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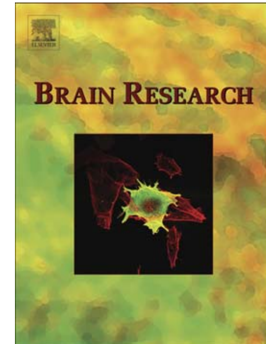

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PII: S0006-8993(10)00439-7
DOI: doi: 10.1016/j.brainres.2010.02.061
Reference: BRES 40137

To appear in: *Brain Research*

Accepted date: 22 February 2010

Please cite this article as: Amanda Freeman, Mallory Bowers, Alysia Vrailas Mortimer, Christina Timmerman, Stephanie Roux, Mani Ramaswami, Subhabrata Sanyal, A new genetic model of activity-induced Ras signaling dependent pre-synaptic plasticity in Drosophila, *Brain Research* (2010), doi: 10.1016/j.brainres.2010.02.061

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A new genetic model of activity-induced Ras signaling dependent pre-synaptic plasticity in Drosophila

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Number of pages: 45
Number of figures and tables: 6

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ABSTRACT

Techniques to induce activity-dependent neuronal plasticity *in vivo* allow the underlying signaling pathways to be studied in their biological context. Here, we demonstrate activity-induced plasticity at neuromuscular synapses of Drosophila double mutant for *comatose* (an NSF mutant) and *Kum* (a SERCA mutant), and present an analysis of the underlying signaling pathways. *comt; Kum* (CK) double mutants exhibit increased locomotor activity under normal culture conditions, concomitant with a larger neuromuscular junction synapse and stably elevated evoked transmitter release. The observed enhancements of synaptic size and transmitter release in CK mutants are completely abrogated by: a) reduced activity of motor neurons; b) attenuation of the Ras/ERK signaling cascade; or c) inhibition of the transcription factors Fos and CREB. all of which restrict synaptic properties to near wild type levels. Together, these results document neural activity-dependent plasticity of motor synapses in CK animals that requires Ras/ERK signaling and normal transcriptional activity of Fos and CREB. Further, novel *in vivo* reporters of neuronal Ras activation and Fos transcription also confirm increased signaling through a Ras/AP-1 pathway in motor neurons of CK animals, consistent with results from our genetic experiments. Thus, this study: a) provides a robust system in which to study activity-induced synaptic plasticity *in vivo*; b) establishes a causal link between neural activity, Ras signaling, transcriptional regulation and pre-synaptic plasticity in glutamatergic motor neurons of Drosophila larvae; and c) presents novel, genetically encoded reporters for Ras and AP-1 dependent signaling pathways in Drosophila.

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Classification Terms

Section: Cellular and Molecular Biology of the Nervous System

Keywords: Drosophila, neuron, plasticity, Ras, Fos, synapse

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INTRODUCTION

Although meaningful changes in neural activity are widely considered to drive neuronal plasticity during learning, very few model synapses allow plasticity pathways to be analyzed *in vivo*. A majority of studies that assay activity-dependent plasticity are performed on cultured neurons, where techniques ranging from elevation of extra-cellular potassium to adding glutamate receptor agonists have been used successfully. In Drosophila, a few "excitability" and signaling mutants have been described that display increased synapse growth and transmitter release (Davis et al., 1996; Keshishian et al., 1996; Rohrbough et al., 2003; Schuster et al., 1996). Most notable examples are a double mutant combination of *eag* and *Shaker* potassium channel mutants (Budnik et al., 1990; Zhong et al., 1992) or "seizure" mutants that approximate conditions of increased neural activity leading to gene expression patterns predicted to mediate changes in synaptic strength and connectivity (Guan et al., 2005). However, plasticity phenotypes in these models are either highly sensitive to genetic modifiers or poorly understood in terms of the participation of key synaptic and nuclear signaling factors.

In order to simulate a generally conserved process of activity-induced synaptic plasticity, we identified and characterized a new robust model of activity-dependent plasticity in Drosophila that also engages a set of core plasticity-related signaling modules. Aiming to maximize the likelihood that the observed plasticity was induced by activity, rather than solely through poorly defined developmental processes (Sigrist et al., 2003; Zhong and Wu, 2004), we established a specific set of criteria that the new model had to fulfill. These included: *i)* these animals should display altered synaptic growth

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and transmitter release, *ii)* these changes must be activity-dependent, i.e. they should be abolished if neural activity is attenuated, *iii)* key signaling cascades such as the Ras/ERK pathway should be operational and required for observed synaptic changes, and *iv)* ideally, these long-term changes should depend on the activity of key transcription factors such as CREB and Fos.

