

Acute Induction of Conserved Synaptic Signaling Pathways in *Drosophila Melanogaster*

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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 *N*-ethylmaleimide-sensitive fusion factor 1 and sarco-endoplasmic reticulum Ca²⁺ ATPase, respectively, result in persistent (>4 hr) activation of neuronal extracellular signal-regulated kinase (ERK). ERK activation at the larval neuromuscular junction 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* (CCAAT element binding protein). The effect of “seizure-stimulation” on ERK activation requires neural activity and is mediated through activation of MEK (MAPK/erk kinase), the MAPKK (mitogen-activated protein kinase kinase) 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.

Key words: neurogenetics; temperature-sensitive paralysis; presynaptic; extracellular-signal regulated kinase; ERK; MAPK; Ras; sarco-endoplasmic reticulum Ca²⁺ ATPase; SERCA; Fasciclin II; Fas II; *Drosophila*; larval neuromuscular junction; NMJ

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 Ca²⁺. 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 have been shown to involve phylogenetically conserved molecules. In particular, the developmental plasticity of the *Drosophila* neuromuscular junction (NMJ), as it expands ~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 (neural cell adhesion molecule) and *Aplysia* ApCAM (*Aplysia* cell adhesion molecule) (Bailey et al., 1992; Mayford et al., 1992; Keshishian et al., 1996; Brunner and O’Kane, 1997), and requires functions of ERK, cAMP response element-binding protein (CREB), and AP1 (activator protein 1) (Davis et al., 1996; Koh et al., 2002; Sanyal et al., 2002).

Early events in the establishment of plasticity, however, 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

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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 either 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 (Barzilay 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* sarcoplasmic reticulum Ca^{2+} 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 downregulation of the synaptic cell adhesion molecule Fas II, and the induction of immediate-early genes (IEGs) Fos and c/EBP in *Drosophila*. To our knowledge, this 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 downregulation in *Drosophila*, the procedure that 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 (t.s.) *comatose* mutant, *comt*^{tp7} [isolated in Tata Institute of Fundamental Research (TIFR), Colaba, Mumbai, India], and a novel, 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 (TIFR, Colaba, Mumbai, India). A temperature-sensitive *paralytic* mutant, *para*^{ts1}, was provided by Barry Ganetzky (University of Wisconsin, Madison, WI). The temperature-sensitive mutant *Resistant to dieldrin*^{MD-RR} (*Rdl*^{MD-RR}) was obtained from the Bloomington *Drosophila* stock center. The wild-type strain, Canton-S (CS), was obtained from D. Brower (University of Arizona, Tucson, AZ).

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 per vial). For seizure induction, flies were heated for 4 min at 40°C in water baths and 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 were recovered at 35°C in water baths.

Electrophysiology. All recordings were made from the dorsal longitudinal flight muscles (DLMs) 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 min before recording. Both the ground and recording electrodes were heat-pulled glass microcapillaries (tip resistance, 3–5 MΩ) 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 after 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 was typically <2–3 min. Data were 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 and then using mechanical decapitation (vortexing) and separation with tissue isolation sieves. For Western blots using larvae, CNSs were dissected out of wandering third-instar larvae in Ca^{2+} -free HL-3 Ringer’s solution. Adult heads or larval CNSs were added to 2× 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 2× 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-dephosphorylated (DP)-ERK monoclonal antibody (1:2000) (Sigma, St. Louis, MO) and anti-β-tubulin (1:4000) (Zymed). Proteins were visualized with peroxidase-conjugated secondary antibodies (1:1000) and developed with an ECL chemiluminescence kit (Amersham Biosciences). Quantitation of band intensities was performed by scanning the developed BioMax autoradiographic films with a UMAX Astra 1220U scanner and then analyzing the densitometric signal of the resultant images with Metamorph (Universal Imaging) image analysis software.

Immunohistochemistry. The following antibodies were used for this study: mouse anti-DP-ERK (1:200; Sigma), rabbit anti-DSYT2 (1:200; H. Bellen, BCM, Houston, TX), rabbit anti-GluRII (1:200; Y. Kidokoro, Gunma School of Medicine, Maebashi, Japan), and rabbit anti-Fasciclin II (1:3000; V. Budnik, University of Massachusetts, Amherst, MA). Appropriate secondary antibodies conjugated to fluorescent Alexa dyes (Molecular Probes, Eugene OR) were used.

