MOLECULAR AND GENETIC ANALYSIS OF SYNAPTIC SIGNALING IN

DROSOPHILA

By

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Taryn C. Jackson: _________________________________
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**ABSTRACT**

Molecular and genetic analysis of synaptic signaling in *Drosophila* has yielded many insights into nervous system development, properties of synaptic transmission, and how long-lasting changes in neurons occur. Synaptic signaling components required for synaptic transmission and pathways leading to nervous system plasticity are typically conserved from insects to humans. The role of proteins and genes in synaptic function in flies can be analyzed from the level of a single synapse to complex behaviors in the whole organism. Because of a fully sequenced genome and the ease of mutagenesis in flies, genetic screens have been useful in identifying novel regulators of synaptic transmission and long-term memory.

In flies, conditional mutations affecting synaptic transmission at nerve terminals often lead to temperature sensitive paralysis. In a screen for mutations that interact with *Drosophila shibire*<sup>ts</sup> mutants, the *stoned* gene was identified as a regulator of synaptic vesicle cycling. *Stoned* encodes two neuronally expressed proteins, stonedA and B, which are required for synaptic vesicle recycling and normal synaptic transmission. However, the exact functions of the two stoned proteins are not fully understood. We investigate distinct roles of the stoned proteins here and show that *stoned* has a novel role in synaptic growth.

Memory in flies can be divided into genetically distinct phases based on the requirement for protein synthesis and activation of the transcription factor CREB. Novel regulators of long-term olfactory avoidance memory were isolated in a mutant screen in
flies. Mutants in the *Drosophila* gene *lk6*, homologous to the translational regulator MNK, have defects in long-term olfactory avoidance memory. We find that *lk6* is highly expressed in the fly nervous system, and is activated by and functions downstream of Ras/ERK signaling in fly neurons. Insights provided here from *Drosophila* add to the evidence that MNK may be the link between ERK signaling and the regulation of translation in long-term plasticity.

Ultimately, understanding synaptic function has therapeutic potential to aid in alleviation of nervous system dysfunction. Insight into the molecular pathways underlying plasticity and long-term memory gained from studies in flies, mollusks, and rodents has been pivotal in the development of potential drugs to aid in memory deficits in humans.
CHAPTER ONE
INTRODUCTION

Analysis of the cellular and molecular basis of synaptic signaling in neurons is important in understanding how the nervous system develops, functions, and changes with experience. Genetic analysis of synaptic signaling has been aided by research using Drosophila melanogaster, a model organism that has proven to be a useful tool to identify and characterize genes required for nervous system and synaptic function. Because of the recently sequenced genome and ease of mutagenesis in flies, large scale genetic screens can be performed to identify novel genes and proteins required for various synaptic functions. Characterization of synaptic signaling components can be done at the level of a single synapse by examining their role at the larval neuromuscular junction. The neuromuscular junction in flies is a glutamatergic synapse used to examine a variety of synaptic functions, including synaptic transmission, neurotransmitter release, and developmental and activity-dependent plasticity. In addition to examining how synapses function at the cellular and molecular level, the behavioral output of nervous system function can also be easily studied in flies. The genes, mechanisms, and processes that govern behavior and synaptic development and function in flies are typically conserved in higher vertebrates, and often give insight into nervous system function and dysfunction in humans. Because of the ability to examine the function of a gene from the cellular to behavioral level, Drosophila provides an excellent model organism for the study of nervous system function.
Synaptic vesicle cycling in *Drosophila*

Chemical neurotransmission requires that neurotransmitter be released from a presynaptic cell and bind to receptors on a postsynaptic cell. Neurotransmitters, such as acetylcholine or glutamate, are released in discrete packets of transmitter filled vesicles. These synaptic vesicles are released when an action potential in the presynaptic cell leads to increases in intracellular calcium. Calcium entry through voltage-gated channels is a trigger to promote synaptic vesicle fusion with the plasma membrane and release of neurotransmitter into the synaptic cleft. Synaptic vesicles are then internalized, or endocytosed from the presynaptic membrane, thus allowing recycling of synaptic vesicle membrane and associated proteins. Appropriate recycling of vesicles following transmitter release is necessary for sustained synaptic transmission and communication between neurons.

Many insights into synaptic vesicle cycling in neurons have come from studies in *Drosophila*, since the processes of exo and endocytosis of vesicles are highly conserved across species. For instance, a screen for temperature sensitive mutations that cause paralysis in flies identified the *shibire* mutant (Holden and Suzuki 1973; Poodry, Hall et al. 1973). The *shibire* mutation (the *shi* gene encodes the fly homolog of vertebrate dynamin, a GTPase involved in vesicle endocytosis) blocks synaptic vesicle internalization causing paralysis at restrictive temperatures. (Kosaka and Ikeda 1983; Kosaka and Ikeda 1983; van der Bliik and Meyerowitz 1991). Another mutation, *stoned*, also leads to temperature-sensitive paralysis in flies (Holden and Suzuki 1973). Furthermore, *stoned* enhances the paralysis of *shibire* mutants, indicating that the protein
product may interact with dynamin and play a role in synaptic vesicle endocytosis (Petrovich, Merakovsky et al. 1993). In flies, the stoned locus encodes two proteins, stonedA and stonedB. Both these proteins are expressed in the nervous system and are required for the regulation of synaptic vesicle recycling (Andrews, Smith et al. 1996; Stimson, Estes et al. 1998; Fergestad, Davis et al. 1999; Stimson, Estes et al. 2001). StonedA and B are also thought to interact with the calcium sensor synaptic vesicle protein synaptotagmin, and mutations in stonedA and B block retrieval of synaptotagmin from the plasma membrane and cause defective synaptic transmission (Stimson, Estes et al. 1998; Fergestad, Davis et al. 1999; Phillips, Smith et al. 2000; Fergestad and Broadie 2001; Stimson, Estes et al. 2001). Although both stonedA and B seem vital for synaptic transmission, (only stonedB is conserved in organisms other than insects) the specific contribution of the two distinct stoned proteins to synaptic vesicle cycling and neural transmission is not fully understood. The existence of powerful genetic tools in Drosophila, permits a dissection of the roles of the stonedA and B proteins in synaptic function.

**Conserved signaling pathways regulate long-term plasticity at the larval neuromuscular junction**

Post-mitotic neurons possess the ability to undergo changes in the strength and arborization of their synaptic connections. Changes in neurons that occur during development of the nervous system often utilize mechanisms similar to those used during experience dependent plasticity. Signaling pathways and cellular changes during these phenomena are broadly conserved in mammalian neurons. For instance, the larval
neuromuscular junction (NMJ) in flies exhibits large increases in growth of synaptic connections and neurotransmitter release throughout development to accommodate a growing postsynaptic muscle. These changes require synaptic activity and signaling pathways that are also required for models of plasticity in other systems, such as long-term potentiation (LTP) in the mammalian hippocampus and long-term facilitation (LTF) at the *Aplysia* sensory-motor synapse. At the NMJ, neural activity recruits signaling through second messenger cascades (such as cAMP and Ca\(^{2+}\)) leading to increased evoked transmitter release and synaptic arborization. Signaling in these neurons finally impinges on kinases (ERK, PKA) and their cognate transcription factors (CREB, AP1 etc.) (Budnik, Zhong et al. 1990; Zhong and Wu 1991; Davis, Schuster et al. 1996; Koh, Ruiz-Canada et al. 2002; Sanyal, Sandstrom et al. 2002). Additionally, the involvement of translation factors eIF4E and PABP activity in specific synaptic domains to regulate synaptic growth and evoked transmitter release, indicates that plasticity at the NMJ is also modulated at the level of translation (Sigrist, Thiel et al. 2000). Similarly, in the mammalian hippocampus and the *Aplysia* sensory-motor synapse, activation of cAMP and Ras/ERK signaling, along with activation of the transcription factor CREB, are also required for long-term changes in synaptic connections (Bartsch, Ghirardi et al. 1995; Martin, Michael et al. 1997; Bartsch, Casadio et al. 1998; Michael, Martin et al. 1998; Barco, Alarcon et al. 2002). Long-term plasticity in the hippocampus and *Aplysia* is also regulated at the translational level, since both the translation of newly transcribed mRNAs and pre-existing mRNAs present at synapses are required for long-term synaptic
changes in neurons (Frey, Krug et al. 1988; Bailey, Montarolo et al. 1992; Frey and Morris 1997; Casadio, Martin et al. 1999).

Conserved molecular mechanisms for long-term memory

Underlying learning, memory, and behavioral plasticity in flies and in other organisms, are changes in the synaptic function and growth of connections in individual neurons or circuits in the brain. Thus, mechanisms that regulate synaptic function and synaptic changes may give insights into the molecular and cellular basis for memory. In support of this, biochemical signaling pathways that govern the control of synaptic function and growth during development are also required for behavioral plasticity. In both vertebrates and invertebrates, short-term memory can be distinguished from long-term memory based on the requirement for new gene expression and protein synthesis (Bailey, Bartsch et al. 1996). In many organisms, long-term memory may be elicited by longer training sessions separated by rest intervals, while single, brief training sessions result in short-term memory. In experimental models of memory in animals, long-term memory elicited by spaced training sessions is blocked by the application of protein synthesis inhibitors. Additionally, cellular models for analyzing the long-term changes in neurons thought to underlie memory such as LTP in the mammalian hippocampus and LTF in the Aplysia sensory motor synapse also exhibit distinct short-term and long-term phases (Kandel 2001). In both systems, spaced stimulation results in long-term strengthening and growth of synapses that can be blocked by protein synthesis inhibitors, while single stimulation results in short-term synaptic strengthening that does not require protein synthesis (Frey, Krug et al. 1988; Bailey, Montarolo et al. 1992). These findings
indicate that short-term changes in synapses result from modification of existing molecules and proteins, while long-term changes require new protein synthesis to occur. Based on the large body of evidence indicating behavioral and cellular differences underlying short and long-term memory, there has been much investigation to identify molecules that may be differentially required for short-term and long-term forms of plasticity and memory.

One of the best studied assays for analyzing memory in *Drosophila* is the associative olfactory conditioning procedure. When an odor is paired with an aversive shock stimulus, flies learn to avoid the odor when later exposed to it without a shock. A single pairing of shock and odor results in short-term memory lasting several hours, while multiple pairings of shock and odor result in long-term memory that can last for days in flies (Figure 1.1) (Dubnau and Tully 1998). A variety of signaling pathways have been shown to be required for the formation of olfactory avoidance conditioning in flies, however, blocking protein synthesis and manipulations in the transcription factor CREB gene have been shown to specifically affect long-term memory. This indicates that short-term and long-term memory are genetically separable phases in *Drosophila*. Feeding of the protein synthesis inhibitor cycloheximide to flies has no effect on memory immediately after training, but significantly decreases memory several hours after a spaced training session (Tully, Preat et al. 1994). Cychoheximide also has no effect on memory several hours after massed training sessions in which flies receive multiple odor and shock pairings that are not separated by rest intervals, indicating that the long-term, protein synthesis dependent phase of memory requires spaced training. Additional
studies showed that overexpression of a CREB isoform that inhibits CREB function in flies reduces memory after spaced training to a similar level as protein synthesis inhibition (Yin, Wallach et al. 1994). Overexpression of a CREB activator that increases CREB function allowed a massed training trial to elicit longer lasting memory that was protein synthesis dependent (Yin, Del Vecchio et al. 1995). These studies indicate in flies, long-term memory is genetically distinct from short-term memory and that there are potentially many genes whose transcription and translation are required in order for long-term memories to form.

**Identification of candidate long-term memory genes using *Drosophila***

Because of the established paradigms for inducing genetically distinct phases of short and long-term memory and the ability to perform large scale mutagenesis screens, *Drosophila* has proven to be useful in discovering novel genes required for long-term memory. Recently, a genetic screen conducted by Tim Tully and colleagues identified 60 *Drosophila* mutant lines that have specific defects in long-term associative olfactory avoidance memory (Dubnau, Chiang et al. 2003). In the candidate long-term memory mutants, the protein synthesis and CREB dependent phase of memory 24 hours after a spaced training procedure was greatly reduced, while memory one hour after training was normal. Additionally, all mutants displayed normal shock and odor reactivity, indicating their deficits were due to the pairing of odor and shock and not an inability to react normally to either stimulus.

In parallel, DNA microarrays were performed to identify candidate memory genes that are differently expressed with long-term memory formation (Dubnau, Chiang et al.
2003). Transcript profiles in fly heads with spaced olfactory conditioning training, resulting in protein synthesis and CREB dependent long-term memory in flies, were compared to transcripts expressed with massed training, which does not lead to long-term memory formation. Using this comparison, 42 candidate memory genes were identified to be transcriptionally regulated in fly heads with long-term memory formation.

Interestingly, many of the candidate memory genes identified using these parallel approaches have known roles in translation of mRNA. For example, both methods revealed that the translational repressor *pumilio* may be specifically required for long-term memory. Additionally, DNA microarrays showed that the translational regulators *staufen* (involved in mRNA localization), *moesin* (required for staufen localization), *orb* (Drosophila CPEB), and *eIF2G* (a translation initiation factor) are expressed during long-term memory formation. In the memory mutant screen, mutants potentially affecting *oskar*, *eIF5C*, and *lk6* were deficient in long-term olfactory avoidance memory. One mutant that displayed memory defects in this screen, named *ikar*, is a mutant in the *Drosophila* gene *lk6*, which encodes a protein kinase homologous to the mammalian translational regulator MNK. *ikar* mutants have memory levels similar to wild type one hour after spaced training, but only 12% of wild type levels at 24 hours after training, indicating that the *lk6* gene may be required for long-term memory in *Drosophila* (Figure 1.2).
Drosophila LK6 is homologous to mammalian MNK, an eIF4E kinase that has a role in translation initiation

In Drosophila, LK6 was identified as a microtubule binding protein in embryos and has been shown to regulate Ras signaling in eye development (Kidd and Raff 1997; Huang and Rubin 2000). Much more is known about the mammalian homolog of LK6, MNK. MNK was identified as a MAPK interacting kinase and was later discovered to have 2 forms, MNK1 and MNK2 (Fukunaga and Hunter 1997; Waskiewicz, Flynn et al. 1997). MNK is phosphorylated by ERK and p38 MAPK, which allows it to phosphorylate eIF4E, a translation factor that initiates translation by binding the 5’ cap of mRNAs (Waskiewicz, Johnson et al. 1999). MNK1 seems to be responsible for inducible phosphorylation of eIF4E, while MNK2 functions to phosphorylate eIF4E at a basal level (Scheper, Morrice et al. 2001; Ueda, Watanabe-Fukunaga et al. 2004). In mammalian systems, MNK1 comes in contact with eIF4E when both are bound to another translation factor, eIF4G (Pyronnet, Imataka et al. 1999). Phosphorylation of eIF4E at Ser209 by MNK1 results in enhanced translation (Scheper and Proud 2002). eIF4E binds to mRNAs and associates with other factors necessary for translation, including the ribosome binding translation factor eIF3 and the polyA binding protein (PABP) that is bound to 3’ polyA tails of mRNAs (Figure 1.3A) (Raught and Gingras 1999; Pyronnet 2000). LK6 in flies has recently been shown to act in a similar way to MNK1. When expressed in human cell lines and Drosophila S2 cells, LK6 binds to ERK, its activity is increased with ERK signaling, and it is able to phosphorylate eIF4E (Parra-Palau, Scheper et al. 2004).
Signaling pathways leading to new protein synthesis in long-term plasticity and memory

Long-term memory and long-lasting modifications in synapses require gene expression and the synthesis of new proteins. New protein synthesis is often regulated at the transcriptional level as indicated by the key role that many transcription factors play in converting short-term to long-term plasticity. In rodents, *Aplysia*, and flies, one of the key regulators of long-term memory and plasticity is the transcription factor CREB. The induction of CREB can often convert short-term plasticity and memory into long-term, and blocking CREB function prevents lasting memory and synaptic change. Increasing evidence has implicated new protein synthesis regulated at the translational level as a mechanism for long-term neuronal plasticity as well. Specifically, local protein synthesis in neurons is thought to be one mechanism by which synaptic activity may mark certain synapses to undergo plastic changes (Martin, Barad et al. 2000). In mammalian neurons, mRNAs are targeted to dendrites and neuronal activity can induce the translation of these mRNAs (Aakalu, Smith et al. 2001). Local dendritic translation occurs even when the cell body is separated from the dendrites and is required for long-term synaptic changes observed in both Aplysia LTF and hippocampal LTP (Frey and Morris 1997; Martin, Casadio et al. 1997; Bradshaw, Emptage et al. 2003).

Cytoplasmic polyadenylation is one proposed mechanism for the regulation of translation of specific local mRNAs (Mendez and Richter 2001). Cytoplasmic polyadenylation elements (CPEs) are found in the 3’ untranslated regions of specific mRNAs (such as CaMKII) and are recognized by cytoplasmic polyadenylation element
binding protein (CPEB). In the case of CaMKII translation, neuronal activity causes the phosphorylation of CPEB by Aurora kinase, which results in polyadenylation of the mRNA and displacement of Maskin, an inhibitor of eIF4E (Wu, Wells et al. 1998). Polyadenylation allows PABP to bind to the polyA tail and associate with 5’ translation factors eIF4E and eIF4G which then initiate translation of CaMKII.