Based on our earlier observations of increased neural activity and acute MAPK phosphorylation in the nervous system of a combination of *comatose* and *Kum* mutants (called CK henceforth), we hypothesized that these animals could fit these criteria (Hoeffer et al., 2003). In the current study we report that in CK mutants there is a substantial increase in growth and transmitter release at the neuro-muscular junction that is abolished through chronic neuronal hyperpolarization. Further, a canonical Ras/MAPK pathway and the transcription factors Fos and CREB are required for observed changes in synapse size and strength. Consistent with a model in which the Ras/ERK pathway acts to stimulate Fos transcription in motor neurons, novel genetically encoded reporters of Ras activation and Fos transcription indicate robustly increased Ras signaling and Fos transcription in *comt; Kum* motor neurons. Beyond development of a new, paradigmatic model for activity-dependent plasticity in a genetically amenable model organism, our results document a signaling pathway from neural activity to transcription and illuminate contextual roles for Ras in long-term plasticity.

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RESULTS

***comt; Kum* double mutants are hyperactive and display activity-dependent synaptic growth and transmitter release.**

We had previously observed increased neuronal ERK phosphorylation in double mutant combinations of *comatose* and *Ca-P60A* (Hoeffer et al., 2003). To test if this is an outcome of increased motor activity in these mutants locomotor activity was measured in adult CK flies using the Drosophila Activity Monitor (DAM) (Zordan et al., 2007). 3-5 day old adult flies were introduced individually in glass vials and fly activity was monitored over a 3 day period under 12 hour light/dark rearing conditions. Figure 1A shows that the total number of beam breaks in the CK group were on an average twice that of control animals (inset). Increased activity was seen throughout a 24 hour period but continued to follow a circadian pattern (graph is a cumulative average of three days of data, see materials and methods for details). These results suggest that under normal rearing conditions CK double mutants display increased locomotor activity.

Next, we hypothesized that increased motor activity in CK mutants might lead to increased synaptic growth and transmitter release. Indeed, there is appreciable synaptic overgrowth at these larval neuromuscular junctions as defined by increased number of synaptic boutons at this synapse (around 150% of controls as measured by counting synaptotagmin labeled pre-synaptic boutons on muscles 6 and 7 in abdominal segment A2; Figure 1B) (Sanyal et al., 2002). For instance, on average, actual bouton counts (including both type Ib and Is boutons) for control Canton-S synapses is 131, while CK mutant synapses have on average 169 boutons. These overgrown synapses also release

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more neurotransmitter per action potential since their average EJC (evoked junctional current) amplitudes are similarly increased when compared to controls (as measured with two-electrode voltage clamp recordings from muscle 6 in abdominal segment 2; Figure 1B; for instance average EJC amplitude for Canton-S is -97 nA, while it is -123 nA for CK animals; see supplementary table 1 for raw bouton counts and EJC amplitudes across genotypes). Since mEJC amplitudes remain unchanged across genotypes, increased EJC amplitudes suggest pre-synaptic mechanisms that lead to stable elevation in transmitter release (data not shown; these measurements are acquired in 1 mM extra-cellular Calcium concentrations; we observe enhanced transmitter release at most commonly used Ca concentrations. However, at lower concentrations of extra-cellular Calcium, these differences in EJC amplitude are likely to be more pronounced). Interestingly, both single mutants individually show elevated transmitter release but no change in synapse size as compared to controls. Together, these observations suggest synergistic mechanisms that change both synapse size (the average number of synaptic boutons) and strength (the average amount of neurotransmitter released per action potential) in CK mutants. Similar synergy has been observed previously with *eag* and *Shaker* mutants and dominant-negative transgenics (Mosca et al., 2005; Zhong et al., 1992). To verify that these synaptic changes are indeed due to increased neural activity, we attempted to chronically hyperpolarize motor neurons (MNs) in a CK background through the expression of an inward rectifier potassium channel ($K_{ir}2.1$) or an open Shaker type mutant channel (termed EKO for electronic knock-out) (Baines et al., 2001; Hartwig et al., 2008; Johnson and Bennett, 2008; White et al., 2001). Expression of $K_{ir}2.1$ in motor neurons using C380-GAL4 (a *futsch* enhancer trap line) caused widespread lethality (Budnik et al.,

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1996; Sanyal et al., 2003; Sanyal, 2009). However, measurement of synapse size and strength in observably healthy EKO expressing animals showed clearly that synaptic phenotypes of CK animals were completely abolished. Both in controls (EKO expression in a wild type background) and CK mutants, expression of EKO constrained synapse size and strength to very similar values (marginally less than wild type animals; Figure 1B and C; synapse size: CK = 153% +/-8; CK+EKO = 70% +/-5; EKO = 67% +/-4. Synapse strength: CK = 168% +/-15; CK+EKO = 70% +/-5; EKO = 70% +/-5). Since EKO is known to hyperpolarize neurons making them less excitable (Hartwig et al., 2008; White et al., 2001), these results, in conjunction with other data presented here, support the premise that synaptic plasticity seen in CK is due to chronic changes in neural activity.