For the examination of the larval CNS, wandering third-instar were dissected in Ca^{2+} -free HL-3 Ringer’s solution (Stewart et al., 1994) and fixed in 3.5% Ca^{2+} -free paraformaldehyde for 3 hr on ice. The larval CNS was then incubated for 2 hr in block (PBS, 0.2% Triton X-100, 2% BSA, and 5% goat serum, pH 7.2) and subsequently incubated with primary antibody overnight at 4°C. The preparations were washed six times, shaking for 20 min each in block, and incubated for an additional 2 hr with secondary antibody. Larval CNSs were then washed six times in PBS-0.2% Triton X-100 and mounted in Vectashield (Vector Laboratories) 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 described previously (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 X-100, 2% BSA, and 5% goat serum for 1 hr. Incubations with primary antibody were performed overnight at 4°C, and secondary antibody incubations were performed for 1.5 hr at room temperature. For immunohistochemical analysis of the *Drosophila* NMJ under neural activity blockade conditions, third-instar animals of the appropriate genotype were treated to induce neural activity but were recovered at 35°C for 15–20 min before dissection. Animals were fixed for 10 min in Bouin’s fixative (standard nonalcoholic) and then washed eight times (10 min each) in wash (PBS, 0.2% Triton X-100, pH 7.2). Incubations with primary and secondary antibodies were performed identical to those 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 RT-PCR were performed as described previously in Sanyal et al. (2002). Briefly, for each treatment and genotype, RNA was harvested from 1- to 2-d-old male *Drosophila*. Separate RNA extractions were performed for each independent experiment (*n*). PCR products obtained from quantitative-PCR reactions were visualized after electrophoresis in 2% agarose and then stained with ethidium bromide. A one-cycle difference represents a twofold 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 nonpermissive temperatures. Many other mutants such as *sei*^{ts} and *Rdl*^{ts} (Jackson et al., 1984; French-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* (Magyar et al., 1995; Periz and Fortini, 1999; S. Sanyal, unpublished observations), showed sustained and particularly long-lasting convulsions (*comt*^{tp7}) or contractions (*Ca-P60A*^{Kum170}) after brief exposure to the appropriate nonpermissive temperature (Fig. 1). After a 4 min exposure to restrictive temperature (35°C), *comt* mutants demonstrate robust seizure-like behaviors lasting >1 hr at room temperature. *Ca-P60A*^{Kum170} mutants exposed to 40°C for 4 min show prolonged (18–48 hr) 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 (data not shown).

To examine the cellular basis for these behaviors, we performed intracellular recordings from adult DLM under permissive and restrictive temperatures (Fig. 2*a*). At normal (permissive) temperatures, 20°C, virtually no spontaneous DLM action potentials are observed via intracellular recordings before heat treatment. Wild-type animals exhibit a slight increase in spontaneous DLM firing after 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. 2*b*, top panel, No HS). After 4 min exposure to nonpermissive temperatures, both mutant *comt* and double-mutant *comt*; *Ca-P60A* flies displayed strong, spontaneous activity for at least 60 min (Fig. 2*b*, middle and bottom panels). Although *Ca-P60A* mutants alone did not display spontaneous activity close to

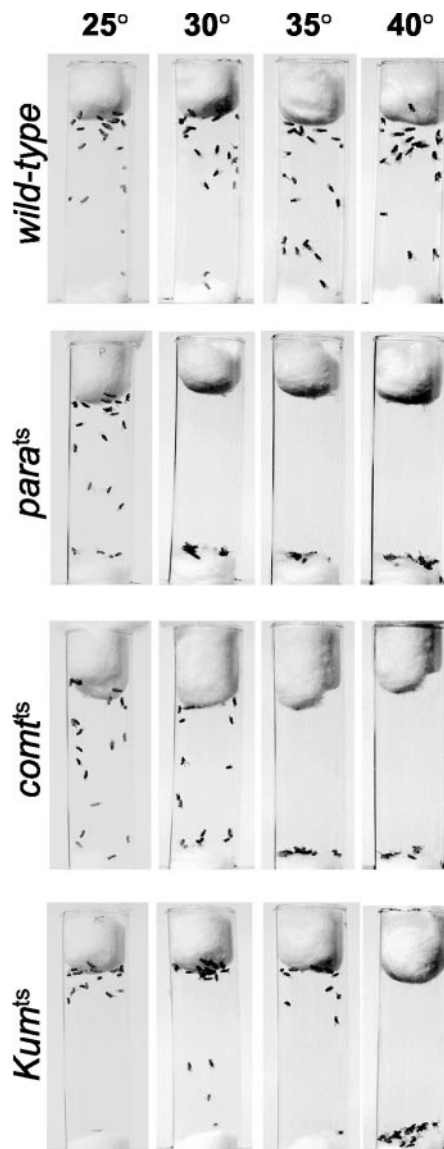


Figure 1. Temperature dependence of paralysis in *Drosophila* Na⁺ channel (*para*^{ts1}), NSF (*comt*^{tp7}), and SERCA (*CaP60A*^{Kum170}) mutants. *Drosophila* were exposed to different restrictive temperatures for 2 min and assayed for paralysis. Tight and distinct restrictive temperatures for *Drosophila* mutants shown in the figure are *para*^{ts1} (30°C), *comt*^{tp7} (35°C), and *Ca60A*^{Kum170} (40°C).