Recent evidence has highlighted a more general mechanism for translational regulation during synaptic plasticity through activation of the translational machinery, namely the translation initiation factor eIF4E. As stated previously, ERK and p38 MAPKs phosphorylate MNK1, which phosphorylates eIF4E and induces translation. Additional regulation of eIF4E occurs through the mammalian target of rapamycin (mTOR) pathway (Figure 1.3B). mTOR phosphorylates eIF4E binding proteins (4E-BPs), which normally bind and inhibit the function of eIF4E (Gingras, Raught et al. 2001). Both ERK and mTOR signaling pathways are induced by neural activity and contribute to synaptic plasticity. ERK is activated in neurons and is required for many types of long-term synaptic plasticity and memory (Thiels and Klann 2001; Adams and Sweatt 2002). Although ERK has typically been shown to regulate long-term plasticity through its activation of transcription factors (Impey, Obrietan et al. 1998), increasing evidence from studies in mammals shows that ERK may also directly control translation in long-term plasticity. A recent study shows that stimulation of hippocampal neurons induces a phase of LTP that requires translation but not transcription which correlates with the activation of eIF4E and is ERK dependent (Kelleher, Govindarajan et al. 2004). Although not investigated directly, MNK1, as the ERK activated eIF4E kinase, is the
most likely link between ERK and the regulation of translation factors in plasticity. In support of this, application of BDNF or activation of NMDA receptors in the hippocampus leads to increases in activation of MNK1 and eIF4E, both of which are ERK dependent (Takei, Kawamura et al. 2001; Banko, Hou et al. 2004). Similarly, late phases of hippocampal LTP and Aplysia LTF can be blocked by rapamycin, indicating that eIF4E regulation by mTOR signaling is also required for long-term plasticity (Tang, Reis et al. 2002).

**LK6 as a potential regulator of translation required for long-term memory and synaptic plasticity**

The ability to study plasticity at the larval NMJ, a neuronal preparation that undergoes synaptic changes in response to neural activity and manipulations in translational regulators, makes *Drosophila* an ideal system for studying novel genes that are required for translational control of synaptic plasticity. The fly larval NMJ is highly accessible to genetic, cell biological, and electrophysiological analysis. The pre and postsynaptic cells can be easily distinguished and separated genetically by overexpressing or deleting genes in neurons or muscles. The ease of genetic manipulation in flies through loss of function mutants and tissue specific transgene expression also enables genetic epistatsis and interaction studies to determine how novel genes fit into known plasticity and translational regulation pathways. Additionally, the ability to activate conserved synaptic signaling pathways in the *Drosophila* nervous system allows both genetic and biochemical studies to determine potential upstream regulators and downstream effectors of novel genes (Hoeffer, Sanyal et al. 2003).
Because of the potential role of LK6 in translation as an eIF4E kinase and the likelihood that it links ERK to translational regulation, it is useful to examine LK6 function in the fly nervous system. I initially found that the long-term memory mutant \textit{ikar} has low levels of \textit{lk6} expression and is deficient in LK6 protein (Figure 1.4A and B). At the NMJ, \textit{ikar} mutants show reduced synaptic size and strength, a phenotype we hoped would give insight into the mechanism underlying the long-term memory deficit in \textit{ikar} mutants (Figure 1.4C). However, I was unable to map the \textit{ikar} synaptic phenotype to loss of \textit{lk6} function, since blocking \textit{lk6} function with other \textit{lk6} mutants and expressing double stranded RNA to knockdown \textit{lk6} transcripts do not have similar synaptic phenotypes at the NMJ (Figure 3.2A). Therefore, we are unable to determine if \textit{ikar} affects long-term memory and synaptic plasticity at the NMJ due to the loss of \textit{lk6} function, or an unknown modifier in the \textit{ikar} mutant background. However, subsequent studies with \textit{lk6} in the fly nervous system have shown that \textit{lk6} likely functions downstream of ERK to regulate synaptic plasticity.

Although a great deal is known about the way neurons communicate, change during development or with experience, and the behavioral output of these synaptic changes, there are still many gaps in our understanding of synaptic function and plasticity in the nervous system. Because of the ease of genetic analysis, conserved synaptic signaling, and ability to observe effects of genetic manipulation from the cellular to behavioral level, \textit{Drosophila} offers a highly tractable system to aid in understanding of nervous system function. Separate genetic screens in flies have identified the \textit{stoned} gene as a potential regulator of synaptic vesicle cycling, and the \textit{lk6} gene as a potential
regulator of synaptic plasticity and long-term memory. Further analysis to examine the role of stoned in synaptic function at the larval NMJ and lk6 in synaptic signaling and plasticity is presented here.

Ultimately, our knowledge of how neurons function and change with experience from model systems is useful not only in understanding normal nervous system function, but also aids in the development of drugs and therapies to treat nervous system dysfunction in humans. The recently expanding knowledge of molecules required for neuronal synaptic changes and memory from mammals, mollusks, and insects has made the development of drugs to treat memory disorders possible. The majority of drug targets are aimed at the CREB pathway, however, upstream regulators and downstream effectors of CREB signaling and other parallel pathways may also prove to be potential targets for memory enhancing drugs. The study of nervous system function and how synapses change with experience and influence behavioral output in flies has and will continue to be critical to the possibility of memory modification in humans in the future.
Figure 1.1
Olfactory avoidance memory in *Drosophila* has both short-term and long-term phases.

When flies are exposed to an odor that is paired with a shock, they will avoid the odor when it is presented later. Single pairings of odor and shock result in a short-term memory that decays within 24 hours and is not blocked by protein synthesis inhibitors and does not require the function of CREB. Multiple pairings of shock and odor without rest intervals (massed training) induces a stronger memory lasting up to 3 days that also does not require protein synthesis and CREB, indicating that olfactory avoidance memory from single pairing and massed training relies on modifications in existing proteins. Multiple pairings of shock and odor separated by rest intervals (spaced training) leads to long-term memory lasting up to 7 days. Olfactory avoidance memory after spaced training is blocked when flies are fed protein synthesis inhibitors, or in flies expressing an inhibitory CREB isoform, indicating that spaced training induces long-term memory that requires gene expression and protein synthesis.
Olfactory Avoidance Conditioning

Odor 1  Odor 2

Shock

- single pairing
- massed training (10 pairings)
- spaced training (10 pairings with rest intervals)

Odor 1 vs. Odor 2
- Modifications in pre-existing proteins

Short-term memory (minutes to hours)

Odor 1 vs. Odor 2
- CREB dependent gene expression and protein synthesis

Long-term memory (hours to days)
Figure 1.2

In *ikar* mutants, long-term memory is disrupted, while short-term memory remains normal. After training in olfactory conditioning, flies are given a choice between the odor that has been paired with a shock and the unpaired odor. A performance index (PI) is calculated based on the distribution of flies between the 2 odors. A PI of zero indicates that flies distribute themselves equally between the 2 odors, while a PI of 100 indicates that 100% of the flies avoid the paired odor. Immediately after spaced training (0 hour retention time), *ikar* mutants display levels of olfactory avoidance memory similar to wild type flies (both show PIs of approximately 80). Olfactory avoidance memory immediately after spaced training is not blocked by protein synthesis inhibitors and does not require CREB. 24 hours after spaced training, *ikar* mutants have only 12% of wild type memory levels, indicating that the mutation in *ikar* interferes with the protein synthesis and CREB dependent phase of long-term memory. Figure modified from Tim Tully.
Figure 1.3

The regulation of eIF4E leading to translation.

A. MNK is phosphorylated by p38 and ERK. Activated MNK comes into contact with eIF4E when they are both bound to eIF4G, which allows MNK to phosphorylate eIF4E. eIF4G is also bound to eIF3. eIF3 recruits the ribosome to begin translation of mRNA. PABP, which is associated with the polyA tail of mRNA, also interacts with eIF4G.

B. Signaling pathways leading to eIF4E activation. ERK signaling regulates the phosphorylation of eIF4E. ERK activates MNK, which allows it to activate eIF4E when both are bound to eIF4G. eIF4E function is also regulated by mTOR signaling. The function of eIF4E is inhibited when it is bound to 4E-BP, which blocks the eIF4G binding site on eIF4E. Phosphorylation of 4E-BP with mTOR signaling causes the release of eIF4E from 4E-BP so that it can associate with eIF4G and bind to the 5’ cap of mRNA to initiate translation. mTOR signaling also activates eIF4G.
Figure 1.4

Synaptic phenotypes of *ikar* mutants.

A. The *ikar* mutation causes reduced levels of *lk6* transcripts. The *lk6* gene produces 2 transcripts, *lk6*-A and *lk6*-B. Using quantitative PCR to measure levels of gene expression in fly heads, *ikar* mutants have decreased levels of *lk6*-A mRNA, but not *lk6*-B when compared to wild type.

B. LK6 protein levels are reduced in *ikar*. The 2 transcripts in *lk6* encode two proteins, LK6-A and LK6-B. In Western blots from fly head extracts, an antibody to LK6 recognizes 2 proteins corresponding to LK6-A and B in wild type flies. The *ikar* mutation decreases LK6-A levels, while LK6-B remains normal. Dynamin is used as a loading control.

C. *ikar* mutants show reduced synaptic size and strength at the larval NMJ. Synaptic size, as measured by bouton number on larval bodywall muscles 6 and 7 of the 2nd abdominal segment A2, is 72% of wild type bouton number (n=22, p=0.002). Synaptic strength, as measured by excitatory junctional potential (EJP) in muscle 6 of segment A2 with stimulation of the motor nerve, is 80% of wild type EJPs (n=9, p=0.03).
CHAPTER TWO

FUNCTIONAL DISSECTION OF A EUKARYOTIC DICISTRONIC GENE:

TRANSGENIC STONEDB, BUT NOT STONEDA, RESTORES NORMAL
SYNAPTIC PROPERTIES TO DROSOPHILA STONED MUTANTS

Note from author: The material in this chapter was derived from work that I participated in but was not primary contributor to. Patty Estes performed much of the work in this study, including generation of transgenic fly lines and immunocytochemistry. My contribution to this study mainly involved electrophysiological analysis of stoned mutants and rescued transgenic lines. I have included text and figures from the original manuscript in this dissertation with permission from the publisher and the article’s primary author.

Abstract

The dicistronic Drosophila stoned mRNA produces two proteins stonedA and stonedB that are localized at nerve terminals. While the stoned locus is required for synaptic vesicle cycling in neurons, distinct or overlapping synaptic functions of stonedA and stonedB have not been clearly identified. Potential functions of stoned products in non-neuronal cells remain entirely unexplored in vivo. Transgene-based analyses presented here demonstrate that exclusively neuronal expression of a dicistronic stoned cDNA is sufficient for rescue of defects observed in lethal and viable stoned mutants. Significantly, expression of a monocistronic stonedB trangene is sufficient for rescuing
various phenotypic deficits of stoned mutants including those in organismal viability, evoked transmitter release and synaptotagmin retrieval from plasma membrane. In contrast, a stonedA transgene does not alleviate any stoned mutant phenotype. Novel phenotypic analyses demonstrate that in addition to regulation of presynaptic function, stoned is required for regulating normal growth and morphology of the motor terminal; however, this developmental function is also provided by a stonedB transgene. Our data, though most consistent with a hypothesis in which stonedA is a dispensible protein, is limited by the absence of a true null allele for stoned and due to partial restoration of presynaptic stonedA by transgenically provided stonedB. Careful analysis of effects of the monocistronic transgenes together and isolation clearly reveals that the presence of presynaptic stonedA is dependent on stonedB. Together, our findings improve understanding of the functional relationship between stonedA and stonedB and elaborate significantly on the in vivo functions of stonins, recently discovered, phylogenetically conserved stonedB homologs that represent a new family of “orphan” medium (μ) chains of adaptor complexes involved in vesicle formation. Data presented here also provide new insight into potential mechanisms that underlie translation and evolution of the dicistronic stoned mRNA.

Introduction

The Drosophila stoned locus generates an unusual dicistronic message with two open reading frames ORF1 and ORF2 that are separated by a 55 nucleotide interval containing termination codons in all alternative reading frames. ORF1 encodes a 850
residue protein termed stonedA and ORF2 a 1260 residue protein termed stonedB (Andrews, Smith et al. 1996). The locus has received considerable attention not only for its curious discistronic organization, but also because stonedA and stonedB proteins appear to have important functions in regulating synaptic vesicle trafficking at the presynaptic terminal (Blumenthal 1998; Fergestad and Broadie 2001; Robinson and Bonifacino 2001; Stimson, Estes et al. 2001).

At nerve terminals, stimulus-evoked calcium entry triggers transmitter release through rapid, regulated exocytosis of readily releasable synaptic vesicles. Vesicle proteins, including fusion proteins and neurotransmitter pumps, so deposited on plasma membrane are then retrieved via endocytosis and recycled locally to form new synaptic vesicles. During membrane retrieval from the plasma membrane, adaptor proteins bind cytosolic tails of synaptic vesicle proteins such as synaptotagmin and cluster them into microdomains from which nascent endocytic vesicles first bud and then detach via sequential and concerted actions of several proteins including clathrin, intersectin/DAP160, Eps15, dynamin and others (Zhang and Ramaswami 1999; Slepnev and De Camilli 2000). The classical plasma membrane adaptor complex AP2 involved in initial recognition of internalized molecules contains two large subunits α and β, a medium subunit μ2 and a small subunit σ2; three other homologous adaptor complexes AP1, AP3 and AP4 are similarly organized as tetramers, each containing subunits homologous to the large, medium and small chains of AP2 (Hirst and Robinson 1998; Robinson and Bonifacino 2001).

The involvement of stoned proteins in membrane retrieval at the nerve terminal is
indicated by several observations. First, stonedA and stonedB are highly enriched at presynaptic nerve endings (Stimson, Estes et al. 1998; Fergestad, Davis et al. 1999; Stimson, Estes et al. 2001). Second, they bind the synaptic-vesicle protein synaptotagmin (Phillips, Smith et al. 2000). Third, mutations in stoned that alter expression of both stonedA and stonedB cause substantial defects in synaptic vesicle recycling: thus, stoned mutations cause defective, delayed retrieval of synaptic-vesicle proteins, aberrant synaptic-vesicle size, and defective synaptic transmission (Stimson, Estes et al. 1998; Fergestad, Davis et al. 1999; Fergestad and Broadie 2001; Stimson, Estes et al. 2001). The mechanism by which stoned proteins participate in vesicle cycling is less clear. While both stonedA and stonedB sequences contain motifs consistent with direct roles in vesicle traffic (Andrews, Smith et al. 1996; Stimson, Estes et al. 1998), only stonedB, with orthologs in C. elegans and mammals (Upadhyaya, Lee et al. 1999; Martina, Bonangelino et al. 2001; Walther, Krauss et al. 2001) is strongly conserved across phylogeny; stonedA is not obviously conserved outside insects and perhaps non-insect arthropods. Together with evolutionary conservation, the very strong homology of stonedB homologs to μ subunits (medium chains) of adaptor proteins has led to an economical, but untested hypothesis that synaptic defects observed in viable and lethal mutant alleles of stoned predominantly reflect cellular functions of stonedB.

StonedB and its homologs, together termed the “stonin” family, are orphan adaptor μ chains. Eukaryotic genomes do not encode obvious partner subunits (similar orphan large and small chains) with which stonins may assemble into a new class of adaptor (Robinson and Bonifacino 2001). Thus, stonedB and its homologs are likely to
function by a mechanism different from conventional μ-chains. Stonins contain extended C-terminal domains conserved among μ subunits of tetrameric AP complexes. In addition, they contain an N-terminal proline and serine rich domain and share a unique central 140 residue “stonin homology domain” not found in μ-chains of the four known adaptor complexes (Martina, Bonangelino et al. 2001). Like the *C. elegans* ortholog encoded by gene C27H6.1 and stonedB, mammalian stonin2 (but not the second homologous stonin1) contains multiple NPF motifs that may mediate its documented binding to EH domains of Eps15, Eps15R and intersectin (Martina, Bonangelino et al. 2001). Like *Drosophila* stonedB, stonin2 also binds to synaptotagmin. Some insight into the mechanism of stonin function in endocytosis is suggested by the observation that human stonin2 facilitates the uncoating of clathrin-coated vesicles *in vitro*, potentially by displacing the adaptor AP2 from its binding sites on vesicle proteins (Walther, Krauss et al. 2001).

Several issues remain to be resolved in order to better understand functions of the stoned gene in particular, and of stonins in general. First, because stonins may be ubiquitously expressed in all cell types (Martina, Bonangelino et al. 2001), it remains unclear if they are general components of endocytosis, or proteins required only for specific forms of neuronal endocytosis. Second, the specific stoned product(s) whose deficiency underlies physiological and morphological defects observed in stoned mutant synapses (Stimson, Estes et al. 1998; Fergestad, Davis et al. 1999; Fergestad and Broadie 2001; Stimson, Estes et al. 2001) has yet to be identified. Finally, the origin, significance and regulation of the discistronic stoned mRNA remains mysterious.
Experiments and observations discussed here address these issues.

**Materials and Methods**

* Cultures and stocks

Drosophila cultures were maintained at 21°. Wild-type was Oregon-R (ORCB; from Danny Brower, University of Arizona). The strain used for the germline transformation was w1118. The strain used for mapping and balancing transgenes: yw67C2; Sp/SM5,Cy; Sb/TM3,Ser was also obtained from the Brower Lab.

Stoned mutants stn+, and stn13-120, were from our collection and stn8P1 was obtained from Norbert Perrimon (HHMI, Harvard Medical School). stn+ was maintained as a homozygous stock whereas stn8P1 and stn13-120 were maintained over the FM7i balancer chromosome (Bloomington Stock Center) which carries the P-element P[w[mC]=ActGFP]JMR3, encoding GFP under the cytoplasmic actin promotor. For larval dissections, stoned males could be distinguished from FM7i males by lack of green fluorescence using a Leica MZ6 stereo microscope outfitted with a GFP fluorescence illuminator (Kramer Scientific Corp., Elmsford, NY). stn8P1 was also maintained over a modified Y chromosome Dp(1,Y)y+mal+ (abbreviated Dp) which contains a stoned duplication.

The neuronal Gal4 driver line elavC155 and the muscle driver line MHC Gal4 were from Corey Goodman’s lab (University of California, Berkeley). The 684 wing disc driver (Manseau, Baradaran et al. 1997) was from Danny Brower’s Lab. We isolated elavC155 stn flies by genetic recombination. The UAS-synaptotagmin transgenic line
UAS-synaptotagmin I (autosomal) was obtained from Troy Littleton (MIT) and the yw;
P[w+UAS-Syt+] (III) line was a gift of Noreen Reist (Colorado State University).