The Ras/MAPK signaling pathway is activated in *comt; Kum* animals and is required for synaptic plasticity.

CK mutants show persistent neuronal ERK phosphorylation upon seizure induction (Hoeffer et al., 2003). We speculated that synaptic changes in CK are driven by increased ERK signaling even under non-seizure inducing conditions, and neural activity might trigger a signaling cascade that impinges on ERK. Since the Ras pathway is known to activate ERK in neurons and has been implicated previously in neuronal plasticity, we asked if synaptic plasticity in CK mutants requires Ras signaling (Arendt et al., 2004; Chen et al., 1998; Finkbeiner and Greenberg, 1996; Kim et al., 1998; Koh et al., 2002; Kushner et al., 2005; Shalin et al., 2006). To directly visualize Ras activation in the nervous system, we adapted a reporter based strategy used previously in yeast and mammalian cultured neurons (Bivona and Philips, 2005; de Rooij and Bos, 1997; Harvey

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et al., 2008; Walker and Lockyer, 2004). We cloned the highly conserved Ras binding domain (RBD) and the cysteine-rich domain (CRD) of Drosophila Raf (*polehole*) fused to a FLAG epitope tag upstream of UAS sites in the Gateway vector pTWF (Tim Murphy: http://www.ciwemb.edu/labs/murphy/Gateway%20vectors.html). This fusion protein, called RBD::FLAG, is expected to bind to activated Ras *in vivo* such that changes in Ras activation can be visualized as altered cellular distribution of FLAG (for example, increased membrane localization of Raf would reflect Ras activation, since membrane bound activated Ras will recruit Raf from the cytosol; Figure 2A) (Bivona and Philips, 2005). As shown in Figure 2B, Western Blot analysis detects the presence of a band of the expected fusion protein size (Raf-RBD-CRD+FLAG) of approximately 18 KD. This reporter, called RBD::FLAG henceforth, faithfully reflects Ras activation in tissues where the pattern of Ras signaling has been documented previously such as the eye imaginal disc (Voas and Rebay, 2004). In the developing eye disc, expression of RBD::FLAG in differentiated neurons using the $elav^{C155}$-GAL4 driver line reveals stereotypic membrane bound FLAG staining in ommatidial clusters (Figure 2E, F and G). Upon co-expression of a form of activated Ras that preferentially targets ERK MAP kinase (UAS-Ras1V12S35), more intense FLAG staining is noticed at the tips of ommatidial clusters, sites of high receptor-tyrosine kinase signaling and Ras activation (Figure 2H, I and J; arrows in inset). Expectedly, RBD::FLAG also functions as a dominant-negative for Ras signaling in the developing eye. Thus, small and rough eyes are observed upon expression of RBG::FLAG in a wild type background (Figure 2 C and D). In sum, these results demonstrate the validity and efficacy of this transgenic reporter protein to detect Ras activity in Drosophila *in vivo*.

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When expressed in neurons using the GAL4-UAS system, the Raf-RBD+CRD::FLAG protein should be predominantly cytosolic unless it binds to activated Ras on the plasma membrane (or intracellular compartments, (Chiu et al., 2002)). Intra-cellular distribution of this reporter in Drosophila motor neuron soma, and thus active Ras signaling, can be determined using antibodies to FLAG. Since Drosophila motor neuron cell bodies are relatively large, differences in membrane localization of RBD::FLAG should be easy to detect with conventional confocal microscopy. Figure 3A and D (inset) show that in wild type animals, RBD::FLAG localizes partially to neuronal membranes (dorsal medial motor neurons are identified through anti-Zfh-1 staining). Consistent with our prediction, membrane bound FLAG staining is significantly enhanced through co-expression of activated Ras (Figure 3B and E; inset). Similarly, in CK animals, increased membrane bound FLAG staining is readily apparent in motor neuron soma as compared to controls (Figure 3C, F; inset). These results suggest that in CK animals, larval motor neurons display consistently elevated Ras signaling and to our knowledge, present the first demonstration of an *in vivo* reporter that can detect changes in endogenous Ras activation.

