ACUTE ACTIVATION OF CONSERVED SYNAPTIC SIGNALING PATHWAYS IN DROSOPHILA MELANOGASTER

by

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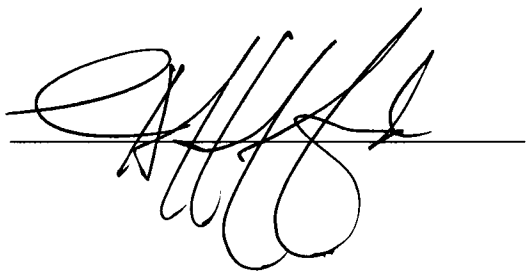
SIGNED: 

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TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

LIST OF ILLUSTRATIONS

LIST OF TABLES

ABSTRACT

Studies of memory have identified several memory classifications: declarative, implicit, working, and anesthesia-resistant. One simple classification that may be applied to the array of model systems now used to explore memory is the requirement for *de novo* gene expression and protein synthesis for the formation of long-term memory (LTM). Short-term memory (STM) appears to require the modification of pre-existing neuronal molecules and is resistant to inhibitors of protein synthesis. These molecules, believed to encode proteins that effect long-lasting neuronal changes likely at the level of the synapse, are manifested behaviorally as memory. Neural activity regulates the cellular decision to synthesize these molecules, yet the identity and function of these molecules are largely unknown. What is known has largely been elucidated by work in mollusks and vertebrates in which procedures have been developed to generate neural activity sufficient to induce long-lasting, protein synthesis-dependent neuronal plasticity. Using these procedures, several key intracellular signaling pathways (Ras/ERK, cAMP/PKA) and important early gene products (arc, zif268, AP1) critical to memory have been identified. Similar procedures are not presently available in *Drosophila.* Establishing these procedures would greatly enhance the *Drosophila* model system for identification of plasticity molecules and mechanisms that control their expression. We have explored the potential of conditional *Drosophila* seizure mutants of *comatose* and *CaP60A* mutants for the development of a neural activity generation paradigm capable of (1) inducing long lasting and robust neural activity; (2) acute and persistent activation of the ERK signaling pathway and induction of *Drosophila* homologs of immediate early genes

known to be involved in plasticity; (3) alteration of synaptic localization of fasciclin II, a known effector of synaptic plasticity. Using these mutants, we have established the conservation in insects of a known neural activity regulated signaling pathway shown to be critical to both long term plasticity and memory. Secondly, we have identified a central role for AP1, a classical activity induced gene, in regulating *Drosophila* neural plasticity. The neural activity paradigm coupled with the identification AP1 dual control of both major branches of long term neuronal change, structural and functional plasticity, provides researchers valuable tools for addressing some the outstanding questions facing the plasticity field today.

CHAPTER ONE

INTRODUCTION

Overview and significance

Memory may be defined as the ability to of an organism to store information about experience through its interaction with the environment. This stored experience is ultimately manifested in the nervous system by the strength and specificity of millions upon millions of connections between neurons, also known as synapses. Neural activity dependent signaling at the synapse can alter the behavior of the neurons in which they reside via the modulation of intracellular signaling pathways that regulate a myriad of neural functions: gene expression, protein synthesis, and localization/degradation of extant synaptic proteins (Bailey et al., 1996; Berridge et al., 2000). A critical signaling pathway underlying many neuronal activities is the mitogen activated protein kinase/extra-cellular signal regulated kinase (MAPK/ERK) signaling pathway.

The ERK signaling pathway is one of the most widely conserved and diversely utilized signaling modules in nature and serves as a critical regulator of cell proliferation, apoptosis, and differentiation (Kolch, 2000). Additionally, it serves a critical role in controlling neural plasticity, learning, and memory (Adams and Sweatt, 2002). The core components of the ERK signaling pathway are found in the insect nervous system (Raabe, 2000) as are numerous molecules involved in the complex upstream signaling leading to ERK activation (Kolch, 2000; Sweatt, 2001), but until recently, it was not known whether the pathway was regulated by neural activity in insects. This fact

highlights a major weakness of plasticity studies' insect model systems when compared to other neural plasticity models. In vertebrates, a number of protocols have been developed for the induction of neural activity, including procedures for the generation of generalized activity (seizures) and physiologically-specific activity as with long-term potentiation (LTP) using both pharmacologically-induced methods and via direct electrophysiological stimulation (Montarolo et al., 1986; Cole et al., 1989; Worley et al., 1993). In the mollusk, *Aplysia californica*, stimulation with application of 5-HT to cultured sensory and motor neurons can induce gene expression, protein synthesis, and pre-synaptic sprouting (Barzilai et al., 1989). As such, these systems have been used to identify neural activity induced genes, analyze critical regulatory pathways, and characterize the roles ultimately of effectors of long lasting plasticity. The fruit fly, *Drosophila melanogaster,* model system lacks similar protocols. Research in *Drosophila* does benefit from excellent genetics, a completely sequenced genome with readily available mutations in a majority of loci, a readily accessible synapse for both structural and functional studies. With the addition of neural activity generation protocols capable of stimulating ERK signaling, *Drosophila* may be used to clarify mechanisms that control the fidelity, duration, and strength of ERK signaling, and very importantly, identify novel ERK effectors involved in long lasting neuronal plasticity. Improved understanding of how neuronal ERK signaling is regulated will undoubtedly benefit our understanding of mechanisms underlying learning, memory, and many classes of neurological disease (Zhu et al., 2002; Jackson and Ramaswami, 2003). In this dissertation I present such a protocol

and evidence for the conservation of neural activity regulation of ERK signaling in *Drosophila.*

The ERK signaling pathway: A Critical Signaling Pathway Involved in Plasticity. Is it a Conserved Plasticity Signal Cascade in Insects?

The (MAPK/ERK) signaling module is composed primarily of an initiating GTPase protein that activates a cascade of three core kinase components composed of sequentially activating dual specificity kinases: a MAPK kinase kinase (MAPKKK) that activates a MAPK kinase (MAPKK) that then activates the final kinase in the cascade, MAPK, which is fully active when dually phosophorylated at activating threonine and tyrosine residues. In the ERK signaling pathway, Ras appears as the initiating GTPase, Raf as the MAPKKK, MAPK/ERK kinase (MEK) as the MAPKK, and ERK as the MAPK (Fig 1.1). This core structure facilitates amplification from upstream signals and provides additional layers of contact for regulation for MAPK signaling duration and strength.

ERK in Long-Term Potentiation

The critical importance of the ERK signaling module has been demonstrated in learning, memory, and neuronal plasticity in both vertebrates and invertebrates (Grewal et al., 1999; Adams and Sweatt, 2002). Long-term potentiation (LTP) can be defined as an enduring increase in excitatory synaptic potential, which depending on preparation and method of induction, may last from minutes to days. LTP has not been directly

demonstrated as a causal mechanism for LTM formation but a considerable body of evidence indicates that a strong link between the two phenomena at present remains the best model for the cellular mechanism by which memory formation takes place (Barnes, 1995; Fanselow and Gale, 2003). LTP may be differentiated into different temporal phases, each with differing requirements for: kinase activity, modification of neuronal membrane receptor composition, gene expression, and protein synthesis. The longest lasting form of LTP, Late LTP or L-LTP is potentiation lasting longer than ~180 minutes. L-LTP requires gene expression and is protein synthesis dependent. L-LTP can be derived via several stimulation protocols. Several L-LTP induction protocols are ERK-dependent as demonstrated by: pharmaceutical blockade of MEK, the sole activating kinase upstream of ERK: (1) NMDA receptor stimulation, using mGluR agonists (Coogan et al., 1999) (2) theta burst stimulation (TBS), which is thought to mimic patterns of neural activity shown by animals performing tasks searching novel environments (Winder et al., 1999; Watabe et al., 2000), (3) high frequency tetanic stimulation (HFS) (English and Sweatt, 1997; Coogan et al., 1999), (4) Blockade of K+ rectifying channels with tetraethylamonium chloride (TEA) (Coogan et al., 1999) and, (5) treatment with brain derived neurotrophic growth factor (BDNF) (Ying et al., 2002).

ERK in Memory

A role for ERK in long term memory formation is clear from a number of behavioral studies in rodents. In contextual fear conditioning experiments, animals are trained by administration of foot-shocks delivered in a specific environment (context).

Mice learn to associate this context with painful stimuli and elicit robust behaviors associated with fear (freezing). In animals trained with foot-shock increased pERK (activated ERK) staining is observed in the hippocampus one hour after training when compared to sham treated (no foot shock same context) (Atkins et al., 1998). Additionally, LTM but not STM, is blocked in rats fed MEK inhibitors systemically or by region specific (intramygdalar or intraventricular) perfusion (Atkins et al., 1998; Schafe et al., 1999; Schafe et al., 2000). Other forms of hippocampal dependent memory are also ERK regulated. The Morris water maze is a task where rodents are trained to find an occluded resting platform in a pool of water using spatial cues. Spatial memory formation in mice treated with the MEK inhibitor, SL237, and then trained using the Morris water maze was inhibited. At the same time, working memory, a measure of learning that takes place during the course of training was normal, demonstrating that short term components of the behavorial task were left intact (Selcher et al., 1999).

ERK signaling is also required for non-hippocampal dependent memory tasks. In the conditioned taste aversion (CTA) assay, animals learn to associate a particular novel food with illness induced by noxious treatment. The noxious experience produces a strong lasting memory manifested by life long aversion to the food item. The insular cortex is widely believed to be important for conditioned taste aversion in rodents. Rats pre-fed MEK inhibitor then trained using CTA paradigm perform normally in STM assays but fail to develop the robust LTM normally associated with the detrimental experience and show reduced pERK insular cortex staining (Berman et al., 1998).

Supporting the body of data derived from pharmaceutical studies, role for the ERK cascade in regulating LTP and LTM has also been determined from genetic studies. Ras-GRF (a calcium responsive activator of Ras activity) knockout mice have deficits in amygdalar dependent LTP and memory tasks (Brambilla et al., 1997). Knockout mice of neurofibromin (NF1), a regulator of Ras activity, show reduced LTM and (Silva et al., 1997; Costa et al., 2002). Heterozygote mice null for K-Ras (a neuronally expressed isoform of Ras) combined with sub-threshold (at concentrations not normally affecting LTP/memory) levels of MEK inhibitor (applied before or at stimulus but not after) show dramatic decreases in early LTP. The authors do not examine LTP beyond 80 minutes but in other experiments where E-LTP has been blocked, L-LTP is also failed to be established (English and Sweatt, 1996, 1997) K-Ras+/- mice also significantly impaired contextual fear conditioning but show normal memory one hour after training (STM) (Ohno et al., 2001). It is interesting to note that in these mutant mice, performance in the Morris water maze (a spatial memory task) was impaired without MEK inhibitor, demonstrating the possibility of cell-specific activation thresholds for ERK cascade. Finally, transgenic mice expressing an inhibitory construct affecting Rap signaling show defects in L-LTP formation (>150 minutes) that were separate of LTP induction. These same mice showed normal contextual fear conditioning immediately after training (STM) but demonstrated a context discrimination task deficit when tested 24 hours later (Morozov et al., 2003).

ERK and Invertebrate Plasticity

Although much of the work establishing the ERK cascade as a key regulator of long lasting forms of neural plasticity has been done in vertebrates, the initial demonstration for the critical importance of the ERK signaling cascade in neuronal plasticity comes from work in *Aplysia californica*. The gill-withdrawal reflex is a classic model for non-associative learning in *Aplysia* where long term facilitation (LTF) of the sensory-motor synapse can be elicited through serotonin release from a regulating interneuron. This response can be duplicated with cultured sensory and motor neurons when coupled with pulsed 5-HT application and is used an assay for LTF. Martin *et al.* showed that presynaptic injection of anti-ApMAPK antibodies or treatment with the MEK inhibitor, PD098059, blocked synaptic enhancement following a normal LTF induction protocol. Over expression of an ApCAM isoform lacking ERK phosphorylation sites or MEK blockade blocks internalization of ApCAM known to be associated with LTF (Bailey et al., 1997). Finally, BDNF induced LTF at the sensory motor synapse is mediated at least in part by MAPK (Purcell et al., 2003).

The role of ERK fruit fly neuronal biology in has been largely restricted to studies examining the development of the *Drosophila* compound eye rather mechanisms of neural plasticity. However, a few studies have provided some direct and indirect evidence for the involvement of ERK signaling in *Drosophila* learning and developmental plasticity. First, deficits in olfactory learning with mutants of the *Drosophila* gene, *leonardo*, points to the possible role of ERK in *Drosophila* learning. The *leo* locus encodes the *Drosophila* homolog of the ζ isoform of a 14.3-3 scaffolding protein. 14.3-3

proteins, particularly ζ forms are known to be involved in upstream signaling preceding the activation of ERK by directly binding ERK, and its upstream activators (MEK) (Freed et al., 1994; Skoulakis and Davis, 1996). Further indirect evidence from studies similar to those in vertebrates (Silva et al., 1997), in which *Drosophila* neurofibromatosis (Nf1) mutants were demonstrated to have impaired learning and short term memory following training in an olfactory avoidance conditioning assay (Guo et al., 2000). More direct evidence comes from a recent study by Koh *et al.* which examined the role of the Ras (a primary GTPase regulator of MAPK) signaling in the regulation of synaptic plasticity at the *Drosophila* larval neural muscular junction (NMJ). Using gain of function Ras mutants, they demonstrated that there was elevated activated synaptic ERK levels and correlated this with increased synaptic size and decreased levels of a neural adhesion molecule, fasciclin II, which is a known ERK target and regulator of synaptic plasticity (Schuster et al., 1996; Koh et al., 2002). As compelling as these results may be, they incompletely address a few key areas. These studies are performed in mutant genetic backgrounds that exert their effects on ERK signaling chronically. Thus, it is impossible to distinguish between the effects these mutations have on ERK signaling during normal developmental plasticity and their role in signaling following *acute* activation of the pathway immediately following a neural activity, i.e., assessing ERK signaling in a fully developed nervous system that is specifically stimulated to undergo plastic change. This is something that is not currently possible in the insect model systems but is possible in vertebrate and *Aplysia* model systems. Secondly, these studies do not directly address the existence of evolutionary conservation of neural activity regulated ERK signal cascade

activation in *Drosophila*, an important prerequisite for more relevant plasticity studies in insects.

A Case for Drosophila

Demonstrating phylogenetic conservation of ERK signaling during regulation of neuronal plasticity in *Drosophila* affords researchers the opportunity to exploit this powerful model system for the examining ERK signaling and plasticity. There are several features of the model system that make it attractive for these types of studies. Firstly, the larval NMJ is a well-characterized synaptic preparation that may be used to examine both neuronal function and strength (Keshishian et al., 1996). Secondly, memory and learning effects can be assessed with the olfactory-avoidance conditioning assay (Tully, 1996). Thirdly, *Drosophila* is a superior genetic model system with thousands of readily available mutants and transgenic reagents. Moreover, the fully sequenced and annotated fruit fly genome has made it possible to create an extensive collection of P-element derived mutants and transgenic constructs that covers nearly every gene in the fruit fly genome, greatly accelerating the pace at which a researcher can examine potential roles ERK regulators or effectors. A final, potentially immense advantage may be derived from the reduced overall complexity (see below) of the ERK signal cascade in *Drosophila* when compared to other excellent genetic systems like the mouse.

The central constituents of the ERK signaling pathway are present in the *Drosophila* and most have been identified as expressed in the nervous system, Table 1.1. The core signaling module in vertebrates contains a large number of closely related

paralogs within each category of the cascade and in many cases, a good deal of functional redundancy among these molecules. For example, the vertebrate Ras family consists of H-Ras, N-Ras, K-Ras (isoforms 4A and 4B); a Ras-like subfamily is composed of R-Ras, TC21 (R-Ras2), M-Ras (Ras-3), Rap1a, Rap 1b, Rap2a, Rap 2b, Ral2A, Ral2B, and RalB (Ehrhardt et al., 2002). In addition to the large number of ERK cascade family members, complexity is further increased with the numerous splice variants available to each subfamily member. Many members of the Ras superfamily are expressed in the same cells and are activated by overlapping sets of signals (Yan et al., 1998). Their close homologies suggest that they could be functionally identical (Yan et al., 1998; Zwartkruis and Bos, 1999). H-, N-, and K-Ras are expressed ubiquitously (Voice et al., 1999; Corbett and Alber, 2001). Only knock out mice of K-Ras are lethal while mutants lacking H- and N-Ras are viable (Umanoff et al., 1995; Johnson et al., 1997; Koera et al., 1997; Esteban et al., 2001). Heterozygote mice null for one copy of K-Ras show normal learning, amygdalar LTP, and fear conditioning LTM. Plasticity effects in these (K-Ras+/-) mice only appear when the animals are also exposed to low levels of MEK inhibitor, pointing out the likely possibility that either H- or N-Ras activities can substitute at least in part for K-Ras in neurons (Ohno et al., 2001). In contrast, *Drosophila* possesses only three *bona fide* Ras molecules D-Ras1, D-Ras2, and D-Ras3 (Segal and Shilo, 1986; Kimchie et al., 1989; Simon et al., 1991) (FlyBase Genome Annotator, 2004). D-Ras3 encoded by the *Roughened* locus possesses three different isoforms but only one is found in the nervous system (Segal and Shilo, 1986).

There are three members of the Raf family in vertebrates: A-Raf, B-Raf, and Raf-1 (also called C-Raf) (Pearson et al., 2001). As with the Ras family, the various Raf isoforms are expressed together in most cell types. Raf-1 is expressed ubiquitously at high levels. B-Raf is highly expressed in the brain and testis, while A-Raf is not. All three types are found in muscle tissue but B-Raf levels are very low (Lee et al., 1996; Chong et al., 2003). Only A-Raf knockout mice survive to birth and have a varying degree of survivability to adulthood (Pritchard et al., 1996), Raf-1 and B-Raf knockout mice are embryonically lethal and die from wide range of developmental abnormalities, revealing essential and non-overlapping roles for them in development (Wojnowski et al., 1997; Wojnowski et al., 1998). However, Raf family members likely complement activity to some extent as the developmental abnormalities in Raf knockouts were greatly enhanced by the loss of single copy of another Raf isoform (Wojnowski et al., 2000). Interestingly, fibroblasts isolated from Raf-1 (-/-) animals show unimpaired ERK activation in response to serum, and redundant compensatory activity from B-Raf likely preserves the cascade (Huser et al., 2001). *Drosophila* possesses two known Raf molecules D-Raf-1 encoded by *pole hole* and, D-Raf-2, described only recently following annotation of the *Drosophila* genome. In the nervous system, D-Raf is expressed by an identified single peptide and most closely resembles vertebrate B-Raf (Fig 1.3) (Mark et al., 1987; Melnick et al., 1993) (FlyBase Genome Annotator, 2004). Continuing down the core-signaling module, there are two closely related MEKs in higher vertebrates, MEK1 and 2. Both are expressed in the nervous system, can fully activate ERK, and appear to be fully functionally interchangeable (Zheng and Guan, 1993; Kolch, 2000; Pearson et al., 2001).

ERK1 (p44) and ERK2 (p42) are both expressed in the vertebrate nervous system and are both activated by a large number of stimuli (Lewis et al., 1998). Although they are almost 90% identical they are differentially activated in response to some stimulation (Fiore et al., 1993; English and Sweatt, 1996). In *Drosophila* there is single MEK, encoded by DSor1 (Tsuda et al., 1993; Karim et al., 1996). ERK1 knockout mice are completely viable and show no apparent defect (Selcher et al., 2001). In fact, paradoxically, in these mutants' learning and memory were enhanced in a region-specific manner (Mazzucchelli et al., 2002). Knockout studies with ERK2, however, resulted in quite different outcomes; ERK2 mice die *in utero* (Adams and Sweatt, 2002; Yao et al., 2003). Conditional ERK2 knockout mice have been generated, but studies with these animals are in the early phases of development (Gary Landreth, personal communication). Analogous the fly MEK, there is single ERK homlog in *Drosophila* encoded by the *rolled* locus (Biggs and Zipursky, 1992). In addition to the principal cascade components (Ras-Raf-MEK-ERK), there are a host of ERK signal cascade-associated proteins such as G-protein signaling modifiers (adapters/enhacers/inhibitors), phosphatases, chaperones and scaffolds (Kolch, 2000; Pouyssegur et al., 2002; Chong et al., 2003). Homologs for the majority of these are found in *Drosophila* (Table 1.1). As with the core signal module, there exist numerous vertebrate paralogs, splice variants, and post-translationally modified isoforms for many of the cascade-associated proteins.

Clearly, studies in higher vertebrates, while highly important and relevant to plasticity studies, are likely to be complicated by the high degree of functional redundancy and overlap among the families of core and accessory ERK signaling

components. Essentially, the *Drosophila* model system may represent a simplified but largely complete neuronal ERK signal cascade suite for study. The powerful combination of genomic resources and neurobiological tools available to *Drosophila* model system coupled with reduced complexity of the ERK signaling cascade may make the model system ideal for addressing the many outstanding questions that remain with respect to ERK signaling and neuronal plasticity. How is the diverse array of potential signaling routes upstream of ERK activation integrated to generate specific neuronal responses? What are the identities of the effectors of ERK activation at the transcriptional/translational level? What mechanisms gate the establishment of transient to persistent ERK signaling in neurons?

Pathways to ERK: Ras, Rap and Raf

Ras activation in neurons

The initial event in the activation of the ERK pathway is the activation of Ras or Ras-like GTPase (Fig 1.1). The Ras protein is a GTPase localized to the plasma membrane that acts as a molecular switch. When bound to GTP, it is functionally active, whereas when it is bound to GDP it becomes inactive. Regulatory proteins such as guanine nucleotide exchange factors (GEF)s promote Ras activity while GTPase stimulating proteins (GAP)s inhibit Ras activity (Boguski and McCormick, 1993; Corbett and Alber, 2001). There are several mechanisms by which Ras can be activated in neurons (Fig 1.2). Ras is a central regulator of ERK activation integrating signals from several signaling pathways (Zwartkruis and Bos, 1999; Iida et al., 2001). Ras activation

has been demonstrated following neuronal depolarization, Ca^{+2}-calmodulin interaction, stimulation of ligand gated channels and integrin signaling complexes as well as activation of a several types of G-protein coupled receptors (Fiore et al., 1993; Rosen et al., 1994; Wang and Durkin, 1995; Finkbeiner and Greenberg, 1996; Gutkind, 2000; Dolmetsch et al., 2001; Hardingham et al., 2001; Schwartz, 2001; Villalonga et al., 2001)

Calcium (Ca^{2+}) is a critical regulator to many neuronal Ras activating pathways direct entry via NMDA or VGCC activation (Cullen and Lockyer, 2002). Additionally, metabotropic glumate (mGlu) or muscarinic acetylcholine (mACh) receptor activation (Agell et al., 2002; Cullen and Lockyer, 2002) can trigger calcium release through a phospholipase-C (PLC)-inositol triphosphate (IP_3) dependent mechanism. Increased intracellular Ca^{2+} may stimulate a variety of proteins to activate Ras. Two neuronal enriched Ras GEFs are Ras-GRP guanine release protein (GRP) and Ras-guanine nucleotide releasing factor (GRF). Ras-GRP binds Ca^{2+} directly through its EF-hand domains. This is mediated by diacylglycerol (DAG) (Ebinu et al., 1998). Perturbation of the DAG binding site blocks Ras-GRP activity. Ras-GRF activates Ras after joining with Ca^{2+}-bound calmodulin (CaM) (Farnsworth et al., 1995). Finally, Pyk2, a cytoplasmic Ca^{2+} dependent kinase can bind and activate another kinase Src. The resultant Pky2-Src complex then recruits Grb2-SOS, a classical Ras GEF (McCormick, 1993). The Grb2-SOS pathway is a well-characterized mechanism for Ras activation. Usually turned on by growth factors (Gutkind, 2000), Pyk2 kinase links this major GEF effector to calcium in neurons (Lev et al., 1995; Dikic et al., 1996). CaM can also stimulate the activity of CaM-dependent kinase II (CaM-KII), leading to the down regulation of the SynGAP, a

neuronal expressed GAP that inhibits Ras activity (Chen et al., 1998). Ca^{2+} may also play a role in inhibiting Ras activity by recruiting RasGAP to the plasma membrane where it can act to block Ras activation (Filvaroff et al., 1992). Transient blockade of Ras/ERK signaling has been observed following high Ca^{2+} stimulation in primary keratinocytes (Chao et al., 1994), even though the mechanism through which this occurs is unknown. At present, however, it is unclear whether such a mechanism exists in neuronal regulation of GAP activity. Nf1 encodes another RasGAP and has been shown to be involved in vertebrate learning and memory (North et al., 1995; Silva et al., 1997). Although its biochemical function has been shown, little is known about its regulation. In fact, down regulation of Ras activity *in vivo* is generally not well understood and remains an interesting question for further study.

Ca^{2+} can also regulate levels of another major intracellular second messenger, cyclic adenosine mono-phosphate (cAMP), to affect Ras activity. CaM can modulate the activity of adenylate cyclase (AC) to elevate cAMP levels. cAMP can regulate Ras activity through cAMP dependent GEF such as Cnras-GEF, a neuronally expressed GEF species which activates Ras after binding cAMP directly. Increased cAMP may also indirectly activate Ras by modulating the G-protein interaction at β2-Adrenergic receptor (β2-AR). Upon activation, cAMP dependent protein kinase (PKA) phosphorylates β2-AR to alter its G-protein binding partner from Gs to Gi. This change allows Gi-βγ subunits (once released by receptor activation) to signal to c-Src and Grb2/SOS to activate Ras (Daaka et al., 1997; Luttrell et al., 1999). A complex relationship between Gi-α and Gs-α exists in neurons to regulate cAMP levels and PKA activity. Gi-α inhibits AC function

while Gs-α promotes it (Simonds, 1999) -- and as illustrated by the previous example, this fine balance strongly influences the downstream effects of GPCR activation in dictating the effects of ERK in neurons (Daaka et al., 1997; Hamm, 1998; Gutkind, 2000). This regulation is further complicated through the specific expression and localization of GPCRs, AC, and G-protein species in different neuronal sub-populations and that maybe additionally regulated by defined plasma membrane micro-domains or "lipid rafts" that can regulate Ras activation and ERK signaling in neurons by virtue of cellular localization of signaling elements (Stork and Schmitt, 2002; Carey et al., 2003).

Rap activation in neurons

Another important member of the Ras-like family of GTPases involved in neural ERK activation is Rap (Fig 1.2). Unlike Ras, Rap is predominantly localized in a perinuclear fashion rather than at the plasma membrane (Mochizuki et al., 2001). Like Ras, Rap is activated by a number of signaling pathways and second messengers such as Ca^{2+}, cAMP, and DAG. This regulation is controlled through a number of Rap-specific GEFs and GAPs. One Ca^{2+} /cAMP mediated pathway to Rap activation in the brain is through the Rap-GEF, C3G. Elevated cAMP promotes PKA activity which in turn activates Src. Src has a number of downstream effectors, one of which is scaffolding protein, Cbl. Once phosophorylated, Cbl forms a complex with C3G and Crk, an adaptor protein named for its Src homology binding domain (SH3). This complex induces Rap activity (Figure 1.3) (Stork and Schmitt, 2002). Another route through which cAMP can influence Rap activity is via the Rap GEF, Epac (de Rooij et al., 1998; Enserink et al.,

2002). There are a number of Epac species, including ones that are restricted to expression in the brain (Kawasaki et al., 1998). This pathway, unlike the C3G-Src mediated mechanism allows Rap activation independent of PKA. PLC, as with Ras, is another major mediator of Rap activity. Increased Ca^{2+} and DAG resulting from PLC activation, promotes Rap activity through CalDAG-GEF, a closely relative of the Ras-GEF, Ras-GRP. Like Ras-GRP, CalDAG-GEF binds Ca^{2+} in a DAG dependent manner and in turn activates Rap (Kawasaki et al., 1998; Bos et al., 2001). Rap, like Ras, is regulated by specific GAPs that inhibit its activity. A number of RapGAPs have been identified, RapGAP1, Spa-1, tuberin, and GAP^{IP4BP}. All of these GAPs are expressed in neurons with only RapGAP1 showing neuronally restricted expression (Rubinfeld et al., 1991; Wienecke et al., 1995; Kurachi et al., 1997; Bottomley et al., 1998). RapGAP1 activity is increased by interaction with Gαi-GTP; this subunit also inhibits AC function and is example of GPCR mediated crosstalk between pathways that activate Rap (Mochizuki et al., 1999). RapGAP1 function is antagonized by Gαo-GDP (inactive form) and can lead to activation of ERK in mammalian PC12 cells (Jordan et al., 1999). GAP^{IP4BP} and its *Drosophila* homolog, Gap1, are particularly interesting because this GAP acts on both Ras and Rap. In addition, it possesses a Ca^{+2} dependent lipid-binding (CaLB) domain which may direct it to the plasma membrane in the presence of elevated Ca^{+2}. It would be interesting to see what role if any this GAP played on determining the effects of upstream signaling that dually activates both Rap and Ras. Finally, Rap possesses lower innate GTPase activity than Ras (Zwartkruis and Bos, 1999), and as

such, RapGAP activity is likely to play a crucial role in regulating Rap mediated cell and induction specific outputs as well as distinguishing Rap effector pathways from Ras.