Construction of stoned transgenes

The complete stoned cDNA was obtained by ligation of fragments from three different cDNA clones. Clone “p33” contained an EcoRI fragment of bases 1-1219 of the published sequence. Clone “fused” contained an EcoRI/PstI fragment of bases 1220-5808 and the third “4.8z3” contained a PstI fragment of bases 5809-8137. The full-length cDNA, named JC9814, was inserted into pBluescript SK+ by ligating p33 into the fused plasmid with EcoRI, and ligating the 4.8z3 sequence in using PstI. For use in transgene rescue, 1.4 kb of the original 1.6 kb 3’ UTR was removed. Briefly, JC9814 was digested with PstI and NotI (from the polylinker) to remove bases 5809-8137. Primers were designed to amplify only from the PstI site at 5809 to base 6714. The reverse primer had an artificial NotI site included for cloning purposes. This amplified product was ligated into the digested JC9814 and was named TM001. This stoned cDNA, containing the entire coding sequence but only 211 bases of 3’ untranslated sequence, was cloned into the transformation vector pUAST (Brand and Perrimon, 1993) in a two-step process using the EcoRI and NotI restriction sites.

Separate stonedA and B constructs were prepared in the laboratory of Kathleen Buckley from TM001. The full-length cDNA was digested with XbaI and NotI, removing all of stonedB sequence and all sequences 3’ of base 2446 in stonedA. Two primers were designed to amplify a product to replace the missing 3’ fragment of stonedA. The forward primer incorporated the 2446 XbaI site and a reverse primer replaced the AclI
site at 2665 and the first termination codon at 2670 with an additional engineered stop codon and a NotI site (tcgaacgttaataagcggcca). We were then able to ligate this entire stonedA sequence directly into pUAST using EcoRI and NotI. The Buckley lab prepared the stonedB fragment by digesting the full-length cDNA with AclI (2665) and Scal (6550), a region which included the five intercistronic termination codons and the entire stoned B coding sequence with 46 bases of 3’ UTR. The sites were then filled to make blunt ends and was cloned using EcoRV. We cloned this sequence into pUAST using EcoRI and NotI sites in the polylinker. We removed the intercistronic region of stop codons from the 5’ end of this construct by amplifying a region from the ATG Start site of stoned B (2724) to the PpuMI site (3962). In this case, the forward primer contained an artificial EcoRI site so that this new fragment could be cloned directly into the plasmid using the existing EcoRI and PpuMI sites. Both the stonedA and stonedB constructs in pUAST were sequenced to confirm that no errors were introduced during PCR amplification or cloning.

The stonedAB, stonedA and stonedB constructs were respectively used to create transgenic $P\{SAB\}$, $P\{SB\}$, and $P\{SA\}$ lines using P-element mediated embryonic germline transformation. Two independent SAB lines were obtained along with 23 SA lines and 22 SB lines. SAB1, SB5, and SA20 were used for the majority of the experiments.

*Experimental and control animals for transgene rescue analysis*

$elav^{C155\text{ lethal}} stn^C/ FM7i$ or $elav^{C155\text{ lethal}} stn^C$ homozygous virgin females were crossed to yw/Y; $P\{stnX\}$ homozygous transgenic males. To analyze phenotypic rescue by a
given transgene, male progeny of the genotype \( \text{elav}^{C155} \text{stn}/Y; P[\text{stn}]/+ \) were selected and studied. In the case of lethal alleles, males carrying the balancer were discarded. These animals were then compared to \( \text{ORCB} \) and \( \text{elav}^{C155} \text{stn}/Y; +/+ \) animals obtained from a cross to \( \text{yw}/Y \) males from the background strain used to establish the transgenic lines. To generate \( \text{stn}^{8P1} \) mutant larvae, \( \text{stn}^{8P1}/\text{Dp} \) males were crossed to \( \text{yfC(1)DX}/Y \) females, yielding males of the genotype \( \text{stn}^{8P1}/Y \). These males survive at a very low frequency (~5 %) and are developmentally delayed and smaller than their female siblings. (We were not able to generate \( \text{elav}^{C155} \text{stn}^{8P1}/Y \) males as escapers were never seen.)

In order to assess rescue using ubiquitous or muscle specific enhancers, we used the \( \text{shi Gal4} \) line (Staples and Ramaswami 1999) and the muscle-specific driver \( \text{MHC Gal4} \) (Sanyal and Ramaswami 2002) lines respectively. For each stoned allele, \( \text{stn}/FM7i; \text{Gal4}/\text{Gal4} \) homozygous lines were generated and crossed to males homozygous for the transgene of interest. Again, efficiency of rescue was determined by comparing \( \text{stn} \) progeny from this cross, with those obtained when similar females were crossed to transgene-free \( \text{yw}/Y \) males.

**Analyzing DsyI transgenes for rescue of stn lethality**

Rescue of stoned lethal phenotypes by neuronal or ubiquitous over-expression of \( \text{Drosophila} \) synaptotagmin I was assessed in \( \text{stn}^{8P1} \) and \( \text{stn}^{13-120} \). Stoned double mutants containing either \( \text{elav}^{C155} \) or \( \text{shi} \text{GAL4} \) were crossed to either \( \text{UAS-synaptotagmin I} \) (autosomal) males or \( \text{yw}; P[w^+UAS-Syt^+] \) (III) males and raised at 25\(^\circ\). The progeny of these crosses were examined for the presence of \( \text{stn}/Y \) males. No adult rescue was detected with either transgene, with either driver, in either mutant background (total
Contrasting observations were previously reported when a different Gal4 driver was used (Fergestad and Broadie 2001).

**Immunocytochemistry and confocal microscopy**

Wandering third instar larvae were dissected to expose the abdominal body wall muscles, as described previously (Estes, Roos et al. 1996; Stimson, Estes et al. 1998; Stimson, Estes et al. 2001). In brief, larval dissections were performed in Ca\(^{2+}\)-free HL3 saline (Stewart, Atwood et al. 1994), containing 0.5 mM EGTA and 21.5 mM MgCl\(_2\), in order to prevent muscle contraction. Analyses were restricted to synapses of muscles 6 and 7 of abdominal segments 2 - 3 (A2-A3). Dissected larvae were fixed in 3.5% paraformaldehyde and processed for antibody staining. Wing discs were prepared from larvae processed as above, except that they were visualized using FITC-conjugated goat anti-rabbit secondary antibodies (ICN Biochemicals, Costa Mesa, CA) at a dilution of 1:200. They were removed from the preparation just prior to imaging and placed on slides treated with a diluted poly-lysine solution (Sigma Chemical Company, St. Louis, MO). A PCM 2000 laser-scanning confocal microscope (Nikon, Melville, NY) and SimplePCI software (C Imaging, Cranberry Township, PA) was used for image acquisition.

StonedA antiserum (Andrews, Smith et al. 1996; Stimson, Estes et al. 1998), StonedB antiserum (3500) (Stimson, Estes et al. 2001) were used at a final dilution of 1:1000. Anti-stonedB and anti-stonedA were visualized using an Alexa 568 goat anti-rabbit antibody (Molecular Probes, Eugene, OR) at a dilution of 1:1000. For examining the distribution of synaptotagmin within synaptic boutons, we stained the larval
preparation with rabbit anti-syt antibody (DSYT2, from Hugo Bellen, Baylor College of Medicine, Houston, TX) at a concentration of 1:200-300 and visualized with Alexa 488 goat anti-rabbit (Molecular Probes, Eugene, OR). Confocal sections, 0.5 μm thick, were collected at 100x power, 3x zoom.

Identification and analysis of satellite boutons

For analyses of satellite boutons, larval preparations were double-labeled with a monoclonal antibody to synaptotagmin (1:500; from Kaushiki Menon and Kai Zinn; California Institute of Technology) visualized with Alexa 568 goat anti-mouse antibody, and FITC- conjugated anti-HRP (1:100; ICN). Any single bouton that was not included in a chain of boutons, but instead appeared to be a lateral sprout, was counted as a satellite bouton. Although not regarded as criteria in this study, satellites typically were observed sprouting from larger boutons rather than from axons, and were usually much smaller than these “parent” boutons.

Electrophysiology and data analysis

For electroretinograms, flies were anesthetized by cooling them briefly on ice and then mounted upright in modeling clay such that the right eye was exposed. The ground electrode, a heat pulled glass capillary filled with 3M KCl, was inserted into the back of the fly's head. The recording electrode, a similarly pulled glass capillary, was advanced until it lightly touched the surface of the fly compound eye. Electrode resistances for both electrodes were between 5-10 MΩ. Flies were routinely allowed to recover in the dark for at least 15 minutes prior to recording. ERGs were induced with flashes of light. Data were acquired using an Axoclamp-2B amplifier (Axon Instruments Inc., Foster City, CA)
and digitized with a Digidata 200 board. All traces were filtered and analyzed using the pClamp6 software (Axon Instruments) and assembled using Adobe Photoshop.

Electrophysiological recordings were made from muscle 6 in the third abdominal segment (A3) in HL3 saline (in mM: 70 NaCl, 5 KCl, 1.5 CaCl₂, 20 MgCl₂, 10 NaHCO₃, 5 trehalose, 115 sucrose, and 5 HEPES, pH=7.3). Excitatory junctional potentials (EJPs) were measured by stimulating the motor nerve with a glass suction electrode. An isolated pulse stimulator (A-M Systems, Everett, WA) delivered 1msec pulses at 1 Hz at a voltage above threshold to stimulate both motor neurons innervating muscle 6. Recordings were taken using an Axoclamp 2B amplifier and pClamp6 software (Axon Instruments, Foster City, CA). Intracellular glass electrodes were pulled using a Sutter Instruments (Novato, CA) electrode puller. Electrodes were filled with 3 M KCL and had resistances of 15-30 MΩ. After electrode insertion into muscle 6, resting membrane potential of muscles measured -60 to -80 mV. EJP amplitude was measured by Mini Analysis software (Synaptosoft, Decatur, GA), which averaged amplitudes from at least 20 evoked responses.

FM1-43 loading

For FM1-43 loading of stn⁻¹/Y synaptic boutons, dissected larvae were placed in normal HL3 saline containing 4 μM FM1-43. The segmental motor nerve was then stimulated at 5 V, 30 Hz for 2 minutes. Immediately following the end of stimulation, non-internalized FM1-43 was rinsed away by several washes in Ca²⁺-free HL3 saline. Stained boutons were viewed using a water immersion lens with a Zeiss Axioscope fluorescence compound microscope (Frankfurt, Germany). Digital images were acquired
with a cooled CCD camera (Princeton Instruments, NJ) controlled by MetaMorph Imaging software (Universal Imaging Corp., West Chester, PA). Immediately after imaging, the preparation was fixed and processed for anti-HRP immunohistochemistry.

**Data analysis and statistics**

The error measurements are reported as standard error of the mean (SEM). Statistical significance was determined by Student’s *t* test.

**Results**

*Defining a dicistronic stoned transgene, stonedAB, that rescues stoned lethality*

To identify minimal coding sequences and promoter elements required for providing essential *stoned* functions, we initially created a dicistronic *stoned* cDNA by appropriately ligating *stoned* coding sequences isolated from partial cDNA clones or amplified cDNA fragments (Materials and Methods; Figure 2.1A). This dicistronic cDNA, termed stonedAB, was cloned into the *Drosophila* transformation vector pUAST, under control of the yeast transcription factor GAL4 (Brand and Perrimon 1993). Through germline transformation, multiple transgenic lines carrying this construct were generated. Experiments described below indicated that this stonedAB cDNA encodes all essential *stoned* functions.

To test the ability of the Gal4-responsive P[stenAB] (“responder”) transgenes to provide *stoned* functions *in vivo*, they were crossed into *stoned* mutant backgrounds either alone or in the presence of “driver” transgenes that drive Gal4 expression ubiquitously (*shi Gal4*), specifically in neurons (*elav*<sup>C159</sup>), or specifically in muscles
(MHC Gal4) (Lin and Goodman 1994; Staples and Ramaswami 1999; Estes, Ho et al. 2000). The ability of the transgenes to rescue lethality of mutants \( stn^{8P1} \) and \( stn^{13-120} \) was analyzed. The \( stn^{13-120} \) allele carries a large insertion in the vicinity of stonedA coding sequences (Andrews, Smith et al. 1996): this mutation disrupts the normal stoned transcript (Andrews, Smith et al. 1996), eliminating all detectable stonedA and stonedB protein in embryonic nervous system (Fergestad, Davis et al. 1999), and causes late embryonic lethality. Although there is no established null allele for stoned, existing data indicate that \( stn^{13-120} \) must be at least a strong hypomorph, causing severely reduced expression of both stoned products (Andrews, Smith et al. 1996). The other allele, \( stn^{8P1} \), molecularly uncharacterized, causes early larval lethality (Miklos, Kelly et al. 1987); it is similar to \( stn^{13-120} \) in reducing presynaptic stonedA and stonedB to levels undetectable levels at motor terminals of rare, escaper, third-instar \( stn^{8P1} \) larvae (Stimson, Estes et al. 2001).

Neural, but not muscle, expression of stonedAB is sufficient to restore complete viability to \( stn^{8P1} \) and \( stn^{13-120} \) (Table 2.1, data shown for line SAB1). This indicates that essential functions of stoned revealed by these mutations are limited to the nervous system. Synapses of \( stn^{8P1} \) and \( stn^{13-120} \) larvae rescued by neural stonedAB expression reveal the presence of wild-type levels of both stonedA and stonedB proteins (Figure 2.1B). Together with the observation that our artificial stonedAB transcript lacking native 5’ and most of the 3’ untranslated mRNA sequences can functionally replace stoned, this suggests that correct translation of the native discistronic transcript does not depend on unique non-coding elements present in these sequences of mRNA.
Neural expression of stonedAB rescues synaptic defects of stoned mutants

To determine cellular functions of *stoned* provided by neuronal stonedAB expression, we analyzed in detail the effects of *P[stenAB] (SAB1)* expression on various previously described electrophysiological and immunocytochemical phenotypes of lethal (*sten*¹⁸P¹ and *sten*¹³-¹²⁰) and viable (*sten*⁵) stoned mutants. At third-instar larval neuromuscular junctions (NMJs), phenotypes in *sten*⁵ and *sten*¹⁸P¹ associated with altered synaptic-vesicle recycling include: i) reduced evoked transmitter release; ii) increased synaptotagmin immunoreactivity on axonal membrane; and iii) reduced levels of stonedA and/or stonedB proteins (Stimson, Estes et al. 1998; Stimson, Estes et al. 2001). Similar phenotypes have been described at *sten*¹³-¹²⁰ embryonic motor synapses (Fergestad, Davis et al. 1999). All of these mutant phenotypes are completely rescued by neural expression of the *SAB1* transgene. Under appropriate conditions, evoked junctional potentials (EJPs) are a good measure of transmitter release (see Materials and Methods). EJP amplitudes, 11.1 +/- 1.3 mV and 5.5 +/- 0.7 mV in *sten*⁵ and *sten*¹⁸P¹, are increased following *SAB1* expression to 42.1 +/- 2.0 mV and 45.8 +/- 2.2 mV respectively (*P*<sub>rescue</sub> < 0.40, 0.63), values indistinguishable from the 44.8 +/- 2.1 mV of the wild-type controls (Figure 2.2A). Similarly, neuronal *SAB1* expression restores normal levels and distribution of synaptotagmin to *sten*⁵ and *sten*¹⁸P¹ nerve terminals (Figure 2.2B). Also significantly, *sten*¹³-¹²⁰ animals rescued by neural expression of stonedAB develop into third-instar larvae (subsequently to adults) with normal viability and synaptic physiology and morphology (Table 2.1, Figure 2.1B, Figure 2.2). These data indicate: first, that stonedAB encodes all functions required for previously described synaptic functions of
stoned; second, that potential stoned expression in postsynaptic muscle is not required for regulating essential aspects of synaptic function.

**Neural stonedB expression restores viability and synaptic transmission to stoned mutants**

Because the rescuing stonedAB transgene encodes both stoned products, it was of particular interest to determine if one or both stoned polypeptides were required for organismal and presynaptic functions of stoned. To address this question, we generated transgenic flies expressing either stonedB or stonedA under Gal4 control (Materials and Methods) and used them to analyze effects of neurally expressing individual stoned products on phenotypes of various stoned alleles.

StonedB expression alone, via the P[stnB] transgene, (SB5) was sufficient to rescue all previously organismal and synaptic defects in stoned alleles we analyzed (Table 2.2, Figure 2.3). Neurally expressed stonedB was as effective as stonedAB in restoring viability to \( stn^{8p1} \) and \( stn^{13-120} \) mutants (Table 2.2). However, in contrast to animals expressing the stonedAB transgene, motor terminals of \( stn^{c} \), \( stn^{8p1} \) and \( stn^{13-120} \) third-instar larvae expressing stonedB showed strong immunoreactivity for stonedB, but greatly reduced stonedA compared to the wild-type (Figure 2.3B). We tested whether synapses with this specific deficit in stonedA showed any physiological or morphological defects. Remarkably, evoked transmitter release as well as the levels and distribution of synaptotagmin in stonedB-expressing \( stn^{c} \), \( stn^{8p1} \) and \( stn^{13-120} \) mutant synapses were completely normal and indistinguishable from wild-type controls (Figure 2.3A, C). EJP amplitudes were 42.9 +/- 2.3, 44.7 +/- 1.8, and 42.8 +/- 3.8 (\( P_{\text{rescue}} < 0.57, 0.85, 0.63 \)) respectively for \( stn^{c} \), \( stn^{8p1} \) and \( stn^{13-120} \) synapses expressing normal levels of stonedB,
but not stonedA, immunoreactivity (Figure 2.2B).

While these data are consistent with stonedB being sufficient to perform all functions of stoned, the slight but unequivocal increase in presynaptic stonedA immunoreactivity in mutant animals expressing SB5 (an issue discussed in more detail in Figure 2.4), is equally consistent with a model in which small amounts of stonedA are also required. Also important, these data alone do not exclude the possibility that stonedA and stonedB have overlapping, redundant functions.