Next, we tested the functional relevance of Ras activation for synaptic plasticity in CK animals. Upon inhibiting motor neuron Ras signaling in a CK background, using a mutant transgene of Ras that has lowered affinity for GTP (UAS-Ras[N17]) (Bergmann et al., 1998), we observed that synaptic changes, both size and strength, in CK were completely attenuated (Figure 3G). Identical results were also obtained upon Raf inhibition (using a UAS-Raf[DN]) in CK animals. Importantly, expression of either dominant-negative Ras or Raf does not affect normal synaptic development, but only

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limit synaptic plasticity in a CK background. Together, these two results support the idea that the Ras/Raf pathway is necessary for synaptic plasticity in CK. Consistent with these findings, expression of a mutant activated form of Ras in motor neurons has been shown previously to cause synapse expansion at the NMJ (Koh et al., 2002). To verify and extend these results, we expressed the same mutant form of Drosophila Ras that is predicted to selectively activate ERK signaling, in motor neurons and found that expression of Ras1V12S35 in motor neurons led to synapse expansion (Figure 4G). Additionally, we also observed a concomitant increase in pre-synaptic transmitter release. These results demonstrate that Ras signaling is sufficient to cause changes in both synapse size and strength at this neuromuscular synapse.

Synaptic plasticity in *comt; Kum* mutants requires the AP-1 transcription factor.

ERK signaling downstream of calcium entry leads to nuclear translocation of ERK and resultant transcriptional activation in neurons (Bading et al., 1993; Feske et al., 2001; Fields et al., 1997; Impey et al., 1998; Martin et al., 1997; Patterson et al., 2001; Roberson et al., 1999; Shobe et al., 2009; Sweatt, 2001; Sweatt, 2004; Thomas and Huganir, 2004). To test if ERK nuclear entry occurs in the larval ventral nerve chord (VNC), we used a transgene that expresses a fusion protein comprising Drosophila ERK, the DNA binding domain of GAL4 and the strong transactivating domain of VP16 (Kumar et al., 2003). When expressed in an animal carrying a UAS-nls-GFP reporter, GFP expression is restricted to cells in which phosphorylated ERK enters the nucleus and the GAL4::VP16 drives transcription of GFP. As shown in Figure 4A, B and C, there is extensive ERK nuclear entry into neurons in the larval brain, and hence GFP expression

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in the VNC and the brain lobes (especially in Kenyon Cells of the Mushroom Body and motor neurons). That nuclear entry of ERK occurs in motor neurons is further established by the observation (Figure 4D, E and F) that several GFP positive nuclei are dually labeled by Elav and Zfh-1 (a marker of VNC motor neurons) (Lee and Lundell, 2007). Prompted by these results, we then asked if two key plasticity-related transcription factors, CREB and Fos are required in CK neurons for synaptic plasticity. Genetic inhibition of either CREB (using a CREB2b transgene, (Davis et al., 1996; Perazzona et al., 2004; Sanyal et al., 2002; Yin et al., 1994)) or Fos (using a bZip only Fos transgene, UAS-Fbz, that has been shown in several studies to function as a Fos dominant-negative protein, (Eresh et al., 1997; Sanyal et al., 2002; Sanyal et al., 2003) inhibited synaptic plasticity in CK neurons and prevented changes in both synapse size and transmitter release (synapse size compared to control: CK = 122%, CK-Fbz = 78%, CK-CREB2b = 75%; synapse strength compared to control: CK = 130%, CK-Fbz = 55%; CK-CREB2b = 70%; Figure 4G). In fact, Fos or CREB inhibition in a CK background resulted in synapses that had identical size and strength when compared to Fos or CREB inhibition in a wild type background (Synapse size: Fbz = 66%, CREB2b = 65%; Synapse strength: Fbz = 79%, CREB2b = 68%). These results provide strong genetic evidence to suggest that Fos and CREB function downstream to activity-dependent signaling mechanisms that are activated in CK neurons. While results with CREB are consistent with previous studies, a necessity for Fos in CK dependent plasticity was also predicted by observations of Fos upregulation following seizure induction in CK animals (Hoeffer et al., 2003).