Rap is conserved in *Drosophila* (Table 1.1) and is quite similar to vertebrate Rap1a (88% identity). Work by Vossler *et al.* 1997 shows that cAMP activation of Rap activity is mediated by PKA. Moreover, Rap may be a critical component to the cAMP dependent activation of Raf in neurons (Figure 1.3) (Vossler et al., 1997). The unique PKA target site that specifies this mode of regulation is conserved in *Drosophila*, Ser-179 (corresponding to Ser-180 in human Rap1a (Quilliam et al., 1991)) (Asha et al., 1999). Unlike vertebrate Rap1a, it does not appear that PKA is required for Rap function in *Drosophila* (Li et al., 1997; Asha et al., 1999). Substitution of Ser site to Ala did not diminish a Rap transgene ability to rescue lethality. Likewise, genetic perturbation of PKA function did not show an interaction between Rap and PKA (Li et al., 1997; Asha et al., 1999). Further confounding is evidence that Rap signaling does not appear to affect Ras mediated signaling pathways in *Drosophila*. Rap1 overexpression did not reduce Ras controlled events in eye development nor did loss of Rap function enhance Ras signaling (Asha et al., 1999). These results were achieved in a developmental biological context and do not exclude the possibility of redundant signaling during development that is independent of PKA function, but still activates Rap in cAMP dependent manner. Furthermore, no studies to date have directly examined the activation of fruit fly Rap, or more specifically Rap activation following neuronal activity. Thus it may be premature to rule out the possibility that Rap may function in *Drosophila* to modulate ERK signaling during the regulation long-term plasticity processes.

Raf and MEK activation

Following G-protein activation, the next major protein in the pathway is a MAPKKK, Raf. Regulation of Raf activity is complex and is affected by host of factors. These include the mechanism of upstream activation, cellular localization of Raf proteins, and cell specific expression the different the Raf subtypes (primarily Raf-1 and B-Raf in neurons) and Raf associated regulators (Kolch, 2000; Pearson et al., 2001). One of principal activators of Raf is Ras. Activation of Raf requires proper localization to the inner leaflet of the plasma membrane of not only itself but also Ras, in order to occur (Kikuchi and Williams, 1994). Ras binds Raf at two domains, the Ras binding domain (RBD) and the cysteine rich domain (CRD), which relieves internal Raf inhibition of the N-terminus on its catalytic C-terminus (Cutler and Morrison, 1997). The CRD is particularly interesting because this domain represents one of the chief differences between Raf-1 and B-Raf (a predominantly neural expressed isoform of Raf). Another interesting difference arises from competition for the CRD binding domain by Rap and Ras: Rap binds the Raf-1 CRD more efficiently, but fails to activate Raf-1 (Vossler et al., 1997; Zwartkruis et al., 1998). This may point to the mechanism of Raf regulation; overly effective binding at Raf-1 CRD inhibits its eventual activation as demonstrated by the drug, ceramide, which greatly enhances Ras affinity to Raf but prevents its activation (Muller et al., 1998). However, Rap1 (or Ras) binding alone to B-Raf CRD is sufficient for its activation. Additionally, not only does Raf-1 require Ras-GTP binding but also the phosphorylation of essential activating residues, Ser-338 and Tyr-341, contained in a Ser/Thr rich conserved region (CR2) of the Raf family of proteins (Chong et al., 2003).

Unlike Raf-1, B-Raf is activated simply by binding GTP loaded Ras (Diaz et al., 1997). This results from subtle changes to B-Raf CR2; Ser-445 (functional equivalent to Ser-338 in Raf-1) is constitutively phosphorylated. B-Raf also has a phospho-mimetic aspartate residue its equivalent Tyr 341 (Mason et al., 1999) allowing it to circumvent this aspect of Raf-1 regulation. B-Raf CRD domain swapping experiments transform Rap1 from a blocker of Raf-1 activity to an activator (Hu et al., 1997) and conversely the addition of the Raf-1 CRD to B-Raf cancels Rap1 activation of B-Raf (Okada et al., 1999). The presence and levels of B-Raf in a cell may represent one of the key pathways regulating ERK in neuronal plasticity (Figure 1.3).

PKA may also regulate Raf/ERK activity directly, but the specific mechanism depends on cell type and manner of upstream regulation. Depending on the cell type and the presence of B-Raf, PKA may promote ERK activation though Rap1. PKA may also act directly on Raf to inhibit its activity in vertebrates. This is mediated by PKA phosphorylation of Raf-1 at a number of Serine residues (43, 259, 621) (Wu et al., 1993; Mischak et al., 1996; Dhillon and Kolch, 2002). Phosphorylation at these sites reduces Raf-1 affinity to Ras-GTP. More detailed site-directed mutagenesis and deletion studies have pointed to Ser-259 as the primary site of PKA regulation of Raf-1 (Mischak et al., 1996; Dumaz et al., 2002). Evidence from *in vitro* studies suggests that this site plays a critical role in regulating protein-protein interactions that normally function to inhibit Raf function. Nonetheless, the exact mechanism(s) by which PKA exerts control on Raf-1 and what roles the other PKA phosphorylation sites play remains incompletely understood. Another remarkable detail in the differences between Raf and B-Raf is the

conserved regulatory site Ser 364 in B-Raf. In B-Raf, PKA phosphorylation at this site does not inhibit its activity but is linked to other forms of kinase regulation (Dhillon and Kolch, 2002). Why PKA does not regulate B-Raf in fashion similar to Raf-1 but is still an important phosphor-regulatory site, is not clear and remains an intriguing question. Other reports have further revealed conflicting information about how PKA may act to regulate Raf. In PC12 cells, growth factor mediated cAMP signaling blocks both Raf and B-Raf activation (Vaillancourt et al., 1994). However, stimulation of PC12 cells or hippocampal cells via depolarization results in robust ERK activation despite Raf-1 inhibition. It was later determined that ERK was activated via Rap1 and B-Raf in PKA dependent manner (Figure 1.3) (Vossler et al., 1997; Grewal et al., 2000b). Levels of B-Raf in a cell can convert cAMP/PKA signals -- from what would otherwise be negative regulation of the ERK pathway -- to robust activation. Since Rap1 is highly expressed in neurons and B-Raf represents the dominant form of Raf in neurons, this route of regulation likely represents a major cellular mechanism for ERK regulation.

Raf is also directly regulated by several other kinases including PKC, Src, and Akt (PKB) (Kolch et al., 1993; Morrison et al., 1993; Diaz et al., 1997; Zimmermann and Moelling, 1999). PKC positively regulates Raf and has been found to phosphorylate Raf-1 at Ser 497 and 499. However, mutations replacing these residues with Ala failed to inhibit PKC activation of Raf-1 (Force et al., 1994). Later studies showed that PKCε also acts on Ser 338. Blocking PKCε blocked this phosphorylation and reduced Raf activation in response to phorbol esters, but when left intact, Raf increased activation in response to growth factor (Barnard et al., 1998). As such, PKC is likely to exert its influence on Raf

through multiple pathways, yet exactly how it does so is still poorly understood. In addition to the role that Src plays in regulating Ras, it has been shown to directly phosphorylate Raf-1 at Ser 338 and Tyr 341 (important CRD residues critical for Ras activation). Even though *in vitro* studies have shown that Src can act to increase Raf activity, this induction following neuronal stimulation has yet to be demonstrated (Dhillon and Kolch, 2002). Furthermore, D-Raf like B-Raf, lacks Tyr 341 and has instead an Asn residue, so even though Src and Raf interact genetically (Li et al., 2000), its exact biochemical role (if any) in *Drosophila* Raf signaling is not clear. Finally Akt kinase has been shown to play an inhibitory role in Raf function for Akt signaling acts on Ser-259 (the same site targeted by PKA) (Zimmermann and Moelling, 1999).

Drosophila only possesses one major Raf isoform, D-Raf, encoded by *polehole* (Table 1.1). Like Raf-1 and B-Raf, several key regulatory features (Ser/Tyr) are conserved between the phyla (Figure 1.4). In D-Raf, Ser 560 is the conserved equivalent of Ser 338, a potential Src site and key residue for mediating Ras-1 function. In B-Raf (Ser 445) this site is constitutively phosphorylated by an as yet unidentified kinase. However, whether or not this true of D-Raf is not known. Ser 621 in Raf-1 is key to interaction of Raf with important scaffold, 14.3-3, in *Drosophila* located at Ser 743. Another key regulatory site that is conserved in D-Raf is Ser 388 (Ser 259 in Raf-1 and Ser 364 in B-Raf). This site is a potential PKA target and is another important residue mediating scaffold protein interactions (Chong et al., 2003). As with most of the D-Raf regulatory sites, whether it is regulated by PKA like Raf-1 or B-Raf has yet to be examined. Ser 43, a putative Raf-1 PKA target site is missing in D-Raf, like B-Raf.

Unlike B-Raf, D-Raf lacks two consensus Akt phorylation sites; sites in B-Raf that when mutated to Ala will generate a constitutive active B-Raf protein. Taken as whole, when compared to the three vertebrate Rafs, D-Raf more closely resembles B-Raf (Figure 1.4) than Raf-1 (40% identity, 61% similarity to the B-Raf isoform). Intriguingly, B-Raf and D-Raf have closely related CRD domains (50% identity and 75% similarity); this may confer to D-Raf regulation with properties similar to those found in regulation of vertebrate Raf by Rap. Currently, however, this is not known.

The most well described Raf substrate is MEK. MEK is dually phosphorylated by Raf to rapidly increase its catalytic activity several thousand-fold (Mansour et al., 1996). Unlike other components of the ERK signaling cascade, MEK appears to have only one known *in vivo* target, ERK. In vertebrates, there are two nearly identical MEKs (MEK1, MEK2) that are functionally interchangeable (Zheng and Guan, 1993) and MEK activation is last step in the ERK activation cascade. Little is known about its regulation outside of its interaction with Raf, however, it is known that Rac (Rho family GTPase) in a Ras/PI3K dependent manner can activate PAK phosphorylation of MEK. This modification has no effect on MEK catalytic activity *per se,* but rather serves to promote its association with Raf (Frost et al., 1997). Whether this form of regulation occurs in neurons or in response to neural activity is not known. Finally, it is worthwhile to mention that a number of useful pharmaceuticals are available to effectively block MEK activity: U0126, PD098059, and SL237. These have been extremely helpful in investigating ERK involvement in neural plasticity (Alessi et al., 1995; Atkins et al., 1998; DeSilva et al., 1998).

Spatial Localization and Ras/Raf/ERK Signaling

Spatial localization is an integral component of controlling and organizing the cellular outputs of ERK activation. Spatial localization of both upstream signaling mechanisms and downstream signal transduction components play a role in shaping the amplitude, duration, and effects of ERK signaling. In the ERK signal cascade, this is accomplished through a number of mechanisms: sub-cellular micro environments; "microdomains" to accelerate, concentrate, and segregate upstream cell surface signals to appropriate components of the ERK signal module; cell-specific expression of ERK signaling components; and finally, scaffolding and adaptor proteins bring together elements of the ERK cascade to create cascade fidelity in the pathway and ensure rapid, efficient transmission of signals from upstream inputs.

Signaling Microdomains

In resting cells, free calcium is found at relatively low concentrations of ~100 nM, depending on the form of stimulation. This concentration can rapidly change by as much a 100-fold. Levels of intracellular calcium are maintained by the delicate equilibrium of entry and removal mechanisms. Calcium entry can arise from VGCC and ligand-gated channels as well as release from IP3 mediated release and calcium-gated calcium channel release from intracellular stores (Blackstone and Sheng, 2002). The function of removal is dictated primarily by Ca^{+2}-ATPases found both at plasma membrane and endoplasmic membranes (Garcia and Strehler, 1999). Calcium binding proteins like calbindin and calretinin also contribute to this removal by binding and sequestering cytosolic calcium that controls its diffusion and range of effect (Lee et al., 2000a; Park et al., 2000).

Intracellular Ca^{2+} is a major contributor to Ras activation and cannot only regulate its initiation but also the duration of its activity (Rosen et al., 1994; Marsh and Palfrey, 1996; Lilienbaum and Israel, 2003). This regulation can be achieved through Ca^{+2} mediated translocation of signaling proteins to the plasma membrane. One such example is Ras-GRF (Fam et al., 1997). How Ca^{+2} signals are manifested and interpreted to control such events may be a function of local calcium "microdomains" defined by specific localization of calcium release machinery, signal transduction components, and removal mechanisms. There is substantial body of evidence supporting the existence and vital function for these specialized cellular regions. Calcium entry from L-type Ca^{+2} channels and NMDA receptors induce different patterns of gene expression (Bading et al., 1993; Hardingham and Bading, 1999). Calcium ionophore and depolarization induced ERK activation in PC12 cells both generate cAMP signaling to PKA activation, but the ionophore mediated pathway controls ERK in PKA independent Ras/Raf dependent pathway while the depolarization induced ERK operates via a PKA-dependent Rap/B-Raf mechanism (Grewal et al., 2000a). Calcium chelator experiments have shown that regulation of IP_3 gated signaling and AC activity at the plasma membrane results from signaling defined in the area immediately proximal to sites of Ca^{+2} influx (Csordas et al., 1999; Fagan et al., 2000). Signaling via mGluR promotes the release of calcium from internal stores; this is a result from the tight physical link of IP_3 receptor clusters and mGlu receptors. This is achieved through multimerization of the scaffold Homer that links mGluR to IP_3-R (Tu et al., 1998). Different domains can be created with the addition of short- tailed Homer isoforms lacking multimerization capacity and decoupling

the mGluR- IP_3-R link, giving neurons that have the capacity to fashion a variety of potential calcium signaling microdomains (Tu et al., 1998). Finally, the IQ domain of the L-type Ca^{+2} channels has been shown to constitutively bind and anchor calmodulin. Removal of this domain has the effect of abolishing sustained ERK activation. Tethering of CaM to the sites of calcium entry potentially generates a signaling microdomain critical to Ras/ERK cascade activation in neurons (Dolmetsch et al., 2001).

Cellular distribution and Scaffolds (14.3-3, KSR, CNK, MP1, RKIP)

The effector pathways influenced by ERK activation may in part be controlled by the sub-cellular distribution of the signal transduction components of the ERK pathway itself. Ras must be localized to the plasma membrane with properly post-translational modification to correctly signal Raf-1 activation (Moodie and Wolfman, 1994). Moreover, differential prenylation of Ras affects its ability to activate ERK and alters the pathways activation kinetics (Apolloni et al., 2000; Chiu et al., 2002). Raf-1 is found in the cytosol, but is also enriched in perinuclear manner, while B-Raf is predominantly located in neuronal processes (Morice et al., 1999). Although somewhat controversial, some studies suggest that Raf activation itself may be dependent on specific recruitment of Raf multi-protein complexes to cholesterol-rich caveolae and lipid "rafts" in the cell membrane (Mineo et al., 1996; Prior et al., 2001). Appropriate targeting of the ERK module components to correct subcellular locations is controlled by a host of important scaffolding and chaperone proteins.

14.3-3 proteins are abundantly and pervasively expressed adapter proteins that specifically bind phospho Ser/Thr residues. This family of proteins controls a variety of cellular activities such as cell division, growth, differentiation, and neural plasticity (Tzivion et al., 2001). 14.3-3 does not activate Raf *in vitro*, and likely serves to promote Raf activation by regulating its association with a Raf activator or an effector. In PC12 cells, 14.3-3 enhances Ras/Raf signaling (Li et al., 1995). In *Drosophila*, overexpression of 14.3-3 function can rescue loss of torso-mediated signaling in a Raf and Ras dependent manner. 14.3-3 serves to enhance Ras signaling as 14.3-3 loss of function mutants rescue rough eye phenotype seen in activated Ras1(V12) (Kockel et al., 1997; Li et al., 1997). *Drosophila* mutations affecting 14.3-3 function, also have affects on olfactory learning and memory (Skoulakis and Davis, 1996). 14.3-3 proteins readily form homo or hetero dimers with other members of the 14.3-3 family (Jones et al., 1995). They are also thought to promote dimer formation with proteins with which they interact, for example 14.3-3 is believed to promote Raf-1 dimer formation, a mechanism by which Raf-1 is maintained in an active state (Luo et al., 1996). In either case, dimerization is critical to proper 14.3-3 function as 14.3-3 dimerization in defective mutants act as dominant negatives preventing Raf-1 activation (Luo et al., 1995). 14.3-3 proteins may act as either inhibitors or activators depending on the context of their binding. 14.3-3 is thought to act as a phosphorylation dependent scaffold bringing PKC in contact with Raf-1, promoting its activation (Van Der Hoeven et al., 2000). 14.3-3 acts as both and inhibitor of Raf-1 and activator depending on phosphorylation state of regulatory serine residues contained in Raf-1 CR2 and CR3 domains (Figure 1.4). Removal of 14.3-3 disrupts both basal and

stimulated Raf-1 activity, supporting the idea that 14.3-3 acts to stabilize Raf-1 activation states (Tzivion et al., 1998). When both Ser 259 and Ser 621 are phosphorylated, 14.3-3 binds Raf-1 and is believed to augment the intramolecular ability of Raf-1 N-terminus to inhibit the activity of its C-terminal catalytic domain. When Ras-GTP binds Raf-1, it can dislodge the Raf CRD from the C-terminal domain as well as 14.3-3 from Ser-259. Under current models of Raf activation, Ser 259 is dephosphorylated by PP2A (Abraham et al., 2000), removing one arm of 14.3-3 from contact with Raf-1. This free domain is then utilized to recruit and localize other proteins to the Ras/Raf/14.3-3 complex at the plasma membrane (Kolch, 2000). Finally, 14.3-3 may play a significant role in determining ERK effector pathway choice in cells. Work from Qiu *et al.* showed that levels of 14.3-3/B-Raf interaction are controlled by Rap/B-Raf mediated ERK activation. In C6 cells, where little 14.3-3 is bound to B-Raf, cAMP acts to inhibit ERK activation. Conversely, in PC12 cells where higher concentrations of 14.3-3/B-Raf were found, cAMP stimulated ERK activation. Overexpression of 14.3-3 rescued the cell-type specific inhibition of cAMP on ERK activation (Qiu et al., 2000). The proteins that make the Raf-1 protein complex at the plasma membrane have not been fully identified. Many questions remain including what features of the complex are altered with respect to upstream activation pathway (i.e., GPCR activation, NMDA mediated Ca^{+2} entry) and whether the stability of complex is maintained over time to sustain ERK activation. The potential for 14.3-3 to form a multitude of different interactions with other 14.3-3 family members -- and other adaptor molecules -- is a poorly understood area of the ERK signaling module and an attractive topic future study.

Another important scaffolding protein involved in ERK regulation is encoded by Kinase Suppressor of Ras (KSR). This interesting protein was first identified in genetic screens to find suppressors of activated Ras because KSR was found to involved in signaling upstream or parallel to Raf and downstream of Ras (Downward, 1995). KSR enhances Raf-1 activation in a Ras dependent fashion. Overexpression of KSR inhibits ERK signaling while reduced expression enhances the cascade (Michaud et al., 1997; Cacace et al., 1999). Its homology and close resemblance to Raf-1 led its discoverers to believe that it was yet another kinase involved in ERK signal cascade (Yao et al., 1995). Intriguingly enough, mutational studies ablating its catalytic domain did not diminish its function. In fact, overexpression of the catalytic domain alone was revealed to be a potent inhibitor of MEK/ERK activation (Therrien et al., 1996; Michaud et al., 1997). Although a ceramide-activated kinase activity for KSR was initially identified (Yao et al., 1995), additional studies support the idea that KSR is not a kinase, but rather a molecular scaffold (Giovannini et al., 2001). KSR associates with Raf-1 at the plasma membrane in a Ras-dependent fashion (Therrien et al., 1996; Xing et al., 1997). KSR has also been shown to bind ERK and MEK1; its interaction with MEK is constitutive, but its association to ERK is dependent on induction of the Ras/ERK signaling pathway (Denouel-Galy et al., 1998; Jacobs et al., 1999; Muller et al., 2000). Therefore, KSR is believed to enhance Raf signaling by bringing all three within close proximity, facilitating activation through the module (Roy and Therrien, 2002). KSR is also likely involved in other scaffolding functions as KSR has five identified domains. CA1.5, CA5 encodes its putative kinase domain and binds MEK, Hsp90 and Raf-1 (Denouel-Galy et

al., 1998). CA4 defines a Ser/Thr rich domain (homologous to the CR2 domain of Raf family members) and this domain binds ERK (Jacobs et al., 1999). A third domain, CR3, interacts with G-βγ subunits linked to GPCR (Bell et al., 1999). Binding G-βγ may be the mechanism that facilitates KSR translocation to the cell membrane. Flanking the CR2 domain are two serine residues that when phosphorylated, are associated with 14.3-3 protein (Cacace et al., 1999). One possible function of this contact is that 14.3-3 keeps KSR locked in an inactive state, sterically preventing G-βγ from associating with CA3, and thus blocking its movement from the cytosol to the plasma membrane where it functions to enhance Raf-1 signaling (Therrien et al., 1996). Alternatively, it may function as a scaffold for 14.3-3 mediated enhancement of Raf-1 activation. Under this model, KSR might act as a "scaffold for the scaffold", facilitating formation of a multi-protein complex that is critical to ERK activation. Although a role for KSR as a scaffold is clear, questions about its function remain. Foremost is whether KSR possesses any catalytic activity (is its putative kinase catalytic domain the very reason it was identified)? If so, what are its substrates and how might they participate in ERK signal transduction?

In a *Drosophila,* a screen for enhancers of a dominant negative KSR developmental pathway, a mutant was discovered that enhanced KSR membrane mediated recruitment of D-Raf (Therrien et al., 1999). The gene, termed connector enhancer of KSR (Cnk), encodes an adaptor protein with several protein-protein interaction motifs (Anselmo et al., 2002). In flies, the C-terminal domain of CNK interacts with D-Raf, blocks activated Ras phenotypes, but had no effect on activated Raf

(Therrien et al., 1999). CNK is also involved in ERK-independent signaling and may be critical linker between ERK dependent and independent signaling pathways. More questions about CNK function arise from an examination of CNK function in vertebrates where researchers were unable to identify any interaction between the vertebrate CNK and Raf-1 (Yao et al., 2000).

Two other scaffolding molecules of note are MEK partner 1 (MP1) and Raf kinase inhibitor protein (RKIP). MP1 is an adaptor protein that preferentially links MEK1 to ERK1 (Schaeffer et al., 1998). The primary feature of interest of MP1 is its preferential association with ERK1. Although it is capable of binding ERK2, it does so with reduced efficacy. This may be example of scaffolding shaping the final output of upstream cascade activation through selective shuttling of ERK1 to activation. The biological implications of this effect are unclear because with the exception of knockout studies, ERK1 and 2 are functionally interchangeable. The final "scaffold" discussed here, RKIP, is perhaps better described as an inhibitory adaptor. RKIP associates with MEK and Raf under either *in vivo* or *in vitro* conditions (Yeung et al., 1999). The binding sites for MEK and Raf overlap and thus simultaneous binding of the two proteins by RKIP is sterically prevented (Yeung et al., 2000). Insofar as this occurs, RKIP disrupts ERK activation by preventing contact between Raf and its downstream target MEK. Growth factor stimulation release keeps RKIP from binding to Raf-1 hence declining signal from growth is correlated with increase RKIP association with Raf-1 (Yeung et al., 2000). It is currently unknown whether RKIP functions in neurons or whether its association with Raf-1 may also be regulated activation signals other than growth factor stimulation. It

would be interesting to determine if RKIP did function in this manner because regulation of its expression provides a simple mechanism for setting activation thresholds for the ERK signal cascade as well as a mode for feedback in regulating the duration of its function.

Persistent ERK activation

There is a gathering body of evidence demonstrating the importance of sustained ERK activation (lasting 60 min or more) in directing cell differentiation, motility, and neuronal plasticity. Persistent ERK signaling is required for differentiation of some cells into post-mitotic cells resembling functional neurons (Marshall and Marshall, 1995). Nerve growth factor (NGF) stimulation results in cell cycle arrest and neurite formation in PC12 cells (Cowley et al., 1994; Marshall and Marshall, 1995). Transient ERK activation in PC12 cells, elicited by epidermal growth factor (EGF) stimulates proliferation and chemotaxis (Marshall and Marshall, 1995; Ho et al., 2001). Also in PC12 cells, Fibronectin and fibroblast growth factor (FGF) synergistically cooperate to promote lasting ERK activation, neural adhesion, and neurite outgrowth (Choung et al., 2002). Stimulating primary astrocytes with isoproterenol, a β-AR agonist, induced maturation to stellate cells. Sustained ERK activation gated by PKA was required for this process (Gharami and Das, 2004). In hippocampal neurons, enduring ERK signaling was required for maintenance of dendrites evoked by neural stimulation with potassium (Wu et al., 2001). Conversely, sustained ERK signaling in hippocampal slices can also induce cell death (Runden et al., 1998).

Sustained ERK signaling is mediated first by the upstream signaling route used to activate the signal cascade. This is initially evidenced by the different ERK signal durations elicited by treatment with growth factors (NGF, PDGF, and EGF). This theme is also borne out in hippocampal neurons as stimulation with glutamate or KCl both evoked robust transient ERK activation (within 5 to 10 minutes) while treatment with KCl alone resulted in lasting ERK phosphorylation (Paul et al., 2003). Pharmacological inhibitors show that initiation of ERK signaling in dendrites requires L-type Ca+2 channels, CAMKII, and Ras, but not PKA (Wu et al., 2001). However, the persistent ERK signaling phase in dendrites was not affected by inhibitors of L-type Ca^{+2} channels, CAMKII, or Ras, but was inhibited by KT5720, an inhibitor of PKA (Wu et al., 2001). Blockade with the MEK inhibitor U0126 showed a continuing requirement for MEK activity in maintaining lasting ERK. Interestingly, U0126 removal allowed ERK activation to return 30' after originally initiated (Wu et al., 2001). Sustained MEK activity was required but the "persistence" signal was located upstream of MEK itself. It was also shown recently, in agreement with hippocampal studies, persistent ERK activation in *Drosophila*, mediated by intracellular Ca^{+2} and neural activity, required continued MEK activity (Hoeffer et al., 2003). Persistent ERK activation is likely broken up into distinct phases and modules, where PKA "gates" the neuronal decision to sustain ERK activation and the persistence module that once activated, preserves and insulates MEK/ERK from the effects of upstream signaling blockade. The ERK cascade likely possesses "biochemical memory" at other levels of the module, as indicated by data showing that pulsed stimulation (four three-minute depolarizations separated by 10

minutes) results in sustained ERK signaling as compared to massed stimulation (a single, 12 minute depolarization) that only evokes transient activation in hippocampal neurons (Wu et al., 2001). Clearly the mode of upstream signaling that ERK activation occurs through plays a key role in determining the period for which cascade is active. What molecular pathways are involved in regulating this process? A few potential mechanisms for determining lasting ERK activation and for regulating ERK signal duration have been identified. One possible mechanism is the differential activation of the Ras-like family members (Ras and Rap) and expression of cell specific cascade members to direct sustained ERK activation. A second mechanism is the regulation of the expression and activity of a number of phosphatases that down-regulate ERK activity.

Rap/B-Raf pathway

In a ground-breaking study, Vossler and colleagues discovered an alternative pathway for ERK activation; one that relied on Rap activation and subsequent activation of B-Raf, the neurally predominant isofom of Raf. Prior studies had revealed only an inhibitory role for Rap, presumably by interference by Ras association with Raf-1 (Kitayama et al., 1990; Cook et al., 1993). Using a Ras dominant negative (RasN17) construct, they showed that cAMP induced maturation of PC12 cells was independent of Ras activity. Following forskolin treatment, B-Raf was activated while conversely Raf-1 was inhibited (Vossler et al., 1997). This suggested a model where levels of B-Raf dictated a cell's response to cAMP. Transfection of B-Raf into a cell line with normally low levels converted PKA into an ERK activator rather than inhibitor. Rap1 is activated

by PKA (Altschuler et al., 1995) and was a possible candidate for B-Raf activation. Again using dominant negative Ras and Rap constructs, they showed that Rap and not Ras was responsible for PKA-dependent B-Raf activation in PC12 cells (Vossler et al., 1997). In a follow up study, York *et al.* expanded on these studies; this time examining NGF mediated sustained ERK activation. They found that sustained ERK activation could be separated into two different phases -- a transient phase (less than 20 minutes) governed by Ras/Raf and a persistent phase controlled by Rap/B-Raf. Furthermore, they observed a requirement for C3G, a RapGEF, and Crk, an adapter protein, for maximal activation of Rap and ERK in transfected reporter cell lines (York et al., 1998).

As attractive a model as it is the original findings have been somewhat difficult to fully reproduce (Garcia et al., 2001; Bouschet et al., 2003) and in some cases, controverted at least to some extent (Zwartkruis et al., 1998). What later studies have made clear is that separate phases of ERK activation exist and that at least in some cell types, the manifestation of these phases is regulated in concert by the activities of Ras, Rap, Raf and B-Raf that can be summed into a modified model (Figure 1.5). Support for this modified model comes from several sources. Work by Bouschet *et al.* showed that following PACAP stimulation of PC12 cells (a treatment capable of inducing persistent ERK activation), Rap-GTP and Ras-GTP are both stimulated robustly at early equivalent time points. Rap-GTP activity was enduring but Ras-GTP was transient. Concurrent with York *et al.*, they found that Ras was required for transient ERK activation but Rap was required for the persistent aspect of the ERK signaling profile. Rap-GTP was not sufficient for ERK activation but in fact required PKA, PKC, and Ca^{+2} signaling to

manifest. Constitutively active Rap constructs were unable to elicit sustained (or transient) ERK activation as well, but sustained signaling using these constructs was potentiated when combined with PKA, PKC, or Ras activation (Bouschet et al., 2003). Differences in subcellular localization of Rap/Ras may help explain the delay in Rap mediated onset of signaling. Ras is localized to the plasma membrane while Rap is localized to the perinuclear compartment (Mochizuki et al., 2001). Although both Ras and Rap are rapidly loaded with GTP, Rap/B-Raf activation of ERK signaling additionally requires translocation to the plasma membrane (Vossler et al., 1997). Additional sequential regulatory steps, ordered phosphorylation events or perhaps recruitment of adaptors that maintain Rap-GTP over time, may be required for the maturation of the "persistence module" and subsequent translocation of Rap-GTP to the plasma membrane where it functions to promote lasting ERK cascade activation. In further support for this model, maturation of megakaryocytes induced by thrombopoietin (TPO) is dependent on sustained ERK activity. Using TPO receptor mutants that are unable to selectively bind Ras and Rap, it was determined that Ras/Raf signaling initiated activation of the ERK gating sustained cascade activation that was dependent on Rap/B-Raf signaling (Garcia et al., 2001). Finally persistent ERK signaling in hippocampal dendrites required that Ras and L-type Ca^{+2} channel function for initiation, but once established, only PKA activity was required. This supports the notion that Ras signaling plus other regulatory activity -- perhaps influencing early Rap signaling -- is required to activate the ERK cascade, then later PKA serves as the gate to persistent activity (Wu et al., 2001).