*Neural expression of stonedA is not sufficient for rescuing stoned phenotypes*

To identify potential synaptic functions of stonedA, we generated *stoned* mutants expressing stonedA transgenes in the nervous system. In contrast to stonedB expression, stonedA did not alter the lethal phenotype of *stn*\(^{8p1}\) and *stn*\(^{13-120}\). This lack of rescue was a common property of 4 independent stonedA transgenes that we tested (*SA1, SA5, SA19, SA20*). Furthermore, ERG recordings (see below) were used as a rapid screen for rescue for 14 additional lines with the same result. The transgenes expressed stonedA protein efficiently in wing discs when crossed to the wing Gal4 driver 684 (Figure 2.4A). Strong stonedA immunoreactivity was observed in a reticulate pattern in expressing cells of wing imaginal discs (Figure 2.4A shows data for the *SA19* and *SA20* transgenes). Similar levels of stoned immunoreactivity, absent in the 684 driver control discs, were also observed when the rescuing *SABI* transgene was crossed to the 684 driver. These data support the conclusion that, in the absence of stonedB, the stonedA protein is translated from its monocistronic mRNA, but thereafter unable to provide essential synaptic functions of *stoned*. 
In order to determine potential contributions of stonedA expression to synaptic functions, we further examined effects of stonedA expression on synaptic physiology and synaptotagmin distribution at stn mutant larval synapses. For reasons likely involving specific genetic backgrounds, we were unable to obtain any viable “escaper” stn\textsuperscript{8Pl} mutant third-instar larvae that expressed stonedA. However, stn\textsuperscript{C} larvae expressing stonedA transgenes were obtained and their synaptic properties compared to stn\textsuperscript{C} mutants alone. In contrast to stonedB, that completely restored mutant nerve terminals to wild-type function and morphology, stonedA expression had no effect on either the mutant EJP or plasma-membrane distribution of synaptotagmin. EJP values for stn\textsuperscript{C} larvae expressing stonedA transgenes were 8.3+/−0.4 mV, not significantly different from the 11.1+/−1.3 mV observed for appropriate stn\textsuperscript{C} controls (Figure 2.4B). The exaggerated plasma membrane distribution of synaptotagmin seen in stn\textsuperscript{C} mutants was also seen in the presence of neurally expressed stonedA (Figure 2.4C). To test whether stonedA might encode a function required at central synapses, that may differ from the NMJ, we compared effects of neural stonedA and stonedB expression on the synaptic on and off transient components of the stn\textsuperscript{C} electroretinogram (ERG), an extracellularly recorded, ensemble response of the adult visual system to a brief light flash (Petrovich, Merakovsky et al. 1993). While stonedB expression completely restored the missing synaptic components of the stn\textsuperscript{C} ERG, stonedA expression had no effect (Figure 2.4D).

These experiments could indicate either that stonedA at nerve terminals cannot restore synaptic functions missing in stn\textsuperscript{C} mutants, or that stonedB is required for the transport, localization or stability of stonedA. To address this issue, we examined
synapses of \textit{stn}\textsuperscript{C} mutants with a neurally driven SA transgene for stonedA and stonedB immunoreactivity. Unexpectedly, presynaptic stonedA was not obviously increased following neural expression of stonedA alone via either \textit{SA5}, \textit{SA19} or \textit{SA20} transgenes. However, when stonedA was co-expressed with stonedB, by combining the \textit{SA5} and \textit{SB15} transgenes in an \textit{elav}\textsuperscript{C155} \textit{stn}\textsuperscript{C} background, substantially elevated levels of presynaptic stonedA were apparent (Figure 2.4E). Thus, the stable presence of stonedA at nerve terminals requires stonedB. For this reason, our studies of stonedA transgenes provide only limited insight into functions of presynaptic stonedA. However, because essential \textit{stoned} functions occur under conditions of highly reduced stonedA, it appears more likely that stonedA functions at synapses are either modest or redundant.

\textit{A novel function for stoned in synaptic growth is also provided by stonedB}

While examining the effect of \textit{stoned} transgenes on various phenotypes associated with altered synaptic vesicle recycling, we observed an unexpected consequence of the \textit{stn}\textsuperscript{8P1} mutation on the structure of presynaptic motor terminals. Dramatic alterations in presynaptic architecture, specifically the abundance and occasional proliferation of small, bud-like boutons from a morphologically normal bouton, are obvious in \textit{stn}\textsuperscript{8P1} (Figure 2.5). Similar unusual, “satellite” boutons emanating from “parent boutons” have been recently observed in \textit{Drosophila} strains overexpressing specific forms of the Alzheimer’s amyloid precursor protein ortholog APPL and are hypothesized to represent early stages of branch formation and activity-dependent synapse growth (Torroja, Packard et al. 1999; Zito, Parnas et al. 1999).
Like satellite boutons in *appl*-overexpressing strains, those in *stn*\textsuperscript{8P1} contain components required for active neurotransmitter release including synaptotagmin (Figure 2.5C) and csp (data not shown). To directly examine whether satellite boutons are functional in *stn*\textsuperscript{8P1}/Y terminals, we used the fluorescent dye FM1-43 that labels actively cycling synaptic vesicles. Both parent and satellite boutons are labeled with FM1-43 in response to nerve stimulation, indicating that all components required for evoked vesicle fusion and subsequent recycling are present in these unusual varicosities (Figure 2.5D). These morphological defects observed in the *stn*\textsuperscript{8P1} mutant strain map to a mutation in the same region as *stoned*, as they are complemented by the duplication *mal*\textsuperscript{+}Y, (Figure 5B). Mutant *stn*\textsuperscript{8P1} terminals show 5.7 +/- 0.51 satellites compared to 2.8 +/- 0.47 in *stn*\textsuperscript{8P1}/*mal*\textsuperscript{+}Y (p < 0.007).

To determine the stoned product(s) involved in regulating bouton morphology, we tested the ability of neurally driven *SAB1* and *SB5* expression to rescue this phenotype (Fig 2.5B). As for other defects in *stn* mutants, the stonedAB and stonedB transgenes completely rescued the aberrant bouton phenotype (satellite bouton frequency 2.3 +/- 0.30 and 3.3 +/- 0.61 corresponding to P\textsubscript{rescue} values of < 0.002 and < 0.03 respectively). As considered below, this provides support for a model in which the *stoned* locus, and stonedB in particular, has previously unappreciated functions in membrane traffic events distinct from synaptic-vesicle recycling.
Discussion

A dicistronic mRNA from the *Drosophila stoned* locus is translated to produce two proteins, stonedA and stonedB. The first is a poorly conserved molecule with no obvious homolog in *C. elegans* and mammals; the second, a founding member of a new, widely conserved family of proteins called stonins. Previous analyses have demonstrated that at least one or both *stoned* products are required for regulating normal synaptic-vesicle recycling and, thereby, synaptic transmission and the distribution of synaptic-vesicle proteins. Experiments described here make three important points. First, *stoned* function is essential only in the nervous system. Second, while stonedA is presynaptically localized, its stable presence at nerve terminals is not only largely dispensible, but also dependent on the expression of stonedB. In contrast, transgenically provided stonedB provides all essential molecular activities missing in viable and lethal *stoned* alleles. Finally, the *stoned* locus, and likely stonedB, has a previously unrecognized function in regulating the structure of synaptic boutons.

These points are discussed below in the context of the genetics of *stoned*, cellular functions of stonins, and the evolution and regulation of the discistronic *stoned* mRNA.

*Insights into molecular functions of stoned and its products*

Complete rescue of *stoned* lethal alleles by neuronal expression of a *stoned* cDNA strongly argues that the major function of *stoned* is in the nervous system. This result is consistent with two previous observations that suggest a neural-specific function for *stoned*. First, in an elegant genetic scheme for generating mosaic (gynandromorph) animals carrying both female *ston*^{13-120}/+ and male *ston*^{13-120} tissue, viable mosaic animals
with large patches of \textit{stn}^{13-120} nervous tissue were never obtained, under conditions where mosaics with large mutant patches of non-neuronal tissue were frequent (Petrovich, Merakovsky et al. 1993). The apparently normal development of non-neuronal mutant tissue argued that \textit{stoned} functions in these cell types must be modest or dispensible (Petrovich, Merakovsky et al. 1993). Second, a recent study observes that over-expression of a synaptotagmin (\textit{DsytI}) transgene in neurons restores partial viability to \textit{stn}^{13-120} (Fergestad and Broadie 2001). This suppression however, is either limited or dependent on the specific transgenes and/or the mutant strain backgrounds that were utilized. Both neural-restricted \textit{elav}^{C155} and ubiquitous \textit{shi} \textit{Gal4} driven expression of \textit{stoned} cDNA completely rescue the lethality of \textit{stn}^{8P1} and \textit{stn}^{13-120}. In contrast, similar expression of \textit{DsytI} transgenes (Littleton, Serano et al. 1999; Mackler and Reist 2001) has no effect on viability of \textit{stn} mutants under conditions used in our experiments (Materials and Methods). Our demonstration that \textit{stoned} cDNA expression in neurons is sufficient for restoring normal viability and synaptic function to \textit{stoned} lethal alleles thus, confirms and extends previous studies of this locus.

A more detailed analysis of artificial monocistronic \textit{stoned} cDNAs encoding either stonedA or stonedB reveals that neural expression of stonedB alone is sufficient to reproduce all of the effects observed with a full-length dicistronic cDNA. Our favored interpretation, that the second cistron of \textit{stoned} encodes all vital and important \textit{stoned} functions, is limited by the absence of a well-defined \textit{stoned} null background in which the transgene analyses should ideally be performed. It could be argued that \textit{stn}^{13-120} retains some residual stonedA activity that contributes to the ability of neurally expressed
stonedB to rescue $\text{stn}^{13-120}$ phenotypes, but two lines of evidence argue against this possibility. First, the $\text{stn}^{13-120}$ mutation comprises an insertion in the 3’ end of the stonedA encoding cistron: thus, the mutation should substantially reduce ORF1 function (Andrews, Smith et al. 1996). Second, immunofluorescence analysis (Figure 3B) demonstrates that stonedB transgene expression in $\text{stn}^{13-120}$ results in viable animals, with morphologically and functionally normal presynaptic terminals that, importantly, are still substantially deficient in stonedA. The same is true of $\text{stn}^{8P1}$ animals rescued by a stonedB transgene. Thus, our data are more consistent with a model in which stonedB alone performs all identified presynaptic and organismal functions of stoned.

What then might be the function of stonedA? Our data indicate that stonedA expression alone is not sufficient to rescue any documented mutant phenotype of stoned and that stonedA is largely dispensible for organismal viability and presynaptic function. However, a direct analysis of stonedA function is limited by our observation that the stable presence of stonedA at presynaptic terminals requires stonedB (Figures 2.3B and 2.4C). Thus, we were unable to assess stonedA functions in the absence of stonedB. Our current analysis does not exclude the possibility that stonedA has molecular functions that overlap with or facilitate those of stonedB. This possibility is consistent with previous co-immunoprecipitation experiments indicating association of stonedA and stonedB in a common molecular complex, and shared association of both stonedA and stonedB with the synaptic vesicle protein synaptotagmin (Phillips, Smith et al. 2000). The issue of stonedA function is further considered in the last section of this discussion.
Functions of the stonin family of proteins

Our analysis of stonedB function is particularly relevant as it constitutes the first in vivo functional analysis of a member of the stonin family of proteins. Our data predict that the stonins in general will be found to regulate endocytosis of synaptic-vesicle proteins, and that stonin-deficient synapses will display phenotypes of stoned mutants. Indeed stonin genes may be good candidates for certain congenital myasthenic syndromes, a class of human genetic diseases that interrupt neuromuscular transmission. Some of these have been associated with morphological defects at the NMJ that are similar to those of stoned mutants (Fergestad, Davis et al. 1999; Maselli, Kong et al. 2001; Stimson, Estes et al. 2001). The underlying mechanism of stonin function at synapses is likely to involve known molecular interactions of stonins with synaptotagmin, Eps15 and intersectin (Phillips, Smith et al. 2000; Martina, Bonangelino et al. 2001). A particularly attractive idea is that it serves as a “pseudoadaptin” that, at a certain stage of vesicle formation competes for the AP2-binding sites on vesicle proteins and, by displacing AP2, facilitates large-scale, sequential changes in the assembly state of endocytic proteins that underlie the ordered progression of events in the endocytic pathway (Walther, Krauss et al. 2001). However, this model is not easily reconciled with the observation that stonedB remains associated with a vesicle fraction isolated from heads of shibire flies that are depleted of synaptic vesicles (Phillips, Smith et al. 2000).

A major issue to be addressed is whether stonedB in particular, and stonins in general, participate in a wide range of endocytic events or only in the relatively rapid and specialized process of synaptic-vesicle endocytosis. Our experiments address this issue
in two ways. First, the observation that stonedB expression in the nervous system restores normal viability to otherwise lethal alleles of stoned, argues for a neural, if not synapse-specific function for the protein. Non-neuronal functions of stonedB, if any, must be dispensible. However, the second observation that stonedB is also required for regulating morphological changes in boutons associated with synaptic growth (Torroja, Packard et al. 1999; Zito, Parnas et al. 1999; Estes, Ho et al. 2000; Roos, Hummel et al. 2000) suggests a role for stonedB in events not limited to synaptic-vesicle recycling.

Satellite boutons similar to those we describe in stn8P1 are found in synapses of Drosophila overexpressing the wild type, but not an endocytosis-defective form of the Drosophila amyloid precursor protein homolog appl (Torroja, Packard et al. 1999). Thus, it is possible that stonedB influences endocytosis of appl or other growth-related cell surface molecules that are part of a normal pathway for structural synaptic change.

Given the reported ubiquitous expression of mammalian stonins in multiple cell types, and the ability of an overexpressed dominant-negative stonin to interfere with endocytosis in non-neuronal cells, it is possible that mammalian stonins have wider functions (Martina, Bonangelino et al. 2001). Perhaps stonins, initially selected for a specialized task like synaptic-vesicle recycling, have since evolved and diversified to be capable of broad, general functions in endocytosis. The concurrent proliferation of synaptotagmin-encoding genes in mammals (Sudhof 2002) may have contributed to diversification of stonin functions in mammalian species.

Evolution and significance of dicistronic organization of stoned

The stoned dicistronic mRNAs in eukaryotes are a genetic oddity whose functions
and evolution are poorly understood (Blumenthal 1998). Unlike most polycistronic mRNAs that are processed to yield individual monocistronic mRNAs, the mature stoned transcript exists in a discistronic form (Andrews, Smith et al. 1996; Blumenthal 1998; Blumenthal, Evans et al. 2002). Potential reasons suggested for this organization of the stoned mRNA include: a) maintenance of stoichiometry; and b) facilitation of dimer formation between the two proteins because of spatially associated translation of the two proteins. Biochemical experiments demonstrating that the two proteins may be found in a single complex, provide some support for these hypotheses (Phillips, Smith et al. 2000).

Neither of these hypotheses are supported by our observations. Our experiments clearly demonstrate that stoichiometry is not an important factor in stoned function. Animals in which stonedA-stonedB stoichiometry is severely altered show completely normal viability and synaptic function. Second, we show the splitting the two cistrons of stoned into the two constituent ORFs encoding stonedA and stonedB separately, allows stonedB-dependent localization of stable stonedA at nerve terminals. This argues that selective pressure to maintain the dicistronic organization of stoned is not particularly strong, and may not be driven by the two previously suggested mechanisms.

Additional data pertinent to the evolution of this dicistronic mRNA are provided by analyzing the conservation of stonedA and stonedB coding sequences in other species. While stonedB is conserved across metazoa, the only clear stonedA homolog known is found encoded in the genome of the mosquito Anopheles gambiae (approximately 45% identical). Like its fruit fly counterpart, mosquito stonedA has five conserved DPF motifs plus a sixth DPF not found in fruit fly. However, the potential leucine zipper motif
of fruit fly stonedA (Stimson, Estes et al. 1998) is not conserved. In mosquito, the stonedA coding cistron lies no more than 39 bases upstream of an identically oriented stonedB coding cistron; thus, the data are consistent with the existence of a conserved dicistronic organization in insects. Because nematode and mammalian genomes have monocistronic orthologs for stonedB but not for stonedA, it is possible that the dicistronic stoned mRNA originated in arthropods some time after divergence from the vertebrate lineage, but before the divergence of Drosophila from Anopheles. Combined with our data, these observations suggest that there may not be strong functional reasons for the evolutionary conservation of stonedA.

One remarkable, conserved feature of stonedA sequence both in mosquito and in Drosophila is the complete absence of internal methionine residues in the coding sequence. In a single 900 amino acid protein the chance of such an occurrence by chance alone is \( \sim 7 \times 10^{-7} \), if one makes the simplistic assumption that all codons occur at an equal frequency (63/64). Given its conservation in mosquito, it appears likely that this unusual feature of stonedA coding sequences is relevant to the mechanism by which the dicistronic mRNA is translated into two different proteins. While our experiments do not address this mechanism, the definition of a single dicistronic cDNA including intercistronic sequences sufficient to direct translation of the two stoned proteins should facilitate, in future, the detailed analysis of molecular mechanisms that allow the unusual translation of this mRNA.
Table 2.1

Neural expression of $P[stnAB]$ alone rescues $stn$ lethal alleles

<table>
<thead>
<tr>
<th>Cross</th>
<th>Actual $stn/Y$ progeny (% of total)</th>
<th>Expected $stn/Y$ assuming normal viability</th>
<th>Total progeny scored</th>
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<tr>
<td>$C155$ $stn^{8PI}/FM7i$ X $yw$</td>
<td>0 %</td>
<td>25 %</td>
<td>510</td>
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<tr>
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<td>25 %</td>
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<td>$C155$ $stn^{13-120}/FM7i$ X $yw$</td>
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<td>551</td>
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<td>$C155$ $stn^{13-120}/FM7i$ X $P[stnAB]$</td>
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Table 2.2

Neural expression of \( P[stnB] \) alone rescues \( stn \) lethal alleles

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<th>Expected ( stn/Y ) assuming normal viability</th>
<th>Total progeny scored</th>
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<tr>
<td>( C155 ) ( stn^{8P1}/FM7i ) ( \times yw )</td>
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<td>( C155 ) ( stn^{13-120}/FM7i ) ( \times yw )</td>
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<td>551</td>
</tr>
<tr>
<td>( C155 ) ( stn^{13-120}/FM7i ) ( \times P[stnB] )</td>
<td>23 %</td>
<td>25 %</td>
<td>500</td>
</tr>
<tr>
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<td>256</td>
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Figure 2.1

Neural expression of a full-length dicistronic stoned cDNA (stonedAB) transgene (P[stnAB]) restores both StonedA and StonedB proteins to stn\textsuperscript{8P1} and stn\textsuperscript{13-120} mutant presynaptic terminals.