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. 2*c*). Our observation that activity is dependent on *para* function (Fig. 2*d*) 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-P60A*^{Kum170} animals, increased synaptic activity induced predominantly by the *comt* mutation

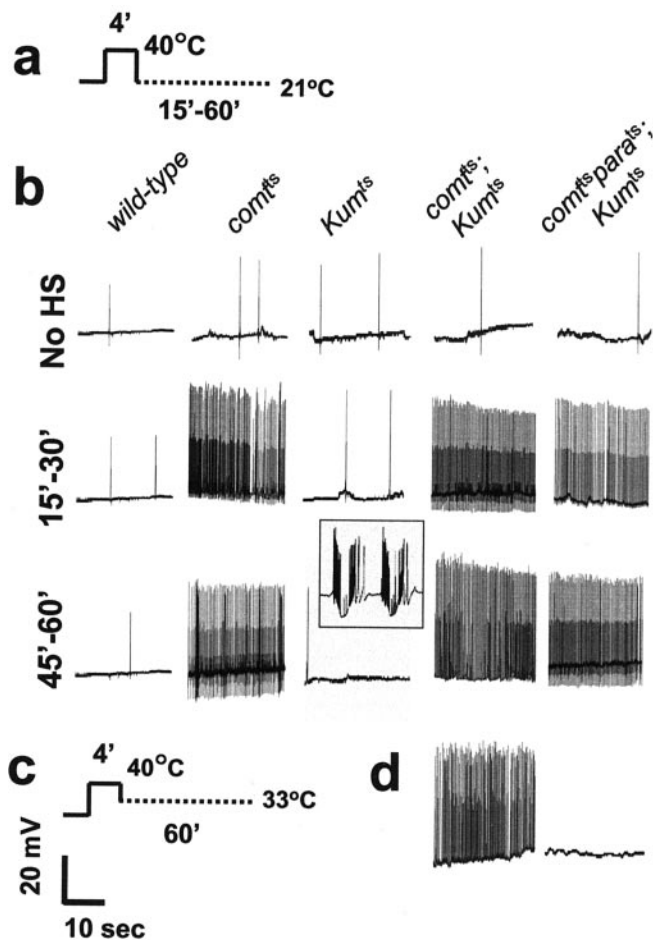


Figure 2. *Drosophila* NSF mutants exhibit spontaneous seizure-like activity after exposure to restrictive temperatures. *a, b*, After a simple heat treatment protocol (*a*), spontaneous seizure-like activity is induced and continues for at least 60 min in *comt*^{tp7}, *comt*^{tp7}; *CaP60A*^{Kum170},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

should be accompanied by substantially enhanced cytosolic Ca²⁺ signaling attributable to reduced SERCA-dependent calcium sequestration in *Ca-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 (>120 min) phosphorylation of 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-P60A*^{Kum170} double mutants would activate ERK signaling in the *Drosophila* nervous system (Fig. 3).

We exposed *comt*^{tp7}; *Ca-P60A*^{Kum170} mutant animals to a 4 min pulse of heat (40°C), and then let them recover at room temperature for 60 min. 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, DP-ERK (Gabay et al., 1997). Treated double-mutant animals showed a large increase in ERK activation after the treatment (Fig. 3*a*). DP-ERK levels in treated *comt*^{tp7}; *Ca-P60A*^{Kum170} animals were observed to increase ~2.5-fold ($p < 0.05$; $n = 6$) when compared with 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. 3*a*); a 1.5 fold increase in DP-ERK levels was observed in treated wild-type animals. 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. 3*a*). ERK activation is readily observed within 15 min (data not shown), peaks at 2 hr ($p < 0.01$; $n = 5$), and persists for at least 4 hr ($p < 0.01$; $n = 6$) (Fig. 3*b*). 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. However, CNS activity induced by *comt*^{tp7} alone does not increase DP-ERK without a concurrent block of calcium sequestration. One possibility is that activity recorded from the DLM in treated *comt*^{ts}, although robust, does not reflect cellular activities in the large majority of CNS neurons that contribute to the DP-ERK signal in our Western blot analysis.