The role of *Drosophila* Rap (*Roughened*) in controlling persistent ERK signaling is unclear. Studies suggest that Rap1 does not function in the regulation of DRaf but instead acts upstream or in parallel to Ras1/DRaf signaling (Asha et al., 1999). *Rap1* in *Drosophila* is required for cell survival, for the loss of function mutations in *Rap1* are lethal (Hariharan et al., 1991; Asha et al., 1999). However, Rap1 function is apparently dispensable in adults, so it is possible that some vertebrate equivalent Rap functions in flies are maintained by as yet uncharacterized members of the *Drosophila* ERK cascade (Asha et al., 1999). Persistent ERK activation is likely evident in *Drosophila* (Hoeffer et al., 2003), so it should be possible to isolate either with genetic screens or candidate gene approach, molecular players that regulate this process.

Phosphatases and the ERK Cascade

To date, ERK and its homologous family members throughout biology have only been shown to be activated by a single upstream effector, MEK. ERK is however the target of multiple phosphatases with respect to its inactivation. These include several dual specificity kinases, serine/threonine kinases, and protein tyrosine family members. ERK itself can promote its downregulation (Figure 1.6). ERK can control occur multiple aspects of phosphatase activity to contribute to its inhibition. MAPK phosphatase-1 and -3 (MKP1, MKP3) are directly phosphorylated by ERK. This modification increases their catalytic activity (Camps et al., 1998b; Slack et al., 2001). ERK can also promote the activity of MKP1, 2 through direct phosphorylation of residues that protect them against proteolytic turnover (Brondello et al., 1999). Finally, ERK is potent activator of MKP

transcription. MKP1 and MKP2 are rapidly and robustly induced by ERK activation in CCL39 cells. Interestingly, although MEK blockers completely block the induction both MKP1 and MKP2, they have different upstream pathways for induction. MKP1, but not MKP2, were also modestly induced by PKA and PKC activators. This may reveal a potential mechanism for crosstalk between upstream activation pathways in the downregulation of ERK signaling (Brondello et al., 1997). It is also notable that MKP activity is induced even in the presence of sustained ERK activation. In a study by Camps *et al.*, NGF stimulation induced MKP3 within one hour of stimulation that peaked after a few hours and was maintained for five days, matching the period over which PC12 differentiate and extend neurites. Sustained ERK signaling was observed and required for this process and MKP3 expression during this period likely acted to fine tune ERK signaling strength, possibly serving an anti-apoptotic role (Camps et al., 1998a). MKPs may play a central role in neuronal survival by preventing sustained ERK activation (Stanciu et al., 2000). What mechanisms prevent induced MKP activity from completely reducing ERK activity under these conditions is unknown.

Another key phosphatase involved in the regulation of sustained ERK activity is striatal enriched tyrosine phosphatase (STEP). STEP is highly expressed in the nervous system and is found in the hippocampus as well as many other structures in the brain (Lombroso et al., 1991; Boulanger et al., 1995). NMDA receptor activation is one of the many neuron cell surface routes that can result in ERK activation. NMDA receptor mediated Ca^{+2} influx into hippocampal neurons can also lead to the activation of STEP (Paul et al., 2003). Additionally, Ca^{+2} entry from L-type Ca^{+2} channels, or release from

intracellular stores, does not activate STEP (Paul et al., 2003). Modulation of STEP activity was linked directly to calcineurin (PP2B) phosphatase activity. Calcineurin, activated via NMDA receptor mediated Ca^{+2}, acts to remove an inhibitory phosphate group from the STEP KIM domain, involved in substrate binding (Paul et al., 2003). Not only does this study demonstrate an important role for STEP in sustained ERK signaling, but it also highlights the critical importance of signaling microdomains in regulating the ERK cascade, not only in its initiation but also its inactivation.

The study by Paul *et al.* is one of the first to link early mechanisms of neural activation, ERK signaling, and the molecular events that control the dueling forces of activation and inactivation. Very little is known about what molecular mechanisms control their phosphatase expression, neuronal localization, or maintenance of basal activity states. What role do phosphatases play in the formation of multi protein complexes, control of phospho-dependent scaffolding (14.3-3), or the inactivation of signaling components upstream of ERK that are acutely regulated by phosphorylation (Raf, Rap)? A number of important phosphatases involved in ERK signaling in *Drosophila* have been identified. Table 1.1 includes *Drosophila* phosphatases known to participate in ERK pathway as well as possible *Drosophila* homologs of vertebrate interactors. One phosphatase, Protein tyrosine phosphatase-ERK/Enhancer of Ras (PTP-ER) functions to negatively regulate Ras1 signaling during eye development (Karim and Rubin, 1999). PTP-ER is a protein tyrosine phosphatase family member and is related to the vertebrate He-PTP, STEP, and PC-PTP phosphatases. Dual specificity kinases in *Drosophila* have also been identified and characterized. DMPK and DMPK-3 (homolog

of vertebrate MKP-3) both bind and act to down regulate the activity of D-ERK *in vitro* (Lee et al., 2000b; Kim et al., 2002). They had no effect on activated versions other *Drosophila* MAPK family members, JNK, or Dm-p38 *in vitro.* DMPK-3 is expressed at low levels throughout the life cycle of the fly with maximal expression in the adult head (Kim et al., 2002). DMPK appears to be primarily expressed in late larval stages and in adulthood (Lee et al., 2000b). It would be interesting to examine whether perturbation of either DMPK would have any effect on olfactory avoidance learning in *Drosophila.* DMKP-3 is found exclusively in the cytosol, suggesting the possibility for compartmental roles for these MPKs in regulating DERK activity. The identification of DMKP and DMPK-3 became possible with the completed fruit fly genomic sequence. In the years prior to the availability of the completed *Drosophila* genome, genetic screens only uncovered a single MKP family member, the JNK phosphatase, *puckered (puc)* (Martin-Blanco et al., 1998). This suggests a good deal of functional redundancy among the MKP family members and should be an important consideration for *Drosophila* studies aimed at examining phosphatase regulation of ERK signal cascade.

Consequences (downstream effects) of ERK signaling

One of the most exciting and expanding areas of ERK signaling biology is the identification of ERK substrates and determination of their roles as effectors of the ERK cascade. ERK is found throughout the cell, and in its inactivated state, it is primarily found in the cytoplasm. Upon activation, it can be quickly localized to a number of cellular locations, such as the plasma membrane and endoplasmic reticulum, and

translocated into the nucleus where it can activate gene expression (Lenormand et al., 1993; Martin et al., 1997). ERK targets a wide array of cellular proteins, membrane-associated proteins (plasma, vesicle), cytoplasmic kinases, cytoskeletal proteins, and nuclear proteins (transcription factors, nuclear filaments) (Grewal et al., 1999; Pearson et al., 2001). Very few *bona fide* ERK targets have been identified in *Drosophila* (Peverali et al., 1996; Ciapponi et al., 2001; Koh et al., 2002), however, with the recent availability of protocols for the activation of neuronal ERK coupled with studies at the larval NMJ, it should be possible to rapidly identify new *Drosophila* ERK substrates and expose their functions in mechanisms of synaptic plasticity.

Membrane Targets of ERK Cascade

ERK targets many proteins localized to the plasma membrane as well as receptors (EGF), voltage gated channels (Kv4.2), neural adhesion molecules (ApCAM), signaling proteins (Phospholipase A2, Phospodiesterase), and vesicular membrane targets (synapsin) (Takishima et al., 1991; Lin et al., 1993; Jovanovic et al., 1996; Martin et al., 1997; Hoffmann et al., 1999). In a few cases, the cell biological effects of ERK activation have been described. ERK activity can promote the uptake of cell surface adhesion molecules, a key early step in synaptic expansion. In ApCAM, the Apylsia homolog of vertebrate, neural cell adhesion molecule (NCAM) is phosphorylated by ApERK and removed from the neuronal surface to protein degradation machinery (Bailey et al., 1997). Similarly, Fasciclin II (Fas II) in *Drosophila* is regulated by Ras/ERK signaling. Increased ERK activity leads to downregulation of synaptically localized Fas II (Koh et

al., 2002; Hoeffer et al., 2003). In addition to synaptic structure, ERK can also directly affect synaptic function through activation of synapsin during high frequency neuronal firing control increasing vesicular availability to exocytotic machinery (Chi et al., 2003). The A-Type potassium channel, Kv4.2, is a substrate for ERK both *in vivo* and *in vitro* (Adams et al., 2000). ERK dependent phosphorylation of the channel C-terminal domain reduces K+ current through the channel (Yuan et al., 2002). This downregulation may represent one of the mechanisms of enhancing dendritic excitability during LTP induction (Frick et al., 2004). Finally, ERK may play a role in penetrating MAG-dependent inhibition of neuronal regeneration by downregulating the activity of plasma membrane associated phosphodiesterase (Gao et al., 2003).

Cytoplasmic Targets of ERK Cascade

Several cytoplasmic proteins are also known substrates of ERK. Most of them are kinases, but recently an ubiquitin ligase, Ub-EDD, was also identified as an ERK target (Eblen et al., 2003). Kinase targets of activated ERK include p90 ribosome S6 protein kinase family members (Rsk-1, 2, 3, and-B), p70 SK61, MAPK interacting kinases (Mnk1, Mnk2), MAPK activated proteins (MAPKAP-2, 3pK), and mitogen-stress activated kinase-1 (Msk-1) (Mukhopadhyay et al., 1992; Stokoe et al., 1992; Ludwig et al., 1996; Zhao et al., 1996; Fukunaga and Hunter, 1997; Deak et al., 1998; Pierrat et al., 1998; Wang et al., 2002). Activated Rsk has targets in both in the nuclear and cytoplasmic compartments. It can reduce the activity of the Ras GEF, Sos, to downregulate upstream ERK activation (Frodin and Gammeltoft, 1999). Rsk can also function in the nucleus: Rsk2 can phosphorylate histone H3 to regulate chromatin

structure and promote gene expression by increasing the accessibility of promoter binding sites (Sassone-Corsi et al., 1999). Another ERK effector, Msk1, has also been shown to phosphorylate histone H3 as well a high-mobility group protein (HMG) also implicated in chromatin accessibility (Thomson et al., 1999). Activated Rsk2 translocated to the nucleus also acts directly on transcription factors to promote their activity, in this manner Rsk2 is believed to act as a proxy ERK regulator of gene expression (Impey et al., 1998). Additional evidence for this relationship has been obtained from contextual fear conditioning experiments. Following training in fear conditioning experiments in mice, p90rsk is rapidly phosphorylated in an ERK dependent fashion (Sananbenesi et al., 2002). Finally, Rsk2 is implicated in the severe cognitive disorder, Coffin-Lawry Syndrome, and ERK function mediated by Rsk activity may be critical to the manifestation of this condition (Zeniou et al., 2002).

A screen for novel ERK substrates revealed Mnk as a target of activated ERK signaling (Fukunaga and Hunter, 1997). This finding may be important as Mnk has a role in regulating eIF4e activity and protein translation. Under the Mnk/eIF4e "clamping" model, increased eIF4e phosphorylation is believed to increase its affinity to mRNA and promote increased rates of translational elongation. Supporting this are observations that increased eIF4e phosphorylation is generally related to increased rates of cellular translation (Kleijn et al., 1998; Raught and Gingras, 1999). Mnk phosphorylates in an ERK dependent manner. eIF4e *in vivo* (Waskiewicz et al., 1997; Waskiewicz et al., 1999) is localized to post-synaptic densities and dendritic lipid rafts (Asaki et al., 2003; Tanoue and Nishida, 2003). Together these findings may demonstrate a role for ERK in

influencing neuronal protein synthesis as one of the primary requirements of long term plastic change in neurons.

Finally, ERK may play a role in modulating the activities of several other cytoplasmic kinases via indirect pathways. One such example is calmodulin-dependent kinase II (CAMKII) whose critical role in plasticity and LTP is well established (Giese et al., 1998; Wu and Cline, 1998; Rongo, 2002). Following TBS stimulation of rat hippocampal slices, persistent CAMKII activity is observed both cytoplasmically in cell bodies and in the dendritic layers. In this stimulation paradigm, ERK function was required for both early and persistent phases of CAMKII activation (Giovannini et al., 2001). Although not predicted to be a direct target of ERK based on sequence, indirect regulation of CAMKII via ERK may highlight a larger role for ERK via intermediate regulators, further expanding the scope of ERK influence in neuronal plasticity.

Nuclear Translocation and ERK Regulated Gene Expression

One the most important cellular processes modulated by ERK activation is gene expression. LTM and long lasting forms of LTP (L-LTP) are both dependent on *de novo* gene expression and protein synthesis. Nuclear ERK translocation is required for LTF in Aplysia (Martin et al., 1997). Nuclear ERK translocation *in vivo* is a regulated process. During forskolin or BDNF mediated L-LTP induction, ERK translocation into the nucleus is gated by cAMP (Patterson et al., 2001). In hippocampal neurons, nuclear trafficking of phospho-ERK and phosphorylated ERK were dependent on PKA activity (Impey et al., 1998). In larval *Drosophila*, perturbation of proper ERK nuclear localization prevents proper eye development (Kumar et al., 2003).

Compartmentalization of ERK activity is an important node in the regulation of ERK signaling because regulation of its entry into the nucleus appears to be critical step in controlling the effector pathways and eventual neuronal outputs of ERK activation.

Once in the nucleus, ERK can directly or indirectly modify transcription factors to regulate gene transcription. One key but indirect target of ERK is cAMP responsive element (CRE) binding protein (CREB). CREB function has been implicated several forms of neuronal plasticity, LTP, LTF, and LTM. CREB functions in both vertebrate and invertebrate plasticity and is thought to be a central molecular switch in regulating long lasting forms of plasticity (Yin et al., 1995; Kogan et al., 1997; Bartsch et al., 1998; Josselyn et al., 2001; Sanyal et al., 2002). CREB is activated by phosphorylation at Ser 133, a target site for a number of kinases (PKA, CAMK, Akt, Msk) (Mayr and Montminy, 2001). Additionally, CREB Ser133 may be phosphorylated by Rsk. ERK and Rsk2 are required for Ca+2 stimulated CREB activation in PC12 and hippocampal cells (Impey et al., 1998). CRE-mediated transcription associated with L-LTP and induction of CREB regulated neuronal targets (c-fos, egr-1, Mkp-1) are inhibited by pharmacological blockade of MEK (Impey et al., 1998; Sgambato et al., 1998).

ERK directly targets several other transcription factors: AP-1 family members (c-fos, c-jun) (Chen et al., 1993; Xia et al., 1996), ETS family members (Elk1, Sap-1, Sap-2, Spi-B) (Gille et al., 1995; Janknecht et al., 1995; Mao et al., 1996; Strahl et al., 1996; Xia et al., 1996; Davis et al., 2000), and others (C/EBP, c-Myc, Pax6) (Chuang and Ng, 1994; Gupta and Davis, 1994; Davis, 1995; Michael et al., 1998; Mikkola et al., 1999). The role in regulating plasticity for a few transcription factors (Elk-1, Fos, Jun, C/EBP) has been

characterized to some extent (Alberini et al., 1994; Sanyal et al., 2002; Thiels et al., 2002). ERK can modulate transcription factor function through simple classical mechanisms, namely phosphorylation driven activation/repression of basal transcription factor function, to control gene expression. ERK can also affect transcription factor rates by more indirect means, through regulation of protein stability. Under basal conditions, c-fos is rapidly turned over by cellular proteolytic machinery. ERK mediated phosphorylation of C-terminal serine residues in c-fos stabilize the protein allowing it to accumulate and then activate gene expression (Murphy et al., 2002). Under conditions where ERK activation is transient, stabilization of c-fos is also brief. On the other hand, when ERK activation is persistent, c-fos levels build. Similar stabilization kinetics has been observed for other ERK targets, c-myc, egr-1, and c-Jun (Murphy et al., 2004). Murphy and colleagues propose this as a potential cellular mechanism for the interpretation of kinase signal strength and duration. For most transcription factors, the role if any, in regulating plasticity and their downstream transcriptional targets is largely unknown. This is region of substantial interest and future studies may reveal transcriptional modules that underlie different aspects of ERK mediated neuronal plasticity or respond differentially to upstream ERK activation pathways. In this respect the discovery of converging signal cascade crosstalk in the regulation ERK nuclear translocation (PKA gating) suggests that multiple signal pathways may converge on transcriptional modules to shape neuronal responses to plasticity inducing stimuli.

Concluding Thoughts

Curiously, few of the contributions to the ERK/plasticity field have come from studies in *Drosophila*. This is perhaps surprising considering that the known core/accessory components of the ERK signal cascade are largely conserved in *Drosophila* and both acute and genetic procedures for controlling the *Drosophila* ERK signaling pathway are available. Hopefully, these combined with the tremendous genomic and proteomic resources available to the fruit fly model system and well characterized synaptic preparation should greatly facilitate progress in important areas that remain inadequately understood. One outstanding area of interest is regulation of pathway crosstalk between the many signaling modules that may lead to ERK activation. What are the different contributions of the upstream signaling pathways with respect to the physiological stimuli invoked to activate ERK signaling? What upstream pathways are primarily responsible for directing persistent cascade activation? Scaffolding proteins are likely to play a critical role in dictating these responses by regulating protein-protein interactions leading ERK activation, restricting the subcellular localization of signaling components, or regulating/maintaining context specific interactions to promote precise neuronal responses. A second major area of interest should be the identification of ERK substrates. Given the great number of input pathways leading to ERK, the number of known substrates is very likely underrepresented. An important consideration in this effort is the likelihood that there exist significant numbers of indirect ERK substrates (CREB, eIF4e) whose neuronal functions are mediated by important proxy kinases like Rsk and Mnk. Invariably, the classification of ERK substrates will lead to a better

understanding of the plasticity mechanisms that ERK regulates. The neuronal ERK cascade is activated by numerous extracellular stimuli that transduce signals to a complex network of intracellular signaling pathways leading to neuronal plasticity. While a great deal has been learned about the mechanisms of ERK activation, only a few neuronal ERK substrates (Adams and Sweatt, 2002) and even fewer neuronal ERK dependent processes (Mayford et al., 1992; Murphy et al., 2004) have been described. Abundant evidence from vertebrate and invertebrate studies establishes a central role for ERK in neuronal plasticity, learning, and memory. The powerful genetic *Drosophila* model system should be brought to bear more fully on these topics to better our understanding of the ERK signal cascade thereby improving treatments for neurological disease, disorder, and injury.

FIGURE 1.1 Core components of the ERK signaling module

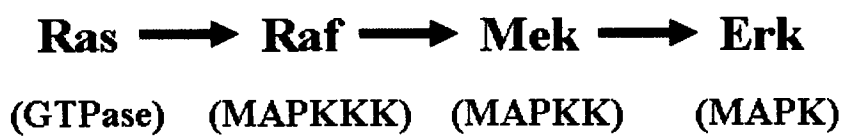

FIGURE 1.2 Neuronal ERK signal cascade is activated by multiple mechanisms including cell surface receptors, channel signaling pathways, and calcium signaling from intracellular stores. See text for description. VGCC, voltage-gated calcium channels, GPCR, G-protein coupled receptor, AC, Adenylate cyclase, PLC, Phospholipase-C, NMDA-R, N-methyl-D-Aspartate receptor, βA-R, β-adrenergic receptor, CaM, Ca^{++}-Calmodulin, DA-R, Dopaminergic and A1 adenosine receptor.

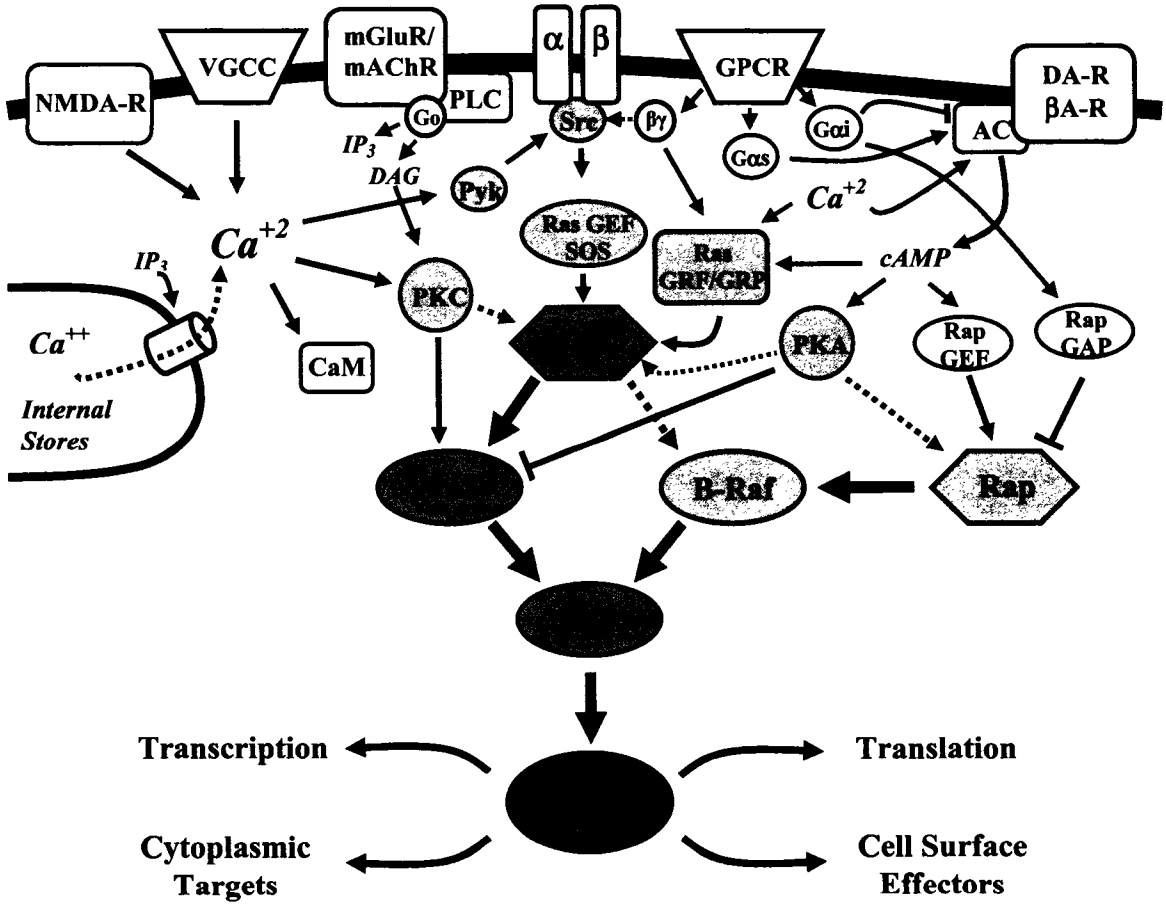

FIGURE 1.3 Pathways leading to Raf activation via Ras-like family members Ras and Rap. In some neurons, PKA can antagonize Ras activation and activate ERK via a Rap/B-Raf dependent pathway. This pathway may also be activated independent of PKA via Ca^{+2} dependent RapGEFs, EPac and CalDAG GEF activities. (See text for description)

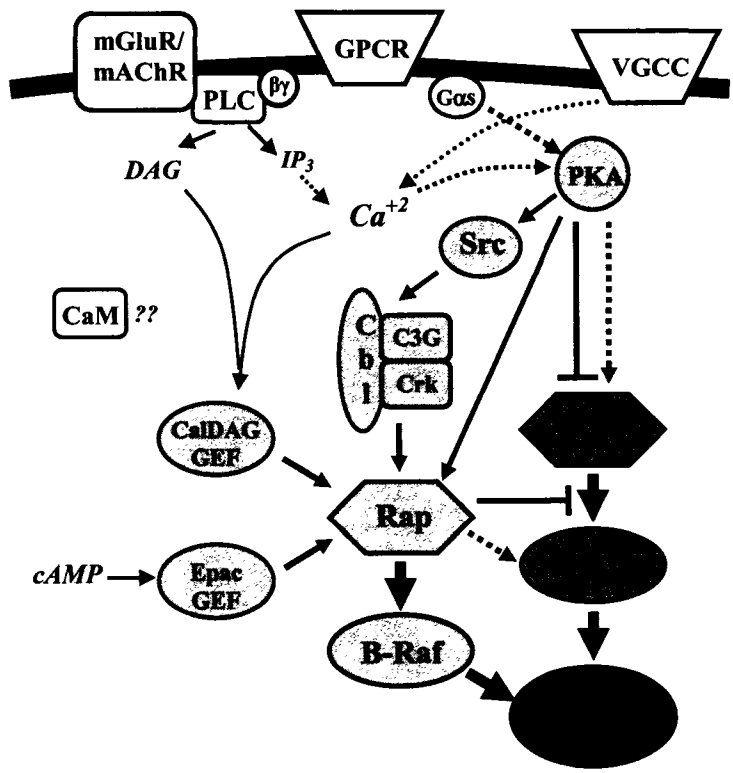

FIGURE 1.4 A Comparison of *Drosophila* Raf (polehole) and major vertebrate Raf isoforms, Raf-1 and B-Raf. All Raf family members, have three conserved regions (CR1.3). CR1 contains the Ras binding domain (RBD) and the cysteine rich domain (CRD). CR2 is a serine/threonine rich domain involved in regulating its activity. The C-terminal end, CR3, contains the catalytic domain, that encodes the Raf kinase activity. D-Raf more closely resembles the vertebrate B-Raf isoform, conserved regulatory residue are shown, unconserved residues are denoted with an (*).

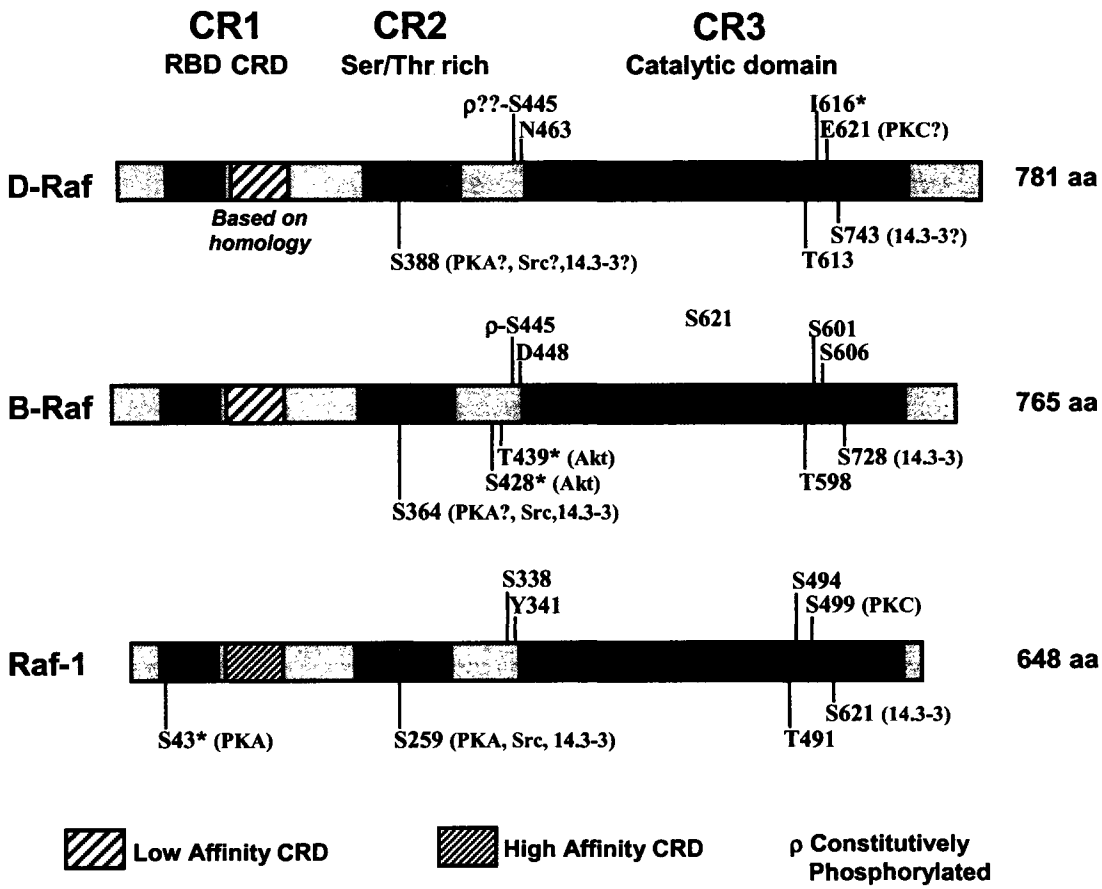

FIGURE 1.5 A model for Ras/Raf and Rap/B-Raf mediated persistent ERK cascade activation. Ras and Rap are localized to different compartments in the neuron. Rap and Ras are activated by numerous pathways. Ras can activate ERK signaling regardless of Rap-GTP activity. Rap-GTP is not competent to activate the ERK pathway and is potentiated by parallel signaling from PKA, PKC and/or activated Ras/Raf. PKA and Ras/Raf signaling are required "gating" activities for Rap/B-Raf signaling activate ERK. (See text description).