A. Organization of the P[stnAB] construct with details of the 55 bp intercistronic element that contains a total of 5 termination codons in all three frames (shown in bold).

B. StonedA and stonedB at presynaptic terminals (top panels), missing in terminals of stn\textsuperscript{8P1} “escapers” (second row of panels and Stimson et al., 2001), are restored (third row) by expressing the P[stnAB] transgene under the control of the neural elav promoter. Similar restoration of stoned proteins is seen for the embryonic lethal allele stn\textsuperscript{13-120} (bottom panels). All stonedA images and stonedB images are taken at identical gain and aperture settings and so the displayed brightness of staining roughly represents the amount of presynaptic protein.
A

\[ \text{stonedA} \quad \text{stonedB} \]

\[
\begin{array}{cccccc}
\text{PUAST} & \text{UAS} & \text{ORF1} & \text{ORF2} & \text{SV40 polyA} & \text{PUAST} \\
\hline
\text{TAAAGATAAAGATAAGACGATTTTAAATCACAAGAAAAGTAAAAGCCGAAATCGAAATG} \\
\end{array}
\]

B

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<th>( \alpha \text{StonedB} )</th>
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<tr>
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<td>![Image of str\text{^<em>} \text{P} \text{^</em>}]</td>
</tr>
<tr>
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<td>![Image of str\text{^<em>} \text{P} \text{^</em>} \text{; P[strAB]}]</td>
<td>![Image of str\text{^<em>} \text{P} \text{^</em>} \text{; P[strAB]}]</td>
</tr>
<tr>
<td>\text{str}\text{^{10^8}} \text{P} \text{^*} \text{; P[strAB]}</td>
<td>![Image of str\text{^{10^8}} \text{P} \text{^*} \text{; P[strAB]}]</td>
<td>![Image of str\text{^{10^8}} \text{P} \text{^*} \text{; P[strAB]}]</td>
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</tbody>
</table>
Figure 2.2

All known synaptic phenotypes of *stn* mutants are rescued by neural (*elav*) expression of *P[stnAB]*.

**A.** Mean EJP amplitudes and representative traces in third instar larval synapses of mutants with and without a rescuing *P[stnAB]* transgene. Evoked transmission is not shown for *stn*^{13-120} that die as late embryos, well before the third instar larval stage. Error bars represent standard error of the means (SEMs).

**B.** *stn*^{c} and *stn*^{8P1} boutons show characteristic mislocalization of synaptotagmin staining to the plasma membrane (Stimson et al., 1998; Stimson et al., 2001); this defect is rescued by neural expression of *P[stnAB]*. The same result is seen in *stn*^{13-120} mutants rescued by the presence of the *P[stnAB]* transgene.
Figure 2.3

Neural expression of a truncated cDNA containing only StonedB coding sequences (a \( P[stnB] \) transgene) is sufficient to rescue mutant synaptic phenotypes in \( stn^C \), \( stn^8P1 \) and \( stn^{13-120} \).

A. Mean EJP amplitudes in mutants before and after rescue with \( P[stnB] \). Error bars represent standard errors of the means (SEMs).

B. StonedB protein levels are restored to wild type in \( stn \) mutants where previously they were undetectable (see Figure 2.1B and 2.4E for pre-rescue levels). Small but unambiguous increases in levels of presynaptic stonedA are also seen in these mutants following neural \( P[stnB] \) expression.

C. Rescue with the \( P[stnB] \) transgene restores wild type localization of synaptotagmin at the larval neuromuscular junction (see Figure 2.2B for pre-rescued localization).
A

![Graph showing EJP amplitude (mV) for different genotypes: wild type, mutant, and +P[stnB].](image)

- wild type
- mutant
- +P[stnB]
- lethal

B

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C

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Figure 2.4

Neural expression of stonedA coding sequences alone does not alleviate any of the known defects in stoned mutants.

A. Lethality of the other stoned alleles, stn$^{8P1}$ and stn$^{13-120}$ was not rescued by the P[stnA] transgene although it expressed stonedA protein in wing discs when controlled with the Gal4-driver 684 (Materials and Methods).

B. Mean EJP amplitudes for wild type and stn$^c$ mutants are shown before and after neural expression of P[stnA]. Stn$^c$ larvae expressing P[stnA] have EJPs with amplitudes indistinguishable from stn$^c$. Error bars represent standard errors of the means (SEMs).

C. P[stnA] does not rescue the synaptotagmin mislocalization phenotype seen in stn$^C$.

D. In electroretinogram recordings, synaptic on/off transients missing in stn$^C$, are restored by P[stnB] transgenes but not by P[stnA].

E. Barely detectable levels of stonedA observed stn$^C$ larval synapses (top two rows; Stimson et al., 1998) are not perceptibly increased after P[stnA] transgene expression in neurons (third row). Remarkably presynaptic stonedA protein is restored to wild type levels when both P[stnB] and P[stnA] transgenes are expressed simultaneously in the nervous system.
**Figure 2.5**

A function for the *stoned* locus in synaptic development is revealed by unique morphological defects in *stn*<sup>8P1</sup>.

**A.** “Satellite boutons” (arrows) in *stn*<sup>8P1</sup> synapses are revealed by anti-HRP staining.

**B.** Histogram of quantified data shows that *stn*<sup>8P1</sup> larvae have significantly more satellite boutons per A3 hemisegment than do wild type. This phenotype is rescued by a duplication on the Y chromosome which carries a wild type copy of *stn*. The phenotype is similarly rescued by the neural expression of either the *P[stnAB]* or the *P[stnB]* transgene.

**C.** Satellite boutons contain functional synaptic vesicles and other components of the synaptic vesicle machinery as they are be labeled with an endocytic tracer FM1-43 dye following nerve stimulation. A bouton with two satellites in a live terminal imaged after loading with FM1-43 (left panel) has been fixed and stained with anti-HRP (right panel).
CHAPTER THREE
LK6 FUNCTIONS DOWNSTREAM OF RAS/ERK TO REGULATE SYNAPTIC GROWTH IN THE DROSOPHILA NERVOUS SYSTEM

Abstract

Cellular changes that underlie long-term plasticity in neurons depend on new protein synthesis. Thus, translation of pre-existing mRNA and new transcripts is tightly regulated during this process. In mammalian systems, there is increasing evidence that ERK signaling may control translation, through the activation of MNK1, a MAPK stimulated, eIF4E kinase. For instance, it has been suggested that during LTP induction in mice, ERK signals to eIF4E and promotes translation. There is also evidence that MNK1 is activated by ERK in NMDA dependent models of LTP in vitro. However, direct proof linking MNK activation during Ras/ERK mediated neural plasticity is lacking. Here, we investigate the role of the lk6 gene, the Drosophila homolog of MNK1, in a model of Ras/ERK driven synaptic plasticity at the neuromuscular junction (NMJ). LK6 has previously been shown to bind both ERK and eIF4E in vitro, and upon activation through ERK signaling, phosphorylate eIF4E. We show first, that both isoforms of LK6, LK6-A and LK6-B, are expressed in the adult and larval nervous system. Second, to determine if LK6 is required for synaptic plasticity in flies, we used mutants to remove expression of either LK6-A (lk6EP886 and lk6KG08062) or LK6-B (lk61), and also generated a UAS-lk6-RNAi transgenic fly to knock down total LK6 protein specifically in the nervous system. While there is no discernible effect of reducing LK6
levels in the nervous system in a wild type background, it significantly suppresses the effect of increased Ras/ERK signaling on the growth of this motor synapse. This suggests that levels of MNK protein are limiting in this context. Finally, to test if *Drosophila* MNK is a direct target of Ras/ERK signaling, we quantified phospho-MNK in the nervous system of heated *Ca-P60A*~Kum170~ mutants (these conditional mutants show robust induction of phospho-ERK in the nervous system). We find that P-MNK levels are increased two-fold as compared to controls in a MEK dependent manner. Our findings that LK6 is activated by ERK signaling and functions downstream of ERK to regulate synaptic growth, along with existing evidence that LK6 functions as an eIF4E kinase, substantially strengthen the idea that LK6 mediates the effect of ERK signaling on the translational regulation of long-term plasticity.

**Introduction**

In a variety of organisms, from mollusks to insects to mammals, long-term memory and long-lasting modifications in synapses require new gene expression and protein synthesis, while short-term forms of plasticity do not (Bailey, Bartsch et al. 1996; Kandel 2001). The synthesis of new proteins required for long-term plasticity is thought to be regulated at the transcriptional level, although there is increasing evidence that direct translational regulation of mRNAs may also mediate long-lasting synaptic changes (Klann and Dever 2004). Additionally, local translation of existing dendritic mRNAs may be a mechanism by which synaptic activity marks specific synapses to undergo plastic changes (Frey and Morris 1997; Martin, Barad et al. 2000; Aakalu, Smith et al.)
One way neuronal activity may lead to translation is through activation of the Ras/ERK pathway. ERK signaling is induced in neurons with activity, and is required for many types of long-term plasticity and memory (Thiels and Klann 2001; Adams and Sweatt 2002). ERK has typically been shown to regulate long-term plasticity through the activation of transcription factors that lead to gene expression changes at the mRNA level (Impey, Obrietan et al. 1998); however, ERK signaling also directly controls translation. Stimulation of hippocampal neurons induces a translation-dependent, transcription-independent phase of LTP that requires ERK and correlates with increased protein synthesis and activation of the translation initiation factor eIF4E (Kelleher, Govindarajan et al. 2004). eIF4E is a rate limiting phospho-protein necessary for protein synthesis that initiates translation by binding to the 5’ cap of mRNAs and forming a complex with other translation factors (Gingras, Raught et al. 1999; Pyronnet 2000). Although the significance of eIF4E activation is not entirely clear, in most cases, phosphorylation of eIF4E is correlated with increased rates of translation (Scheper and Proud 2002). The ERK stimulated, eIF4E kinase MNK1 (MAPK interacting kinase) is the most likely candidate linking ERK signaling to eIF4E activation and translational regulation (Fukunaga and Hunter 1997; Waskiewicz, Flynn et al. 1997; Waskiewicz, Johnson et al. 1999; Scheper and Proud 2002). Stimuli that induce long-term plasticity in mammalian hippocampal neurons, including the application of BDNF or activation of NMDA receptors, lead to ERK dependent activation of MNK1 and eIF4E, indicating that MNK1 may mediate ERK’s regulation of translation underlying long-term synaptic changes (Banko, Hou et al. 2004).
To discover more about the function of MNK in neurons and to further establish that MNK is required for Ras/ERK dependent regulation of translation in long-term plasticity, we chose to examine the role of MNK in Ras/ERK mediated synaptic growth in *Drosophila*. The *Drosophila* protein kinase LK6 is homologous to mammalian MNK1. As the fly homolog of MNK1, LK6 also functions as an ERK dependent, eIF4E kinase. There is evidence for genetic interaction between *lk6* and *ras* during fly eye development, and when expressed in human cell lines and Drosophila S2 cells, LK6 binds to ERK, its activity is increased with ERK signaling, and it is able to phosphorylate eIF4E (Huang and Rubin 2000; Parra-Palau, Scheper et al. 2004; Arquier 2005).

Synaptic plasticity in flies can be readily assayed at the larval neuromuscular junction (NMJ). This genetically accessible synapse shows activity-dependent changes in synaptic size and strength throughout its development (Budnik, Zhong et al. 1990). These developmental changes utilize biochemical pathways similar to those involved in other models of long-term synaptic plasticity and memory such as LTP in the mammalian hippocampus and LTF in Aplysia neurons (Zhong and Wu 1991; Davis, Schuster et al. 1996; Koh, Gramates et al. 2000). Notably, synaptic growth at the NMJ is positively regulated by Ras activation, and enhancement of Ras/ERK signaling in motor neurons leads to significant increases in synaptic arborization (Koh, Ruiz-Canada et al. 2002).

In this study we aimed to investigate if Ras/ERK regulation of translation occurs via the MNK kinase. For this, we generated reagents that, in addition to existing ones, allow us to disrupt MNK function in the nervous system. We further used the known effects of increased Ras/ERK signaling at the motor synapse to test if MNK activity is
required during this process. We show that LK6 is highly expressed in the fly nervous system and functions downstream of Ras/ERK to regulate synaptic growth at the larval neuromuscular synapse. Finally, we demonstrate that acute induction of ERK in the nervous system, accomplished by eliciting seizures in *Drosophila Ca-P60A*\textsuperscript{Kam170} mutants (Hoeffer, Sanyal et al. 2003), induces phosphorylation of MNK. Additionally, we demonstrate that LK6 is activated by neural activity in an ERK dependent manner. These findings are strongly consistent with a model wherein LK6/MNK is the link between ERK signaling and regulation of translation during long-term synaptic plasticity.

### Materials and Methods

*Drosophila strains and culture conditions*

Flies were reared in standard culture conditions at 25\textdegree C for synaptic growth experiments, and 21\textdegree C for seizure induction. The following fly strains were used in these studies: *lk6* mutants *lk6\textsuperscript{1} and lk6\textsuperscript{2}* were provided by Pierre Leopold (CNRS, France), the P-element line *lk6*\textsuperscript{EP886} was obtained from Exelixis (San Francisco, CA), and *lk6*\textsuperscript{KG08062} was obtained from the Bloomington Stock Center (Indiana U., Bloomington, IN). The UAS *lk6* RNAi transgenic line was generated by Paul Etter in the Ramaswami lab. The RNAi construct that can be expressed under control of the upstream activating sequence (UAS) of the yeast transcriptional activator GAL4 was created according to procedures previously described (Lee and Carthew 2003). The double-stranded hairpin construct targets 561 base pairs in exons 4-6 of both LK6 transcripts. The UAS *Ras\textsuperscript{V12-S35}* line and the Gal4 motorneuron driver *C380* were obtained from Vivian Budnik (U. of
Massachusetts, Worcester, MA). The nervous system Gal4 driver \textit{C155} was obtained
from Corey Goodman. The temperature-sensitive mutant \textit{Ca-P60A}^{Kum170} was isolated by
S. Sanyal, A. Basole, and K. S. Krishnan (TIFR, Mumbai, India). Wild-type strains used
were Canton-S (Danny Brower, U. of Arizona) and \textit{w}^{118}.

\textit{Immunohistochemistry}

Wandering 3\textsuperscript{rd} instar larvae were dissected in \textit{Ca}\textsuperscript{2+} free HL3 saline and fixed in
3.5\% paraformaldehyde for 30 minutes. Fixed larvae were then blocked for 2 hours in
PBS block (PBS, 0.3\% Triton, 2\% BSA, and 5\% goat serum) and then incubated with an
antibody to csp (1:25) (Konrad Zinsmaier, U. of Arizona) overnight at 4\textdegree. After three 10
minute washes with block, larval preps were incubated with secondary antibody for one
hour at room temperature, and then washed in PBS with 2\% Triton twice for 10 minutes
each, and finally once in PBS for 10 minutes. Dissected larvae were mounted in
Vectashield (Vector Laboratories, Inc.) on glass slides.

Fluorescently labeled larval neuromuscular synapses at segment A2, muscle 6 and
7 were imaged using a laser scanning confocal microscope (Nikon). Maximum
projections were obtained from serial sections of each sample. The number of boutons
labeled with csp at each synapse were counted using Metamorph imaging software
(Universal Imaging). Area of muscles 6 and 7 was also measured and calculated using
Metamorph. For statistics, the student’s \textit{t} test was used to compare differences in bouton
number between genotypes.
**Generation of seizures**

One day old adult flies were placed in clean, disposable, borosilicate glass vials with strips of Whatman filter paper and heated in a water bath for four minutes at 40°C. Flies were allowed to recover at room temperature for 60 minutes before heads were removed and frozen.

**MEK inhibitor feeding**

One day old flies were starved for 9 hours in clean glass bottles containing no food. Flies were then placed in glass tubes overnight containing filter paper soaked with a 1 mM concentration of the MEK inhibitor U0126 (Cell Signaling) in a 2% sucrose, 5% yeast solution with red food coloring. Flies were heat shocked for 4 minutes at 40°C the following day. Heads of flies with red abdomens indicating ingestion of MEK inhibitor were removed and frozen after heat shock.

**SDS Page and Western blotting**

Protein extracts from fly heads were obtained by anesthetizing adult flies with CO₂, removing their heads, and freezing in liquid nitrogen. 10 heads per sample were frozen in plastic tubes, which were then placed in SDS-PAGE buffer at 10 uL per head and homogenized with a motorized pestle. For protein extracts from the larval CNS, 3rd instar larvae were dissected in Ca²⁺ free saline and brains were removed and placed in SDS-PAGE buffer. Protein lysates were separated on a 12 % or 7.5% acrylamide gel by loading 10 uL of sample per lane. Blots were probed with antibodies to DP-ERK (1:2000) (Cell Signaling), P-MNK1 (1:500) (Cell Signaling), or LK6 (1:1000) (Jordan Raff) and dynamin (Jack Roos, UCSF) or β-tubulin (Chemicon) as loading controls.
Proteins were visualized with HRP secondary antibodies (1:10,000) and developed with an ECL chemiluminescent kit (Amersham). Quantification of band intensities was performed by scanning the images on Kodak Biomax films with a UMAX Astra 1220U scanner and analyzing with ImageJ software.