The observed ERK activation after our treatment could have resulted either from 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 after 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 threefold increase in DP-ERK in treated *para*^{ts1} *comt*^{tp7}; *Ca-P60A*^{Kum170} ($p < 0.01$; $n = 6$) under activity-permissive conditions (Fig. 3*a*). However, under conditions nonpermissive 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 and less than half that of similarly treated *comt*^{tp7}; *Ca-P60A*^{Kum170} animals ($p < 0.05$; $n = 4$) (Fig. 3*c*). Thus, ERK activation observed after 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: (1) reduced turnover of ERK; (2) downregulation of phosphatase activity targeting DP-ERK (Brondello et al., 1999; Bhalla et al., 2002); or (3) upregulation of 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 (Fig. 3*a*). 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 was reduced substantially ($p < 0.01$; $n = 4$) when compared with control, sham-fed *Ca-P60A*^{Kum170} *Drosophila*. DP-MAPK levels in treated, MEK-inhibited *Ca-*

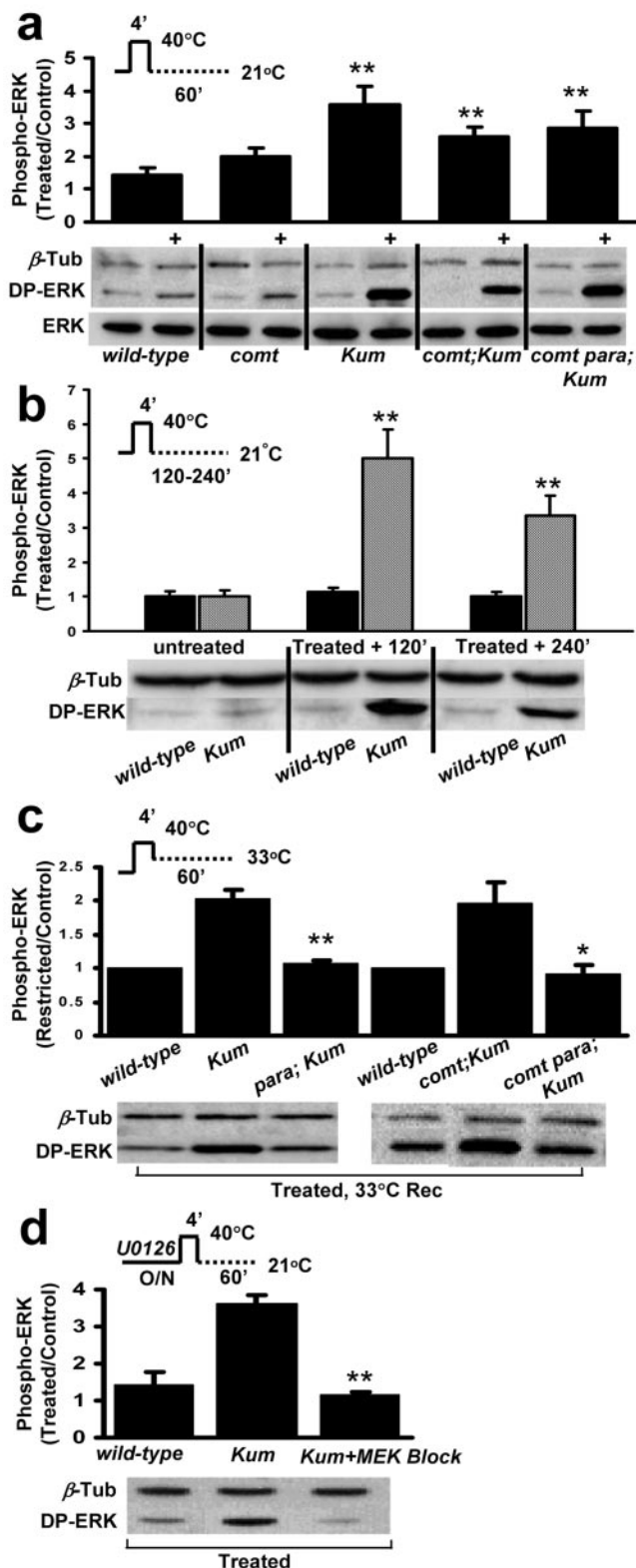


Figure 3. A brief temperature pulse induces persistent activation of ERK in *comt*^{tp7}; *CaP60A*^{Kum170} mutants through a neural activity, MEK, and *CaP60A*^{Kum170}-dependent pathway. *a*, After a brief 40°C pulse shown in the top trace, ERK activation in various strains is shown 60 min after treatment. The histogram indicates the ratio [treated (heated) vs (untreated) controls] of P-ERK immunoreactivity from densitometric scans of Western blot data shown in the bottom panels. When compared with 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 ; $p = 0.006$) (*b*). ERK activation persists