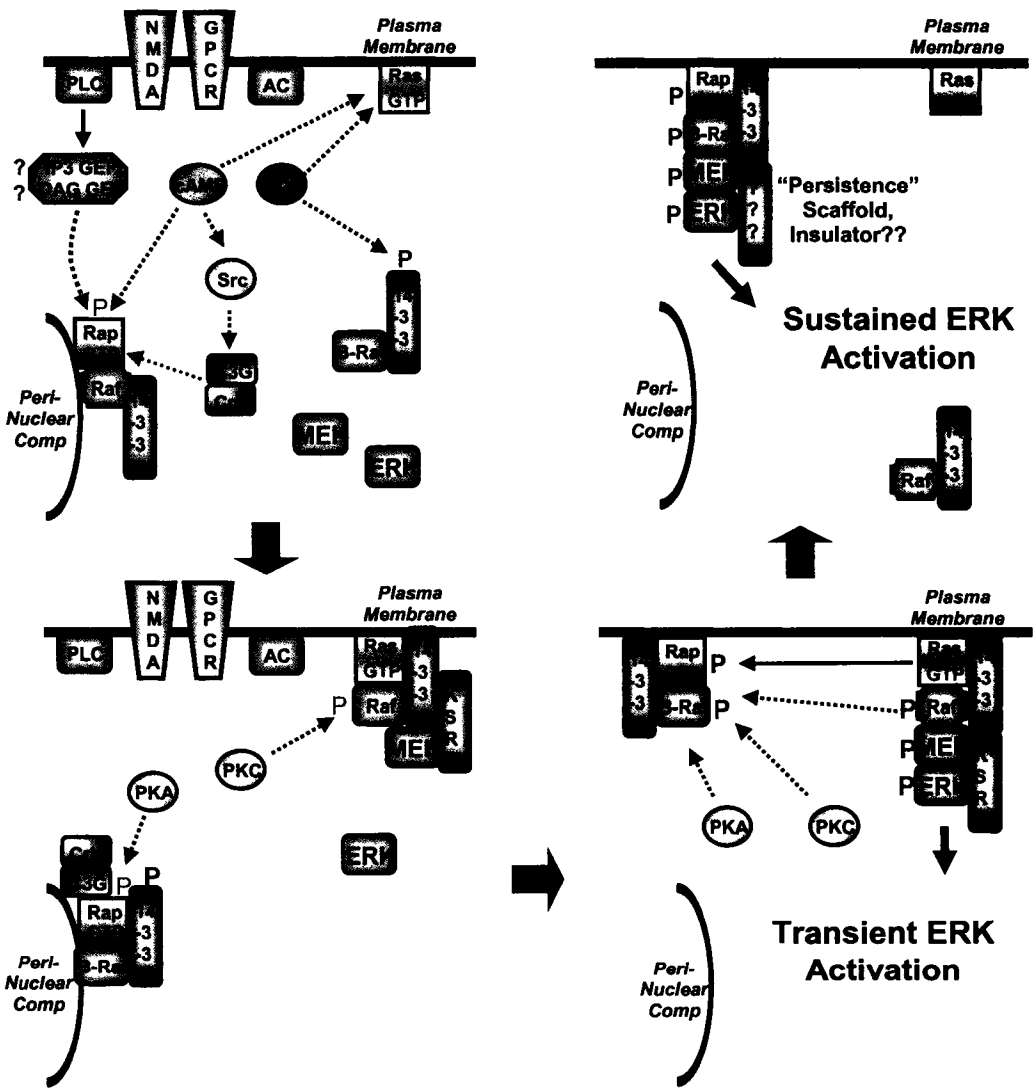

FIGURE 1.6 Mechanisms of phosphatase downregulation of ERK. Striatal enriched tyrosine phosphatase (STEP) is activated by phosphate removal by calcineurin (CaN). Active STEP can antagonize MEK activation of ERK. ERK can directly affect the activities of MAPK phosphatases via three modes of regulation; (1) phosphorylation of MKP, which promotes catalytic activity (2) phosphorylation of MKP, which confers proteolytical protection (3) transcriptional up regulation of MKP. (See text for further description).

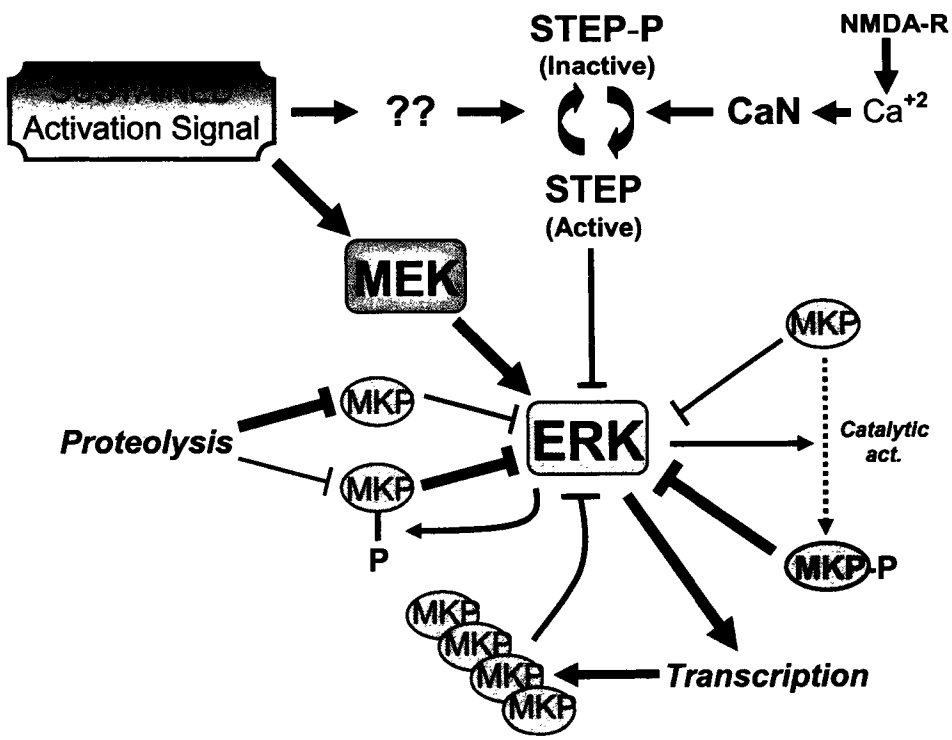

TABLE 1.1 *Drosophila* ERK Signaling Cascade Components

Cascade Component	Vertebrate Homolog	Proposed Function	Selected References
G-proteins			
Ras1	H-,N-,K-Ras (A/B)	Activation of D-Raf	[Simon 1991, Koh 2002]
Rap (Roughened)	Rap1a	Activation of D-Raf	[Hariharan 1991, Asha 1999]
MAKKK			
D-Raf (polehole)	B-Raf (Raf-1)	Activation of D-Sor	[Dickson 1992, Melnick 1993]
MEK			
D-Sor1	MEK1/2	Activation of rolled	[Tsuda 1993, Karim 1996]
ERK			
rolled (Dm-ERK)	ERK1/2	Numerous targets, Gene Expression, Translation, Protein Turnover	[Biggs 1992, Brunner 1994]
Ras GEF			
Sos	Ras GEF	GEF, promotes Ras activity	[Olivier 1993, Karlovich 1995]
CG5522	Ras GRF (cdc25)		None
Ras GAP			
NF1	Neurofimbrin	RasGAP, reduces Ras activity	[Guo 2000]
Gap1	p120 GAP, GAP1IP4BP	RasGAP	[Powe 1999]
Gigas	Tuberin	Ras/RAP GAP	[Acebes 2001, Tapon 2001]
Rap GEF			
dPDZ-GEF	PDZ-GEF	Rap GEF	[Lee 2002]
dC3G	C3G	Rap GEF	[Ishimaru 1999]
Gef26?? Gap1	CalDAG GEF??	Rap GEF (Ca^{+2} dep GAP)	None
Rap GAP			
RapGap1	RapGap1	Rap GAP	[Chen 1997]
Epac	Epac	Rap GAP	[Brody 2002]
AE003758	Spa1	Rap GAP	[Bos 2001]
Scaffolds, Adapters			
leonardo	14.3-3ζ	Scaffold, promotes/Inhibits Raf activation	[Li 1997, Cacace 1999, Chang 1997]
KSR	KSR1,2	Scaffold, promotes Raf translocation to PM	[Therrien 1995]
drk	Grb2	Adapter	[Simon 1993, Luschnig 2000]
Crk	Crk	Adapter	[Galletta 1999]
CG5110	MP1	Facilitates Raf-MEK interaction	[Anslemo 2002]
CG10298	RKIP	Disrupts Raf MEK interaction	None
CNK2A(B)	Maguin-1	Adapter	[Therrien 1998, Lanigan 2003]
dos	Gab2	Adapter	[Raabe 1996, Herbst 1996]
Kinases			
Src64B	c-Src	Ras, Raf activation, ERK signal maint	[Dodson 1998, Dubnau 2003]

Dco	PKAc	cAMP dep kinase activation/inhibtion	[Kalderon 1988]
PKAr	PKAr	cAMP dep kinase regulatory subunit	[Kalderon 1988, Goodwin 1997]
Pkc98E	PKCε	Ca^{+2} dep Kinase ε isoform	[Schaeffer 1989]
Pkc53E	PKCα	Ca^{+2} dep Kinase α isoform	[Rosenthal 1987, Schaeffer 1989]
Akt1	PKB	Dep. Kinase, Raf activation	[Staveley 1998]
PI3K	PI3K	Phosphatidylinositol kinase	[Prober 2002]
PI3K-59F	PI3K	Phosphatidylinositol kinase	[Linassier 1997]
PI3K-68D	PI3K	Phosphatidylinositol kinase	[Leevers 1996]
PI3K-92E	PI3K	Phosphatidylinositol kinase	[MacDougall 1995]
Pak	Pak	Src, Raf activation	[Harden 1996, Hing 1999]
Src42A	v-yes-1, Fyn	Src kinase	[Lu 1994, Lu 1999]
Fak56D	Praline rich tyrosine kinase-2 (pyk-2) ???	Tyrosine Kinase	[Fox 1999, Fujimoto 1999]
Abl	Praline rich tyrosine kinase-2 (pyk-2) ???	Tyrosine Kinase	[Comer 1998, Elkins1990]
Channels, Receptors			
Sev	**EGFR**	RTK	[Hafen 1987]
Ca+2 channel β-subunit	L-Type Ca+2 channel	Ca^{+2} entry	[Littleton 2000]
cacophony (cac)	Voltage gated Ca+2 channel	Ca^{+2} entry	[Kawasaki 2000, Littleton 2000]
GluR-A	mGluR1	Glutamate Receptor	[Sigrist 2002, DiAntonio 1999]
mAchR-60C	mAchR	Muscarinic Acetylcholine Receptor	[Blake 1993, Brody 2000]
NMDAr1	NMDA-R	Ligand gated Ca^{+2} Channel	[Betz 1993, Volkner 2000]
Phosphatases			
D-MKP	MSG5	ERK phosphatase	[Lee 2000]
D-MKP3	MKP3	ERK phosphatase	[Kim 2002, Kim 2004]
Csw	SH2-PTP	RTK inactivation	[Perkins 1996, Herbst 1996]
CG32697	STEP??	Phosphatase,	None
PTP-ER	MEG1, STEP, He-PTP	Phosphatase, ERK inactivation	[Karim 1999]
Dm-NIPP1	NIPP1	Nuclear Inhibitor of PP1	[Parker 2002]
D-PP1c	PP1c	Protein Phosphatase1 cat. subunit	[Parker 2002]
microtubule star (mts)	PP2Ac	Phosphatase cat subunit, Raf activation, ERK inactivation	[Cohen 1993, Wassarman 1996]
widerborst (wdb)	PP2Ar	Phosphatase reg subunit	[Hannus 2002]
Other			
D-Cbl	Cbl	Ubiquitin Ligase	[Pai 2000, Hime 2001, Robertson 2000]
Cno	AF-6	Rap effector, cytoskeleton binding	[Boettner 2003]
msk (moleskin)	Importin 7	Nuclear Importin	[Lorenzen 2001]
Rasputin (rin)	Rin	RasGAP binding protein	[Pazman 2000]
Plc-21C	PLC	Phospholipase	[Kim 1995, Thackeray 1998]

CA P60A	SERCA	Calcium ATPase, Ca^{+2} Sequestration	[Sanyal 2004, Hoeffer 2003]
Hsp83	Hsp90	Chaperone, stabilizes KSR	[Cutforth 1994]

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CHAPTER TWO

ACUTE INDUCTION OF CONSERVED SYNAPTIC SIGNALING PATHWAYS IN *DROSOPHILA MELANOGASTER*

ABSTRACT

Analyses of early molecular and cellular events associated with long-term plasticity remain hampered in *Drosophila* by the lack of an acute procedure to activate signal transduction pathways, gene expression patterns, and other early cellular events associated with long-term synaptic change. Here, we describe the development and first use of such a technique. Bursts of neural activity induced in *Drosophila comatose*^{ts} and *CaP60A*^{Kumts} mutants, with conditional defects in NSF1 and SERCA, respectively, result in persistent (>4hrs) activation of neuronal extra cellular-signal regulated kinase (ERK). ERK activation at the larval neuromuscular junction (NMJ) coincides with rapid reduction of synaptic Fasciclin II; in soma, nuclear translocation of activated ERK occurs together with increased transcription of the immediate early genes Fos and c/EBP. The effect of "seizure-stimulation" on ERK activation requires neural activity and is mediated through activation of MEK, the MAPKK that functions upstream of ERK. Our results: 1) provide direct proof for the conservation of synaptic signaling pathways in arthropods; 2) demonstrate the utility of a new genetic tool for analysis of synaptic plasticity in *Drosophila*; and 3) potentially enable new proteomic and genomic analyses of activity-regulated molecules in an important model organism.

INTRODUCTION

Experience-dependent modification of the nervous system underlies learning and memory (Bailey et al., 1996). Synaptic activity promotes changes in intracellular concentrations of important second messengers such as cAMP and Ca^{++}. Appropriate levels and dynamics of these second messengers activate signal transduction modules that direct gene expression changes underlying long-term plasticity (Sweatt, 2001; West et al., 2001). One signaling module critical to this process is the Ras/extracellular signal-regulated kinase (ERK) pathway (Atkins et al., 1998; Orban et al., 1999; Manabe et al., 2000; Ohno et al., 2001). ERK signaling is a potent regulator of gene expression associated with long-term plasticity (Martin et al., 1997; Dolmetsch et al., 2001). In addition to its role in regulating gene expression, the Ras-ERK pathway has additional functions outside the nucleus; e.g., to control synaptic structure by regulating the internalization of synaptic cell-adhesion molecules (Mayford et al., 1992; Bailey et al., 1997; Koh et al., 2002).

Despite the emerging outline for signaling pathways that regulate long-term synaptic change, several components remain unknown and the hypothesized functions of known components are often inadequately tested *in vivo*. These issues may be particularly well addressed in a genetic model organism like *Drosophila* in which long-term behavioral and synaptic plasticity has been shown to involve phylogenetically conserved molecules. In particular, the developmental plasticity of the *Drosophila* neuromuscular junction (NMJ) as it expands about 50 fold from a small embryonic synapse to a mature third instar NMJ, involves processes that function during the

establishment of late-long-term potentiation (L-LTP) in mammals and long-term facilitation (LTF) in *Aplysia*. Thus, *Drosophila* NMJ development is neural activity dependent, is negatively regulated by synaptic levels of the cell adhesion molecule Fasciclin II (Fas II), a *Drosophila* homolog of mammalian NCAM and *Aplysia* ApCAM (Bailey et al., 1992; Mayford et al., 1992; Keshishian et al., 1996; Brunner and O'Kane, 1997) and requires functions of ERK, CREB, and AP1 (Davis et al., 1996; Koh et al., 2002; Sanyal et al., 2002).

However, early events in the establishment of plasticity, e.g. the sequence of molecular events that lie between synaptic activity and the initial synaptic and nuclear responses, remain essentially unstudied in *Drosophila*. This lacuna derives from the absence of procedures, similar to those described in mollusks and vertebrates (Montarolo et al., 1986; Cole et al., 1989; Worley et al., 1993), to acutely induce neural activity patterns that lead to long-term plasticity. In vertebrates, seizures, induced pharmacologically with kainate or by direct electrical stimulation have been used extensively to identify activity-regulated genes such as Arc and c-Fos (Cole et al., 1989; Curran et al., 1990; Lyford et al., 1995). Similarly, spaced neural stimulation procedures in vertebrates, hippocampal cell culture, and mollusks have allowed several analyses of the signaling pathways and cellular mechanisms that initiate long-lasting synaptic change (Barzilai et al., 1989; Woo et al., 2000; Colicos et al., 2001; Wu et al., 2001). Guided by analyses in vertebrates (Charriaut-Marlangue et al., 1988; Ben-Ari and Represa, 1990; Contzen and Witte, 1994), we identified, characterized, and used specific conditional and neural excitability mutants in *Drosophila* for induction of neural "seizures" and thereby,

synaptic signaling pathways associated with long-term neuronal change. We demonstrate that the mutants *comatose*^{ts} (*comt*^{ts}), in the vesicle fusion protein NSF (Pallanck et al., 1995), and *Ca60A*^{Kumts}, in the *Drosophila* SR/ER Ca^{++} ATPase SERCA (Magyar et al., 1995, Periz and Fortini, 1999, S. Sanyal, unpublished observations), allow experimental induction of neural seizures. Resulting neural activity induces persistent phosphorylation of neuronal ERK, ERK translocation into nuclei, rapid down regulation of synaptic cell adhesion molecule Fas II, and the induction of immediate early genes Fos and c/EBP in *Drosophila*. This, to our knowledge, is the first direct demonstration that these synaptic signaling events are conserved in arthropods. As we demonstrate by MEK perturbation analyses, and by experiments that substantially tighten the temporal link between synaptic signaling, ERK activation and Fas II down regulation in *Drosophila*, the procedure we describe should enable wider and deeper analyses of mechanisms that underlie long-term plasticity.

MATERIALS and METHODS

Drosophila strains and culture conditions

Flies were reared in standard conditions at 21° C. We used the following strains for the experiments conducted in this study: a temperature-sensitive *comatose* mutant, *comt*^{tp7} (isolated in TIFR, Mumbai), and a novel a temperature-sensitive *Ca-P60A* mutant, *Ca-P60A*^{Kum170} (*Kum*), isolated by one of us (S. Sanyal) in collaboration with A. Basole and K.S. Krishnan in TIFR Mumbai. A temperature sensitive *paralytic* mutant, *para*^{ts1}, was provided by Barry Ganetzky (U. Wisconsin Wisconsin, Madison). The

temperature sensitive mutant *Resistant to dieldrin*$^{MD\text{-}RR}$ (*Rdl*$^{MD\text{-}RR}$) was obtained from the Bloomington *Drosophila* stock center. The wild type strain, Canton-S (CS) was obtained from D. Brower (University of Arizona).

Paralysis/Seizure Generation

One-day-old adult flies were placed in clean, disposable borosilicate glass vials with Whatman 3M strips for scaffolding under non-crowded conditions (~30 animals/vial). For seizure induction, flies were heated for four minutes at 40°C in water baths, then removed and allowed to recover at 22-25°C. For "restricted recovery" experiments in which neural activity was blocked in a *para*^{ts1} background, adult flies were recovered at 33° C and larvae at 35°C in water baths.

Electrophysiology

All recordings were made from the dorsal longitudinal flight muscles (DLM) in the fly thorax. Flies were anesthetized lightly by cooling on ice for a few minutes. Anesthetized flies were mounted upright in modeling clay such that the thorax was exposed for electrode penetration. Flies were allowed to recover for at least 10 minutes before recording. Both the ground and recording electrodes were heat-pulled glass micro-capillaries (tip resistance, 3-5 M) filled with 3 M KCl. The ground electrode was inserted into the head, and the recording electrode was inserted through the thoracic cuticle into the DLMs. The typical firing pattern of the thoracic muscles was used to confirm the position of the recording electrodes (Ikeda and Kaplan,

1970). Electrode tip resistance was essentially unchanged upon muscle penetration and muscle resting membrane potentials were less than -50 mV in all cases. Stage temperature was controlled using a peltier device. Ramping time for temperature changes were typically less than two to three minutes. Data was acquired using an axoclamp2B amplifier (Axon Instruments) and a digidata 1200 digitizer board. Data were visualized and processed using pClamp6 software.

SDS-PAGE and Western blotting

Adult *Drosophila* heads were isolated by snap freezing whole flies in liquid nitrogen then using mechanical decapitation (vortexing) and separation with tissue isolation sieves. For westerns using larvae, CNSes were dissected out of wandering third instar larvae in Ca^{++}-free HL3 Ringers. Adult heads or larval CNSes were added to 2X SDS protein extraction buffer (50 mM Tris-HCl ph 6.8, 1.6% SDS, 8% Glycerol, 4% β-mercaptoethanol, 0.04% Xylene Cyanol/Bromophenol Blue, including 2X Complete Mini Roche Protease Inhibitor) and homogenized using a motorized pestle. Protein lysates were separated on a 12% acrylamide gel. Blots were probed with anti-DP-ERK monoclonal antibody (1:2000) (Sigma), anti-β-Tubulin (1:4000) (Zymed). Proteins were visualized with peroxidase-conjugated secondary antibodies (1:1000) and developed with an ECL chemiluminescent kit (Amersham). Quantitation of band intensities was performed by scanning the developed BioMax autoradiographic films with a UMAX Astra 1220U scanner then analyzing the densiometric signal of the resultant images with Metamorph (Universal Imaging) image analysis software.

MEK inhibitor delivery

The MEK inhibitor U0126 was obtained from Cell Signaling technologies. A 200 mM stock of the drug was prepared by diluting the lyophilized drug in DMSO, storing the stock at -20 C° protected from light. For drug delivery, a solution of 5% Sucrose, 2% yeast (solid) was prepared in ddi H_20, the stock U0126 was then added to this solution to a final concentration of 1mM. Groups of ~50-100 *Drosophila* were starved 6-8 hours and then added to disposable, sterile glass test tubes layered with 3M Wattman paper soaked in the working drug solution. The animals were then added to the drug containing vials and allowed to feed overnight (in an incubator at desired temperature condition). The next day, drug fed flies were treated then positively selected for U0126 ingestion based on the dark gray to bluish staining of the abdomen indicative of drug consumption. Alternatively, a few drops of red non-toxic food coloring may be added to the drug solution to facilitate identification of the animals exposed to drug (useful for drugs such as KN-093 where no staining is evident after feeding).

Immunohistochemistry

The following antibodies were used for this study: mouse anti-DP-ERK (1:200; Sigma), rabbit anti-DSYT2 (1:200; H. Bellen), rabbit anti-GluRII (1:200; Y.Kidokoro), and rabbit anti-Fasciclin II (1:3000; V. Budnik). Appropriate secondary antibodies conjugated to fluorescent Alexa dyes (Molecular Probes, Eugene OR) were used.

For the examination of the larval central nervous system (CNS), wandering third-instar were dissected in Ca^{++}-free HL3 Ringers (Stewart et al., 1994) and fixed in 3.5%

Ca^{++}-free paraformaldehyde for three hours on ice. The larval CNS was then blocked for two hours in in block (PBS, 0.2% Triton, 2% BSA and 5% goat serum, pH 7.2) and subsequently incubated with primary antibody O/N at 4° C. The preparations were washed six times shaking for 20 minutes each in block and incubated for additional two hours with secondary antibody. Larval CNSes were then washed six times in PBS-0.2% Triton and mounted in VECTASHIELD (Vector Laboratories Inc.) mounting media on Superfrost+ (VWR) and visualized in a manner identical to the NMJ (see below).

The immunohistochemical procedures used for the analysis of the *Drosophila* NMJ in this study were as previously described (Sanyal et al., 2002). Briefly, wandering third-instar larvae were dissected and fixed in 3.5% calcium-free paraformaldehyde and blocked in PBS containing 0.15% Triton, 2% BSA and 5% goat serum for one hour. Incubations with primary Ab were performed overnight at 4°C and secondary antibody incubations were performed for 1.5 hours at room temperature. For immunohistochemical analysis of the *Drosophila* NMJ under neural activity blockade conditions, third instar animals of the appropriate genotype were treated induce neural activity (as earlier described) but were recovered at 35° C for 15-20 minutes before dissection. Animals were fixed for 10 minutes in Bouin's fixative (standard non-alcoholic) then washed eight times (10 minutes each) in wash (PBS, 0.2% Triton, pH 7.2). Incubations with primary and secondary antibodies were performed identical as described above. Activity blockade experiments were performed on single animals to minimize the time between blockade at restrictive temperature and fixation.

To quantify DP-ERK and Fasciclin II levels, synapses were fluorescently labeled and imaged using a laser scanning confocal microscope (Nikon). Maximum projections were obtained from serial sections of each sample. All images for comparison were from identically processed preparations and were obtained using matching settings during the same session. The images were analyzed with Metamorph imaging software (Universal Imaging). After background subtraction, the average pixel intensity of scanned boutons was measured and analyzed.

RNA extraction and Quantitative RT PCR

RNA extraction and Quantitative PCR was performed as previously described in Sanyal et al., 2002. Briefly, for each treatment and genotype RNA was harvested from 1.2 day old male *Drosophila.* Separate RNA extractions were performed for each independent experiment (n). PCR products obtained from Q-PCR reactions were visualized after electrophoresis in 2% agarose then stained with ethidium bromide. A one-cycle difference represents a two fold difference in starting template concentration.

Statistics

Student's T-Test was used for most comparisons. For the analysis of gene expression a one-way ANOVA was performed comparing the cycle difference in target gene expression between treated wild-type and mutant genotypes.

RESULTS

Induction of seizures using comt^{tp7} and CaP60A^{Kum170} Drosophila

In the mammalian brain, pharmacologically or electrically induced seizures trigger not only activity-induced gene expression (Dragunow and Robertson, 1987; Gall et al., 1990), but also long-lasting structural alterations (i.e. formation of additional synaptic contacts) in the nervous system (Ben-Ari and Represa, 1990; Nicoll and Malenka, 1995). In an attempt to similarly trigger activity-mediated processes in the *Drosophila* CNS, we examined the possibility that conditional *Drosophila* mutants with inducible seizure-like behaviors might serve as viable seizure models. To test this idea, we first examined the behaviors of a panel of published and recently isolated, unpublished, *Drosophila* temperature-sensitive paralytic mutants.

Several t.s. paralytics, like the sodium channel mutant *para*^{ts1} (Suzuki et al., 1971; Loughney et al., 1989; Budnik et al., 1990), showed flaccid paralysis when shifted to non-permissive temperatures. Many other mutants such as *sei*^{ts} and *Rdl*^{ts} (Jackson et al., 1984; Ffrench-Constant, 1994) showed behavioral convulsions after brief exposure to elevated temperature (data not shown). However, among these, two mutants, *comt*^{ts} (Pallanck et al., 1995) and *Ca-P60A*^{Kum170}, one of three novel, dominant t.s. alleles of the *Drosophila* SERCA gene *Ca-P60A* (Sanyal et al., in preparation; Magyar et al., 1995; Periz and Fortini, 1999) showed sustained and particularly long-lasting convulsions (*comt*^{tp7}) or contractions (*Ca-P60A*^{Kum170}) after brief exposure to the appropriate non-permissive temperature (Fig 1). Following a four-minute exposure to restrictive temperature (35°C), *comt* mutants demonstrate robust seizure-like behaviors lasting more

than an hour at room temperature. *Ca-P60A*^{Kum170} mutants exposed to 40°C for four minutes show prolonged (18-48 hour) paralysis, punctuated by uncoordinated twitches, muscle contraction and infrequent but intense bouts of seizure–like behavior followed by a return to a state of severely restricted movement (not shown).

To examine the cellular basis for these behaviors, we performed intracellular recordings from adult dorsal flight muscle (DLM) under permissive and restrictive temperatures (Fig. 2a). At normal (permissive) temperatures, 20°C, virtually no spontaneous DLM action potentials are observed via intracellular recordings prior to heat treatment. Wild-type animals exhibit a slight increase in spontaneous DLM firing upon heating, in *comt* mutants this effect is much more robust and in both cases this observed increase is blocked by severing the DLM motor axon and is thus derived from increased neural activity (Kawasaki and Ordway, 1999). All mutants showed wild-type levels of activity at permissive temperature (Fig. 2b, top panel). After four minute exposure to non-permissive temperatures, both mutant *comt* and double-mutant *comt; Ca-P60A* flies displayed strong, spontaneous activity for at least 60 minutes (Fig. 2b, middle and lower panels). While, *Ca-P60A* mutants alone did not display spontaneous activity close to the levels observed in *comt*, prolonged recordings indicated sporadic bursts of activity not observed in wild-type controls (data not shown). Triple mutant *comt para; Ca-P60A* flies were identical to *comt* and *comt; Ca-P60A* under these experimental conditions.

To test whether spontaneous muscle activity at high temperature was driven by motor neuron activity, we tested whether action potential firing in muscles could be reversibly blocked by inhibiting neuronal Na^+ channel function in *comt para; Ca-P60A*

mutants by raising the *Drosophila* to temperatures restrictive for *para* (Fig. 2c). Our observation that activity is dependent on *para* function (Fig. 2d) demonstrates that the observed firing of the flight muscle is synaptically driven by spontaneous neural activity.