**RNA extraction and quantitative PCR**

Total RNA was extracted from 200-300 heads of 1-3 day old flies of the given genotype using the RNeasy kit (Qiagen). Equal amounts of total RNA (4 ug) for all samples were purified from genomic DNA with the DNA-free DNase kit (Ambion) prior to oligo(dT)-primed cDNA synthesis using the Omniscript cDNA synthesis kit (Qiagen). The cDNA was diluted 1:20 with nuclease-free H₂O (Invitrogen) for Q-PCR reactions performed on a Cepheid SMARTCycler using QuantiTect SYBR Green kit (Qiagen). Transcript levels were determined using primer sets specific for *lk6-A* or *lk6-B* (details available on request). Each PCR reaction was repeated in triplicate for 3 independent RNA preparations comparing transcript levels in *lk6* mutants to a white-eyed control strain. Expression differences are shown as the average change in cycle number at which PCR product (determined by fluorescent signal) is detected as statistically significant above background. This is referred to as the crossing threshold and the more cDNA template present at the start of the reaction, the fewer number of cycles it takes to reach this point. A one-cycle difference represents a two-fold difference in starting template concentration between samples. All transcript levels are normalized to the control gene, ribosomal protein 49 (*rp49*), as previously described (Sanyal, Sandstrom et al. 2002). Cycle differences were tested against the value of 0, expected if there were no change in
expression levels, using the Student’s \( t \)-test. Only results showing a \( P \)-value <0.05 were considered statistically significant.

Results

**LK6 is expressed in the Drosophila nervous system**

The \( lk6 \) gene predicts two transcripts, \( lk6 \)-A and \( lk6 \)-B, both of which are expressed in flies (Arquier 2005) (Figure 3.1A). We further show that both \( lk6 \) transcripts are expressed in the nervous system. Using quantitative PCR probes to identify the presence of \( lk6 \)-A and \( lk6 \)-B mRNA, we find that both transcripts are expressed in fly heads (Figure 3.1B). The \( lk6 \) loss of function alleles show altered expression of \( lk6 \) mRNA. \( lk6^{EP886} \) and \( lk6^{KG08062} \) mutants show lower levels of \( lk6 \)-A mRNA expression, while \( lk6^1 \) shows severely reduced \( lk6 \)-B mRNA levels (Figure 3.1B). The \( lk6 \)-A transcript is decreased 2 fold in \( lk6^{EP886} \), 32 fold in \( lk6^{KG08062} \), while \( lk6 \)-B is reduced 362 fold in \( lk6^1 \).

The \( lk6 \) transcripts are predicted to produce two proteins: \( lk6 \)-A encodes a larger 1142 amino acid protein, while \( lk6 \)-B encodes a smaller 1090 amino acid protein (Figure 3.1A). Both proteins are expressed in the Drosophila nervous system. In Western blots of adult fly heads and larval central nervous systems, an antibody to LK6 recognizes two major protein bands at 200 and 180 kD, which correspond to the LK6-A and LK6-B proteins (Figure 3.1C). Depending on the location of the mutation in the loss of function \( lk6 \) mutants (Figure 3.1A), they show different effects on LK6 protein expression; mutants affect the presence of LK6-A (\( lk6^{EP886} \) and \( lk6^{KG08062} \)) or LK6-B (\( lk6^1 \)) (Figure
Interestingly, mutations which decrease the levels of one \(lk6\) transcript have normal protein levels of the other, and often increased expression of the unaffected transcript (Figure 3.1).

The presence of multiple \(lk6\) transcripts and proteins indicates that the two isoforms may have redundant functions, therefore it is necessary to knockdown both proteins to examine \(lk6\) function. The null allele for LK6-A and B, \(lk6^{2}\) is a P-element excision that removes the last 6 exons of the \(lk6\) gene (Figure 3.1A) and is homozygous lethal in adults with only few larvae surviving until 1\(^{st}\) instar (Arquier 2005), indicating that \(lk6\) is critical for survival. Due to the unavailability of viable null mutations, we found it difficult to examine the effect of completely knocking out \(lk6\) function in 3\(^{rd}\) instar larvae and adults. To remedy this and to have better spatial control in our experiments, we generated a \(lk6\) RNAi transgene under UAS control to express double stranded RNA and silence expression of both \(lk6\)-A and B transcripts in the tissue of choice. When expressed in the nervous system, UAS \(lk6\) RNAi decreases both LK6-A and B protein in fly heads (Figure 3.1C) and causes a reduction in the levels of both \(lk6\)-A and \(lk6\)-B transcripts, 6 fold and 5 fold respectively (Figure 3.1B).

\textit{LK6 functions downstream of Ras/ERK to regulate synaptic growth at the NMJ}

In mammalian systems, ERK signaling may control translation underlying synaptic plasticity through the activation of the eIF4E kinase MNK1, although direct proof that MNK1 is required for Ras/ERK induced synaptic plasticity has not been well established. Similarly, activating Ras/ERK signaling in motor neurons (using transgenic expression of Ras1\(^{V12-S35}\) that specifically activates ERK) leads to large increases in...
synaptic size at the NMJ (Koh, Ruiz-Canada et al. 2002). If LK6 functions downstream of ERK to regulate synaptic growth, it is possible that reducing LK6 function in an activated Ras background will attenuate the effect of enhanced Ras/ERK signaling on synaptic growth. In a wild type background, blocking function of one or both of the lk6 transcripts has no significant effect on synaptic growth (Figure 3.2A). Synaptic size, as measured by bouton number in muscles 6 and 7 of segment A2 in Drosophila larvae, is not significantly altered in the LK6-A mutants \(lk6^{EP886}\) (n=16, p=0.679) and \(lk6^{KG08062}\) (n=5, p=0.954) or in the LK6-B mutant \(lk6^I\) (n=9, p=0.179) compared to wild type (n=10). Inhibiting both LK6-A and B function in the nervous system by expressing lk6 RNAi with a pan-neuronal GaL4 driver (C155 Gal4) has no effect on bouton number at the larval NMJ compared to Gal4 alone (n=10, p=0.508).

We next tested if MNK is required for Ras dependent synaptic expansion. When Ras\(^{V12-S35}\) is expressed in motorneurons with C380-GAL4, a motor neuron specific GAL4 driver (Koh, Ruiz-Canada et al. 2002), larval NMJ synapses are 25% larger than C380 alone (n=20, p=0.0000015). Inhibiting \(lk6\) function suppresses the effect of increased Ras/ERK signaling on the growth of the NMJ (Figure 3.2B). Expression of UAS lk6 RNAi in the C380-UAS Ras\(^{V12-S35}\) background leads to synapses that are significantly smaller than C380-UAS Ras\(^{V12-S35}\) (n=15, p=0.021). Expression of UAS lk6 RNAi with C380 has no significant effect on synaptic size compared to C380 controls (n=6, p=0.214), indicating that \(lk6\) is not required in motorneurons for synaptic growth in a wild type background.
These findings suggest that while there is no discernible effect of reducing LK6 levels in the nervous system on growth of the neuromuscular synapse, LK6 may function downstream of the Ras/ERK pathway to regulate NMJ plasticity. Although not investigated directly, LK6, as a potential eIF4E kinase that functions downstream of ERK, may link Ras/ERK signaling to translational regulation in long-term plasticity.

*LK6 is activated by neural activity in an ERK dependent manner*

In non-neuronal mammalian cells, ERK phosphorylates MNK1 at Thr197 and 202 (Fukunaga and Hunter 1997; Waskiewicz, Flynn et al. 1997). The ERK phosphorylation sites are conserved in LK6, and there is evidence that LK6 can be activated by ERK in *Drosophila* cell culture (Parra-Palau, Scheper et al. 2004). To determine if LK6 is activated by ERK in the fly nervous system, we utilized temperature sensitive seizure mutants in dSERCA (*Ca-P60A*Kum170). In these animals, heating to restrictive temperatures results in increased Ca\(^{2+}\) levels and seizure-like spikes of activity in the nervous system. Previous work from our lab has shown that exposing *Ca-P60A*Kum170 flies to a 40°C heat pulse for 4 minutes induces high levels of phosphorylated ERK in fly heads that increases rapidly and is persistent over time (Hoeffer, Sanyal et al. 2003). We tested if increased ERK activation in these mutants leads to detectable changes in the phosphorylation of MNK. For this, we used a mouse antibody that recognizes both ERK phosphorylation sites in MNK1, on Western blots of proteins isolated from fly head lysates. Increased levels of phospho-MNK1 are observed 60 minutes after heating *Ca-P60A*Kum170 flies, which correlates with increases in phospho-ERK levels (Figure 3.3A). P-MNK1 levels increase 2 fold in *Ca-P60A*Kum170 heated flies compared to heated wild
type (n=7, p=0.001) (Figure 3.3B). The P-MNK1 band runs at the same size as LK6 on a western blot (200 kD) indicating that the P-MNK1 antibody is likely recognizing phosphorylated LK6. To determine if the phosphorylation of LK6 is dependent on, and not just correlated with ERK phosphorylation, we fed flies a MEK inhibitor, U0126, that specifically blocks the activity of the upstream kinase of ERK. Inhibiting MEK in Ca-P60A\textsuperscript{Kum170} heated flies blocked the increase in both P-ERK and P-LK6, indicating that the LK6 activation is likely due to ERK signaling (Figure 3.3A). In flies fed MEK inhibitor, P-MNK1 is only increased 1.19 fold in heated Ca-P60A\textsuperscript{Kum170} compared to wild type (n=3, p=0.042) (Figure 3.3B). With these results, we provide in vivo evidence that LK6 is a potential ERK target in flies and further proof that Ras/ERK/LK6 signaling occurs to regulate plasticity in the fly nervous system.

**Discussion**

Recent studies have suggested that ERK signaling regulates translation required for long-term plasticity through the activation of MNK1 and eIF4E. For instance, stimuli that induce LTP in the mammalian hippocampus correlate with MNK1 and eIF4E activation and enhanced translation rates in an ERK dependent manner (Banko, Hou et al. 2004; Kelleher, Govindarajan et al. 2004). Our current study provides evidence that LK6, the fly homolog of MNK1, is 1) required for Ras/ERK mediated synaptic growth and 2) phosphorylated by ERK in the fly nervous system in vivo. Below we discuss these findings in the context of signaling during neural plasticity and the potential effect on eIF4E activation and regulation of translation.
Ras/ERK mediated synaptic growth is MNK dependent

ERK has been shown to positively influence NMJ growth in Drosophila based on the finding that motorneuron overexpression of Ras (V12-S35) that specifically activates ERK, causes large increases in synaptic size (Koh, Ruiz-Canada et al. 2002). Our results indicate that LK6 is required for this Ras/ERK mediated synaptic growth. Specifically, blocking \textit{lk6} function using RNA interference in a C380; Ras\textsuperscript{V12-S35} background, results in significantly smaller synapses than C380; Ras\textsuperscript{V12-S35} alone. Thus, we show for the first time that loss of LK6 function has an effect on synaptic plasticity in a Ras/ERK activated background. This lends credence to the idea that Ras/ERK dependent synaptic plasticity may function at least in part, through LK6, and ultimately regulate translation.

It has previously been shown that LK6 is phosphorylated by ERK and is able to phosphorylate eIF4E in \textit{Drosophila} cell culture and in fly ovaries (Parra-Palau, Scheper et al. 2004; Arquier 2005). But is LK6 an ERK target in neurons? By inducing neural activity in \textit{Drosophila} \textit{Ca-P60A}\textsuperscript{Kam170} mutants, we find that phosphorylation of both ERK and LK6 is induced in fly heads. This activity induced phosphorylation of ERK and LK6 is blocked by administration of a MEK inhibitor, indicating that LK6 phosphorylation is dependent on, and not simply correlated with, ERK activation. To our knowledge, this is an initial demonstration that LK6 is downstream of ERK in the fly nervous system and is activated following neural activity. Taken together, these results imply that MNK may be important for activity-dependent synaptic signaling, probably functioning downstream to ERK during plasticity regulation.
Potential routes to translation regulation through eIF4E modulation

Although not investigated directly in this study, there is substantial evidence that MNK may regulate translation through phosphorylation of the translation initiation factor eIF4E. MNK phosphorylates eIF4E in vitro, and eIF4E activation is correlated with enhanced ERK and MNK signaling (Wang, Flynn et al. 1998; Waskiewicz, Johnson et al. 1999). Additionally, in both mice and flies that are mutant for MNK and LK6, eIF4E phosphorylation is drastically reduced (Ueda, Watanabe-Fukunaga et al. 2004; Arquier 2005). In most cases, increased eIF4E phosphorylation is correlated with increased rates of translation (Scheper and Proud 2002), presumably through an increase its 5’ cap binding affinity. However, recent findings indicate that eIF4E phosphorylation may actually decrease its ability to bind mRNA (Minich, Balasta et al. 1994; Knauf, Tschopp et al. 2001; McKendrick, Morley et al. 2001; Scheper, van Kollenburg et al. 2002; Zuberek, Wyslouch-Cieszynska et al. 2003). Based on the current model, it is likely that unphosphorylated eIF4E binds to the 5’ mRNA cap to initiate translation, but that eIF4E must be phosphorylated and removed from the 5’ mRNA cap to be able to initiate translation of other mRNAs (Scheper and Proud 2002).

The regulation of eIF4E phosphorylation in long-term synaptic plasticity may come into play when neural activity signals to ERK to promote gene expression or local protein synthesis, and there are many new transcripts that require translation. Under these conditions regulation of MNK function becomes critical for proper translation initiation of new transcripts (Arquier 2005), and may be a mechanism for fine-tuning of eIF4E function (Gingras, Rauth et al. 2001). The possibility that under basal conditions
eIF4E phosphorylation by LK6 is not critical for translational regulation may contribute to the lack of synaptic growth phenotypes when LK6 function is decreased in the nervous system. In both mammals and flies, deleting MNK1 or LK6 often does not cause any detectable biological defects. In mice, animals that are mutant for both MNK1 and MNK2 have no detectable phosphorylated eIF4E protein, yet they are viable, fertile, and grow and develop normally (Ueda, Watanabe-Fukunaga et al. 2004). Although it has been shown that eIF4E phosphorylation and \textit{lk6} is required for growth in flies (Lachance, Miron et al. 2002; Arquier 2005), in another study, \textit{Drosophila lk6} mutants have aberrant growth phenotypes only when they are raised on food with a 30\% yeast content, but not on 100\% yeast (Reiling 2005). This indicates that LK6 is a positive regulator of growth under low nutrient conditions. The lack of growth effects in \textit{lk6} mutants at 100\% yeast may be explained by a model in which high nutrients allow mTOR induced phosphorylation of 4E-BP, which triggers the release of eIF4E and allows it to initiate translation. The phosphorylation of eIF4E by LK6 may be important when nutrients and mTOR signaling are low and the majority of eIF4E is still bound to and inhibited by 4E-BP (Gingras, Raught et al. 2001; Reiling 2005).

Our findings show that LK6 has a role in Ras/ERK mediated synaptic growth when it is blocked presynaptically. However, previous work at the NMJ has shown that postsynaptic alterations in eIF4E lead to enhanced synaptic function and arborization (Sigrist, Thiel et al. 2000). It is likely that ERK/LK6/eIF4E signaling occurs both pre- and post-synaptically to regulate synaptic growth at the NMJ. Further experiments are required to qualitatively discriminate between these two possibilities.
A model for ERK signaling during long-term plasticity

It is widely accepted that ERK is an important signaling factor required for learning, memory, and neuronal plasticity in both vertebrates and invertebrates (Impey, Obrietan et al. 1999; Sweatt 2001). Exactly what happens downstream of ERK signaling is not as well understood. ERK has many targets in neurons that may mediate its regulation of long-term synaptic changes, including modifications in existing proteins and the activation of gene expression. Translocation of ERK to the nucleus initiates gene expression through the activation of the transcription factor CREB, a key molecular switch that is necessary for converting short-term to long-term memory (Impey, Obrietan et al. 1998). However, translation of transcripts is also critical for long-term plasticity and is regulated by ERK signaling. This is supported by recent studies indicating that ERK dependent activation of eIF4E and translation correlates with late LTP and long-term memory in rodents (Kelleher, Govindarajan et al. 2004).

At the fly NMJ, there is evidence that the activation of Ras/ERK signaling at least partially mediates synaptic growth through reduction of the cell adhesion molecule Fas II (Koh, Ruiz-Canada et al. 2002). It is likely that Ras/ERK signaling also causes increased synaptic growth by regulating gene transcription. In addition, our studies show that the translational regulator LK6 is also required for Ras/ERK mediated synaptic growth. It is of note that inhibiting LK6 function in our experiments only partially suppressed the effect of increased ERK signaling. This indicates that other downstream effectors may also contribute to ERK’s regulation of synaptic growth. In a generalized model of plasticity (Figure 3.4), ERK may regulate existing proteins, gene expression, and the
synthesis of new proteins simultaneously. Thus, both translation of pre-existing synaptic mRNAs and newly transcribed mRNAs may be regulated by ERK signaling. Our findings support ERK’s role in promoting translation through LK6 activation, but do not distinguish between translational regulation of newly transcribed mRNAs or pre-existing “local” transcripts. It is possible that ERK dependent LK6 activation may promote a general increase in translation of new transcripts, since eIF4E regulation is required for all cap-dependent translation. However, the ERK/LK6/eIF4E pathway may also be responsible for enhanced protein synthesis of local, synapse specific mRNAs that may be necessary for long-term synaptic changes. It is also unclear whether ERK dependent translation occurs concurrently with ERK’s regulation of transcription, or is a primary step to generate new proteins that must be synthesized for transcription to occur. The temporal hierarchy of these two related phenomena are likely to form the focus of future experiments. However, we provide an important link between ERK and MNK, which strengthens the idea that ERK regulates general translation through the activation of eIF4E.

It is also likely that LK6 has other functions in flies than eIF4E phosphorylation. Drosophila LK6 is a much larger protein than mammalian MNK, and binds to microtubules and contains PEST sequences indicating that LK6 may have functions other than regulation of eIF4E phosphorylation (Kidd and Raff 1997).

In conclusion, although specifics about the regulation of translation and eIF4E activation by ERK and MNK are not very well understood, our findings provide evidence that MNK functions downstream of ERK to regulate synaptic growth, and that ERK
dependent activation of MNK occurs in the nervous system. Our findings are consistent with a model in which neuronal activity or synaptic signaling to induce long-term plasticity turns on ERK signaling, which can then regulate transcription through CREB activation (via Rsk phosphorylation) and also stimulate translation of pre-existing or newly synthesized mRNAs through activation of MNK and eIF4E (Figure 3.4). Our current experiments further establish that MNK is the link between ERK and translational regulation of plasticity, however, further studies on the role of ERK, MNK, and eIF4E in long-term plasticity and translational regulation using both vertebrate and invertebrate models will be useful in strengthening this link.
Figure 3.1

LK6-A and B are expressed in the *Drosophila* nervous system.