If CK animals activate neuronal Ras signaling and also require Fos and CREB for plasticity, then we should detect a functional necessity for CREB and Fos downstream of

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Ras signaling. To test this idea, we inhibited Fos or CREB in neurons that express an activated form of Ras that is known to stimulate the ERK pathway (previous section, and (Koh et al., 2002)). Either Fos or CREB inhibition in this background using the UAS-CREB2b or UAS-Fbz transgene completely inhibits Ras dependent synaptic changes and no increase in synaptic growth or transmitter release is observed (synapse size as compared to controls: Ras = 131%. Ras-Fbz = 79%, Ras-CREB2b = 86%; synapse strength as compared to controls: Ras = 139%, Ras-Fbz = 48%, Ras-CREB2b = 74%; Figure 4G). Again, Fos or CREB inhibition in a Ras over-expressing or control background produces identical synaptic phenotypes, confirming that Fos and CREB function downstream to Ras signaling (synapse size: Fbz = 76%, CREB2b = 81%; synapse strength: Fbz = 42%, CREB2b = 81%). Interestingly, the interaction between Ras and CREB was found to be specific to the context of neuronal plasticity. Our experiments in the fly eye, for example, showed that a Ras-dependent overgrowth phenotype described previously is not suppressed by either Fos or CREB inhibition (Figure 4H). This apparent context specificity is not due to the relatively inefficient inhibition of these transcription factors in the eye, as evidenced by the observation that Fbz expression in a wild-type eye produces small malformed eyes (Cerrato et al., 2006; Franciscovich et al., 2008; Hyun et al., 2006). Together, these observations collectively support a model in which plasticity of motor synapses in CK animals requires Ras signaling upstream of the transcription factors Fos and CREB (Figure 4I).

***In vivo* reporters document stimulation of neuronal Fos transcription in *comt; Kum* mutants.**

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While CREB function downstream of Ras/ERK in plasticity has been investigated extensively, the involvement of Fos in this pathway is less well understood (Curtis and Finkbeiner, 1999; Fields et al., 1997; Hoeffer et al., 2003; Sweatt, 2004). Potential mechanisms that increase Fos activity in neurons are suggested by the long known transcriptional regulation of Fos in various models (Fields et al., 1997; Ghosh et al., 1994; Hughes and Dragunow, 1995; Morgan and Linnoila, 1991; Nestler et al., 2001; Sheng et al., 1990). Significantly, in a previous study we had also demonstrated through quantitative RT-PCR that seizure induction in CK animals led to an upregulation of Fos mRNA in adult Drosophila brains (Hoeffer et al., 2003). To directly detect Fos transcription *in vivo*, we constructed a transgenic reporter of Fos transcription based on the prediction that a 5 Kb region of genomic DNA upstream of the Drosophila Fos gene (*kayak*) might regulate Fos transcription (Materials and Methods). We cloned this DNA upstream of an nls-tagged GFP coding region in the vector p-Stinger (Barolo et al., 2000). GFP expression and nuclear accumulation should report both basal and stimulated Fos expression. Using a similar strategy, we also cloned the same putative enhancer region upstream of the GAL4 gene in the vector pPTGAL (Sharma et al., 2002). We first tested if this reporter is expressed in tissues where previous studies have documented the presence of the Fos protein, such as in the leading edge cells of Drosophila embryos (Figure 5A, B and C, arrows mark leading edge in embryos at three different developmental stages) (Riesgo-Escovar and Hafen, 1997; Zeitlinger et al., 1997). We then imaged expression from this reporter as compared to anti-Elav staining (to label all post-mitotic nuclei) in the larval CNS and found extensive co-localization including the identifiable dorsal medial motor neuron clusters in the VNC (Figures 4D-F) (Sanyal et

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al., 2003; Sanyal, 2009). To further confirm that this reporter accurately reflects the *kayak* (dFos) expression pattern in the larval brain, we co-stained a Fos-reporter line with antibodies against Drosophila Fos (Zeitlinger et al., 1997). As shown in Figure 5G-I, nls-lacZ expression from Fos-GAL4 reporter largely co-localizes with anti-Fos staining (inset shows close-up of dorsal medial motor neurons). To the best of our knowledge, this is the first time that Fos expression has been studied in Drosophila brains using either an anti-Fos antibody or an *in vivo* reporter. These observations suggest that a Fos transcriptional reporter faithfully detects Fos expression in Drosophila tissues and significantly augments our ability to detect dynamic changes in Fos transcription *in vivo*.

To determine if Fos transcription is enhanced in CK larval motor neurons, we visualized reporter expression in wild type and CK backgrounds in the larval ventral nerve chord (VNC). In wild type VNC, Fos expression is detectable in and co-localizes with Elav staining in both the medial and lateral MNs. Importantly, representative images in Figure 6A-H also show that there is increased GFP expression from the Fos reporter in CK nerve chords as compared to relevant genetic controls. We further predicted that seizure induction in CK animals might induce Fos transcription consistent with previous RT-PCR results (Hoeffer et al., 2003). This is indeed true (Figure 6I-P) and Fos transcription is strikingly induced following mild heat pulse induced seizures in CK animals as compared to similarly heated wild type controls (40°C for 3 minutes followed by 60 minutes of recovery at room temperature). These results importantly document the pattern of endogenous Fos expression in the larval VNC and also show its modulation by neural activity in CK animals.