Taken together, these observations suggested to us that, in double-mutant $comt^{tp7}$; $Ca\text{-}P60A^{Kum170}$ animals, increased synaptic activity induced predominantly by the *comt* mutation should be accompanied by substantially enhanced cytosolic Ca^{++} signaling due to reduced SERCA-dependent calcium sequestration in $Ca\text{-}P60A^{Kum170}$. Together, increased synaptic activity and enhanced calcium signaling could be expected to activate neural signaling pathways that initiate synaptic plasticity.

Persistent neuronal ERK activation by an activity and MEK dependent mechanism.

Treatments that induce plasticity-associated gene expression in mollusks and vertebrates also induce sustained (more than 120 minutes) phosphorylation of the extracellular-signal regulated kinase, ERK (English and Sweatt, 1996; Martin et al., 1997; Impey et al., 1998; Wu et al., 2001). With this in mind, we examined whether brief heat treatment of $comt^{tp7}$; $Ca\text{-}P60A^{Kum170}$ double mutants would activate ERK signaling in the *Drosophila* nervous system (Figure 3).

We exposed $comt^{tp7}$; $Ca\text{-}P60A^{Kum170}$ mutant animals to a four-minute pulse of heat (40°C) then recovered at room temperature (R.T.) for 60 minutes. To evaluate ERK activation in neurons after this "treatment", we performed a western blot analysis of proteins isolated from head lysates using an antibody specific to the activated form of ERK, diphosphorylated-ERK (DP-ERK) (Gabay et al., 1997). Treated double mutant

animals showed a large increase in ERK activation after the treatment (Fig 3a). DP-ERK levels in treated *comt*^{tp7}; *Ca-P60A*^{Kum170} animals was observed to increase approximately 2.5 fold ($p<0.05$; n=6) when compared to untreated animals of identical genotype. A 3.5 fold DP-ERK increase was observed with treated *Ca-P60A*^{Kum170} animals alone ($p<0.01$; n=6) (Fig 3a), a 1.5 fold increase in DP-ERK levels in treated wild-type animals was observed, this slight increase was consistent with previous studies examining ERK activation in response to heat shock (Chen et al., 1995). Basal total ERK levels were unchanged between treated and untreated lysates as well as other genotypes (Fig 3a). ERK activation is readily observed within 15 minutes (data not shown), peaks at two hours ($p<0.01$; n=5) and persists for at least four hours ($p<0.01$; n=6) (Fig 3b). Thus, consistent with activation of neuronal plasticity pathways, a brief temperature exposure to either *comt*^{tp7}; *Ca-P60A*^{Kum170} or *Ca-P60A*^{Kum170} mutants, is sufficient to induce strong and sustained ERK activation in the *Drosophila* head.

The observed ERK activation following our treatment could have resulted from either neural-activity dependent signaling generated during the treatment or via intracellular signaling pathways initiated by SERCA inhibition that are independent of increased neuronal activity. To distinguish between these two possibilities, we examined ERK activation following treatment under conditions in which neuronal action potentials are permitted (21°C) or inhibited (33°C) in *para*^{ts1}; *Ca-P60A*^{Kum170} double mutants and *para*^{ts1} *comt*^{tp7}; *Ca-P60A*^{Kum170} triple mutants. At temperatures permissive for *para*^{ts1}, ERK activation in either genetic background was not affected. Thus, we observed an approximately three-fold increase in DP-ERK in treated *para*^{ts1} *comt*^{tp7}; *Ca-P60A*^{Kum170}

($p<0.01$; n=6) under activity-permissive conditions (Fig 3a). However, under conditions non-permissive for *para*, ERK activation was blocked. Treated *para*^{ts1}; *Ca-P60A*^{Kum170} or *para*^{ts1} *comt*^{tp7}; *Ca-P60A*^{Kum170} animals under activity-restrictive conditions had DP-ERK levels nearly identical to treated wild-type controls, less than half that of similarly treated *comt*^{tp7}; *Ca-P60A*^{Kum170} animals ($p<0.05$, n=4) (Fig 3c). Thus, ERK activation observed following seizure induction in *comt*^{tp7}; *Ca-P60A*^{Kum170} or *Ca-P60A*^{Kum170} *Drosophila* is dependent on neuronal activity.

The observed ERK activation from our treatment could be a result of several neural activity dependent mechanisms: i) reduced turnover of ERK; ii) down regulation of phosphatase activity targeting DP-ERK (Brondello et al., 1999; Bhalla et al., 2002); or iii) up regulation of MAPK-kinase (MEK) activity (Atkins et al., 1998). The first possibility is argued against by our observation that treated and untreated animals show nearly identical levels of total ERK protein (Figure 3a). To distinguish between the next two possibilities, we tested whether ERK activation during our procedure could occur under conditions of MEK inhibition (Martin et al., 1997). MEK was pharmacologically inhibited by feeding animals U0126, a selective inhibitor of MEK activity (English and Sweatt, 1997). Under these conditions, ERK activation in *Ca-P60A*^{Kum170} animals substantially reduced ($p<0.01$, n=4) when compared to control, sham-fed *Ca-P60A*^{Kum170} *Drosophila*. DP-MAPK levels in treated, MEK-inhibited, *Ca-P60A*^{Kum170} animals were the same as in wild type controls (Fig 3d). Results similar to those described above from analysis of adult *Drosophila*, were also obtained from western analyses of CNSes dissected from similarly treated third instar larvae (data not shown). These observations

led us to conclude that persistent MEK signaling, and not decreased dephosphorylation, was responsible for the observed sustained activation of ERK.

ERK activation occurs in CNS neurons

Being based on analyses of CNS lysates, the above experiments did not identify the specific cell type in which ERK activation occurs. To address this issue, we performed immunohistochemical studies to confirm that ERK activation following seizure induction occurs in neurons. Because increased ERK activation in treated $comt^{tp7}$; $Ca\text{-}P60A^{Kum170}$ appears to derive almost completely from the $Ca\text{-}P60A^{Kum70}$ mutation (Fig 2), and because single mutant work offers some technical advantages, we used $Ca\text{-}P60A^{Kum170}$ alone for these studies.

We analyzed the brains of dissected third-instar larvae before and after one hour of seizure induction. As expected, a substantial increase in activated ERK could be detected in larval CNS neurons (Figure 4 A-D). In untreated control or $Ca\text{-}P60A^{Kum170}$ animals, we found low levels of diffuse DP-ERK reactivity throughout the brain with higher reactivity in some regions of the central brain (data not shown). As seen in Fig 4 panel D, a robust increase in DP-ERK immunoreactivity in the central brain regions and in ventral ganglia of treated $Ca\text{-}P60A^{Kum170}$ larvae (n=6) was observed when compared to either similarly treated wild-type controls (Fig 4 panel C) or unheated mutant animals (data not shown). That ERK activation occurs in neurons is indicated by co-localization of strong DP-ERK immunoreactivity with a marker for neuronal neuropil (nsyb-GFP) (Fig 4E and 4F). As in adults, ERK activation in larval brains is completely blocked with

treatment of larvae with U1026 prior to treatment (not shown). Significantly, the regions of greatest DP-ERK induction are in the functionally mature, central regions of the larval brain (Fig 4 E-F), outside the still developing optic lobes regions (Hanson, 1993; Truman et al., 1993; Meinertzhagen et al., 1998). This is consistent with our earlier observation that induction of DP-ERK is driven by neural activity.

Cytosolic activation and nuclear translocation of activated ERK in Ca-P60^{Kum170} mutants

In order to identify subcellular domains where ERK activation occurs, and to better place ERK signaling in a functional context, we examined ERK signaling in the *Drosophila* larval NMJ, particularly convenient for fine localization studies (Estes et al., 1996). An important, untested prediction from the observation that ERK and Ras are present at the synapse (Koh et al., 2002) is that local Ras/ERK signaling should be responsive to synaptic activity. To examine this hypothesis and characterize the extent and location of synaptic ERK activity, we analyzed treated (1 hour) and untreated, wild-type and *Ca-P60A*^{Kum170} *Drosophila* NMJs double-stained with antibodies recognizing synaptotagmin (Syt), a presynaptic marker, or DP-ERK.

As expected, Syt levels were identical among treated or untreated wild-type or *Ca-P60A*^{Kum170} control animals (Fig 5A and 5C). Similarly basal DP-ERK levels were also identical at NMJs of untreated wild-type and *Ca-P60A*^{Kum170} larvae (data not shown). Prior to treatment, activated ERK in boutons of the NMJ was primarily localized in small regions (termed "hot spots" by Koh et al., 2002). However, 40 minutes after a brief exposure to high temperature, substantially increased ERK activation is observed at *Ca-*

P60A^{Kum170}, but not in control neuromuscular preparations. Increased presynaptic and muscle DP-ERK immunoreactivity are both clearly evident (Figure 5B, 5D; See also Fig 6).

A second aspect of ERK signaling required for long-term plasticity, the regulation of gene expression is dependent on nuclear translocation (Martin et al., 1997; Patterson et al., 2001). Nuclear translocation of activated ERK is postulated to be a key step in determining the type of cellular response of a given system to a stimulus; indeed, nuclear translocation can transform the ERK-signaling response from graded to switch-like (Ferrell, 1998). In the case of neurons, the switch-like, all-or-nothing cellular response to upstream ERK signaling may be the activation of transcriptional factors that gate long-term plasticity (Martin et al., 1997; Patterson et al., 2001). In light of these previous studies, it was particularly striking to observe strong nuclear translocation of DP-ERK in post-synaptic muscle, within 40 minutes of heating *Ca-P60A*^{Kum170} larvae (Fig. 5D).

As with adult heads and larval brains, pre- and postsynaptic ERK activation was MEK dependent as evidenced by its complete block with U0126. Inhibition of MEK also reduced nuclear translocation of postsynaptic DP-ERK following treatment (Fig 5F). Finally, to corroborate the results from earlier western analyses and to examine whether synaptic ERK activation was neural activity dependent, we tested ERK activation under conditions in which neural activity was blocked. Treated *para*^{ts1}; *Ca-P60A*^{Kum170} larvae recovered at temperatures restrictive for *para* function had significantly reduced levels of synaptic and muscle ERK activation when compared to similarly treated *Ca-P60A*^{Kum170} animals (Fig. 5b Panel B-D).

Taken together, these data indicate that synaptic signaling at the *Drosophila* NMJ can result in MEK-dependent local activation of presynaptic and postsynaptic ERK. Levels of signaling induced in *Ca-P60A*^{Kum170} mutants are sufficient to direct the translocation of DP-ERK into nuclei of postsynaptic cells.

Acute ERK activation at larval NMJs is associated with rapid reduction of synaptic Fas II

Experiments in *Aplysia* suggest that a local function of activated ERK at synapses is to phosphorylate and thereby negatively regulate levels of the cell adhesion molecule ApCAM that inhibits synaptic expansion (Mayford et al., 1992). The conservation of this signaling module has been largely inferred in *Drosophila* from two striking, but relatively indirect observations. First, Fas II, an ApCAM homolog, also negatively regulates synapse expansion in *Drosophila* (Schuster et al., 1996). Second, chronic induction of ERK signaling in motor neurons is associated with reduced synaptic Fas II and increased synaptic size (Koh et al., 2002).

If local ERK activation, rather than temporally distant consequences of ERK signaling, were sufficient for regulating synaptic FasII, then we predicted acute ERK activation accomplished by shifting *Ca-P60A*^{Kum170} to non-permissive temperatures could result in rapid, quantifiable reduction of Fas II. In order to test this idea, we quantified synaptic Fas II and DP-ERK levels in *Ca-P60A*^{Kum170} larvae after seizure induction (Figure 6). We analyzed preparations 100 minutes after initial heat exposure to allow sufficient time for Fas II turnover.

After treatment, synaptic DP-ERK immunoreactivity at *Ca-P60A*^{Kum170} synapses was ~175% higher than those of untreated animals ($p<0.01$, n=64, four separate experiments); Fas II levels at the same treated synapses were reduced to levels ~78 (+/-4)% of that found in untreated *Ca-P60A*^{Kum170} mutants ($p<0.01$, n=64, four separate experiments) (Fig. 6). In contrast, levels of DP-ERK and synaptic FasII in treated wild-type animals were very similar to those observed in untreated controls (Fig 6).

This observation of reduced Fas II levels in less than 100 minutes of ERK activation substantially tightens the temporal link between ERK activation and Fas II down-regulation in *Drosophila*, and strengthens the evidence for phylogenetic conservation of the ERK/CAM signaling module as first described in *Aplysia* (Bailey et al., 1992). This is particularly significant given the emerging evidence for a likely parallel or positive-feedback pathway in vertebrates where internalization of the NCAM/L1 protein has been postulated to turn on ERK signaling (Schaefer et al., 1999; Schmid et al., 1999; Kolkova et al., 2000).

Immediate early gene expression in comt^{tp7}*; Ca-P60A*^{Kum170} *Drosophila*

A biochemical consequence of synaptic signaling pathways leading to long-term plasticity is altered nuclear gene expression (Bailey et al., 1996; Kandel, 2001). In both vertebrates and *Aplysia*, nuclear translocation of activated ERK, is associated with CREB-dependent expression of activity-regulated immediate-early genes (IEGs) such as Fos and c/EBP (Ginty et al., 1994; Bartsch et al., 1998).

We tested whether t.s. seizures that cause activation and nuclear translocation of ERK in *comt*^{tp7}; *Ca-P60A*^{Kum170} also caused expression of *Drosophila* homologs of the plasticity associated genes, DFos, DJun, and Dm-C/EBP one hour after seizure induction (Figure 7). Using real-time quantitative RT-PCR analysis, we found that DFos mRNA levels in adult head increased ~2.7 fold compared to treated wild-type animals ($p<0.05$ n=11) and Dm-c/EBP levels increased ~2.1 fold ($p<0.05$ n=10) within one hour of treatment (Fig 7). Expression of DJun was unchanged between treated *comt*^{tp7}; *Ca-P60A*^{Kum170} and wild type *Drosophila* (Table 1). In contrast to ERK activation that occurred in *Ca-P60A*^{Kum170} alone, Fos and c/EBP induction required the presence of both mutations (Table 1). Our observations on Fos and c/EBP expression are consistent with the idea that following brief inactivation of *comt* and *CaP60A* function in *Drosophila* neurons, activity-dependent signaling pathways achieve qualitative and quantitative features required to stimulate at least the early stages of plasticity-associated neuronal gene expression.

DISCUSSION

Through a new characterization of *Drosophila* temperature-sensitive (t.s.) paralytic mutants, we developed and examined consequences of a procedure that causes high levels of sustained neural activity and synaptic signaling *in vivo*. Brief exposure of specific t.s. *Drosophila* "seizure" mutants to non-permissive temperature causes persistent activation of the neuronal ERK/MAP kinase cascade through prolonged upstream MEK signaling. Using this procedure, we observe ERK activation in at least

two cellular locations: first, at the synapse where it causes down regulation of the cell adhesion molecule Fas II, and second, in the nucleus where it potentially modulates functions of activity-regulated transcription factors. Together with ERK activation, we also observe transcriptional up-regulation of DFos and Dm-c/EBP, *Drosophila* homologs of well-described neural activity regulated genes involved in long-term plasticity.

Results summarized above make three contributions. First, by acutely inducing and analyzing synaptic signaling pathways in insects, they directly demonstrate phylogenetic conservation of activity-regulated ERK signaling in insect neurons. Second, by more tightly defining the temporal relationship between the induction of activity, ERK activation, Fas II down regulation and immediate-early gene expression in *Drosophila*, results presented here extend and substantiate previous functional analyses at the *Drosophila* neuromuscular junction (Schuster et al., 1996; Koh et al., 2002). Finally, by providing a method to acutely induce plasticity pathways, they allow novel experimental access in *Drosophila* to early signaling events and components involved in long-term plasticity.

How do comt and Ca-P60A mutations induce ERK and consequences of its activation?

We report that the $comt^{tp7}$ and $CaP60A^{Kum170}$ t.s. paralytic mutants in *Drosophila* exhibit temperature-inducible neural seizures that are useful for initiating and analyzing activity-induced signaling pathways in neurons. At present, only plausible explanations exist for the mechanisms by which the conditional mutations cause seizures *in vivo*. The origin of behavioral seizures observed in *comatose* animals is particularly unclear. In

comt^{tp7} mutants at non-permissive temperatures, inactivation of the fusion ATPase NSF reduces the efficiency of neurotransmitter release and synaptic-vesicle fusion (Pallanck et al., 1995; Kawasaki and Ordway, 1999). This would be expected to cause reduced synaptic activity, not an increase. The unambiguous observation that seizures *do occur* (Siddiqi and Benzer, 1976; Ordway et al., 1994) indicate either that NSF has neuronal functions of which we are not yet aware, or possibly that this isoform of *Drosophila* NSF (dNSF1) functions predominantly in inhibitory neural systems. Independent of the explanation, increased neural activity does occur in the mutant and here, we harness it to induce and analyze activity-regulated gene expression. The prolonged contraction and increased activity in conditional SERCA mutants can be explained by SERCA's role in intracellular calcium sequestration. Inhibition of proper neuronal calcium sequestration that results in elevated cytosolic calcium could increase neural and synaptic activity. Elevated intracellular calcium that results from either enhanced synaptic activity and/or from altered sequestration may act directly to activate signal transduction pathways (Bito et al., 1997; Gutkind, 2000; Blackstone and Sheng, 2002). A more detailed mechanistic understanding of the neural origins of behavioral seizures is lacking not only for these *Drosophila* mutants, but also in several vertebrate seizure models that are the focus of intense research (Puranam and McNamara, 1999).

Phylogenetic conservation of neuronal signaling to ERK

To our knowledge, this is the first demonstration that neuronal activity in arthropods, results in ERK phosphorylation, nuclear localization, and increased

expression of conserved immediate early genes. Influential demonstrations of these phenomena have been performed largely in mollusks and mammals that, in evolutionary terms, are equally distant from arthropods (Fields et al., 1997; Martin et al., 1997; Vanhoutte et al., 1999; Dolmetsch et al., 2001). While synaptic signaling underlying long-term plasticity is poorly studied in insects, a rich tradition of behavioral studies in a variety of insect groups, especially honeybees and social insects, moths and the *Diptera*, have uncovered many long-lasting forms of behavioral change (Menzel and Muller, 1996, Collett et al., 2001). Our demonstration that specific, important events in the molecular pathway to long-term plasticity conserved in insects increases the likelihood that long-term behavioral changes will be found to occur through evolutionarily conserved mechanisms. Establishing this conservation in *Drosophila* is particularly important because it not only validates untested assumptions in the field, but also extends the experimental resources and advantages of *Drosophila* to studies of early cell biological events in the regulation of long-term plasticity.

Acute activation of plasticity pathways in Drosophila

Drosophila has emerged as an important model organism in which to analyze mechanisms of long-term plasticity. Influential behavioral experiments have previously demonstrated the broad conservation of transcriptional regulator function between *Drosophila* and mammals (Bailey et al., 1996; Silva et al., 1998). At a cell biological level, the conservation of signaling pathways upstream of transcriptional regulators such as AP1 and CREB has been largely inferred by experiments in which chronic

manipulations of synaptic signaling components (e.g. potassium channels, cAMP, adenylate cyclase, Ras, ERK and CREB) in motor neurons result in synaptic changes predicted by analyses in other species (Budnik et al., 1990; Zhong et al., 1992; Davis et al., 1996; Koh et al., 2002). However, lack of control over the inducing neuronal stimulation combined with the poor temporal resolution of these analyses, typically three to five days, have been insufficient to demonstrate: a) that activation of these signaling components in *Drosophila* can indeed be driven synaptic activity; and b) the sequential or temporal relationship between neural activity, ERK phosphorylation, Fas II down regulation, and immediate early gene expression. Thus, this study potentially fills an important gap in the field. In addition, it allows a new experimental tool to analyze pathways and mechanisms associated with the establishment of long-term plasticity.

The ability to analyze early signaling events that initiate long-term plasticity

By providing an assay for synaptically driven activation of ERK, the *Drosophila* seizure paradigm described here provides genetic access to a major issue in synaptic plasticity. Protein synthesis dependent, long-term plasticity is believed to be gated by ERK signaling (Sweatt, 2001). Therefore, identifying and understanding the signaling components that determine not only ERK activation, but also the duration and subcellular localization of the ERK signal, is particularly significant. Stimuli that result in persistent ERK activation and regulate its nuclear translocation are associated with activity-regulated gene expression and long lasting structural changes at synapses (Wu et al., 2001; Murphy et al., 2002). In *Aplysia*, pulsed 5-HT treatment sufficient to induce LTF

also promotes activation of ApMAPK (ApERK) (Michael et al., 1998); activated ApERK is translocated to the nucleus through a cAMP regulated process and this translocation is required for ERK's established functions in activating CREB, AP1, and other transcription factors (Bailey et al., 1997; Martin et al., 1997). We demonstrate using pharmacological, rather than genetic, inhibition of MEK that synaptically induced ERK activation that we observe occurs through MEK activation. The assay for synaptically driven ERK activation described here should enable similarly designed genetic experiments to analyze how poorly studied, candidate synaptic signaling pathways (Dolmetsch et al., 2001; Patterson et al., 2001) and candidate components of nuclear translocation (Johnson Hamlet and Perkins, 2001; Lorenzen et al., 2001) interact to achieve appropriate levels and localization of the ERK signal *in vivo*.

Identifying early components of the activity-response in Drosophila

The ability to initiate synaptic signaling on a relatively large scale in the *Drosophila* nervous system enables cell biological, biochemical, and genomic experiments to identify processes and molecules that are rapidly regulated by synaptic signaling. Some examples of such potential analyses are outlined below: 1.) At a cell biological level, modulation of ion channel localization and function have been shown to be regulated by kinases that are potentially turned on by neural activity (Yuan et al., 2002), it is of obvious interest to ask whether indeed these and/ or other channel modulations occur *in vivo* in response to synaptic stimulation. Such questions may be asked by electrophysiological and anatomical studies before and after seizure stimulation.

2.) At a biochemical level, the activation of ERK (and other kinases) by synaptic activity should result in altered phosphorylation of a large number of neural proteins. At one level, it is of interest to test whether known phosphoproteins, like Fas II for instance, are modified in response to stimulation procedures described here, and to then analyze the biochemical consequences of altered phosphorylation state. At a more global level, large scale two-dimensional gel and mass spectrometry analyses (Joubert et al., 2001; van Rossum et al., 2001), particularly powerful in animals with small sequenced genomes, should allow identification of novel neuronal proteins whose levels or phosphorylation states are rapidly altered by synaptic activity *in vivo*. 3.) At a genomic level, microarray and SAGE analyses (Brenman et al., 2001; Jasper et al., 2001) could be used to potentially make substantial additions to the relatively small panel of known activity-regulated genes (Worley et al., 1993; Alberini et al., 1994; Lyford et al., 1995; Nedivi et al., 1996; Brakeman et al., 1997; Bartsch et al., 2000; Guzowski et al., 2000). Functional studies of novel proteins identified from genomic or proteomic screens have the potential to add significantly to our knowledge of plasticity regulation.

Finally, it is important to acknowledge that processes other than synaptic plasticity that may be initiated in response to the stimulation procedures we have described. We demonstrate that subsets of critical events that underlie long-term plasticity regulation are triggered by these procedures. However, signaling pathways, molecules, and processes that regulate other neural responses to activity – cell death for instance – may also be triggered under conditions we have outlined (Meldrum, 2002). More comprehensive analyses of conditional mutants in *Drosophila* (Palladino et al.,

2002) may yield additional or complementary tools for analyzing the activity response of nervous systems.

FIGURE 2.1

Temperature-dependence of paralysis in *Drosophila* Na^+ channel ($para^{ts1}$), NSF ($comt^{tp7}$) and SERCA ($CaP60A^{Kum170}$) mutants. *Drosophila* were exposed to different restrictive temperatures for two minutes and assayed for paralysis. Tight and distinct restrictive temperatures for *Drosophila* mutants shown in the figure are: $para^{ts1}$ [30°C]; $comt^{tp7}$ [35°C]; and $Ca60A^{Kum170}$ [40°C].

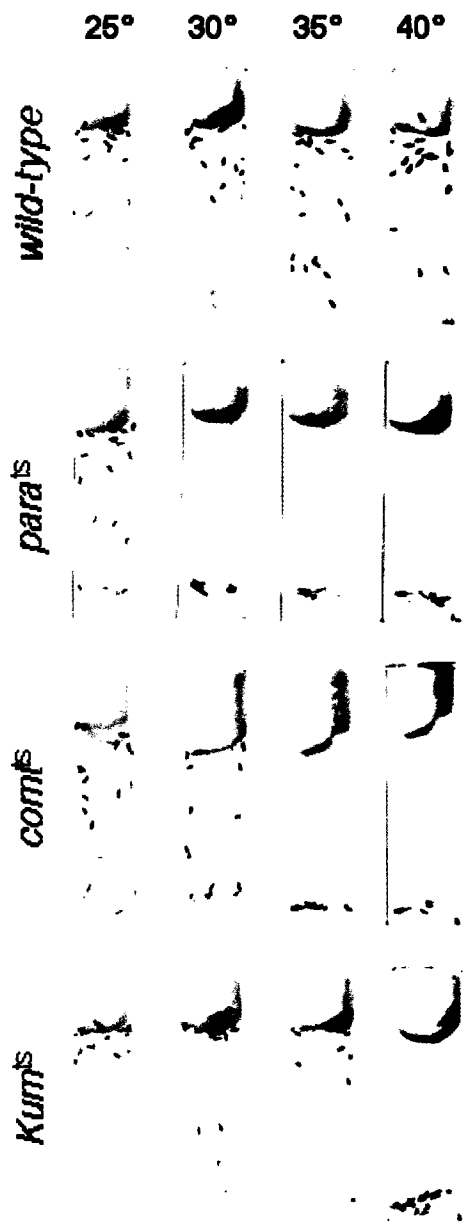

FIGURE 2.2

Drosophila NSF mutants exhibit spontaneous seizure-like activity following exposure to restrictive temperatures. (2a and 2b) Following a simple heat treatment protocol (2a) spontaneous seizure-like activity is induced and continues for at least 60 minutes in $comt^{tp7}$, $comt^{tp7}$; $CaP60A^{Kum170}$ and $comt^{tp7}$; $CaP60A^{Kum170}$ mutants (2b). The activity displayed in the inset for treated $CaP60A^{Kum170}$ mutant was observed in nearly all animals tested but was seen infrequently. (2c and 2d) Seizure-like activity was reversibly precluded (2d) under conditions in which Na^+ channel activity was blocked using the $para^{ts1}$ allele restricted at 33°C (2c). $comt^{tp7}$ $para^{ts1}$; $CaP60A^{Kum170}$ animals, that show no spontaneous activity at 33°C, show the expected firing following treatment at temperatures permissive for $para^{ts1}$ function (2b).

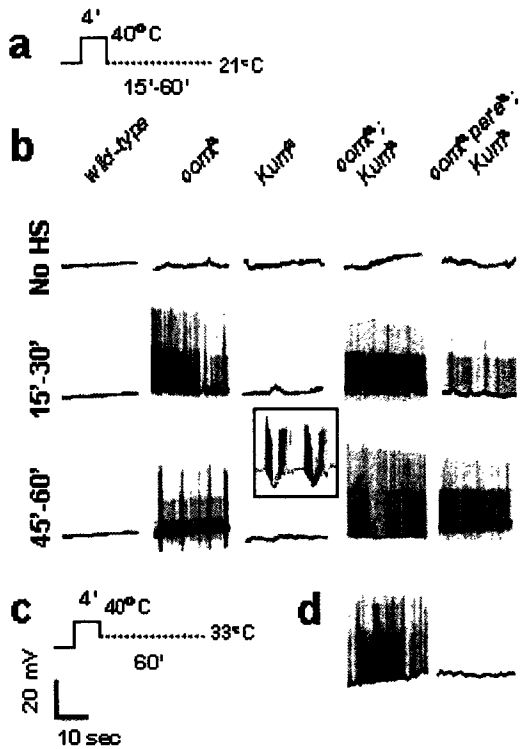

FIGURE 2.3

A brief temperature pulse induces persistent activation of ERK in *comt*^{tp7}; *CaP60A*^{Kum170} mutants through a neural activity, MEK, and *CaP60A*^{Kum170}-dependent pathway. (a) Following a brief 40°C pulse shown in the top trace, ERK activation in various strains is shown 60 minutes post-treatment. The histogram indicates the ratio [treated (heated) vs (untreated) controls] of P-ERK immunoreactivity from densitometric scans of western blot data shown in the lower panels. When compared to treated wild-type, these ratios are: *comt*^{tp7} [2.0±0.24 p=0.077], *Ca60A*^{Kum170} [3.5±0.56 p=0.010]; *comt*^{tp7}; *Ca60A*^{Kum170} [2.5±0.31 p=0.013], *comt*^{tp7}*para*^{ts1}; *Ca60A*^{Kum170} [2.9±0.53 p=.006]; (b) ERK activation persists for more than 4 hrs in treated *Ca60A*^{Kum170} animals. When compared to similarly treated wild-type animals at two hours [5.0±0.82 p=0.005] and at four hours [3.3±0.58 p=0.006]; (c) ERK activation seen in either *CaP60A*^{Kum170}or *comt*^{tp7}; *CaP60A*^{Kum170} is completely precluded by blocking neural activity in a *para*^{ts1} background. Treated *para*^{ts1}; *CaP60A*^{Kum170} or *comt*^{tp7}*para*^{ts1}; *CaP60A*^{Kum170} recovered at a temperature restrictive for *para* (33°C) show no activation beyond that of similarly treated wild-type. *CaP60A*^{Kum170} [2.03±0.11], *para*^{ts1}; *CaP60A*^{Kum170} [1.06±0.05 p=0.001], *comt*^{tp7}; *CaP60A*^{Kum170} [1.97±0.30], *comt*^{tp7}*para*^{ts1}; *CaP60A*^{Kum170} [0.92±0.12 p=0.0238] (d) ERK activation following seizure induction requires MEK activity. Treated animals pre-fed the MEK-inhibitor drug U0126 show no increase in DP-ERK following treatment. *CaP60A*^{Kum170} [3.62±0.21] U0126- *CaP60A*^{Kum170} [1.16±0.07 p=0.0003].