A. The *Drosophila* *lk6* gene is predicted to produce two transcripts, *lk6*-A encodes a larger 1142 amino acid protein while *lk6*-B encodes a smaller 1090 amino acid protein. We used mutants with the p-element insertions shown here, *lk6*\(^{EP886}\), located 5’ of the first exon in *lk6*-A, and *lk6*\(^{KG08062}\), located in the first exon of *lk6*-A. Two deletions used in this study are also shown, *lk6*\(^{l1}\) deletes the first exon of *lk6*-B, while *lk6*\(^{l2}\) deletes the entire coding sequence downstream of *lk6*-B. To knock down expression of both LK6 isoforms, we also generated a *lk6* RNAi transgene under UAS control using a sequence from exons 4 through 6 present in both *lk6*-A and *lk6*-B transcripts.

B. Using quantitative PCR to examine gene expression, both *lk6* transcripts are expressed in the fly nervous system. Primers were designed to sequences present in *lk6*-A or *lk6*-B. Mutants with insertions located upstream of or within the first exon of *lk6*-A, *lk6*\(^{EP886}\) and *lk6*\(^{KG08062}\), show lower levels of *lk6*-A expression (n=3, p=0.039 for *lk6*\(^{EP886}\), p=0.0004 for *lk6*\(^{KG08062}\)) and higher levels of *lk6*-B expression (n=3, p=0.014 for *lk6*\(^{EP886}\), p=0.019 *lk6*\(^{KG08062}\)) in fly heads compared to wild type. Flies with the *lk6*\(^{l1}\) deletion show lower levels of *lk6*-B expression in heads compared to wild type (n=3, p=0.0002). When the lk6 RNAi transgene is expressed in the nervous system using the C155 Gal4 driver, transcript levels of lk6-A (n=3, p=0.02) and lk6-B (n=3, p=0.0006) are both significantly reduced compared to non-driven controls. One cycle difference in transcript level corresponds to a two-fold change.
C. In Western blots of adult fly heads and larval central nervous systems, an antibody to LK6 recognizes two major protein bands at 200 and 180 kD, which correspond to the LK6-A and LK6-B proteins. In \( lk6^{EP886} \) and \( lk6^{KG08062} \) fly heads, the LK6-A protein band is missing, while in \( lk6^{1} \), the LK6-B protein band is missing. Levels of both LK6-A and LK6-B proteins are reduced when UAS \( lk6 \) RNAi is expressed in the nervous system with C155 Gal4. Dynamin is used as a loading control.
A. Illustrated diagram with gene expression levels and RNAi sequence.

B. Bar graph showing cycle difference for Ik6 alleles: Ik6^{EP88}, Ik6^{KG08062}, Ik6^{1}, and C155-Ik6 RNAi. Comparison between Ik6-A and Ik6-B with significant differences indicated by asterisks.

C. Western blot analysis: LK6 band profiles for wild type, Ik6^{EP88}, Ik6^{KG08062}, Ik6^{1}, and C155-Ik6 RNAi, with loading controls for adult head and larval CNS at 200 kD and 185 kD.
Figure 3.2

LK6 functions downstream of Ras/ERK to regulate synaptic growth at the larval NMJ.

A. LK6 is not required for synaptic growth at the larval NMJ in a wild type background. Synaptic size, as measured by bouton number in muscles 6 and 7 of segment A2 in *Drosophila* larvae, is not significantly altered in the LK6-A mutants *lk6*<sup>EP886</sup> (n=16, p=0.679) and *lk6*<sup>KG08062</sup> (n=5, p=0.954) or in the LK6-B mutant *lk6<sup>1</sup>* (n=9, p=0.179) compared to wild type (n=10). Inhibiting LK6-A and B function in the nervous system by expressing lk6 RNAi with C155 Gal4 has no effect on bouton number at the larval NMJ compared to Gal 4 alone (n=10, p=0.508).

B. Inhibiting LK6 in motorneurons by expressing UAS lk6 RNAi with C380 Gal4 suppresses C380-UAS Ras<sup>V12-S35</sup> overgrowth. When Ras<sup>V12-S35</sup> is expressed in motorneurons with C380, larval NMJ synapses are 25% larger than C380 alone (n=20, p=0.0000015). Expression of UAS lk6 RNAi in the C380-UAS Ras<sup>V12-S35</sup> background leads to synapses that are 14% smaller than C380-UAS Ras<sup>V12-S35</sup> (n=15, p=0.021). Expression of UAS lk6 RNAi with C380 has no significant effect on synaptic size compared to C380 controls (n=6, p=0.214).
Figure 3.3

LK6 is activated downstream of Ras/ERK in the *Drosophila* nervous system.

A. Generation of neural activity by exposing the temperature sensitive mutant *Kum*<sup>170</sup> to a 4 minute heat shock at 40°C induces activation of ERK and LK6 60 minutes after heating, as measured by Western blots of adult fly heads probed with antibodies to phosphorylated ERK and MNK1. *Kum*<sup>170</sup> flies that are fed the MEK inhibitor U0126 prior to heat shock do not show activation of ERK or LK6. Dynamin is shown as a loading control.

B. The histogram shows quantification of the Western blot data as a ratio of heated *Kum*<sup>170</sup> (treated) to heated Canton-S (control) flies. When compared to heated Canton-S flies, phospho-MNK1 levels in heated *Kum*<sup>170</sup> are increased 2.16 to 1, while the dynamin loading control ratio is 1.05 to 1 (n=7, p=0.001). When compared to heated Canton-S flies, phospho-MNK1 levels in heated *Kum*<sup>170</sup> that are fed MEK inhibitor have a ratio of 1.19 to 1, while the dynamin loading control ratio is 0.86 to 1 (n=3, p=0.042 comparing p-MNK1 to p-MNK1 with MEK inhibitor).
Figure 3.4

Mechanisms of ERK dependent protein synthesis in long-term plasticity. Ras/ERK signaling is induced with neuronal activity. ERK regulates new gene expression by phosphorylation of CREB via Rsk. ERK signaling also activates MNK, which phosphorylates eIF4E. Activated eIF4E can initiate translation of pre-existing transcripts, or can enhance translation of newly transcribed mRNAs. Both of these processes may be required for new protein synthesis necessary in long-term synaptic plasticity.
CHAPTER FOUR

PROSPECTS OF MEMORY-MODIFYING DRUGS THAT TARGET THE CREB PATHWAY

Note from author: Much of the text and figures contained in this chapter were published in an article written by myself with help from my advisor, Mani Ramaswami.


Abstract

The quest to find safe and effective drugs to treat memory disorders and decline in humans has been aided by the understanding of the molecules underlying memory formation. Studies from both vertebrates and invertebrates have identified molecules that are specifically required for converting memories into a long-lasting and stable form. Due to the fact that activation of the transcription factor cAMP-responsive element binding protein (CREB) is critical for converting short-term to long-term memory, the molecules required for CREB activation and function are potentially important targets for memory modifying drugs. The progress, prospects, challenges and limitations of drugs targeting the CREB pathway are reviewed here in context of molecular mechanisms and the biology of memory.
Introduction

Genetic factors, age, injury and disease often produce memory deficits in humans. Despite their considerable therapeutic potential, safe and effective drugs to enhance memory are not yet available. Recent insights into molecules and pathways that underlie memory formation have helped identify potential drug targets; in addition they have enabled rapid screens for molecules that modulate mechanisms of neural plasticity (Barco, Pittenger et al. 2003; Tully, Bourtchouladze et al. 2003). Central to this new informed approach have been insights gained from genetic, cell biological and behavioral analyses of memory mechanisms in the mouse, the marine mollusk *Aplysia*, and the fruit fly *Drosophila melanogaster* (Kandel 2001). In each organism, similar, evolutionary conserved molecular pathways have been outlined that likely underlie distinct phases of memory first defined by early experimental psychologists. In particular, long-term memory, first defined by Hermann Ebbinghaus as a process requiring temporally separate episodes of “spaced” training, has been shown to be a distinctive protein synthesis-dependent form of memory initiated, in mammals, mollusks, and insects, through the transcription factor CREB (Yin and Tully 1996). We discuss below how this greater understanding of the molecular basis of long-term memory formation has opened up the opportunity to identify memory enhancing drugs. Prospects for drugs that inhibit long-term memory formation, potentially useful for preventing long-term consequences of psychological trauma, are also briefly considered. Finally, we discuss important biological unknowns and complexities that make a search for memory modifying drugs a particularly challenging goal in contemporary biological and pharmaceutical research.
Models for analyzing the mechanisms of long-term memory

In the mammalian brain, the hippocampus, a structure in the medial temporal brain lobe, is most often cited as the anatomical region where memory formation occurs. Hippocampal lesions, famously of the patient H.M., have demonstrated its requirement for consolidation of declarative memories in humans and other animals (Squire 1992). More instructively, neurons in the hippocampus grow new synaptic connections and change the strength of existing connections in response to specific forms of behavioral or synaptic stimulation (Colicos, Collins et al. 2001; Wu, Deisseroth et al. 2001). Long-term potentiation (LTP), the strengthening of synaptic connections after stimulation, can be produced in several neural pathways in the hippocampus (Bliss and Collingridge 1993). While the range of hippocampal functions are poorly understood, its role in storing spatial information has been extensively studied and beautifully documented. Not surprisingly, genetic mutations that affect LTP formation often also cause defects in the acquisition of spatial memory (McHugh, Blum et al. 1996). In addition to the hippocampus, the amygdala is required for learning associations with emotional significance, such as fearful or painful stimuli (McGaugh 2000). While inputs to the hippocampus from the amygdala may allow the amygdala to modulate hippocampal dependent memories, LTP in the amygdala has been tightly correlated with fear conditioning (Rogan, Staubli et al. 1997), a phenomenon in which rodents learn to associate a tone with unpleasant electrical stimulation of their feet (footshock). Thus, in mammals, mechanisms of memory have been primarily elucidated by analyzing effects of
molecular perturbations on hippocampal and amygdalar LTP or spatial memory and fear conditioning.

Remarkably, several molecules involved in behavioral and synaptic plasticity in mammals are also required for homologous processes in mollusks and flies. In all three systems, growth of new synaptic connections has been correlated with long-term, but not short-term forms of plasticity (Bailey and Chen 1988; Koh, Gramates et al. 2000; Colicos, Collins et al. 2001). In *Aplysia*, the activation of CREB by appropriate synaptic signals is a critical and essential process for long-term sensitization (enhanced gill withdrawal following mild siphon stimulation) and underlying long-term facilitation (LTF) of the sensorimotor synapse that controls this behavior (Bartsch, Ghirardi et al. 1995). Similarly, in *Drosophila*, CREB activation is required for long-term olfactory associative memory and activity-dependent motor neuron plasticity (Yín, Wallach et al. 1994; Davis, Schuster et al. 1996).

**Molecular pathways to long-term synaptic and behavioral change**

Several molecules have been identified that seem to be required for conserved pathways to long-term plasticity in various animal and cellular models of plasticity (Kandel 2001) (Figure 4.1). When integrated, information from these models indicates a framework in which synaptic activity, signaling through G-protein coupled receptors or ion channels, leads to increases in second messengers such as Ca\(^{2+}\) and cAMP. Specific spatial and temporal features of Ca\(^{2+}\) and cAMP transients then cause activation of several downstream protein kinases. The best established memory kinases that function
in the CREB pathway include the calcium-calmodulin dependent CaM Kinases, the cAMP responsive Protein Kinase A (PKA), Protein kinase C (PKC), and the Mitogen Activated Protein (MAP) kinase, ERK. These kinases can cause short-term modifications in the strength and growth of synaptic connections by modifying existing proteins such as ion channels and cell adhesion molecules (Bailey, Kaang et al. 1997). Persistent activation of ERK that, like long-term memory, usually requires spaced stimulation protocols (Michael, Martin et al. 1998; Wu, Deisseroth et al. 2001), induces gene expression and synthesis of new proteins required for longer-term modifications of synapses (Adams, Roberson et al. 2000). Two important issues in synaptic signaling considered later in the article are, therefore: a) why and how spaced stimulation procedures cause persistent kinase signaling; and b) why and how persistent but not transient kinase activity triggers meaningful new gene expression. However, before addressing these issues it is important to appreciate that synaptically driven gene expression is controlled by the activation of several transcription factors including the cAMP responsive element binding factor (CREB) that is phosphorylated by ERK, as well as accessory transcription factors, Fos, Jun, c/EBP and others, that may or may not be responsive to synaptic signaling (Bito, Deisseroth et al. 1996; Yamamoto, Hegde et al. 1999; Sanyal, Sandstrom et al. 2002).

CREB has emerged as a central integrator of synaptic signaling, a molecule proposed to gate the transition between long-term and short-term memory (Lonze and Ginty 2002). It is likely that all neurons contain at least two opposing isoforms of CREB; one that when phosphorylated acts as a transcriptional activator, and another opposing
isoform than when phosphorylated acts as a transcriptional repressor. As demonstrated in *Drosophila* (Yin, Wallach et al. 1994; Yin, Del Vecchio et al. 1995), *Aplysia* (Bartsch, Ghirardi et al. 1995), and mouse (Bourtchouladze, Frenguelli et al. 1994; Barco, Alarcon et al. 2002), increased levels of the CREB repressor inhibit initiation of long-term plasticity; in contrast, induction of the CREB activator significantly reduces the number of training trials required for long-term memory formation and, thus, enhances initiation of long-term synaptic change. Together these observations establish that a stimulus could induce long-term plasticity by: a) inhibiting CREB repressor; b) turning on the CREB activator; or c) both. Dramatic enhancement of long-term memory observed following CREB activation underscores the significance of the CREB activating pathway for the development of memory enhancing drugs. The balance between inhibitory and activating signals, described above for CREB, is a integral element of intracellular signaling pathways of profound importance for drug design. Drugs that antagonize inhibitory molecules are likely to be more commonly found than those that stimulate activators. Thus molecules that inhibit long-term plasticity, particularly at rate-determining steps in the signaling pathway, should represent particularly promising drug targets.

**Established targets for memory enhancing drugs**

As shown in Figure 4.1, the pathway from synaptic activity to CREB phosphorylation involves multiple molecules, from neurotransmitter receptors to CREB itself. Genetic or pharmacological manipulations of several of these molecules (listed in Table 4.1) have been shown to either enhance memory formation in normal animals or to
alleviate memory deficiencies. Significant side effects of these manipulations, generally poorly studied, are only briefly considered later in this article.

**Neurotransmitter receptors**

Receptors for glutamate, the primary neurotransmitter in the mammalian central nervous system, have been manipulated to enhance memory formation. The activation of both AMPA and NMDA subtype of glutamate receptors is required for LTP in the hippocampus. AMPA receptor activation is necessary to allow sufficient depolarization in neurons to activate NMDA receptors. NMDA receptor activation is required to allow $\text{Ca}^{2+}$ to enter neurons, which may turn on $\text{Ca}^{2+}$ dependent signaling cascades and lead to the activation of kinases that regulate synaptic changes (Nicoll and Malenka 1999).

Mouse knockouts of the NMDA receptor have memory defects as expected, but, more striking, overexpression of NMDA receptor in mice enhances learning and memory in a variety of tasks (Tsien, Huerta et al. 1996; Tang, Shimizu et al. 1999). Consistent with these genetic studies, drugs that act as NMDA receptor agonists can increase synaptic transmission and enhance memory (Pussinen, Nieminen et al. 1997). Enhancement and changes in LTP are also responsive to increased AMPA receptor number or function; increased AMPA receptor activity reduces the requirement for neuronal depolarization needed to activate NMDA receptors. Thus, ampakines, drugs that can positively modulate depolarizing currents from AMPA receptors enhance synaptic plasticity, memory in rats, and memory scores in humans (Lynch 2002). Benzothiadiazides also increase AMPA receptor activity, LTP, and spatial memory in rats (Lynch 2002). Companies such as
Pharmacia and Cortex Pharmaceuticals are investigating the use of substances that enhance NMDA and AMPA receptor function as potential memory enhancing drugs.

*Cytoplasmic signaling components*

Downstream of second messengers Ca\(^{2+}\) and cAMP are the kinases such as CaM kinases, PKC, PKA, and ERK that also regulate a wide variety of neural processes unrelated to synaptic function and/or plasticity. Inhibiting cAMP, CaM kinases, PKC, PKA, and ERK have all been shown to regulate several forms of cellular and behavioral plasticity (Abeliovich, Paylor et al. 1993; Martin, Michael et al. 1997; Atkins, Selcher et al. 1998; Kang, Sun et al. 2001). While increasing the activity of atypical (poorly understood) PKC has recently been shown to enhance long-term memory formation in *Drosophila* (Drier, Tello et al. 2002), in general, behavioral consequences of directly increasing basal activity of these kinases have not be systematically assessed. In some cases, increasing concentrations of second messengers or kinase activity actually decreases the capacity for nervous system plasticity and memory (Zhong and Wu 1991). In contrast to manipulations that directly increase levels of these signaling kinases, genetic perturbation of inhibitory inputs into the signaling pathway has been shown to cause memory enhancement (Abel, Martin et al. 1998). These observations could indicate that local relief of inhibition, rather than global activation, contributes substantially to native synaptic signaling events that underlie long-term plasticity.

Protein phosphatases such as calcineurin and protein phosphatase 1 (PP1) can negatively regulate memory by dephosphorylating proteins that have been phosphorylated by kinases. Calcineurin can dephosphorylate PKA, a kinase critical for
synaptic plasticity and memory. Genetically inhibiting calcineurin in mice, which may relieve its repression on kinases such as PKA, results in enhancements in LTP and spatial memory (Malleret, Haditsch et al. 2001). Calcineurin may also negatively regulate memory by dephosphorylating “Inhibitor 1”, an endogenous inhibitor of PP1. One of PP1’s potential targets for dephosphorylation is CREB (Alberts, Montminy et al. 1994). Dephosphorylation of CREB by PP1 has been shown recently to normally inhibit memory formation. Mice overexpressing inhibitor 1 which have decreased PP1 activity are able to obtain maximum levels of memory with a single training session in an object recognition task that normally requires spaced episodes of training (Genoux, Haditsch et al. 2002). This enhancement of memory after spaced training is correlated with both an increase in CREB phosphorylation and CREB dependent gene expression to levels that are usually only achieved after spaced training. From these findings, it appears that PP1’s normal role is to suppress CREB activation and memory formation; relief of this repression facilitates formation of long-term memory. Drugs that inhibit the function of calcineurin, PP1, and other negative regulators of memory are potentially very useful therapeutic tools in enhancing CREB function and memory.