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DISCUSSION

In this study we describe a new model for activity-dependent pre-synaptic plasticity in Drosophila. In the double mutant combination of *comt* and *Kum*, sustained elevation of neural activity (potentially including seizure-like motor neuron firing at under normal rearing conditions) results in the expansion of motor synapses with concomitant increase in transmitter release. These synaptic changes are mediated by the Ras/ERK signaling cascade and the activity of at least two key transcription factors, CREB and Fos. *In vivo* reporter assays also directly demonstrate Ras activation and enhanced transcription of Fos in the nervous system. To our knowledge CK is the only genetic model of synaptic plasticity in Drosophila in which pre-synaptic plasticity has been correlated with the Ras/ERK signaling cascade. This result is especially relevant given the wide conservation of the Ras/ERK signaling cascade in plasticity and recent demonstrations of the involvement of this signaling cascade in learning behavior in flies (Godenschwege et al., 2004; Moressis et al., 2009). Significant insights into Ras mediated regulation of both synapse growth and transmitter release are also presented.

Comparison with other methods to alter neural activity plasticity in Drosophila

Non-invasive methods to manipulate neural activity in select neurons continue to be an important experimental target in plasticity research. In Drosophila, combinations of the *eag* and *Shaker* potassium channel mutants have long been used to chronically alter neural activity and study downstream cellular events (Budnik et al., 1990; Mosca et al., 2005; Schuster et al., 1996). In recent years, transgenic expression of modified Shaker

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channels have also been generated and used to alter excitability in both neurons and muscles (Hartwig et al., 2008; Mosca et al., 2005; White et al., 2001). However, we opted to develop the CK model of activity-dependent plasticity since in our hands we found that synaptic changes in CK were consistently more robust than *eag Sh* and core plasticity related signaling components were activated in a predictable manner in CK mutants (Hoeffer et al., 2003). Another advantage with CK is the option of acutely inducing seizures as has been used to identify activity-regulated genes (Guan et al., 2005). CK thus combines advantages of both *eag Sh* and seizure mutants, and as is shown in this study, leads to an activity-dependent increase in synaptic size and transmitter release. We believe that this model will prove highly beneficial to the large community of researchers who investigate synaptic plasticity in Drosophila. The utility of more recent techniques (such as the ChannelRhodopsin or the newly reported temperature sensitive TrpA1 channel transgenes) to induce neural activity dependent synaptic plasticity at Drosophila motor synapses has not been tested yet and it will be interesting to see if these afford greater experimental flexibility in the future (Hamada et al., 2008; Hornstein et al., 2009; Schroll et al., 2006).

Ras signaling in synaptic plasticity

Signal transduction through the Ras cascade has been shown to affect both dendritic and pre-synaptic plasticity in invertebrate and vertebrate model systems (Alpar et al., 2008; Arendt et al., 2004; Finkbeiner and Greenberg, 1996; Koh et al., 2002; Komiyama et al., 2002; Krapivinsky et al., 2003; Kushner et al., 2005; Rumbaugh et al., 2006; Seeger et al., 2005; Shalin et al., 2006; Wang et al., 2004). In mammalian neurons,

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Ras signaling has been linked to hippocampal slice LTP, changes in dendritic spine architecture and plasticity of cultured neurons. In this context, Ras signaling has been shown to impinge on downstream MAP kinase signaling, thus implicating a canonical signaling module already established as a mediator of long-term plasticity in vertebrates. In Drosophila, expression of a mutant constitutively active Ras that is predicted to selectively target ERK leads to synapse expansion and increased localized phosphorylation of ERK at pre-synaptic terminals (Koh et al., 2002). In light of these observations, we tested if Ras signaling was necessary and sufficient for synaptic plasticity in CK. Our results suggest that synaptic changes in CK are driven by stimulated Ras/ERK signaling in Drosophila motor neurons, and these can be replicated by directly enhancing Ras signaling in these cells. Furthermore we find that Ras activation is sufficient to cause stable elevation in pre-synaptic transmitter release. Finally, we also provide evidence to show that synaptic effects of Ras activation require the function of both Fos and CREB in motor neurons. We feel that the consistency of signaling events in CK with those observed in mammalian preparations makes this a more useful and generally applicable genetic model of synaptic plasticity.