FIGURE 2.3

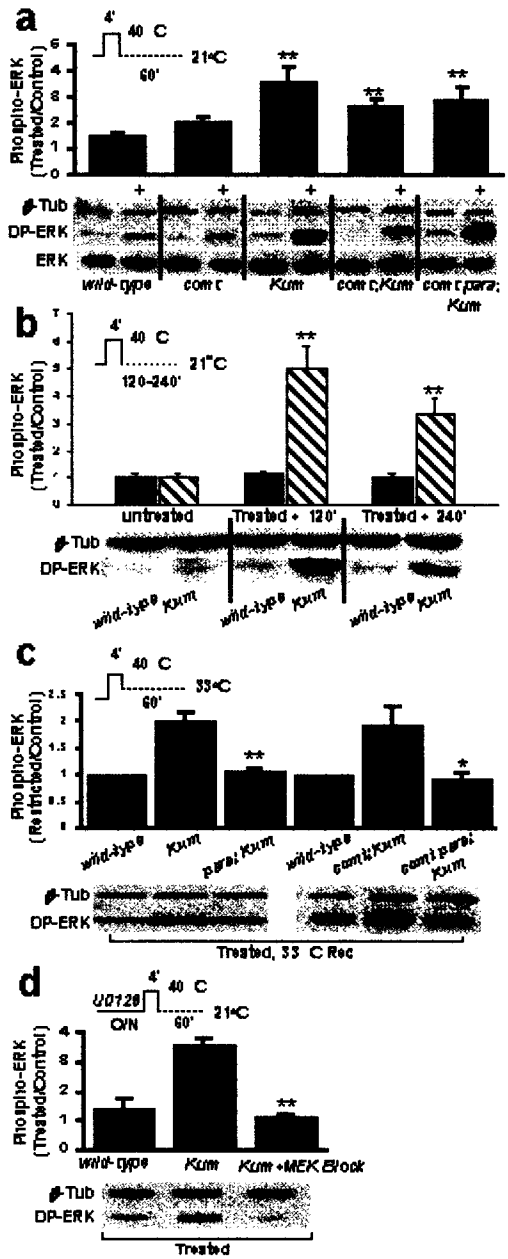
a
4'
40 C
21°C
60'
Phospho-ERK (Treated/Control)
β-Tub
DP-ERK
ERK
wild-type
com1
Kum
com1;Kum
com1;para; Kum
b
4'
40 C
21°C
120-240'
Phospho-ERK (Treated/Control)
untreated
Treated + 120'
Treated + 240'
β-Tub
DP-ERK
wild-type
Kum
c
4'
40 C
33°C
60'
Phospho-ERK (Restricted/Control)
wild-type
Kum
para1;Kum
wild-type
com1;Kum
com1;para1; Kum
β-Tub
DP-ERK
Treated, 33 C Rec
d
4'
40 C
U0126
O/N
21°C
60'
Phospho-ERK (Treated/Control)
wild-type
Kum
Kum +MEK Block
β-Tub
DP-ERK
Treated

FIGURE 2.4

Activity-driven increase in DP-ERK activity in *Drosophila* larval CNS neurons as by detected by confocal microscopy (Panels A-F). Immunohistochemical evidence for ERK activation in the brain lobes and ventral ganglia of treated *CaP60A*^{Kum170} (Panel D) but not wild-type (Panel C) *Drosophila* larvae. Panels A-B, neurally driven synaptobrevin-GFP (labels synaptic regions); Panels C-D, DP-ERK (activated ERK) staining throughout the larval CNS. Panels E and F, merged images of treated wild-type and mutant larvae showing increased DP-ERK activity in both cells and neuropil of the larval CNS.

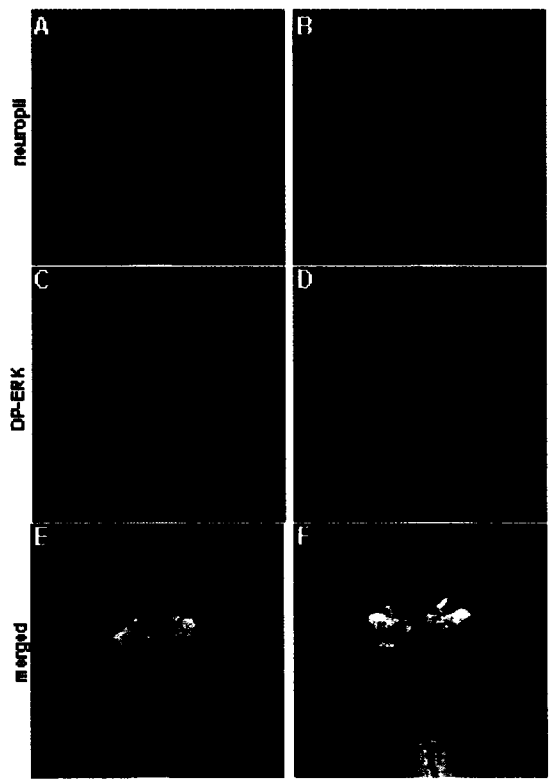

FIGURE 2.5

(a) Activity-and MEK-dependent induction of cytosolic and nuclear DP-ERK 60 minutes after a 4 minute temperature pulse to *CaP60A*^{Kum170} larvae. DP-ERK immunoreactivity at the larval neuromuscular junction (segment A2, muscles 6 and 7) labeled with Synaptotagmin I to visualize presynaptic nerve endings. Panels A, B show treated wild type animals. Panels C, D show treated *CaP60A*^{Kum170} animals. Panels E, F show treated *CaP60A*^{Kum170} animals fed the MEK-inhibitor U0126 prior to treatment. (b) DP-ERK immunoreactivity following treatment and neural activity blockade at the larval neuromuscular junction (segment A3, muscles 6 and 7) labeled with Synaptotagmin I to visualize presynaptic nerve endings. Treated *CaP60A*^{Kum170} (Panels A,B) and *para*^{ts1}; *CaP60A*^{Kum170} (Panels C,D) recovered under conditions where neural activity is blocked (35°) in *para*^{ts1} background following treatment, the recovery temperature, fixation conditions and time of recovery differs from the protocol used for 5a (see Methods). Green=DP-MAPK, Red= Synaptotagmin.

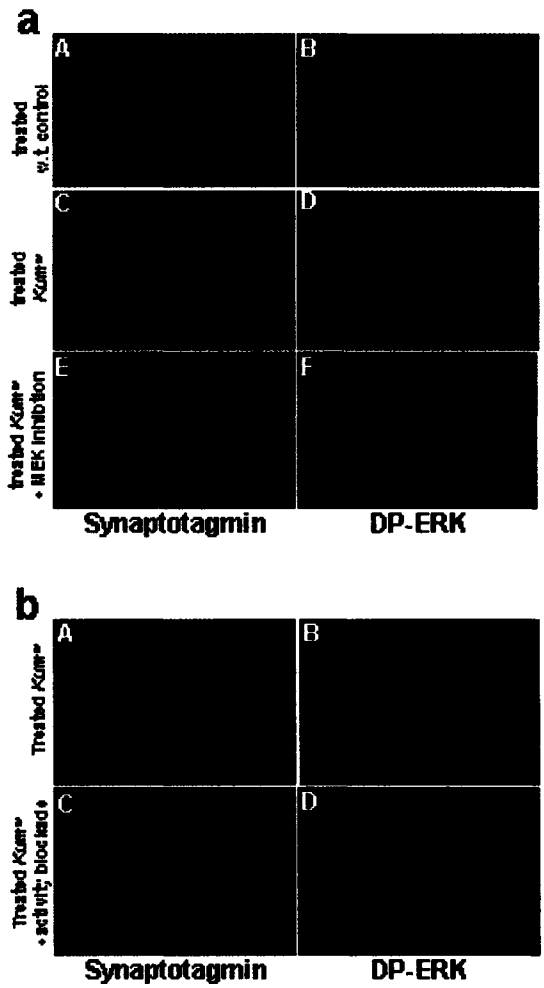

FIGURE 2.6

Increase in activated synaptic ERK is correlated with the rapidly reduced levels of Fasciclin II at the *Drosophila* larval NMJ. Fluorescence intensity for DP-ERK and Fas II was measured from treated and untreated *Ca60A*^{Kum170} animals, then compared after values were normalized to untreated animals ($p<0.01$, n=64, N=4 independent experiments). Bouton images taken from treated and untreated wild type and *Ca60A*^{Kum170} animals; Scale bar = 5 μM.

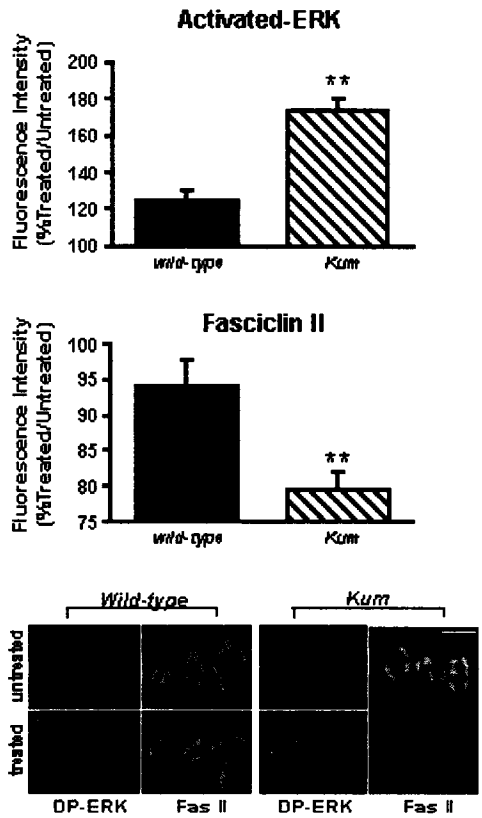

FIGURE 2.7 Induction of *Drosophila* homologs of the immediate-early genes Fos (*kayak*) and c/EBP (*slbo*) following seizure induction and ERK activation in *Drosophila*. (a) In mRNA extracted from entire fly heads, DFos is increased 2.7 fold in treated $comt^{tp7}$; $CaP60A^{Kum170}$, *Drosophila* when compared to wild-type animals (n=11 p=0.004). (b) Dm-c/EBP is increased 2.1 fold in $comt^{tp7}$; $CaP60A^{Kum170}$, *Drosophila* when compared to wild-type animals (n=10 p=0.017). The gel bands beneath each graph show RT-PCR products at identical cycles just as the Q-PCR reactions entered log-linear growth phase for each comparison. (+=treated, -=untreated). See Table 1 for additional data.

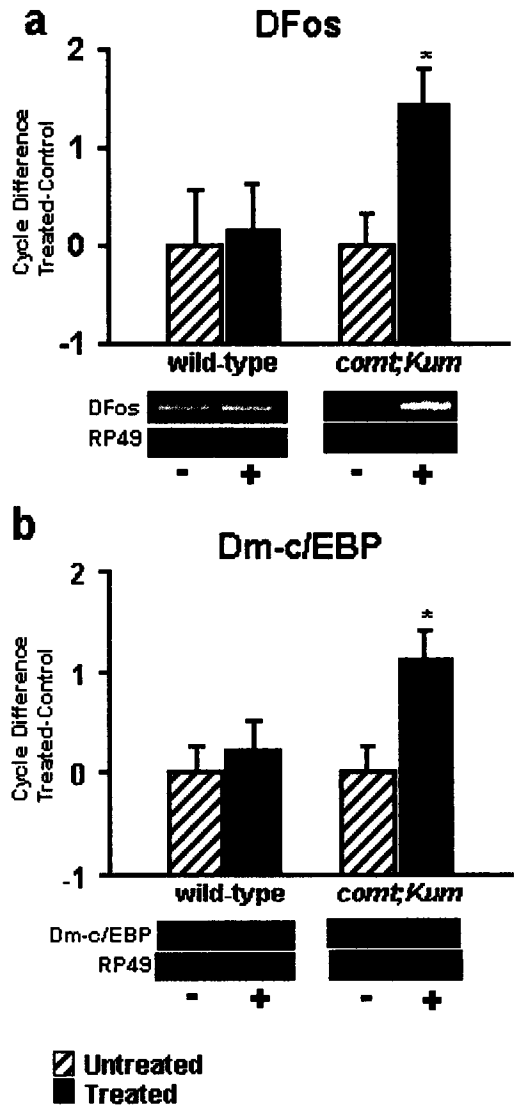

TABLE 2.1: Gene Expression following Seizure Induction in *comt; CaP60A* mutants

	Gene	Mean Fold Gene Expression (+ vs -)	S.E.	C.V.	p-value	n
wild-type						
	DFos	**1.1(1.6-0.8)**	**0.519**	**45.07**	**0.68**	**11**
	c/EBP	**1.2(1.6-0.9)**	**0.427**	**35.66**	**0.77**	**11**
	DJun	**1.0(1.4-0.7)**	**0.529**	**53.06**	**0.99**	**9**
	Gapdh1	**1.0(1.2-0.9)**	**0.226**	**22.03**	**0.89**	**6**
comt; Ca60A						
	DFos	**2.7(3.6-2.1)**	**0.380**	**13.85**	**0.004**	**10**
	c/EBP	**2.1(2.4.1.7)**	**0.254**	**12.62**	**0.017**	**10**
	DJun	**0.9(1.0-0.8)**	**0.128**	**15.02**	**0.52**	**9**
	Gapdh1	**1.0(1.1-0.9)**	**0.121**	**12.11**	**0.98**	**7**

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CHAPTER THREE

FOS AND JUN FUNCTION UPSTREAM OF CREB TO CONTROL PLASTICITY AT THE DROSOPHILA LARVAL NEUROMUSCULAR JUNCTION.

Note from Author: *The material in this chapter was derived from work that I participated in but was not the primary contributor to. I have included text and figures from the original manuscript in this dissertation with permission from the article's primary authors.*

ABSTRACT

Activity-regulated gene expression mediates many aspects of neural plasticity including long-term memory. In the prevailing view, patterned synaptic activity causes kinase-mediated activation of the transcription factor CREB. Together with appropriate cofactors, CREB then transcriptionally induces a group of "immediate-early" transcription factors and, eventually, effector proteins that establish or consolidate synaptic change (Bailey et al., 1996). Here, using a *Drosophila* model synapse, we analyze cellular functions and regulation of the best-known immediate-early transcription factor, AP1, a heterodimer of the basic leucine zipper proteins Fos and Jun (Curran and Morgan, 1995). We observe that AP1 positively regulates both synaptic strength and synapse number, thus showing a greater range of influence than CREB (Davis et al., 1996). Observations from genetic epistasis and RNA quantitation experiments indicate that AP1 acts upstream of CREB, regulates levels of CREB mRNA, and functions at the

top of the hierarchy of transcription factors known to regulate long-term plasticity. A Jun-kinase signaling module provides a CREB-independent route for neuronal AP1 activation; thus, CREB regulation of AP1 expression (Sheng et al., 1990) may, in some neurons, constitute a positive feedback loop rather than the primary step in AP1 activation.

INTRODUCTION

Neuronal activity-regulated gene expression is controlled by signaling pathways that impinge on a transcription factor cascade including the cAMP-responsive transcription factor, CREB, and probably several other rapidly induced "immediate-early" transcription factors such as Fos and Jun, c/EBP and Zif-268 (Bailey et al., 1996). Downstream genes regulated by these and other transcription factors act predominantly to alter either synapse structure or function (Curran and Morgan, 1995; Bailey et al., 1996; Davis et al., 1996). A role for CREB at the top of the transcriptional cascade is strongly argued by demonstrations that induction of an activator isoform of CREB substantially enhances long-term memory (LTM) in *Drosophila* and rodents, and long-term facilitation (LTF) in the sea slug Aplysia (Yin et al., 1995; Bartsch et al., 1995; Josselyn et al., 2001). These findings together with substantial data to indicate the CREB-responsiveness of several immediate-early genes (Sheng et al., 1990; Sheng and Greenberg, 1990) has led to a framework in which immediate-early transcription factors act downstream of CREB to effect enduring synaptic change (Bailey et al., 1996).

The first activity-regulated transcripts to be identified in vertebrates encode c-Fos and c-Jun, DNA binding proteins that heterodimerize to form the transcription factor AP1 (Curran and Morgan, 1995). Induction of ΔFosB, a naturally occurring dominant-negative isoform of FosB in mammals, has been shown to underlie long-term sensitization to cocaine (Kelz et al., 1999). However, the existence, in mammals, of multiple Fos and Jun isoforms with pleiotropic and potentially overlapping activities has so far precluded a detailed analysis of the positive functions of AP1 in neuronal plasticity (Brown et al., 1996; Karin et al., 1997). In *Drosophila*, Fos and Jun are encoded by single genes, D-Jun and D-Fos, each highly conserved with respect to its vertebrate orthologs and expressed in the *Drosophila* nervous system (Perkins et al., 1990). Detailed analysis of a Fos-, Jun- and Jun Kinase (JNK) signaling module's function in *Drosophila* embryonic and pupal dorsal closure provides a genetic framework (Noselli et al., 1999) for a similar dissection of AP1 function during synaptic plasticity at the *Drosophila* larval neuromuscular junction (NMJ). The maturation of this synapse as it grows in size (number of boutons) and strength (stimulus evoked response) during the three instars of larval growth involves activity-dependent mechanisms that also underlie long-term plasticity and memory (Davis et al., 1996). Thus, genetic manipulation of neuronal activity or cAMP levels results in predictable alterations in synaptic size and strength (Budnik et al., 1990; Zhong and Wu, 1991; Zhong et al., 1992; Cheung et al., 1999; Sigrist et al., 2000). Hyperactivity- or cAMP-induced increases in synaptic strength is CREB-dependent; increases in bouton number occur via a reduction in levels of Fasciclin II, a synapse cell adhesion molecule whose *Aplysia* ortholog is rapidly

downregulated following induction of LTF (Bailey et al., 1992; Davis et al., 1996; Schuster et al., 1996).

RESULTS & DISCUSSION

Our results show that AP1 positively regulates both bouton number and synaptic strength; such extensive control of plasticity processes is unique among known transcription factors. Increased neural expression of both Fos and Jun, but not of either alone, results in a 30% potentiation in evoked junctional currents (EJC) and a parallel increase in the number of presynaptic boutons (Figure 1A-B). Inhibition of either protein, via targeted neural expression of Fbz and Jbz, dominant-negative forms of Fos and Jun respectively (Eresh et al., 1997; Brand et al., 1993), causes opposite phenotypes at the larval NMJ: a roughly 30% reduction in bouton number and synaptic strength (Figure 1A-B). The similarity and specificity of these effects argue that Fbz and Jbz act by specifically inhibiting endogenous AP1 activity, a premise supported by previous demonstrations that developmental phenotypes caused by Fbz or Jbz induction are identical to those caused by loss-of-function mutations in D-Fos or D-Jun (Zeitlinger et al., 1997). The observation that Fos and Jun positively regulate synapse plasticity in a manner that requires the presence of both molecules, but is sensitive to inhibition of either, indicates Fos and Jun function as a heterodimer to control synapse plasticity. This is significant, given various data to suggest that AP1 is a heterogenous collection of molecularly distinct transcription factors (Karin et al., 1997). Expression of AP1, Fbz or Jbz via a motor-neuron restricted promoter (Cheung et al., 1999) was sufficient for the

observed effects; perturbation of AP1 activity in postsynaptic muscle had little or no effect on the synaptic parameters analyzed (data not shown). Together these data are consistent with a cell-autonomous role for AP1 in regulating neural plasticity.

AP1 regulates synapse strength by controlling the quantal content (number of vesicles fusing per stimulus) of presynaptic transmitter release (Fig 1C). Thus, while AP1 manipulations affect evoked junctional currents (EJCs), responses to individual synaptic vesicles (mEJC amplitudes) remain unchanged (Figures 1A, 1C). AP1 mediated synapse expansion is accompanied by reduced (~25%) levels of synaptic Fasciclin II; Fbz- or Jbz-mediated reductions in synapse size are accompanied by increased (~35%) levels of synaptic Fasciclin II (Figure 1E). This is consistent with a model in which AP1-mediated changes in synapses occur via the classical activity-regulated pathway for synapse plasticity (Bailey et al., 1992).

Previous work has demonstrated a requirement for CREB in determining synaptic strength but not bouton number at the *Drosophila* larval NMJ (Davis et al., 1996). Our observed consequences of AP1 manipulations were not consistent with a simple model of CREB exerting its effects via Fos and Jun expression. Rather, they suggested a model in which CREB acts downstream of AP1 in the pathway leading to altered synaptic strength. We tested this hypothesis by asking whether induction of a CREB-blocker (Yin et al., 1994) transgene (*hs-CREB2b*) would inhibit AP1-induced synaptic changes. Induction of *hs-CREB2b* (Davis et al., 1996) had no effect on synaptic size and strength in a control genetic background; however, it specifically blocked the AP1 induced increase of

synaptic strength, without affecting the AP1 induced increase of bouton number (Figure 2). This specific effect of CREB blocker strongly suggests that AP1's effect on synaptic strength occurs through a pathway that involves that activation of CREB; however, its effect on bouton number occurs via the CREB-independent pathway suggested by previous studies.

One formal explanation for these observations is that AP1 induction in neurons results in general hyperexcitability; thus, it simply acts to turn on the activity- and cAMP-dependent signaling pathway in neurons. In this case, reduction of neuronal cAMP via targeted overexpression of the *dunce* cAMP phosphodiesterase would be expected to block all effects of AP1 overexpression. This was not the case. Neural induction of cAMP phosphodiesterase did not alter AP1's influence on bouton number (Figure 2B); this is consistent with AP1 function downstream of the cAMP-regulated stage in structural synaptic plasticity. In contrast, reduced neural cAMP completely blocked AP1's effect on synaptic strength. As with CREB-blocker, identical induction of cAMP phosphodiesterase had no effect on synaptic size or strength in a control genetic background; thus, low cAMP specifically affects the AP1 induced potentiation of synaptic strength. The simplest interpretation of these observations is that AP1's activity is not substantially reduced at low levels of cAMP achieved in our experiment; however, activity of a downstream molecule required for AP1's effect on synaptic strength is inhibited under these conditions. These observed consequences of cAMP and CREB inhibition on AP1 effects confirm and extend previous studies demonstrating independent control of synapse size and strength (Davis et al., 1996). The similarity of low cAMP

and CREB-inhibition effects on AP1-induced plasticity (Figure 2; Figure 5A) is consistent with a model in which the downstream target of cAMP signaling is CREB itself (Figure 5B).

One potential mechanism for AP1 regulation of CREB is suggested by the presence of consensus AP1-binding sites in the *Drosophila* CREB2 promoter. Consistent with a direct transcriptional control mechanism, we observed a robust increase in CREB mRNA levels following a brief pulse of increased neural AP1 expression. Brief induction of AP1 in the *Drosophila* nervous system was achieved using the *elavGsGGal4* transgene, which causes pan-neural expression of a steroid-dependent, inactive variant of the Gal4 transcription factor. In *elavGsGGal4; UASAP1* animals, a roughly 9-fold increase in levels of neural AP1 is induced by a 6-hour incubation on food doped with the steroid analog Ru486 (Figure 2C) (Osterwalder et al., 2001). Under these conditions, CREB RNA levels are increased about 3-fold. That this increase is caused by AP1, rather than ill characterized consequences of Ru486 feeding for instance, is evidenced by the unchanged levels of CREB RNA in Ru486-fed animals expressing FBZ under *elavGsGGal4* control (Figure 2D).

Our proposal that AP1 functions upstream of CREB to regulate synaptic plasticity, predicts the existence of a CREB-independent signaling pathway that can act as a primary activator of neuronal AP1. One candidate is the Jun kinase (JNK) pathway, known to regulate AP1 function in several contexts in mammals and *Drosophila* (Riesgo-Escovar et al., 1996; Sluss et al., 1996; Chang and Karin, 2001). Several reports (Yang et

al., 1997; Schwarzschild et al., 1997; Martin-Blanco et al., 1998) point to the existence of a Ca^{++}-dependent route to JNK activation in the nervous system. Two critical elements of the JNK pathway expressed in the *Drosophila* nervous system are *basket* (*bsk*) which encodes JNK (Riesgo-Escovar et al., 1996), and *puckered* (*puc*), which encodes a phosphatase that, by dephosphorylating JNK, negatively regulates JNK signaling (Stimson et al., 2001) (Figure 3A). We tested how altered JNK signaling affected synapse plasticity. Reduced JNK signaling by neural overexpression of either Puckered or dominant-negative Basket (Bsk^{DN}), mimicked the synaptic effects of AP1 inhibition. Thus both treatments result in a roughly 30% decrease in synapse strength and bouton number (Figure 3B). Although overexpression of Basket JNK causes no phenotype on its own, it completely neutralizes the effect of Puckered overexpression; this indicates that effects of Puckered on synapse plasticity occur through its documented inhibition of JNK signaling (Figure 3B). In contrast to reduced cAMP (Figure 2), reduced JNK signaling (achieved by neural expression of Puckered) blocked effects of AP1 on both structural and functional synaptic plasticity (Figure 3B). This is consistent with a model in which JNK signals through AP1 to regulate synaptic change.

To further ascertain that JNK effects on synaptic change are mediated by AP1, we studied the effects of increased JNK signaling on synapses inhibited for AP1 (Figure 4). If JNK acts by increasing Jun function, then increased JNK signaling should alleviate consequences of AP1 inhibition that derive from reduced Jun activity. We increased JNK signaling by using a genetic background heterozygous for puc^{E69}, a loss-of-function allele

of *puc*. Although the resulting enhancement of JNK signaling was not sufficient to drive synaptic change in a wild-type background, it completely suppressed effects of neural Jbz expression (Figure 4). This important observation, that reduced endogenous *puc* function can compensate when neuronal AP1 is inhibited, demonstrates that the neural AP1 activity *in vivo* is positively regulated by JNK signaling.

In conclusion we demonstrate that AP1, under JNK regulation, functions upstream of CREB to control a wider range of plasticity processes than anticipated; indeed our data are consistent with a model in which AP1 activation is sufficient for transcriptional control of long-term plasticity. Our finding that AP1 activates CREB probably by regulating CREB mRNA levels, contrasts with previous demonstrations that CREB positively regulates AP1 transcription (Sheng et al., 1990). These apparently conflicting observations may be rationalized if CREB-induction of AP1 is considered as part of a positive feedback loop rather than the primary mechanism for AP1 activation (Figure 5B). Observations presented here clearly demonstrate unanticipated functions of AP1 and expose limitations of current models for transcriptional control of long-term plasticity. For at least one synapse analyzed here, AP1 rather than CREB appears to be the critical transcriptional regulator of long-term plasticity. Further studies in other neuronal subtypes are required to establish the generality of our observations.

METHODS

Drosophila strains and genetic controls

Fly stocks were reared in vials on medium consisting of instant fly food, agar and oatmeal with added yeast (Stimson et al., 2001). Experimental larvae were reared in vials at low density at 25° C. Strains were generously provided by the following sources: Oregon-R, the wild-type strain used, by Dr. Danny Brower (University of Arizona); Gal4-responsive *UAS-Fos*, *UAS-Jun*, *UAS-Fbz* and *UAS-Jbz* transgenes (Bailey et al., 1992) by Dr. Marianne Bienz (Cambridge University, Cambridge UK); *hs-CREB2b* strains by Jerry Yin and Tim Tully; neural Gal4 line, *C155 (P{GawB}elav^{C155})* and muscle line *MHC-Gal4* by Corey Goodman (UC Berkeley); muscle and neural Gal4 lines - respectively *24B* and *elav Gal4* by Liqun Luo (Stanford University); a motor-neuron restricted Gal4 line *D42* by Gabrielle Boulianne (University of Toronto); *UAS-bsk* and *UAS-bsk*^{DN} strains by Marek Mlodzik (Mt Sinai Medical Center, New York); *UAS-puc* and *puc*^{E69} strains by Alfonso Martinez Arias (Cambridge University). *ElavGsGal4* lines[26] were kindly provided by Thomas Osterwalder and Haig Keshishian. In general, experimental animals were generated by crossing males homozygous for UAS transgenes with virgin females homozygous for the Gal4 driver; wild-type controls consisted of the Gal4 driver crossed to the wild type (Oregon-R). In cases where this was not feasible, larvae of the same sex as the experimental animals, from closely matched genetic backgrounds, served as controls. For inducing the CREB-blocker transgene, *hs-CREB2b*, larvae were grown at 27°C and heat-pulsed for 2 hours daily at 38°C as previously

described (Davis et al., 1996). For inducing Gal4 activity in adult *Drosophila* expressing the *elavGsGal4* transgene, 1.3 day old flies were starved for 6 hours and transferred to glass bottles with standard food containing 0.02mg/ml of Ru486 (Sigma) for 6 hours prior to decapitation and RNA extraction.