P60A^{Kum170} animals were the same as in wild-type controls (Fig. 3*d*). Results similar to those described above from analysis of adult *Drosophila* were also obtained from Western analyses of CNSs 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

Because they were 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 after seizure induction occurs in neurons. Because increased ERK activation in treated *comt*^{tp7}; *Ca-P60A*^{Kum170} appears to derive almost completely from the *Ca-P60A*^{Kum170} mutation (Fig. 2) and because single mutant work offers some technical advantages, we used *Ca-P60A*^{Kum170} alone for these studies.

We analyzed the brains of dissected third-instar larvae before and after 1 hr of seizure induction. As expected, a substantial increase in activated ERK could be detected in larval CNS neurons (Fig. 4*A–D*). In untreated control or *Ca-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 Figure 4*D*, a robust increase in DP-ERK immunoreactivity in the central brain regions and in ventral ganglia of treated *Ca-P60A*^{Kum170} larvae ($n = 6$) was observed when compared with either similarly treated wild-type controls (Fig. 4*C*) or unheated mutant animals (data not shown). That ERK activation occurs in neurons is indicated by colocalization of strong DP-ERK immunoreactivity with a marker for neuronal neuropil (neuronally driven synaptic green fluorescent protein) (Fig. 4*E, F*). As in adults, ERK activation in larval brains is completely blocked with treatment of larvae with U1026 before treatment (data 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 lobe regions (Hanson and Meinertzhagen, 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

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, which is 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.

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for >4 hr in treated *Ca60A*^{Kum170} animals. When compared with similarly treated wild-type animals at 2 hr (5.0 ± 0.82 ; $p = 0.005$) and 4 hr (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 after seizure induction requires MEK activity. Treated animals pre-fed the MEK-inhibitor drug U0126 show no increase in DP-ERK after treatment. *CaP60A*^{Kum170} (3.62 ± 0.21) U0126-*CaP60A*^{Kum170} (1.16 ± 0.07 ; $p = 0.0003$).

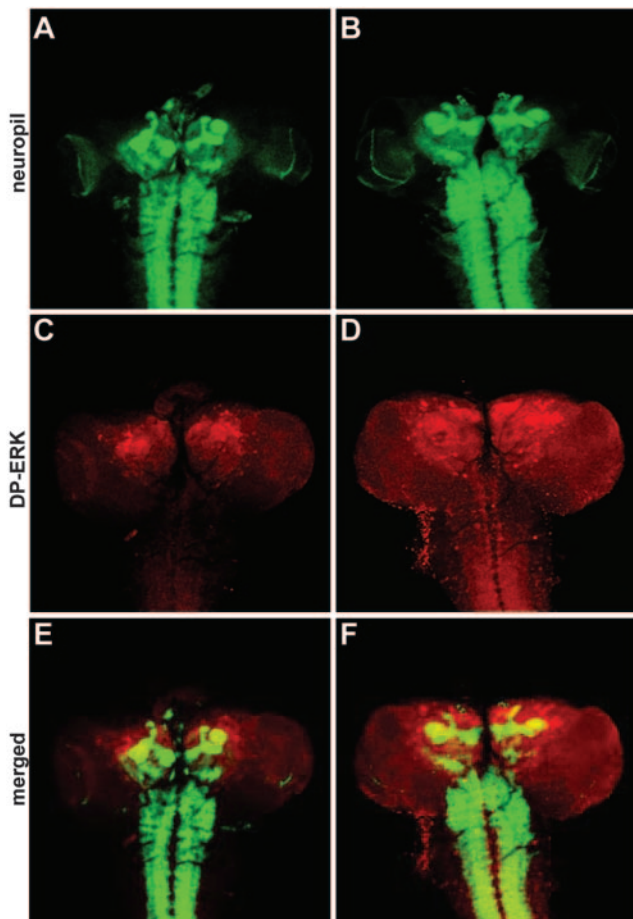


Figure 4. Activity-driven increase in DP-ERK activity in *Drosophila* larval CNS neurons as detected by confocal microscopy (A–F). Immunohistochemical evidence for ERK activation in the brain lobes and ventral ganglia of treated *CaP60A*^{Kum170} (D) but not wild-type (C) *Drosophila* larvae. A, B, Neurally driven synaptobrevin-green fluorescent protein (labels synaptic regions). C, D, DP-ERK (activated ERK) staining throughout the larval CNS. E, F, Merged images of treated wild-type and mutant larvae showing increased DP-ERK activity in both cells and neuropil of the larval CNS.