In vivo reporters of activity-dependent plasticity

In vivo reporters of neural activity have been difficult to design but offer better experimental resolution and flexibility over standard immuno-histochemical or RNA *in situ* methods to detect changes in gene expression in the brain. Thus, a good reporter permits increased temporal and spatial resolution, the option of live imaging (for fluorescent reporters) and in the case of transcriptional reporters, better understanding of

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cis-regulatory elements that control activity-dependent gene expression. In this manuscript we have described two genetically encoded reporters with utility clearly beyond the current study; a Raf based reporter to detect Ras activation in neurons and an enhancer based reporter to detect transcription of Fos.

The Ras binding domain of Raf has been used previously to detect Ras expression in yeast, mammalian cell lines, and recently in hippocampal neuron dendrites *in vitro* (Bivona et al., 2006; Chiu et al., 2002; Harvey et al., 2008; Walker and Lockyer, 2004; Yasuda et al., 2006). We used a similar strategy to model our reporter using the conserved Ras binding domain and the cysteine rich domain (RBD+CRD) from Drosophila Raf, under the reasonable assumption that this would provide sensitive reporter activity in neurons. To our knowledge this is the first time that a Ras reporter has been utilized in an intact metazoan organism to measure changes in endogenous Ras activity. In addition to confirming Ras activation in CK brains, we expect this reporter to find widespread use in tracing Ras activation in multiple tissues through development and in response to signaling changes in the entire organism. Since the reporter is based on the GAL4-UAS system, it can be expressed in tissues of choice, limiting reporter activity to regions of interest (Brand and Perrimon, 1993). Indeed, our experiments with the eye-antennal imaginal disc illustrate the utility of this reporter in identifying regions of activated Ras signaling during eye development.

Our Fos transcriptional reporter is one of very few activity regulated reporters in existence in Drosophila and we expect it to find broad acceptance as a tool to map neural circuits in the fly brain that show activity-dependent plasticity. We believe that the reporter is reasonably accurate since it is expressed in expected tissue domains

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(embryonic leading edge cells, for instance), and also co-localizes extensively with anti-Fos staining in the larval brain. There are several recognizable transcription factor binding motifs that can be detected in this 5 kb region of DNA (including binding sites for CREB, Fos, Mef2 and c/EBP). Which of these transcription factors regulate activity-dependent Fos expression from this enhancer is currently unknown. However, future experiments that dissect functional elements in this large enhancer region are expected to refine and identify these regulatory elements. Such studies are likely to lead the way in the development of a new generation of neural activity reporters in the brain.

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EXPERIMENTAL PROCEDURES

Drosophila stocks, genetics and fly rearing. Flies were grown in standard rearing conditions on cornmeal-dextrose-yeast medium at 22° – 25°C. The *comatose*^{tp7} and *Ca-P60A*$^{Kum[170]}$ stocks have been described previously (Hoeffer et al., 2003; Sanyal et al., 1999; Sanyal et al., 2005; Tolar and Pallanck, 1998). UAS-EKO were obtained from Haig Keshishian (White et al., 2001), UAS-Ras185DV12S35, UAS-Ras185DV12C40, Ras185DV12G37 and UAS-Ras185DN17 from Vivian Budnik (Bergmann et al., 1998; Koh et al., 2002), UAS-Fbz from Marianne Bienz (Eresh et al., 1997; Sanyal et al., 2002) and UAS-Creb2b is an unpublished reagent from Jerry Yin. The hs-Rolled::GAL4::VP16 reporter was from Kevin Moses (Kumar et al., 2003) and UAS-nls-GFP flies were from the Drosophila Stock Collection at Bloomington, IN . The Canton-Special strain (CS) was used as a wild type control throughout and is the genetic background in which both *comatose* and *Ca-P60A* mutants were originally isolated. The *elav*^{C155}-GAL4 line was used for pan-neuronal expression and the ey3.5-GAL4 was used for expression in the developing eye (Franciscovich et al., 2008; Lin et al., 1994). In all experiments with CK, *comatose* females were crossed with males of specific genotypes and male progeny were analyzed. In other experiments, GAL4 females were crossed to males of responder transgenes. All experimental crosses were grown at 25°C with the exception of CK crosses (and relevant controls) that were reared at 21°C.