Immunocytochemistry and antibodies

Preysnaptic boutons of ventral longitudinal muscles 6 and 7 were visualized with a rabbit antibody against *Drosophila* synaptotagmin (DSYT2, kindly provided by Hugo Bellen) as described previously (Stimson et al., 2001). Bouton number in segment A2 was assessed by counting Syt-positive varicosities in a projection of optical sections through the entire junction as previously described (DeZazzo et al., 2000). Muscle surface areas were not significantly different in all genotypes analyzed and so, no correction factor was applied to the measured bouton number. To quantify FasII levels, synapses were first labeled with 1D4 antibodies (from Corey Goodman). In fluorescently stained preparations, three terminal Type 1b boutons of segment A2 or A3 were imaged at maximum resolution (68nm pixel diameter) using a cooled CCD camera (Princeton Instruments) and Metamorph imaging software (Universal Imaging). After subtracting background fluorescence measured at non-synaptic muscle surface, the average pixel intensity in these boutons and surrounding subsynaptic reticulum was measured and analyzed.

Quantitative PCR (Q-PCR)

For Q-PCR, ~50 fly heads were collected for each sample; Total RNA was extracted using the RNAeasy kit (Qiagen) and purfied from genomic DNA using the DNA-free DNAse kit (Ambion) and instructions provided by the respective manufacturers. 2 ug of total RNA, quantified by spectrophotometry, were used to synthesize oligodT-primed cDNA with the Omniscript cDNA synthesis kit (Qiagen). The cDNA synthesis reaction was incubated at 37C for 1 hour then diluted 1:5 with nuclease-free H20 (Invitrogen) for Q-PCR reactions. Q-PCR was performed the Cepheid SMARTCycler using reaction ingredients and the standard protocol from the Quantitech kit (Qiagen). Identical levels of mRNA in control and experimental samples were further ensured by Q-PCR reactions using intron-spanning primers to the housekeeping gene RP49. Transcript levels were determined using primer sets specific to Dfos, Djun and dCREB2. Q-PCR primer sets: RP49 forward primer, atgaccatccgcccagcatac, RP49 reverse primer, gagacgcaggcgaccgttgg, DFos/Fbz forward, accaacgagctcaccgag, DFos/Fbz reverse, agcatgtcggagcgaatc, DJun forward, attgacatggaagcgcagg, DJun reverse, ggttcttcacgatgctagcc, CREB forward, atccggctgcagaagaacag, CREB reverse, ttcagctcctcgatgagcg. Each Q-PCR reaction was repeated three times. For gel visualization, experimental and control RT-PCR reactions were stopped at the same cycle during the log-linear phase of growth determined by monitoring the reactions in real time (Figure 2c&d). Products were taken at cycle (X and cycle X+2), where X is the cycle at which RT-PCR product derived from experimental animals enter the log-linear growth phase. PCR products were visualized after 2% agarose gel electrophoresis. For statistical

comparisons of Q-PCR data, cycles at which RT-PCR products from Ru486-induced and uninduced control samples entered the exponential phase were compared. The difference in PCR cycles required for identical levels of RT-PCR product (at early log-linear growth phase) from experimental and control animals is plotted in Figures 2E and F. A one cycle difference represents a two-fold difference in starting template concentration. For instance the RT-PCR reaction for Fos entered the exponential phase 3.2 cycles earlier in AP1-induced samples than in controls; this translates into a $(2)^{3.2}$ or roughly nine-fold increase in Fos mRNA by our induction procedure. Each sample set was compared using the student's t-test and only results with a p value $<.05$ were considered statistically significant.

Electrophysiology and data analysis

Synaptic currents were recorded from muscle 6 in segment A2 using two-electrode voltage clamp, as described previously (Stimson et al., 2001). Recordings were performed in HL-3 saline (Stewart et al., 1994). In all experiments, the CNS was removed to eliminate spontaneous activity, and electrical stimuli were applied to the segmental nerve, which contains the axons of the two motoneurons innervating muscle 6, via a glass-tipped suction electrode. Stimuli consisted of 0.2 ms pulses from an isolated pulse stimulator (AM systems 2100, Everett, WA), gated by pClamp software (Axon Instruments, Foster City, CA). Intracellular electrodes were pulled from thin-walled borosilicate glass (Frederick and Dimmock, NJ), and filled with 3 M KCl (voltage monitor electrode, Re = 7-10 MΩ) or saturated potassium citrate and 3 M KCl (current

injection electrode, Re = 18-25 MΩ). Data were acquired at a resting $V_{command}$ of -70 mV, using an Axoclamp 2B amplifier (Axon Instruments), and recorded with pClamp software. Data were filtered at 1 kHz before analysis. EJC amplitude was determined from 22-25 contiguous stimuli from a 1 Hz train. mEJC parameters were measured from one to two 41 second records, depending on the frequency of spontaneous events using the minianalysis software (Jaejin Software Inc.) mEJC amplitude was determined by fitting gaussian curves to amplitude distribution histograms. Differences between means were compared using either t-tests (paired comparisons) or ANOVA (multiple comparisons) in Microsoft Excel.

FIGURE 3.1 Neuronal AP1 activity controls both synapse size (the number of presynaptic boutons) and strength (EJC – evoked junctional current) at the *Drosophila* larval neuromuscular junction. A. EJC and mEJC traces, and B. histogram representation of EJC and bouton counting data, from larvae in which either wild-type or dominant-inhibitory forms of Fos (Fbz) or Jun (Jbz) are expressed. Fbz causes a 30% ($P<0.001$) and Jbz a 25% ($P<0.03$) reduction in synapse size and strength. While expression of Fos and Jun cause an increase of 30% ($P<0.001$), Fos or Jun alone have minimal or no effects. The number of larvae examined (n) is indicated for each genotype. C. mEJC sizes in all genetic combinations tested are not significantly altered; thus, alterations in synapse strength arise from reduced quantal content (number of synaptic vesicles per action potential) as shown in D. E. Changes in synapse size are accompanied by parallel and predictable alterations in FasII immunoreactivity in terminal boutons. Top panel: Pseudocolor indicates levels of FasII. Bottom panel: quantitative fluorescence measurements showing that Fas II is increased in Fbz ($p<0.001$) and Jbz ($p<10^{-5}$) expressing preparations, and decreased in AP1 induced preparations ($p<0.004$) compared to control. The number of larvae examined for each genotype is indicated below the histogram bars. For 1E, the number of type1b termini (4-6 per larva) is indicated. Scale: vertical bar, 40 nA for EJC and 2 nA for mEJC; horizontal bar, 25 ms for EJC and 500 ms for mEJC.

FIGURE 3.1

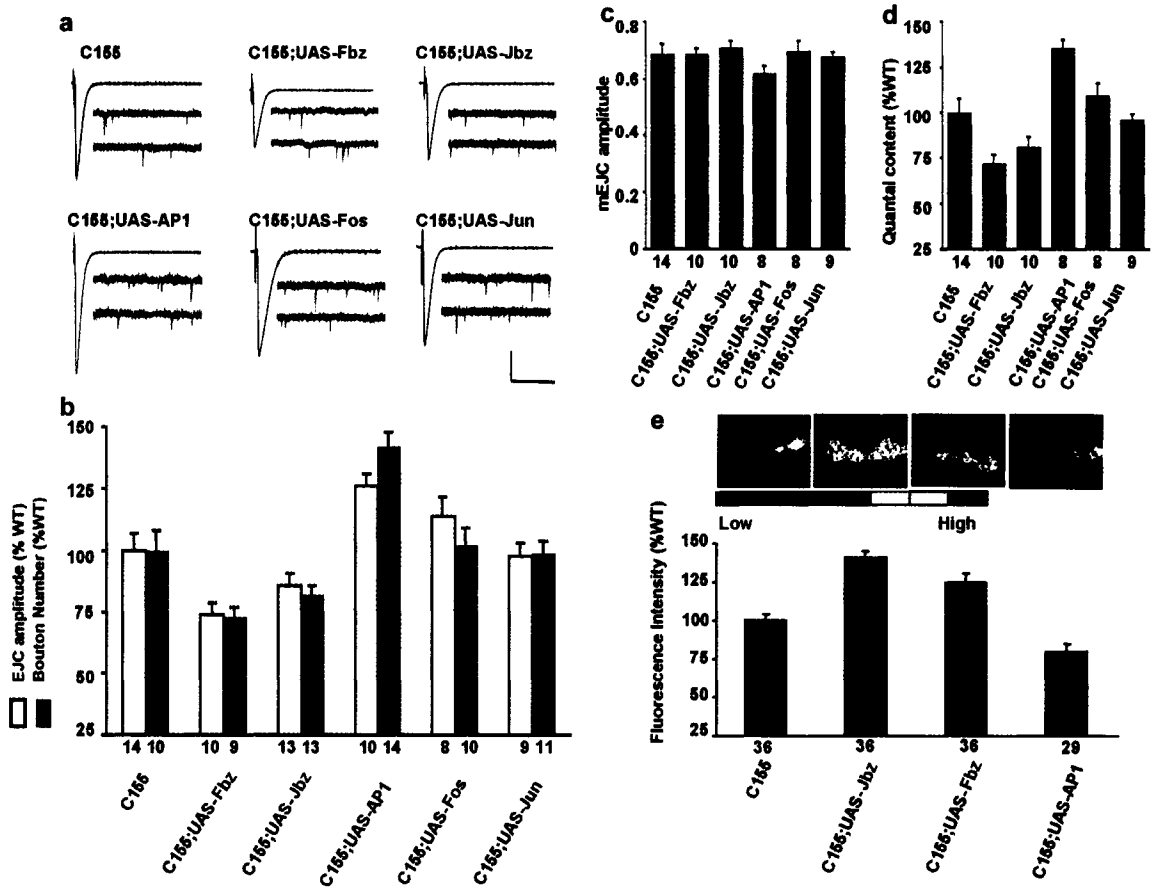

a
C155
C155;UAS-Fbz
C155;UAS-Jbz
C155;UAS-AP1
C155;UAS-Fos
C155;UAS-Jun
b
EJC amplitude (% WT)
Bouton Number (%WT)
14 10
10 9
13 13
10 14
8 10
9 11
C155
C155;UAS-Fbz
C155;UAS-Jbz
C155;UAS-AP1
C155;UAS-Fos
C155;UAS-Jun
c
mEJC amplitude
14 10 10 8 8 9
d
Quantal content (%WT)
14 10 10 8 8 9
e
Low
High
Fluorescence Intensity (%WT)
36 36 36 29
C155
C155;UAS-Jbz
C155;UAS-Fbz
C155;UAS-AP1

FIGURE 3.2 CREB and a cAMP-dependent function are required downstream of AP1 to regulate synapse strength but not bouton number. **A.** In a genetic background where AP1 is induced in neurons, induction of either an inhibitory isoform of CREB (via *hs-CREB2b*), or cAMP phosphodiesterase (via *UAS-dnc*) does not perturb the effect of AP1 on bouton number. In contrast both *hs-CREB2b* ($P<0.02$) and *UAS-dnc* ($P<0.001$) significantly inhibit AP1's effect on synaptic strength. **B.** In a wild-type background, identical induction of *hs-CREB2b* or neuronal expression of *UAS-dnc* has no effect on either synapse size or strength. Thus, reduced CREB and cAMP activity specifically inhibit the AP1-induced increase in synaptic strength. **C. D, E, F**: AP1 induction in neurons leads to rapid 3-fold upregulation of CREB2 mRNA. **C.** CREB2 transcript levels are elevated in AP1-induced neurons when compared to uninduced control animals (total adult head RNA; AP1 induction is accomplished using an Ru486 inducible transgene expression system [Osterwalder 2001] and 6 hours or Ru486 feeding). **D.** Ru486 induction of Fbz does not significantly alter levels of Jun and CREB2 mRNA. **E.** and **F**: A quantitative comparison of mRNA levels by Q-PCR. Following induction of AP1, the average number of PCR cycles (x) required for identical levels of CREB2 RT-PCR product (detected in the long-linear phase of the reaction) decreases by 1.7. This translates to a 3-fold increase ($p<0.02$) in CREB2 mRNA levels in heads from AP1-induced animals relative to uninduced controls. **F.** Following Fbz induction, Fbz transcript levels are increased 12 fold while the average levels of Jun and CREB2 remained the unchanged when compared to mRNA from uninduced controls. 5 (or 4 in one instance) independent cDNA preparations were performed for each experiment.

FIGURE 3.2

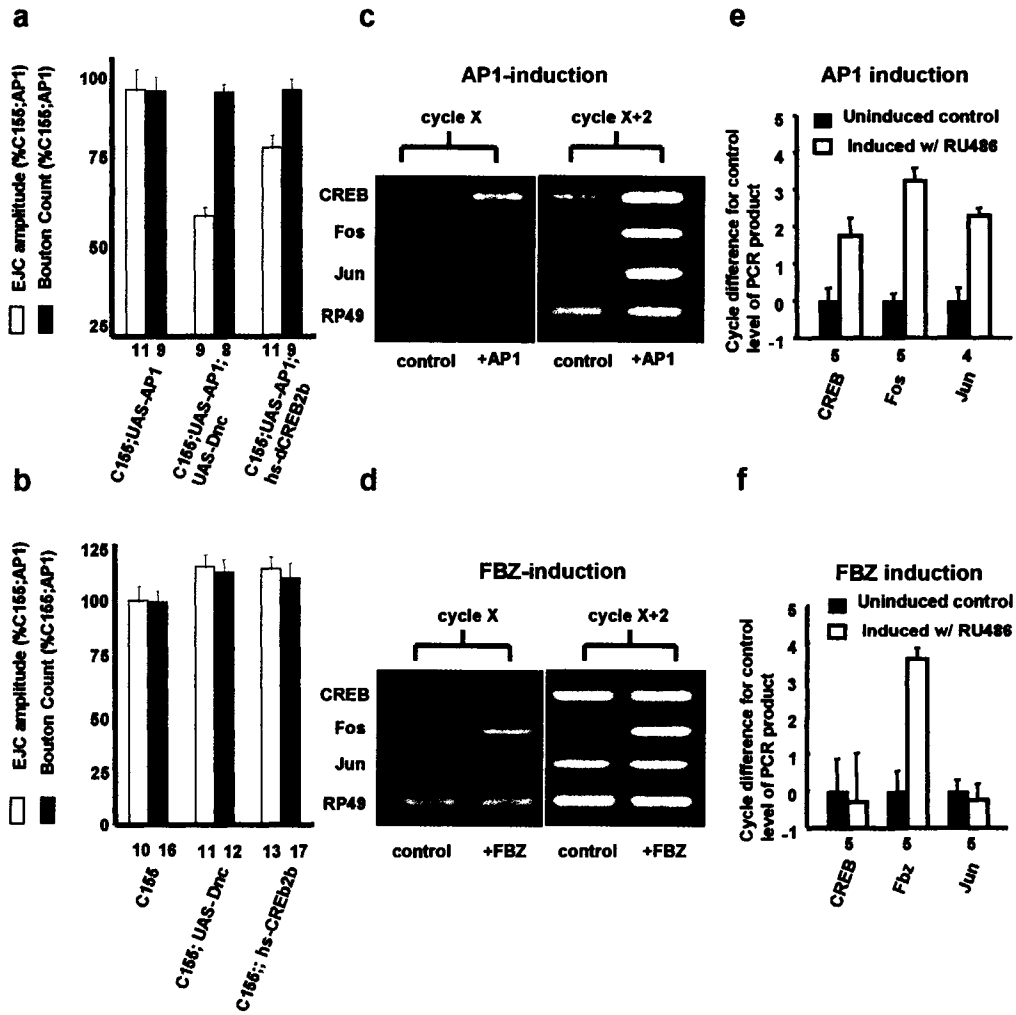

a
c
e
EJC amplitude (%C155;AP1)
Bouton Count (%C155;AP1)
100
75
50
25
11 9
9 8
11 9
C155;UAS-AP1
C155;UAS-AP1; UAS-Dnc
C155;UAS-AP1; hs-dCREB2b
AP1-induction
cycle X
cycle X+2
CREB
Fos
Jun
RP49
control
+AP1
control
+AP1
AP1 induction
Uninduced control
Induced w/ RU486
Cycle difference for control level of PCR product
5
4
3
2
1
0
-1
5
5
4
CREB
Fos
Jun
b
d
f
EJC amplitude (%C155;AP1)
Bouton Count (%C155;AP1)
125
100
75
50
25
0
10 16
11 12
13 17
C155
C155; UAS-Dnc
C155;; hs-CREb2b
FBZ-induction
cycle X
cycle X+2
CREB
Fos
Jun
RP49
control
+FBZ
control
+FBZ
FBZ induction
Uninduced control
Induced w/ RU486
Cycle difference for control level of PCR product
5
4
3
2
1
0
-1
5
5
5
CREB
Fbz
Jun

FIGURE 3.3 **A.** An outline of proximal components of the Jun Kinase (JNK) signaling module in *Drosophila* [Noselli 1999]. JNK (*bsk*) phosphorylation of Jun activates AP1-regulated gene expression. The pathway is antagonized by the *puckered* (*puc*) JNK-phosphatase which dephosphorylates and so inactivates JNK (bsk). **B.** The JNK signaling pathway positively regulated both structural and functional synaptic plasticity. JNK inhibition via neural expression of either a dominant-negative JNK (C155; UAS Bsk[DN]) or wild-type JNK-phosphatase (C155; UAS Puc[WT]) reduces synapse size and strength by about 25%-30%. The effects of Puc expression are completely suppressed by levels simultaneous overexpression of wild-type JNK (C155; UAS Bsk [WT]; UAS Puc[WT]). Decreased JNK via neural overexpression of Puc neutralizes the effect of AP1 on both synapse size and synapse strength (C155; UAS AP1; UAS Puc[WT]). This contrasts with the effect of *dnc* expression shown in Figure 2. % (P values: Bsk[DN] reduces bouton number $p< 0.0001$; and EJC amplitude $p< 0.04$. Puc reduces bouton number $p<0.0001$; EJC amplitudes $p< 0.03$. Puc suppresses AP1 effects on bouton number $p< 0.01$; on EJC amplitude $p< 0.01$).

FIGURE 3.3

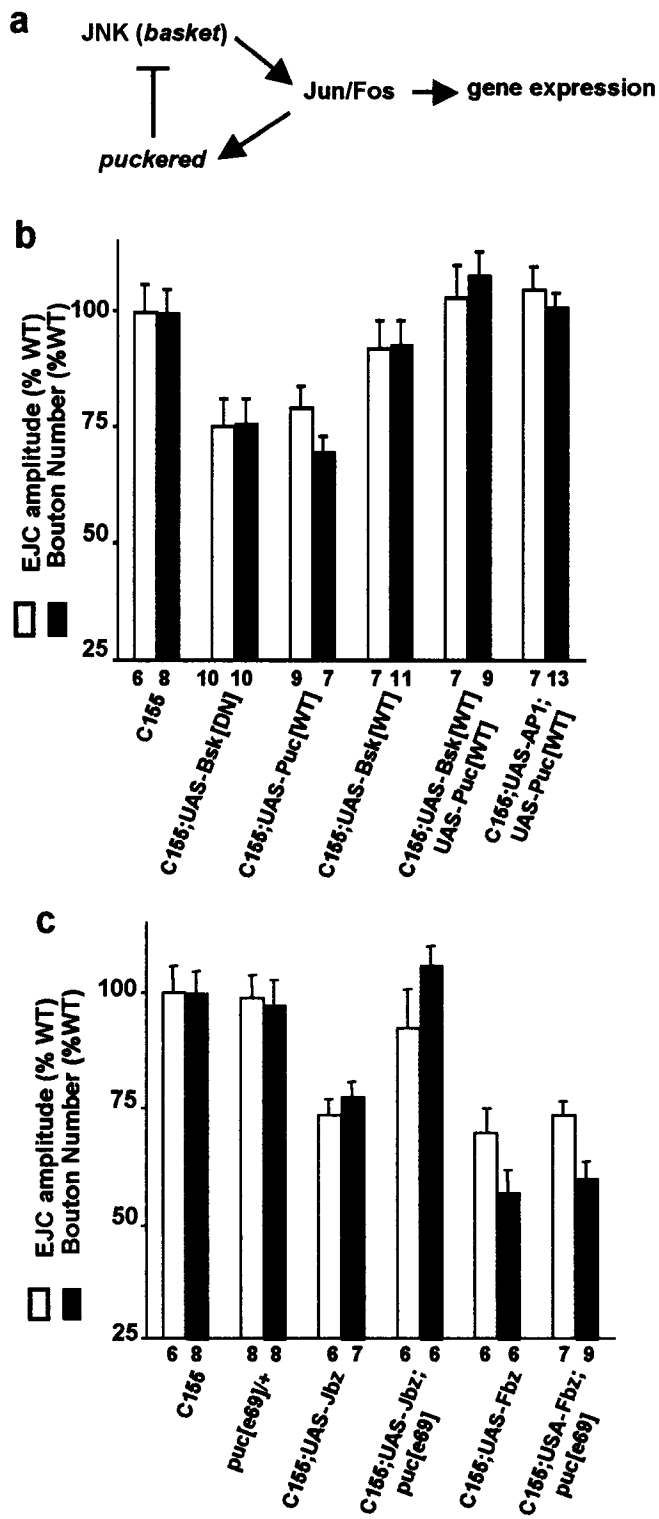
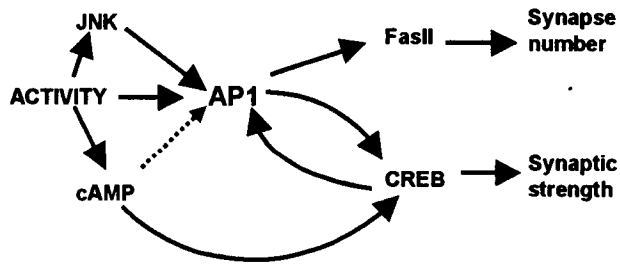

a
JNK (basket)
Jun/Fos
gene expression
puckered
b
EJC amplitude (% WT)
Bouton Number (%WT)
100
75
50
25
6 8
10 10
9 7
7 11
7 9
7 13
C155
C155;UAS-Bsk[DN]
C155;UAS-Puc[WT]
C155;UAS-Bsk[WT]
C155;UAS-Bsk[WT] UAS-Puc[WT]
C155;UAS-AP1; UAS-Puc[WT]
c
EJC amplitude (% WT)
Bouton Number (%WT)
100
75
50
25
6 8
8 8
6 7
6 6
6 6
7 9
C155
puc[e69]/+
C155;UAS-Jbz
C155;UAS-Jbz; puc[e69]
C155;UAS-Fbz
C155;USA-Fbz; puc[e69]

FIGURE 3.4 Jun Kinase regulation of plasticity occurs via neuronal AP1. Genetic reduction of JNK phosphatase in flies heterozygous for a *puc* hypomorphic allele (puc^{e69}/+) has no observable effect in a wild-type background. However, this slight increase in JNK signaling restores normal bouton number and EJC amplitude to Jun-inhibited motor neurons. That puc^{E69}/+ does not suppress effects of Fbz induction may have a trivial explanation; for instance, Fbz induction more effectively inhibits neural AP1). It is also consistent with JNK control of AP1 function occurring via positive regulation of Jun, a process that may be redundant under conditions where Fos activity is limiting. Scale: vertical bar=20 nA; horizontal bar = 25 ms.



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CHAPTER FOUR

FUTURE DIRECTIONS

The primary goal of the studies detailed in this body of work was the establishment a procedure for the acute production of neural activity sufficient to promote activation of fundamental phylogenetically conserved neural activity-dependent plasticity signaling pathways in the genetic model organism *Drosophila melanogaster*. Now that this has been largely accomplished; a natural follow-up to these studies would be to now show the procedure as also capable of regulating neural plasticity. Once this is established it might then be possible to harness the paradigm to address outstanding issues in field of neuronal plasticity, learning and memory utilizing. Early studies designed to examine this issue are promising Adding this dimension to the procedure would greatly enhance these studies and add significantly the importance and utility of future applications of the neural activity generation paradigm by expanding the relevance and importance of the work from an assay of evolutionary conservation to laboratory method. Acute manipulation of neural plasticity in *Drosophila* may be brought to bear on a number of outstanding issues in the plasticity field. Of particular interest and relevance to the work thus far would be the identification and characterization of effectors and regulators of the ERK signaling cascade. In this section I will focus primarily two lines of work this neural activity generation paradigm may be used to address this topic and add to this largely unexplored aspect of neuronal plasticity. (1) A two-dimensional proteomics approach designed to identify ERK regulators/effectors (2) the creation of a

Kum^{ts} ERK reporter line for use in genetic screens designed to isolate genetic components regulating the traffic of activated ERK into the nuclei of post-synaptic cells.

Acute physiological regulation of neuronal plasticity using seizure mutants

The first extension of these studies should be to test the acute physiological manipulation of plasticity via the neural activity generation model; thus providing evidence of a causal link between the conservation of neural activity-dependent signaling pathways and the actual regulation of synaptic plasticity. As an initial starting point, the background effects of the different permissive temperature conditions on the *comt*^{ts} and *Kum*^{ts} mutations were examined. This is critical because as is often the case, temperature sensitive mutations that do not exhibit noticeable phenotypes under permissive conditions and appear "normal", may have other underlying defects that are not seen. Indeed, this is demonstrated from our own studies of the *para*^{ts1} mutation where the temperatures for restrictive behavioral phenotypes and restrictive molecular signaling differed by several degrees (Hoeffer et al., 2003)(Fig 2.1, Fig 2.3).

The approach used to preliminarily address this issue was simple; *Drosophila* were raised in range of permissive temperatures and then synaptic strength and structure (via analyses of the larval NMJ) were examined. The experimental design for these initial sets of experiments is shown on Fig. 4.1. The wild-type and mutant backgrounds examined under these growth conditions showed no apparent increase in larval mortality and exhibited no detectable paralysis suggesting no obvious neuronal impairment at these permissive albeit elevated temperatures (data not shown). The resulting synaptic size for

wild-type (Canton-S) and the seizure mutant, *Kum*^{ts}, grown under these conditions is shown in Fig. 4.2. In *comt*^{ts}; *Kum*^{ts} animals we observed increased synaptic size compared to wild-type or singly mutant *Kum*^{ts} animals under all permissive temperature rearing schemes. These data also show that increased growth temperature may be correlated with increased synaptic size in both wild-type and *Kum*^{ts} animals. In contrast, the total level of increase in *comt*^{ts}; *Kum*^{ts} animals did not differ significantly between the 21°C and 29°C rearing conditions suggesting that the molecular events underlying the observed increases are already saturated in the double mutants but retain signaling amplitude in wild-type or singly mutant animals. The signaling underlying these changes is likely the result of increased neural activity in response to increased rearing temperature. This idea is supported by another study showing that increased rearing temperature is correlated with increased neural activity and a corresponding increase in synaptic size (Zhong and Wu, 2004). Similarly, one possible explanation for the observed increase in synaptic size in seizure double mutants is that even under behaviorally permissive (non-seizure) conditions *comt*^{ts} and *Kum*^{ts} gene products are still partially restricted. This restriction in function results in increased neural activity and/or intracellular signaling upstream of plasticity pathways which in turn signals increases in synaptic size. To further explore this notion, this initial study was extended to examine synaptic strength in these mutant backgrounds and to examine the possibility that the *comt*^{ts} mutation was solely responsible for the observed increase in synaptic size. Conversely, if the observed plastic changes were in fact derived from altered neural activity/signaling resulting in part to direct functional deficits arising from the *comt*^{ts} gene

product, NSF-1, then restoring a wild-type copy of the gene would be predicted to rescue the enhancement. This experiment was facilitated using a *Drosophila* mutant bearing neural specific GAL4 driver (c155) coupled with the $comt^{ts}$ mutation and a UAS-NSF-1 transgene. Since the greatest range of effect between the seizure mutants and wild-type was observed under low temperature permissive conditions, this condition was selected for additional study. Several interesting things were learned from these experiments. First, the presence of the $comt^{ts}$ mutation alone was not sufficient to produce increased synaptic size (Fig 4.3) indicating that both seizure mutations were required to increase synaptic size. Secondly, the neuronal expression of a functional copy of NSF-1 rescued the enhancement of synaptic size observed in $comt^{ts}$; Kum^{ts}, arguing that plastic changes are specific to the gene rather than nebulous effect of genetic background. Thirdly, synaptic strength in doubly mutant $comt^{ts}$; Kum^{ts} *Drosophila* was increased. Remarkably however, synaptic efficacy in the single seizure mutant backgrounds ($comt^{ts}$ or Kum^{ts}) was also enhanced. Furthermore, the observed enhancement was to the same level observed in the double mutants once indicating the possibility that ceiling for strength enhancement is reached in either single mutant background that is not further enhanced by altered signaling derived from the presence of the other seizure mutation. The observed increase in synaptic strength in all mutant backgrounds contrasts with what is seen with synaptic structure. It is possible that the events that regulate synaptic strength have a lower threshold for perturbation then those underlying synaptic structure or that synergy between multiple signaling pathways (presumably activated in the double

mutant) are required for altering synaptic structure while those upstream of synaptic strength may be activated in parallel and independent of each other with equal efficiency.

The evidence so far supports the idea that the seizure mutant background promotes neural activity dependent signaling to regulate plasticity but it the hypothesis still requires more rigorous examination. One critical aspect of the premise is the notion that increased neural activity derived signaling present in the seizure mutant backgrounds leads to observed increases in synaptic strength and size. One experiment to test this idea would be the addition of another mutation to the genetic background that suppresses basal neural activity. The nap^{ts1} mutation may be used for this purpose, the nap^{ts1} mutation blocks increased synaptic size and strength phenotypes observed in the neuronal hyperactivity *eag Sh* mutants (Budnik et al., 1990). To accomplish this *Drosophila* bearing the nap^{ts1} mutation may be recombined onto Kum^{ts} chromosomes, the resulting animals may then be crossed to $comt^{ts}$; nap^{ts1} mutants to generate a triple mutant $comt^{ts}$; Kum^{ts} nap^{ts1}/nap^{ts1} *Drosophila.* If increased neural activity is indeed critical for the observed increases in synaptic strength and size, the presence of the nap^{ts1} mutation should occlude either observed enhancement.