To examine this hypothesis and characterize the extent and location of synaptic ERK activity, we analyzed treated (1 hr) 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. 5*a*, A, C). Similarly, basal DP-ERK levels were also identical at NMJs of untreated wild-type and *Ca-P60A*^{Kum170} larvae (data not shown). Before treatment, activated ERK in boutons of the NMJ was localized primarily in small regions [termed “hot spots” by Koh et al. (2002)]. However, 40 min 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 (Figs. 5*a*, B, D, 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

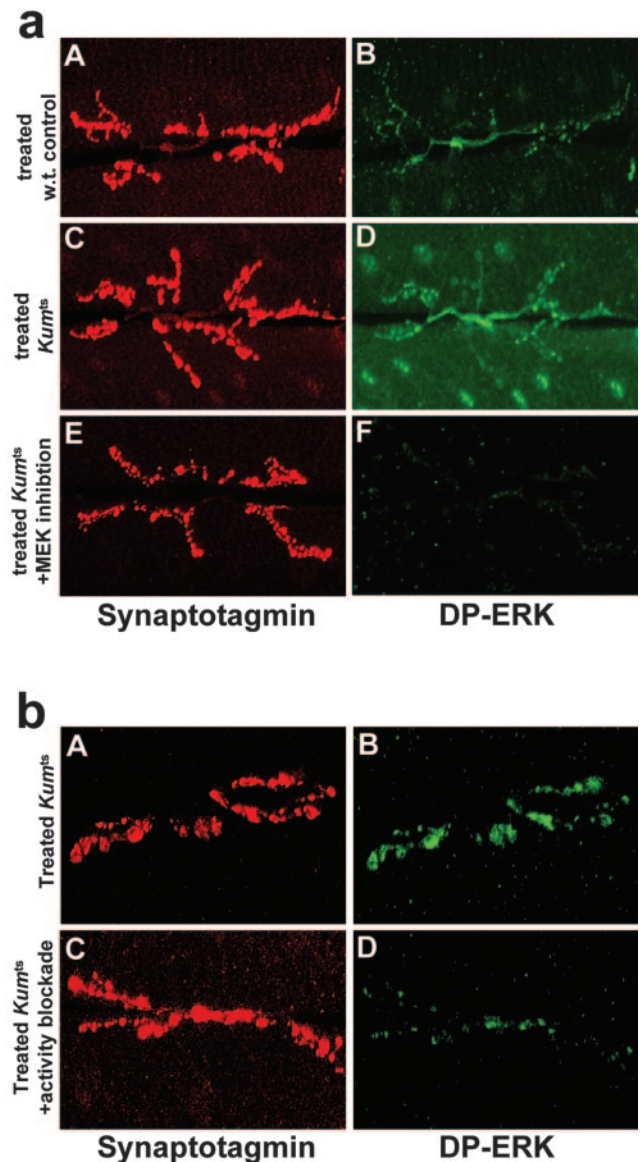


Figure 5. *a*, Activity- and MEK-dependent induction of cytosolic and nuclear DP-ERK 60 min after a 4 min temperature pulse to *CaP60A*^{Kum170} larvae. DP-ERK immunoreactivity at the larval neuromuscular junction (segment A2, muscles 6 and 7) is labeled with Synaptotagmin I to visualize presynaptic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

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 postsynaptic muscle, within 40 min of heating *Ca-P60A*^{Kum170} larvae (Fig. 5*a*, D).

As with adult heads and larval brains, presynaptic and postsynaptic ERK activation was MEK dependent, as evidenced by its complete block with U0126. Inhibition of MEK also re-