The ERK signaling pathway is another potential modulator of CREB activation that may be a target for memory enhancing drugs. Activated ERK phosphorylates CREB through the protein kinase RSK, which leads to CREB dependent gene expression (Impey, Obrietan et al. 1998). In neurons, activation of ERK can occur either transiently or, following spaced stimulation, for longer, more sustained periods of time. In non-neuronal systems sustained ERK phosphorylation allows activated ERK to phosphorylate
and so stabilize and/or active transcription factors downstream of CREB, that require more than 40 minutes to be synthesized (Adams, Roberson et al. 2000; Wu, Deisseroth et al. 2001; Murphy, Smith et al. 2002). Thus, a plausible mechanism exists by which the duration of ERK activation may be interpreted by the cell. Evidence of ERK signaling in non-neuronal systems suggests that transient and sustained ERK activation may result from two separate signaling pathways (Figure 4.1). The activation of Ras by Ca^{2+} may lead to transient ERK phosphorylation, while cAMP activation may mediate a parallel pathway via Rap1 that can lead to ERK phosphorylation for longer periods of time (Stork and Schmitt 2002). Additionally, differences in upstream signals can activate Ras in different locations in the cell, creating the potential for spatial regulation of ERK signaling (Bivona, Perez De Castro et al. 2003). While little is currently known about the pathway to persistent ERK activation, it is nevertheless possible that drugs targeting inhibitory inputs into this pathway may enhance long-term plasticity and memory. More interesting is the possibility that drugs targeting positive signaling molecules in the pathway to persistent ERK could act as specific inhibitors of long-term memory. Because such drugs may ignore mechanisms that underlie transient ERK activation, the non-specific effects of perturbing general ERK signaling may be minimized.

In addition to its regulation of CREB dependent gene expression, ERK signaling has recently been shown to regulate the translation of mRNAs required for long-term memory. In non-neuronal cells, ERK controls translation through the phosphorylation of MNK, which activates eIF4E, a mRNA binding protein that is necessary for translation initiation (Waskiewicz, Flynn et al. 1997; Waskiewicz, Johnson et al. 1999; Pyronnet
ERK dependent activation of eIF4E is induced in the hippocampus with contextual fear conditioning in mice and with stimulation leading to a translation dependent, transcription independent form of late LTP (Kelleher, Govindarajan et al. 2004). Additionally, ERK dependent activation of MNK and eIF4E occurs in the hippocampus with stimuli that induce long-term plasticity, including application of BDNF or the activation of NMDA receptors (Banko, Hou et al. 2004).

Targeting kinases downstream of ERK that regulate translation dependent long-term memory and synaptic plasticity, such as MNK and eIF4E, may be one way to specifically enhance memory and plasticity related ERK signaling, without interrupting general ERK signaling in other systems. However, MNK and eIF4E regulate translation in non-neuronal cells, and are activated with stress and required for normal cell growth, so specific neuronal targeting may be necessary to limit effects of drugs to neural plasticity and memory (Wang, Flynn et al. 1998; Lachance, Miron et al. 2002; Parra-Palau, Scheper et al. 2004).

While a variety of instructive data indicate a positive role for ERK in synaptic signaling, biological complexities in ERK regulation and function in vivo are indicated by an observation of enhanced synaptic plasticity and memory in the striatum of mutant mice lacking the ERK1 isoform (Mazzucchelli, Vantaggiato et al. 2002). This may be explained either by postulating isoform specific functions for ERK (Morozov, Muzzio et al. 2003), or by increased levels of ERK2 resulting from genetic inhibition of ERK1 (Mazzucchelli, Vantaggiato et al. 2002). If the latter explanation is true, then it is conceivable that pharmacological inhibition of ERK1 may inhibit plasticity, a biological
effect that contrasts with consequences of the ERK1 mutation. Such a dichotomy between the effects of analogous pharmacological and genetic perturbations has been previously observed for CaM Kinase II (Lisman, Malenka et al. 1997).

**CREB and its partners**

The most direct target for memory enhancement is CREB itself, whose transcriptional activity may be the limiting component required to convert short-term to long-term memory (Bito, Deisseroth et al. 1996). A large body of evidence implicates CREB in the conversion from short-term memory to long-term memory. In particular, overexpression of the CREB gene in flies can allow a single training session to result in protein-synthesis dependent long-term memory (Yin, Del Vecchio et al. 1995). Similarly, expression of an active form of CREB in rodent hippocampus facilitates the late, protein synthesis dependent phase of LTP (Barco, Alarcon et al. 2002), and inhibition of the CREB repressor protein reduces the number of stimuli required for long-term facilitation at the *Aplysia* sensorimotor synapse (Bartsch, Ghirardi et al. 1995).

Despite the promise of CREB as a target for a memory enhancing drug in humans, no direct CREB regulating drugs have yet been isolated. As for several other components of the CREB pathway, pleiotropic functions of the molecule are a significant barrier to a safe and specific therapy. Increasing CREB function leads not only to enhanced plasticity and memory in the nervous system, but has wide-ranging effects in other nervous system processes and non-nervous system functions (Lonze and Ginty 2002). Enhancing upstream regulators of CREB such as PKA or ERK has even wider ranging biological effects, as these kinases can phosphorylate substrates in signaling
cascades that underlie numerous cellular functions. As discussed later, this lack of specificity of CREB and regulators of CREB function is a significant constraint for the development of memory enhancing drugs that target the CREB pathway.

While molecules upstream of CREB, the main focus of this review, are clearly important in long-term plasticity and memory, several downstream transcription factors that function with CREB may also be potential targets for memory enhancing drugs. For instance, in *Aplysia*, the transcription factor Ap-AF is downstream of CREB and is required for LTF and its induction substantially enhances long-term facilitation (Bartsch, Ghirardi et al. 2000). Thus, other transcription factors such C/EBP, AP1, and Ap-AF that act along with CREB, and signaling pathways that regulate these CREB-partners may also emerge as targets for memory enhancing drugs.

**New screens for modifiers of long-term memory**

Two companies, Helicon Therapeutics and Memory Pharmaceuticals are attempting to develop drugs affecting the CREB pathway that have specific effects on memory enhancement. Helicon Therapeutics has searched a large library of small molecules, built on a backbone that supports transport across the blood brain barrier, for drugs that enhance CREB activity only in certain conditions, eg. subthreshold activation of specific signaling pathways. A cell line containing a CREB-responsive fluorescent reporter serves as a surrogate for the brain, and reporter activity serves as a surrogate for CREB activity. In screening for drugs that do not increase CREB activity in isolation, but do so in combination with the adenylate cyclase activating drug forskolin, several
compounds were identified that act as inhibitors of phosphodiesterase IV (PDE IV) (Tully, Bourtchouladze et al. 2003). PDE IV inhibitors may enhance CREB function because PDE IV normally hydrolyzes cAMP, therefore inhibiting it leads to higher levels of cAMP and thus increases in CREB phosphorylation. PDE IV inhibitors have also been shown to increase the persistence of LTP and facilitate long-term memory in mice (Barad, Bourtchouladze et al. 1998) and enhance object recognition memory in mutants for a CREB binding protein, a memory deficient mouse that is a model for a human mental retardation syndrome (Bourtchouladze, Lidge et al. 2003).

A recent genetic screen in Drosophila for mutations that specifically inhibit 24 hour memory has identified about 60 genes whose perturbation apparently leaves initial learning intact, but specifically reduces longer-term retention of olfactory aversive conditioning in response to 10 spaced training trials (pairings of odor with footshock) (Dubnau, Chiang et al. 2003). A significant number of these mutations could identify proteins with conserved and specific functions in long-term memory. Similarly, large scale screens for mouse mutations in long-term memory are also in progress (Mayford, Mansuy et al. 1997; Sayah, Khan et al. 2000). New insight into signaling pathways and mechanisms of LTM gained from their analyses may identify new targets for memory modifying drugs.

**Potential limitations or challenges to a CREB pathway limited approach**

Successful development of memory modifying drugs that act via the CREB-pathway faces at least two major challenges. First, as for all drugs, side effects of the
drugs on non-memory associated processes must be avoided. Second, the relative efficacy of a drug on different memory pathways must be carefully assessed: eg. a drug that strongly potentiates fearful memories but acts weakly, or ineffectively, on other declarative memories may not be optimal.

**Specificity for memory/plasticity**

Signaling pathways that underlie memory function in multiple cell types. The Ras/ERK pathway not only participates in a wide range of biological processes, but its activation is also correlated with carcinogenesis (Johnson and Lapadat 2002). There are nevertheless two reasons to be optimistic that brain specific signaling pathways can be successfully targeted.

First, most signaling components exist as multiple isoforms, and for several, brain-specific isoforms exist. Thus, a drug that targets a brain-specific isoform has a greater chance of specifically targeting pathways to neural plasticity. The distribution of different isoforms for signaling enzymes, both across all tissue and within the brain, though relatively poorly characterized so far, is likely to be particularly significant for drug development.

Second, greater specificity for neural pathways may be achieved by combination therapy in which multiple pathway components are stimulated (or inhibited) at levels too low to cause side effects on their own. Thus, for instance, partial inhibition of PDE IV and partial inhibition of CREB-repressor may have no effect when individually applied, but in combination may enhance memory without affecting non-neural processes.
Combination therapy is now in vogue for both intellectual and economic reasons in fields ranging from oncology to cognitive therapy.

**Targeting specific neural pathways**

Will all neurons in the brain respond identically to a drug that affects the CREB pathway? Might hippocampal neurons, for instance, show different sensitivities to these drugs than say neurons in the amygdala? While there are little available data to answer this question, it is likely that a wide range of varying sensitivities will be shown by diverse neuronal cell types. In the framework of the CREB pathway, a cell with low levels of a CREB repressor protein but high levels of CREB activator is likely to be highly responsive to ERK/Rsk stimulation, but only poorly responsive to an inhibitor of the CREB repressor. In addition, recent data suggest that in some neurons (Sanyal, Sandstrom et al. 2002), successful activation of the CREB pathway itself may not be sufficient for all plasticity associated gene expression (Barco, Alarcon et al. 2002).

The most elegant mechanism by which drugs could be targeted to specific functional circuits is based on two principles. First, it is important to emphasize that CREB-dependent gene expression is not sufficient to induce synaptic plasticity; CREB-induced gene products act only on synapses that have been transiently “tagged” by (even low levels) of local activity (Frey and Morris 1997). Thus, a drug that activates a CREB pathway will affect only those synapses activated during the presence of the therapeutic compound. Second, subthreshold activation of the CREB pathway can, without inducing plasticity, reduce the level of activity required for initiation of long-term synaptic change. Thus, at appropriate low doses, the effect of a CREB pathway drug can again be targeted
to active neuronal circuits. These observations have led to a proposal for “augmented cognitive training,” especially useful during stroke rehabilitation for instance, in which CREB pathway stimulants are provided together with appropriate brain exercise (Tully, Bourtchouladze et al. 2003). Thus, beneficial effects of exercise are augmented by a CREB pathway stimulant, while pharmacological side effects are minimized.

A second issue concerns the centrality and sufficiency of ERK dependent CREB activation for all gene expression that underlies long-term plasticity. While several experiments have come close to demonstrating the sufficiency of CREB (Barco, Alarcon et al. 2002), more recent studies indicate that CREB may not be required for plasticity in all neuronal subtypes or all types of memory (Balschun, Wolfer et al. 2003). In the glutamatergic fly motor neuron, CREB activity appears to be dispensable for activity-induced synaptic growth, but only required for activity-regulated stable changes in synaptic strength (Davis, Schuster et al. 1996). More puzzling is the observation that the CREB-responsive immediate early transcription factor AP1 (a dimer of Fos and Jun) appears necessary, and its induction sufficient, for triggering synaptic growth as well as increased synaptic strength, the latter by a CREB-dependent pathway. Thus AP1 in some neurons can both recruit CREB and influence CREB-dependent and independent aspects of synaptic plasticity (Sanyal, Sandstrom et al. 2002). While the generality of this analysis requires extensive further studies, it nevertheless serves to emphasize the need for more models for long-term plasticity in which signaling pathways from activity to gene expression may be analyzed and manipulated.
The hippocampus and amygdala mediate declarative memories or memory of facts and events. Other brain regions, such as the basal ganglia and cerebellum, mediate procedural memory of skills. Because the extent of the CREB pathway’s involvement in other memory systems, such as motor skill learning, language, perceptual or emotional learning, has yet to be determined, the utility of CREB-based memory enhancing drugs for these forms of memory remains to be validated (Figure 4.2). As new studies correlate plasticity in the striate, sensory and motor cortex with specific forms of declarative and non-declarative memory, constraints and flexibility in signaling modules that regulate long-term plasticity will undoubtedly be revealed.

**Concluding remarks**

Drugs that target modulatory inputs from the amygdala may provide an indirect route to enhancing CREB-dependent memory. Emotional arousal increases the significance and strength of memories. The amygdala, with inputs to the hippocampus and various brain regions where CREB dependent plasticity occurs, mediates this effect of emotional content on memory by stress hormones and neuromodulators. Local infusions of epinephrine, corticosterone, noradrenergics, and glucocorticoids in the amygdala or near its targets can enhance memory by increasing the perceived emotional significance of an experience; drugs that effect these modulators increase memory (McGaugh 2000).

Independent of the approach followed, there are obvious concerns and challenges to the development of memory enhancing drugs. Not the least, pharmacological
interference may, by interfering with normal memory filters, result in memories of non-significant stimuli and associations. Further molecular complexities associated with memory are indicated by recent studies showing that long-term fear conditioning in rodents is extinguished if protein synthesis in the amygdala is blocked during a subsequent pairing between tone and shock (Nader, Schafe et al. 2000). It remains unclear if CREB is required for protein synthesis associated with such reconsolidation of memories. Despite these and other uncertainties, current knowledge of CREB, its regulators and function, offer the most promising route towards new, useful therapies for cognitive problems associated with neural plasticity.
Table 4.1

Molecules whose genetic or pharmacological manipulation leads to enhanced memory.

<table>
<thead>
<tr>
<th>Validated Drug Targets</th>
<th>Potential drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMDA receptors</td>
<td>D-cycloserine (Temple and Hamm 1996)</td>
</tr>
<tr>
<td>AMPA receptors</td>
<td>Ampakines (Hampson, Rogers et al. 1998)</td>
</tr>
<tr>
<td></td>
<td>Benzothiadiazides (Staubli, Rogers et al. 1994)</td>
</tr>
<tr>
<td>cAMP phosphodiesterase</td>
<td>Rolipram and other PDE IV inhibitors (Barad, Bourtchouladze et al. 1998)</td>
</tr>
<tr>
<td>CREB activator</td>
<td>No known drugs</td>
</tr>
<tr>
<td>(Yin, Del Vecchio et al. 1995)</td>
<td></td>
</tr>
<tr>
<td>CREB repressor</td>
<td>No known drugs</td>
</tr>
<tr>
<td>(Yin, Wallach et al. 1994)</td>
<td></td>
</tr>
<tr>
<td>Calcineurin</td>
<td>No known drugs</td>
</tr>
<tr>
<td>(Malleret, Haditsch et al. 2001)</td>
<td></td>
</tr>
<tr>
<td>Protein phosphatase 1</td>
<td>No known drugs</td>
</tr>
<tr>
<td>(Alberts, Montminy et al. 1994)</td>
<td></td>
</tr>
<tr>
<td>Atypical PKC (Drier, Tello et al. 2002)</td>
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</tbody>
</table>
Figure 4.1

The molecular pathway underlying CREB-dependent long-term memory. Synaptic activity causes glutamate release from the presynaptic neuron. Glutamate activates AMPA and NMDA receptors, which allow Ca\(^{2+}\) entry postsynaptically. Ca\(^{2+}\) can activate protein kinase C (PKC), Ca\(^{2+}/\)calmodulin-dependent protein kinases (CaMK), and adenylyl cyclase (AC). AC causes an increase in cAMP levels, which leads to activated protein kinase A (PKA). Through its effect on PKA and Ras, Ca\(^{2+}\) can also activate ERK, which translocates to the nucleus to activate CREB via RSK. Phosphorylation allows CREB to induce transcription of genes required for long-term synaptic changes. Studies in neuronal and non-neuronal cells suggest that ERK is activated by two separate pathways shown in blue. Ca\(^{2+}\) activation of Ras leads to a rapid but transient ERK activation, while cAMP causes a delayed and sustained ERK signaling via PKA and Rap1. Molecules negatively regulating the CREB pathway are shown in red. Phosphodiesterase (PDE) metabolizes cAMP. Calcineurin (CN) dephosphorylates PKA and inhibitor 1. Inhibitor 1 can inactivate protein phosphatase 1 (PP1), which dephosphorylates CREB.
Synaptic activity

Glutamate release

AMPA

NMDA

GPCR

PKC

CaMK

Ca²⁺

cAMP

PKA

CaMK

ERK

RSK

CREB

CRE

Glu

Glu

Glu

Ras

Rap1

Inhibitor 1

PP1

PDE

CN

CREB

RSK

PKC

CaMK
Figure 4.2
Will the CREB pathway prove to be involved in perceptual learning? The ease with which the odd contour of a “2” in A pops out from a field of “5”s derives from past perceptual learning as revealed by the relative difficulty in identifying the odd one from a set of elements in B, identical to A except for a 90 degree rotation. Such memory of learned contours is associated with plasticity in the visual cortex and may be independent of the hippocampus. The figure is modified from (Wang, Cavanagh et al. 1994; Gilbert, Sigman et al. 2001).
REFERENCES