Ideally, the phenotypic effects derived from altered signaling in seizure mutants should be regulated by the same molecules known to function upstream of other aspects of neural activity-dependent plasticity (Fig 3-4). Since one of the major goals of the procedure is to establish it as a means of studying general neuronal plasticity mechanisms, it is critically important to verify that conserved components of these *bona*

fide plasticity pathways are utilized rather than signaling via alternative and perhaps physiologically irrelevant pathways. A number of transgenic reagents exist that may be used to address these questions. Some preliminary experiments have already been performed to examine the contributions of key transcriptional regulators during seizure derived neural plasticity. The transcription factors activator protein-1 (AP1) and cAMP responsive element binding protein (CREB) regulate important aspects of plasticity at the larval NMJ (Davis et al., 1996; Schuster et al., 1996; Sanyal et al., 2002). AP-1 is a heterodimer formed by two bZIP transcription factors, Fos and Jun. Neuronal over expression of AP1 increases both synaptic strength and size, while over expression of a dominant negative construct (Fbz) decreases synaptic size and strength. Simultaneous expression of AP1 and repressor isoform of CREB (Creb2b) blocks AP1 mediated enhancement of synaptic strength but does not block AP1 mediated enhancement of synaptic size (Sanyal et al., 2002). A single cross with *c155 comt^{ts}; Kum^{ts}* and UAS-Fbz enables an experiment to determine whether seizure mutant mediated plasticity is transduced via AP1 signaling. The results from this experiment are detailed in Figure 4.4. Neural expression of Fbz blocks the effects of *comt^{ts}; Kum^{ts}* on synaptic size, reducing the synaptic size to ~70% of that observed in similarly reared wild-type animals and 50% of that seen in *comt^{ts}; Kum^{ts}* animals. This observation is consistent with the idea that seizure mutants mediate synaptic structure via the same pathways downstream of neural activity and cAMP mediated signaling. Another prediction that can be made based off the AP1/CREB model is that over expression of the repressor isoform CREB2b should block the seizure mediated effects on synaptic strength but not structure. Preliminary results

from overexpressing UAS-Creb2b the *c155 comt^{ts}; Kum^{ts}* background does not follow this prediction. Instead, a small but statistically insignificant increase in synaptic size is detected in *c155 comt^{ts}; Kum^{ts}/UAS-CREB2b* when compared to wild-type animals. There are many reasons this may explain this observation. First, the basal synaptic size for the *c155 comt^{ts}; Kum^{ts}* mutant background must be determined. It may also be that the presence of neuronal driver exerts effects on synaptic structure that act to reduce the robustness *comt^{ts}; Kum^{ts}* mutant phenotypes. Although unlikely, it is a formal possibility that may easily be explored. More complicated explanations are likely to be the source of the difference. In addition to transcriptional regulation of the CREB gene, the functional activities of the various CREB isoforms (activators and repressors) are regulated by direct phosphorylation (PKA, CAMK) (Mayr and Montminy, 2001). As such, it is possible that differential or more robust upstream signaling to CREB protein exists in the seizure mutant background when compared AP1 expression lines. Under these conditions, it is possible that signaling via CREB repressor in this background exceeds the levels achieved via heat shock (hs) mediated expression of CREB2b used in previous blockade experiments, potentially revealing CREB regulation of neuronal structure that was originally masked. One simple explanation presents itself immediately; the possibility that seizure mediated signaling through CREB2b may act to inhibit AP1 function through a negative feedback loop thereby reducing synaptic size (Fig 3-4).

Although promising, the paradigm is still hampered by incomplete characterization and additional experiments are required to allow full utilization of the seizure mutants in plasticity studies. First, it would be important to discover whether or

not UAS-Fbz was capable of blocking the enhanced synaptic strength phenotypes seen in all seizure mutant backgrounds. This would further support the idea that seizure mutant mediated plasticity signals through AP1, and therefore utilize conserved pathways controlling plasticity. Second, the potential role of CREB2b in regulating structural plasticity must be further explored. It is also important to determine if CREB2b activity is greater in seizure mutant backgrounds when compared to wild-type. This might be addressed with immunoprecipitation experiments where the CREB protein is pulled down from three different situations: (1) wild type and hsCREB animals grown under blockade conditions (Davis et al., 1996; Sanyal et al., 2002) (2) c155 background (3) $comt^{ts}$; Kum^{ts} background. CREB protein would be immunoprecipitated using the anti-CREB antibody (generously supplied by J.Yin). Then relative CREB phosphorylation state would be tested with commercially available phosphor-Ser antibodies. In this way it would be possible to not only determine relative levels of neurally expressed CREB2b but also indirectly the activity of CREB in the different genetic backgrounds. If CREB does in fact exert control on structural plasticity that one other prediction would be that CREB2a over expression may have the opposite effect when expressed in seizure mutant backgrounds, enhancing synaptic size. One interesting experiment might be to express CREB2a under singly mutant conditions (wither $comt^{ts}$ or Kum^{ts} alone), conditions where changes to synaptic size have not been observed. Under these conditions, the neurons may be "sensitized" to plastic change and the additional CREB2a may provide the necessary signals to enhance synaptic size that are not present in the singly mutant seizure backgrounds alone. This experiment begins to address the potential interesting

and important observation that all seizure mutant backgrounds enhance synaptic efficacy while only in combination, act to affect synaptic structure. This raises an interesting issue of hierarchy in regulation of neural plasticity; are there gating steps defining one aspect of plasticity from another? Are the mechanisms that regulate synaptic structure more tightly regulated than those regulating synaptic strength or is the threshold for effective signaling simply lower? Finally, what are underlying mechanisms behind the observed effects on plasticity detected in seizure mutants? One likely source of regulation involved is the ERK signal cascade. Experiments to test this idea might include the addition of MEK inhibitor U1026 to the growth media during rearing at permissive growth temperatures to pharmacologically block ERK activity. This approach, while technically simple suffers from a lack of tissue specificity. ERK inhibition in this manner would act on a wide range of tissues in the developing animal; this alone may affect neural plasticity in ways that would be very difficult to dissect. A more attractive alternative, possible due to the excellent conservation between vertebrate and *Drosophila* MEKs, (Kelleher et al., 2004) would be the generation of inducible MEK dominant negative constructs. This would allow the rapid and reversible ERK inhibition in tissue specific manner. Regardless of the seizure activity paradigm characterization results, the extensive and largely phylogenetically conserved nature of ERK involvement in neural plasticity makes an inducible *Drosophila* MEK-DN very useful for studying the involvement of the ERK cascade in already well established plasticity signaling mechanisms.

Identification of neuronal effectors of the ERK signaling pathway

Regardless of the final outcome of confirming the seizure mutant paradigm as a procedure for acute manipulation of plasticity, one result from this work is quite unambiguous; Following disruption of SERCA function, ERK is activated in *Drosophila* neurons for long periods of time (>120 min) in a neural activity dependent fashion. This finding provides the basis for an interesting set of screening experiments allowing insight into gaps in our current knowledge of neuronal ERK signaling. While the involvement of ERK in several forms of long lasting neuronal plasticity is well established (Adams and Sweatt, 2002), our understanding of downstream effectors of ERK signaling and the modulators regulating ERK signaling remains poorly understood. With a few experiments to strengthen the theoretically basis for use of the Kum^{ts} mutation for neuronal ERK activation it should be possible to explore these questions and identify novel effectors and critical regulators accessory to neuronal ERK signaling cascade.

The experimental system would require some further characterization (independent of those described in the previous section) to allow its fuller application to problem ERK effectors and regulators. First, it needs to be established whether the neuronal disruption of SERCA function truly acts to promote long term ERK activation. Our current understanding of the seizure induction paradigm does not allow us to delineate between activation of signaling involved in persistent signaling and the continuous activation of transient mechanisms leading to ERK activation. Since it well established that these distinct pathways (Wu et al., 2001). ERK activation in Kum^{ts}

animals requires neural activity (Hoeffer et al., 2003) is this prerequisite for neural activity only required at initiation or throughout the time course. One experiment that may be used to distinguish between these two possibilities utilizes the *para*^{ts1} mutation to selectively block neural activity at specific time points following induction of seizures in *Kum*^{ts} animals using methods identical to those described in Hoeffer et al. 2003. If continuous neural activity is required for prolonged ERK activation then one prediction should be that blockade shortly after seizure induction should block ERK activation seen later time points (>120 min). Should neural activity be dispensable after a short period of uninterrupted activity then it is possible that pathway(s) associated with persistent signaling have been activated. This result may be juxtaposed with experiments designed to establishing the temporal window in which neural activity is critical to the induction of persistent signaling. Second, is the issue of neuronal specificity; in its current form the seizure paradigm uses a *Kum*^{ts} mutation that would affect signaling in all cell types. The effect of this on our current results has been mitigated thus far by selective enrichment of neuronal tissue (see Methods Chap 2) for analysis. Genetically constraing *Kum*^{ts} signaling strictly to the nervous system or a subset of neurons would vastly improve the resolution of the data obtained from experiments. This should be possible using a UAS-*Kum*^{ts} transgenic construct (already available) in combination with neural specific GAL4 driver (several drivers including pan-neural and those specific to neuronal subtypes are freely available). There is some preliminary evidence indicating that strong expression of the UAS-*Kum*^{ts} gene product is required to manifest paralysis (Sanyal *et al.* in press) it is not known whether the same is true of ERK activation. Should this be the case then it may be

necessary to create neural drivers capable of stronger expression. Strategies for this might include the creation of chromosome bearing multiple copies of a neural specific driver such as elav-GAL4 or a neural driver in combination along with UAS-GAL4 construct. Ideally, a transgenic animal bearing the neural driver and the UAS-*Kum*^{ts} on the same chromosome should be created to facilitate ease the difficulty of crossing the different genetic backgrounds. Should initial experiments be successful the system could be further improved by substituting the GSG-GAL4 for the wild-type GAL4 allowing temporal control in addition to tissue specificity. The addition of this aspect to the experimental design affords a significant improvement in signaling specificity to *Kum*^{ts} mediated ERK activation, a factor may serve ultimately to increase the importance of any findings in the experiments described in the proceeding section.

In addition to the numerous cellular substrates phosphorylated by ERK, its activation is also believed to promote protein translation (Kleijn et al., 1998; Raught and Gingras, 1999; Wang et al., 2002). While a role for protein synthesis in neuronal plasticity has been well established, not much is known about identities of these molecules or more importantly their function in affecting plasticity. *Kum*^{ts} mediated ERK activation may provide the means to gain some insight in this gap in our current information. It should be possible to perform a comparative screen to isolate and identify proteins synthesized in response in signaling activated by *Kum*^{ts} perturbation. One simple experimental design would simply be to treat both wild type and *Kum*^{ts} animals and then isolate proteins following treatment at selected time points. This would control for differences due to initial heat shock. Isolation of *Drosophila* heads coupled with selective

expression of Kum^{ts} in the nervous system should help to restrict ERK mediated effects to the nervous system. The proteins would then be analyzed using two-dimensional electrophoresis. Protein levels may be determined by the relative presence/absence of peptide "spots". To ameliorate problems with gel comparison and interpretation of the protein spot profiles, focused protein samples should be run simultaneously on the same gels. This helps resolve most of the difficulties encountered by small variations in gel composition, running conditions and staining method. One gels are prepared, protein differences between experimental treatments and/or levels may be detected using one of many possible peptide visualization methods (Commassie, silver, or SYBRO staining). All of these current staining techniques for visualizing peptides are compatible with MALDI analysis allowing for rapid identification of proteins of interest. As a proof of principle experiment, several likely protein candidates are available. One strong candidate is MAPK phosphatase (MKP), a protein known to be rapidly translated in response to ERK activation (Brondello et al., 1997). At least three different MKPs are found in *Drosophila* (DMKP); two are known to be expressed in the head (Lee et al., 2000; Kim et al., 2002). Since antibody for DMKP is available, it should be possible to this protein to validate that theory using less labor intensive one dimensional protein blotting. The involvement of translation may be determined by prefeeding *Drosophila* a protein synthesis inhibitor prior to treatment (cycloheximide). Translated proteins of interest would then be tested for ERK dependency by adding a pretreatment with MEK inhibitor U1026. Rapid confirmation peptide up-regulation would be most easily achieved by follow analyses with 1D analysis for those peptides for which primary

antibodies are available. More sensitive detection of protein expression changes can be achieved by employing Cy3/Cy5 protein labeling followed by simultaneously loading of treated and untreated lysates. This technique while more challenging and labor intensive affords the greatest ability to detect subtle differences in protein expression that may be missed with conventional protein staining techniques.

Once established, there are many possible experimental avenues that may be explored using this method. The contribution of other signaling pathways to the regulation of peptides of interest may be examined with prefeeding of pharmacological blockers (KN-093, Wortmannin). Genetic perturbation of signaling with either null/hypomorphic alleles of regulators on interest is also possible. Transgenic disruption of signaling is also a possibility under scenarios where either a neural driver is used in the experimental Kum^{ts} background. This line of inquiry would address the contribution of other signaling pathways to neuronal peptide synthesis but also indirectly asks the question of whether in other important signaling pathways are activated in response to Kum^{ts} disruption. In addition to classification of ERK regulated translated products, the analysis process will also yield proteins that degraded (diminished or missing spot signatures) as well peptides that are not translated but rather stabilized in an ERK dependent fashion i.e. c-Fos (Murphy et al., 2002; Murphy et al., 2004). While not translation regulated effectors, these proteins may represent an important class of effectors involved in the persistent maintenance of the ERK signaling and the modulation of neural plasticity.

ERK translocation to the nucleus

Activated ERK is translocated into the nucleus where it can regulate the activities of a number of transcription factors (CREB, AP1, and Ets family members) (Treisman, 1996; Sweatt, 2001). A critical regulatory step in this process is the translocation of ERK into the nucleus where it can exert its control over transcription. Since ERK lacks a classical nuclear localization signal (NLS), ERK must be transported by another mechanism of active transport to get across the nuclear membrane, this process is poorly understood. There is some evidence that this process is regulated or "gated" by the activities of PKA signaling but its exact role or even its necessicity is somewhat controversial (Martin et al., 1997; Horgan and Stork, 2003). Other questions about the regulation of ERK nuclear translocation also exist. For example, MEK binding to ERK is one mechanism of cytoplasmic "trapping" by which ERK is sequestered from nuclear transport (Fukuda et al., 1997). The MEK/ERK interaction is reduced via MEK activation releasing ERK for transport into the nucleus. Do scaffolds such as 14.3-3 or KSR that mediate MEK/ERK interaction and subcellular localization of cascade components also play a role in nuclear translocation? MEK activation is required for some forms of ERK translocation into the nucleus (Khokhlatchev et al., 1998; Lenormand et al., 1998) but ERK nuclear entry can occur via alternative pathways. There is also a pathway for shuttling of ERK back and forth from the nucleus to the cytoplasm that occurs even in presence of U0126, which blocks MEK activity, showing the existence of at least two different mechanisms of ERK translocation and constitutive shuttling pathway and ERK dependent inducible pathway (Pouyssegur et al., 2002). The identities of the proteins

involved in these two pathways are unknown. Finally, it is known that during transient activation of ERK, nuclear export of activated ERK occurs rapidly but following sustained activation of the ERK cascade, activated ERK is accumulates in the nucleus (Lenormand et al., 1998). This nuclear trapping of activated ERK is known to require ERK dependent transcription but the identities of these molecules; presumably encoding nuclear anchoring proteins, is unknown.

The results of our study show that ERK is translocated into the nuclei of muscle cells in response to activity induction in conditional seizure mutants (Hoeffer et al., 2003). This finding makes a possible a screen designed to located genes involved in the cellular mechanisms controlling the ERK nuclear transport process. Disruption of Kum^{ts} alone is sufficient to observe nuclear translocation into the nuclei of larval muscles; this should allow a relatively easy assay for nuclear ERK transport. Combining Kum^{ts} with a *Drosophila* MAPK-GFP (or other fluorescent protein) fusion construct in conjunction with a muscle specific driver or expression construct would provide the means to easily observe nuclear ERK translocation in the living animal and rapidly screen for genetic effectors of nuclear transport. An identical construct has been made for use in vertebrate cell culture, this construct (owing to relatively high phylogenetic conservation) or one based on the insect sequence may be used to synthesize such a reporter. It is highly probable that the construct will maintain not only its function but also the ability to be actively transported across the nuclear membrane as its vertebrate equivalent and a similarly themed *Drosophila* fusion construct have already been shown retain this capacities (Adachi et al., 1999; Kumar et al., 2003). A similar strategy was recently

employed to examine the translocation of activated MAPK into the nuclei of the cells in the developing eye. In this study they fused a number of different signaling peptides to MAPK to develop a nuclear reporter system ERK translocation (Kumar et al., 2003). While it would be feasible to adapt their reporter system to screens designed to isolate macromolecules involved in ERK nuclear transport, the creation *Kum*^{ts} MAPK::GFP construct on a single chromosome would greatly facilitate genetic screens using this tool. An initial proof of principle experiment could be to combine the reporter system with DIM-7 mutants which have already been shown to affect nuclear ERK transport in cultured *Drosophila* cells (Lorenzen et al., 2001). The same basic approach may used to performa an initial pilot screen examining strong candidates that are believed to be be critical to nuclear transport and anchorage (α/β-importins, CAN/Nup214, MKPs) (Lorenzen et al., 2001; Matsubayashi et al., 2001; Pouyssegur et al., 2002). Novel molecules may be identified with screens be designed for use classical EMS mutagenesis strategies, or with the freely available *Drosophila* P-element insertion mutant library.

FIGURE 4.1 Growth conditions for *Drosophila* seizure mutants to examine effects variable permissive temperature conditions on basal synaptic strength and size. Egg laying females were permitted to lay eggs for one day and the lower end of the permissive growth range then vials were cleared and transferred to different permissive temperatures. (A) *Drosophila* were grown at either low permissive (21°C) or high permissive (29°C) until they reached wandering third larval instar. (B) *Drosophila* were grown at low permissive (21°C) temperature throughout their development but were exposed twice a day to two 30' minute sub-restrictive temperature pulses (32°C) then returned to low permissive temperatures. This was repeated until the animals reached the wandering third larval instar stage.

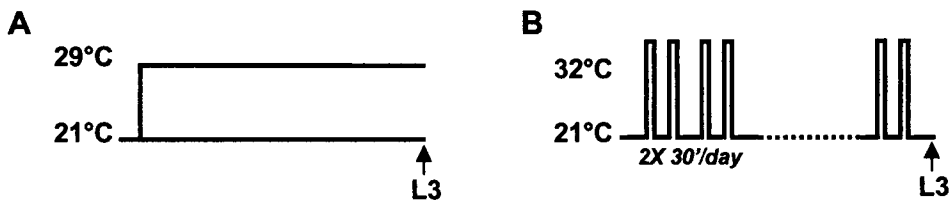


FIGURE 4.2 Synaptic size of wild type *Drosophila* and seizure mutants under different permissive growth conditions. Increased temperature is correlated with increased synaptic size in wild-type and Kum^{ts} *Drosophila*. Conversely, double $comt^{ts}$ and Kum^{ts} mutants exhibited increased synaptic size under all conditions examined. Larval bouton counts were obtained via SYT staining of the larval NMJ and segment A2, muscles six and seven. Bouton counts were normalized to muscle area for all animals examined [p-value from Student's T-test: 21°C p=0.0014, 29°C p=0.045, 32°Cx2 p=0.0039]. N values for each condition within bars.

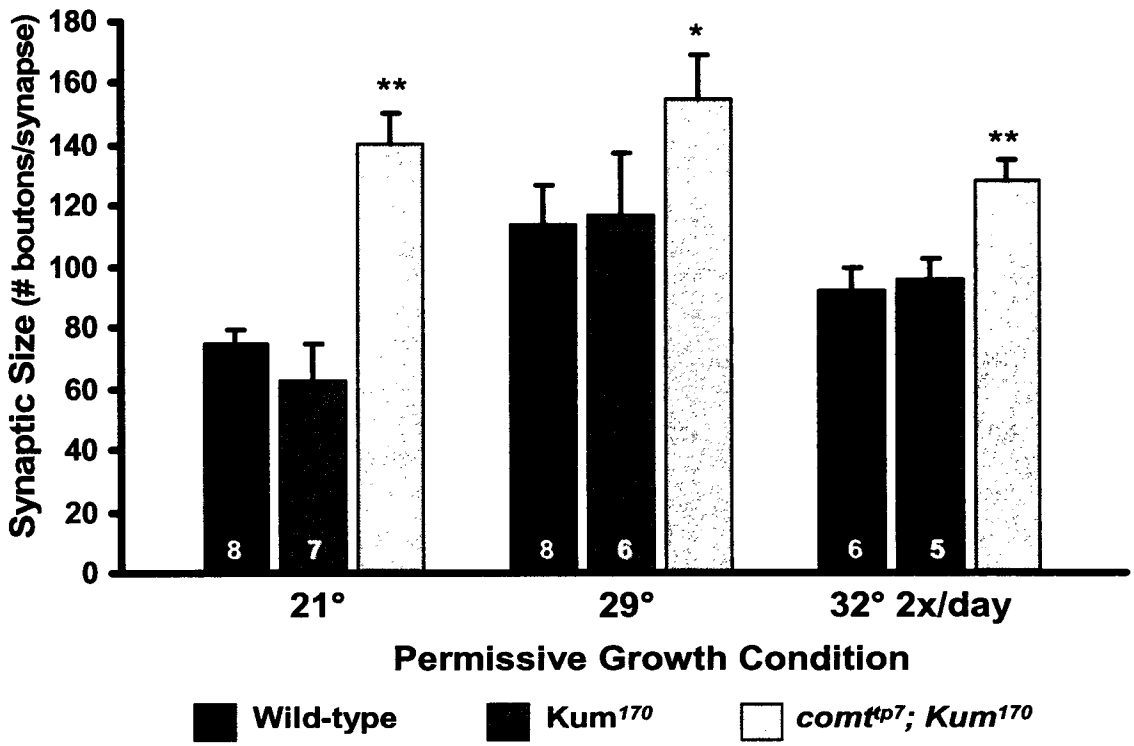


FIGURE 4.3 (A) Synaptic size and strength for *Drosophila* seizure mutants grown under low temperature permissive conditions. Under low temperature permissive conditions synaptic size is increased in $comt^{ts}$; Kum^{ts} double mutants animals when compared to either wild-type or singly mutant $comt^{ts}$ or Kum^{ts} controls [w.t. 100±2.93; Kum^{ts} 102±4.45; $comt^{ts}$ 97±3.49; $comt^{ts}$; Kum^{ts} 154±8.00; *c155* $comt^{ts}$; Kum^{ts}/*UAS-NSF1* 92±4.82, p-value w.t vs $comt^{ts}$; Kum^{ts} = 6.47e-6 p-value $comt^{ts}$; Kum^{ts} vs. *c155* $comt^{ts}$; Kum^{ts}/*UAS-NSF1*=1.41e-9]. All values have been normalized to muscle size then to arbitrary units based wild-type averages. **(B)** Synaptic strength in seizure mutant backgrounds. Unlike synaptic size, strength is enhanced for all genotypes examined [p-value for all comparisons <0.01] N for each genotype in column.

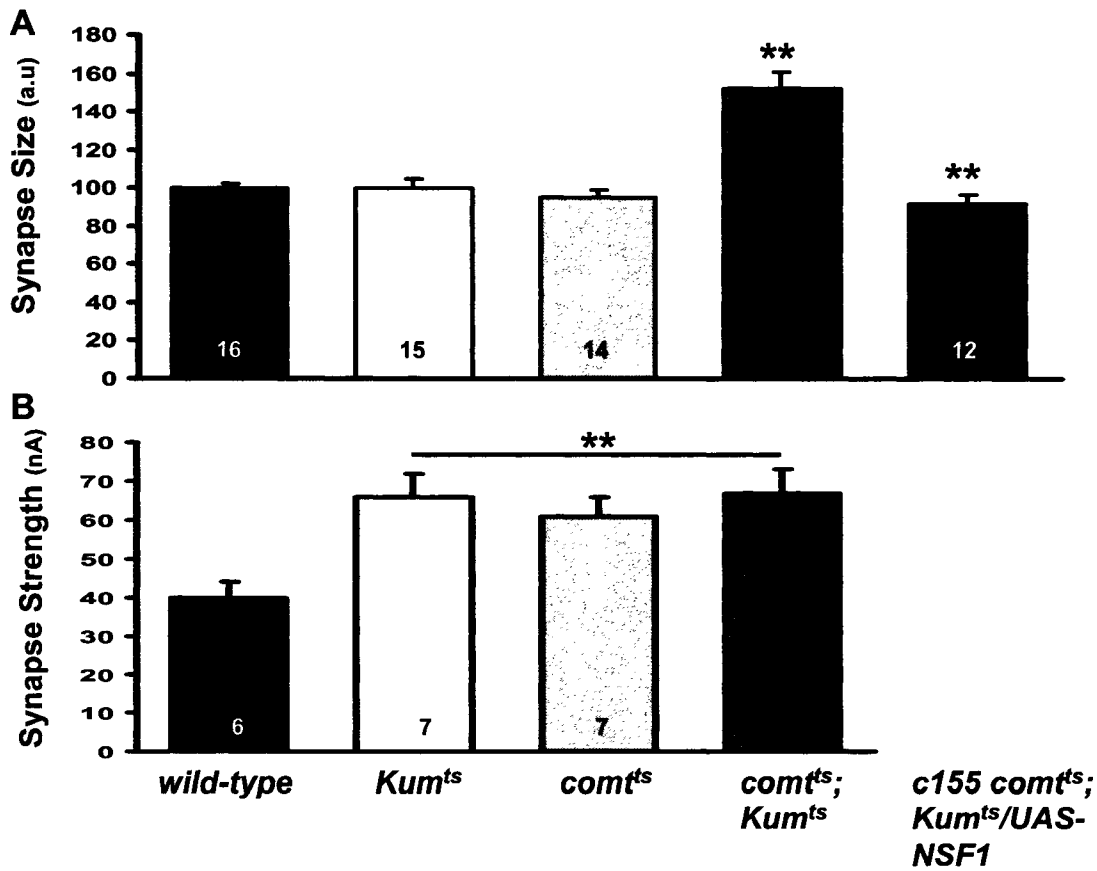
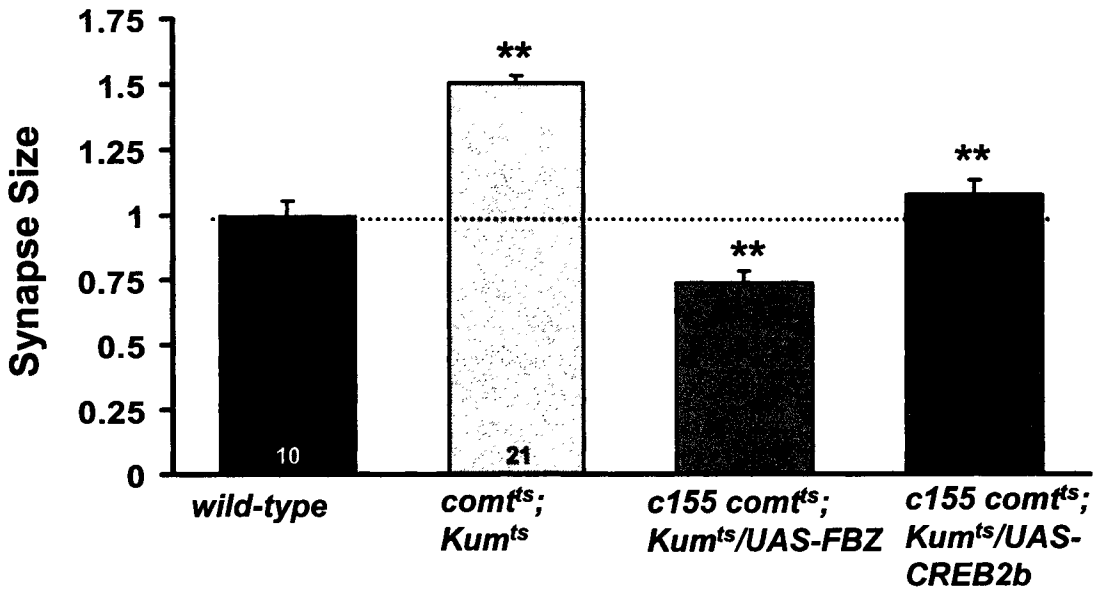


FIGURE 4.4 Synaptic size of seizure mutants in the presence of neurally expressed transgenes. Neural expression of a dominant negative isoform of Fos (Fbz) not only blocks the increase in synaptic size seen in *comt^{ts}; Kum^{ts}* mutants. Neural over expression of a repressor isoform of CREB (CREB2b) reduces the synaptic size of *comt^{ts}; Kum^{ts}* seizure mutants to the levels seen in wild type animals. P-values [wild type vs *comt^{ts}; Kum^{ts}* p=1.412e-9, wild type vs. *c155 comt^{ts}; Kum^{ts} /UAS-Fbz*, p=0.007, wild type vs. *c155 comt^{ts}; Kum^{ts} /UAS-CREB2b*, p=0.370, *comt^{ts}; Kum^{ts}* vs. *c155 comt^{ts}; Kum^{ts} /UAS-CREB2b*, p=4.89e-5]. Dashed line corresponds to wild type size levels, for comparison. N values for each condition within bar.



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