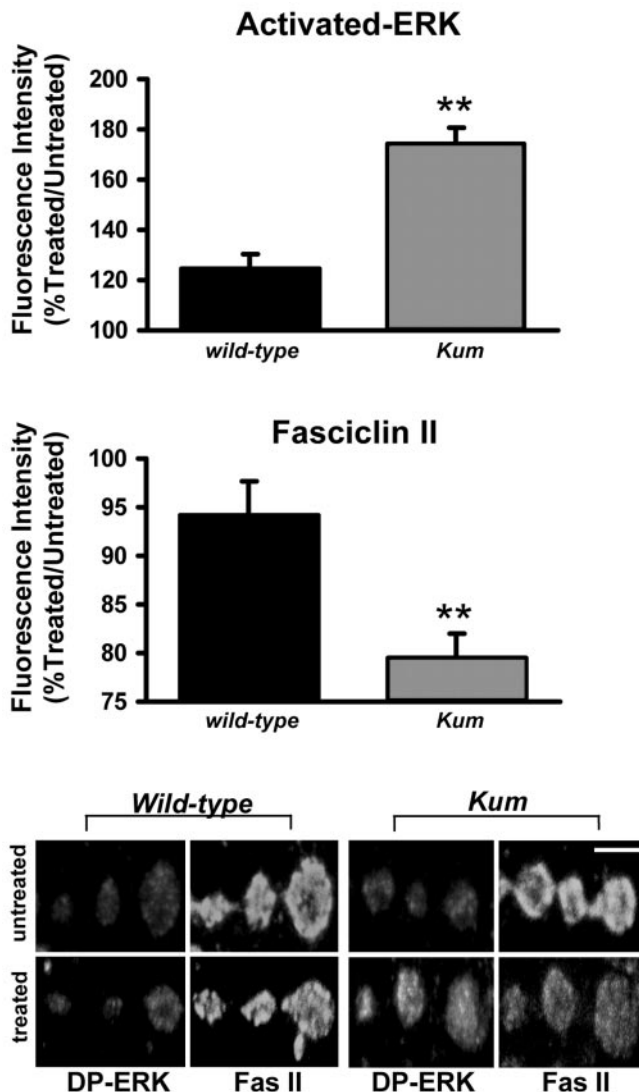


Figure 6. Increase in activated synaptic ERK is correlated with the rapidly reduced levels of Fasciclin II at the *Drosophila* larval NMJ. Fluorescence intensity for DP-ERK and Fas II was measured from treated and untreated *Ca60A^{Kum170}* animals and then compared after values were normalized to untreated animals ($p < 0.01$; $n = 64$; 4 independent experiments). Bouton images were taken from treated and untreated wild-type and *Ca60A^{Kum170}* animals. Scale bar, 5 μ m.

duced nuclear translocation of postsynaptic DP-ERK after treatment (Fig. 5*a, f*). 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 with similarly treated *Ca-P60A^{Kum170}* animals (Fig. 5*b, 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 reg-

ulate levels of the cell adhesion molecule ApCAM, which inhibits synaptic expansion (Mayford et al., 1992). The conservation of this signaling module has been inferred primarily 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 nonpermissive temperatures could result in rapid, quantifiable reduction of Fas II. To test this idea, we quantified synaptic Fas II and DP-ERK levels in *Ca-P60A^{Kum170}* larvae after seizure induction (Fig. 6). We analyzed preparations 100 min 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 that of untreated animals ($p < 0.01$; $n = 64$; four separate experiments); Fas II levels at the same treated synapses were reduced to levels $\sim 78 \pm 4\%$ 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 <100 min of ERK activation substantially tightens the temporal link between ERK activation and Fas II downregulation 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 in which 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 IEGs such as Fos and *c/EBP* (CCAAT element binding protein) (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*, 1 hr after seizure induction (Fig. 7). Using real-time quantitative RT-PCR analysis, we found that DFos mRNA levels in adult head increased ~2.7-fold compared with treated wild-type animals ($p < 0.05$; $n = 11$), and Dm-*c/EBP* levels increased ~2.1-fold ($p < 0.05$; $n = 10$) within 1 hr 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 after brief inactivation of *comt* and *CaP60A* function in *Drosophila* neurons, activity-dependent signaling pathways achieve qualitative and quantitative features required

