophila* larval NMJ, whole-cell spontaneous and evoked currents using two-electrode voltage clamp technique should be done to compare with measurements of whole-cell postsynaptic potentials presented in this study. The whole-cell measurement of both evoked potentials and currents could further be improved by measuring postsynaptic responses due to the recruitment of Type Ib axons by themselves. It has previously been shown that Type Ib motor neurons are usually recruited by lower voltage stimuli and give rise to smaller postsynaptic responses compared to their Type Is counterparts (Kurdyak et al., 1994). Since Type Ib varicosities are the commonly physiologically-studied varicosities using the focal loose-patch extracellular recording technique, studying whole-cell potentials and currents that are attributed to neurotransmitter release from Type Ib varicosities alone could be important. This way, one can attempt to correlate physiological data collected at individual Type Ib varicosities with whole-cell responses attributed to the collective recruitment of the same type of nerve ending varicosities. This allows for separately accounting for properties of morphologically- and physiologically-distinct types of varicosities through combined observations at the electrophysiological, and light and electron microscopical levels. Any correlations of findings from the employment of these techniques would be more meaningful when related to specific types of nerve endings.

The electrophysiological experiments suggested above would provide an additional opportunity to assess alterations in amplitude and kinetics of synaptic
responses associated with the presumed reduction of cAMP due to $dnc$ overexpression. In addition, pharmacological application of cAMP analogs in specimens where $dnc$ has been overexpressed, provides for an opportunity to examine whether the pharmacological augmentation of cAMP would mask some of the effects that are being attributed to a transgenic reduction of cAMP.

Further, it should be pointed out that previous studies focusing on whole-cell postsynaptic currents in $dnc$ null mutants did not account for possible differences in the kinetics of these responses. Possible alterations in the muscle fiber properties have not been addressed in these earlier studies either. This would also be important to assess in the $dnc$ null mutants since preliminary evidence from this study (Appendix 1) and omissions from previous work (Petersen et al., 1997; Davis et al., 1998) suggest that the role of possible alterations in the muscle fiber properties should be given more attention when evaluating the mechanisms that underly perturbations in synaptic strength at the $Drosophila$ larval NMJ.

Renger and colleagues (1998) have provided electron microscopical evidence suggesting that null mutations in $dnc$ (and hence elevated cAMP levels) result in more docked vesicles at the synapses in the presynaptic varicosities. They suggest that elevated cAMP levels may have a mobilizing effect on synaptic vesicles (possibly by phosphorylation of synaptic vesicle associated proteins such as synapsin I; shown by Huttner et al., 1983).

It would be interesting to assess whether the postsyaptic or the conjoint pre- and postsynaptic overexpression of $dnc$ would alter the ultrasructural composition of
presynaptic varicosities. If it does, such evidence would strongly complement the physiological evidence presented here, and could indicate that the normal expression of dnc (and hence optimal cAMP levels) plays a pivotal role in the normal formation of synaptic contacts and in normal whole-cell synaptic transmission.
Chapter Five: References


Jan LY, Jan YN (1976b) L-glutamate as an excitatory neurotransmitter at the *Drosophila* larval neuromuscular junction. J. Physiol. 262:215-236.