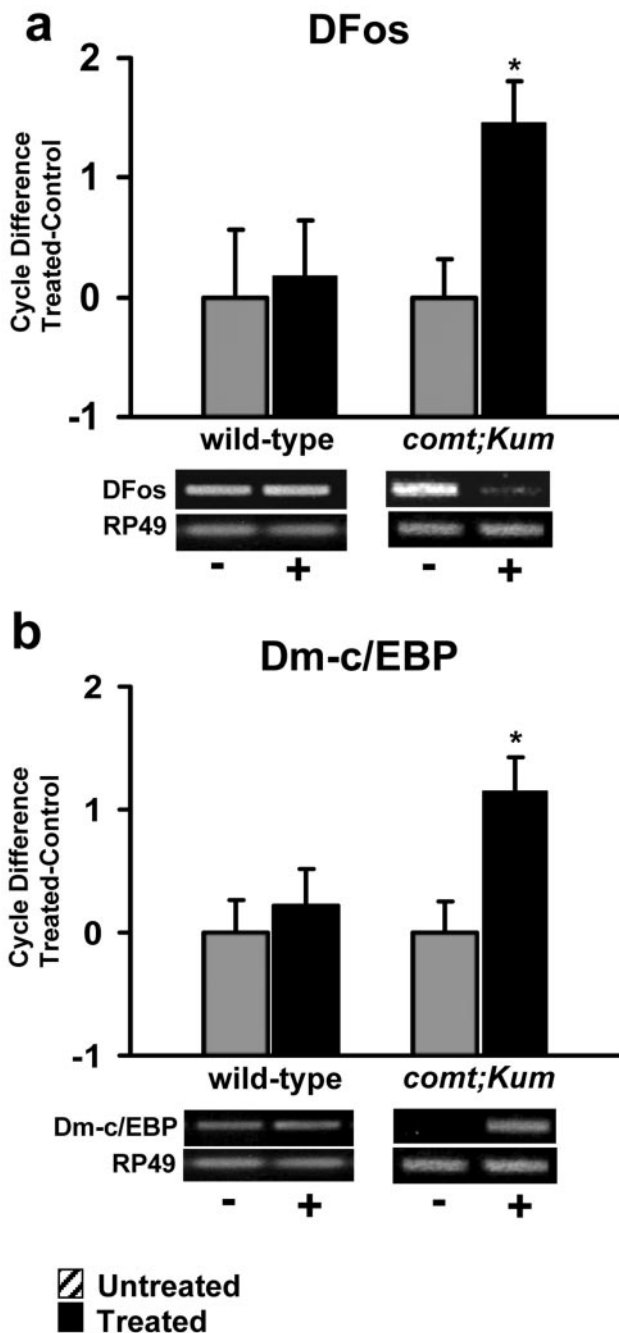


Figure 7. Induction of *Drosophila* homologs of the immediate-early genes Fos (*kayak*) and c/EBP (*sibo*) after seizure induction and ERK activation in *Drosophila*. *a*, In mRNA extracted from entire fly heads, DFos is increased 2.7-fold in treated *comt*^{tp7}; *CaP60A*^{Kum170} *Drosophila* when compared with 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 with 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.

to stimulate at least the early stages of plasticity-associated neuronal gene expression.

Discussion

Through a new characterization of *Drosophila* 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

nonpermissive temperature causes persistent activation of the neuronal ERK/MAP kinase cascade through prolonged upstream MEK signaling. Using this procedure, we observed 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 observed transcriptional upregulation of DFos and Dm-c/EBP, *Drosophila* homologs of well described neural activity-regulated genes involved in long-term plasticity.

The results summarized above make three contributions. First, by acutely inducing and analyzing synaptic signaling pathways in insects, the results 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*, the 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, the results 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 the 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 nonpermissive 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) indicates either that NSF has neuronal functions of which we are not yet aware or possibly that this isoform of *Drosophila* NSF 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 the role of SERCA 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 either from enhanced synaptic activity or from altered sequestration, or both, 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

Table 1. Fold induction of four genes in *Drosophila* head, 56 min after a 4 min exposure to 40°C

	Gene	Mean fold gene expression (+ vs –)	SE	CV	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
<i>comt; Ca60A</i>	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

been performed primarily in mollusks and mammals, which in evolutionary terms are equally distant from arthropods (Fields et al., 1997; Martin et al., 1997; Vanhoutte et al., 1999; Dolmetsch et al., 2001). Although synaptic signaling underlying long-term plasticity is poorly studied in insects, a rich tradition of behavioral studies in various 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 is 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 demonstrated previously the broad conservation of transcriptional regulator function between *Drosophila* and mammals (Bailey et al., 1996). At a cell biological level, the conservation of signaling pathways upstream of transcriptional regulators such as AP1 and CREB has been inferred mostly 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 3–5 d, have been insufficient to demonstrate (1) that activation of these signaling components in *Drosophila* can indeed be driven synaptic activity and (2) the sequential or temporal relationship between neural activity, ERK phosphorylation, Fas II downregulation, 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, identification of and understanding the signaling components that determine not only ERK

activation but also the duration and subcellular localization of the ERK signal are 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 the established functions of ERK 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 the 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 other channel modulations occur *in vivo* in response to synaptic stimulation. Such questions may be answered 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 the 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 the levels or phosphorylation states of which are rapidly altered by synaptic activity *in vivo*. (3) At a genomic level, microarray and SAGE analyses (Brennan 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 may be initiated in response to the stim-

ulation procedures that 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 that 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.

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