Antibodies, immunohistochemistry and confocal miscroscopy. Larval dissection, staining and confocal microscopy were performed according to standard protocols (Franciscovich et al., 2008). Briefly, larvae were dissected in Ca^{2+} free HL3 ringer’s solution, fixed in

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4% Paraformaldehyde, stained with primary antibody overnight, followed by incubation in Alexa Fluor conjugated secondary antibody. For CNS staining, antibody incubation and washes were in a modified phosphate buffer (Sanyal et al., 2003). Mouse anti-Elav (partially purified IgG; DSHB, Iowa) was used at 1:100, mouse anti-syt (ascites, DSHB, Iowa) at 1:1000, rabbit anti-GFP (Molecular Probes) was used at 1:1000, mouse anti-FLAG (Sigma) was used at 1:500, guinea pig anti-Zfh-1 (James Skeath, Washington University, St. Louis) was used at 1:200 and rabbit anti-Fos (Dirk Bohmann, University of Rochester, Rochester) was used at 1:50. Cy-5 conjugated Phalloidin (Jackson Laboratories) and all Alexa Fluor conjugated secondary antibodies (Molecular Probes) were used at 1:200. An inverted 510 Zeiss LSM microscope was used for imaging. For quantitative fluorescence care was taken to prepare samples identically. Samples were imaged and analyzed double blind, and all imaging was interleaved such that control and experimental samples were imaged alternately on the same day. Synaptic size was counted as the number of Syt labeled boutons on muscles 6 and 7 (VL1 and VL2) in abdominal segment A2. Only one synapse was counted from one animal and at least 13 animals were counted for each genotype. Numbers were not normalized to muscle surface area since muscle sizes were independently determined to be comparable across genotypes. Data were analyzed using unpaired Student's t-test.

Electrophysiology. Two-electrode voltage clamp (TEVC) experiments were performed as described previously (Franciscovich et al., 2008; Sanyal et al., 2002). Briefly, larvae were dissected in normal HL3 ringer's solution (Stewart et al., 1994) with 1 mM Ca^{2+}. Both recording and current injecting electrodes were filled with 3M KCl. Only those

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recordings were used where the voltage deflection following nerve stimulation could be clamped to within 5 mV. Muscle 6 (VL2) in abdominal segment A2 was used for all recordings and muscles were clamped at -70mV. A train of 25 supra-threshold stimuli at 0.5Hz was delivered in each experiment from which mean peak EJC values were obtained by averaging the last 20 traces. 8-10 separate animals were used for each genotype. A two minute continuous recording was used to measure mini frequency and amplitude. Traces were analyzed using Clampfit or Mini-Analysis programs (Synaptosoft). 8-10 animals were analyzed for each genotype. Significant differences were identified using an unpaired Student's t-test.

Molecular biology, cloning and transgenic flies. An approximately 5 Kb region upstream of the dFos transcription start site was amplified from genomic DNA using PCR and cloned into the pStinger Vector (Barolo et al., 2000) using primers containing restriction enzyme sites compatible with the multiple cloning site in pStinger. This produced the Fos-nlsGFP reporter transgene. The Fos-GAL4 transgenic construct was made from the same 5 Kb PCR product cloned upstream of the GAL4 coding region in the vector pPTGAL (Sharma et al., 2002). Transgenic flies were generated through standard microinjection techniques by Genetic Services Inc. The Ras binding domain (RBD) and the cysteine rich domain (CRD) from the Drosophila Raf gene (*polehole*) were amplified with PCR and cloned into the Gateway cloning entry vector pENTR-D (Invitrogen). This entry clone was then recombined into the pTWF destination vector such that the coding region for a FLAG epitope is in frame with the RBD+CRD. The resulting amino acid sequence of this fusion protein was: ISGVRLCDALMKALKLRQLTPDMCEVSTTHS

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GRHIIPWHTDIGTLHVEEIFVRLLDKFPIRTHIKHQIIRKTFFSLVFCEGCRRLLFTG FYCSQCNFRFHQRCANRVPMLCKGGRADPAFLYKVVSSATMDYKDHDGDYKD HDIDYKDDDDKHRST (FLAG epitope is underlined) with a predicted molecular weight of approximately 18 KD. Trangenic flies were generated by Genetivision Inc. and produced flies that expressed a C-terminal fusion of RBD+CRD with FLAG.

Drosophila activity monitoring. Locomotor activity was monitored for three consecutive days using the Drosophila Activity Monitor (DAM; TriKinetics, Waltham, MA). Individual male flies 3-5 days old (n=64 for each genotype) were placed in 65x5mm polycarbonate tubes (TriKinetics) containing standard fly food. Flies were housed in a 25ºC chamber with a 12hr:12hr light:dark cycle. Activity recordings were initiated following an 18 hour acclimatization period and monitored using the number of infrared beam breaks in 15 second bins. For analysis and graphing, 15 second recording bins were pooled into 30 minute bins across each 24 hour period for three consecutive days.

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ACKNOWLEDGEMENTS

We acknowledge members of the Ramaswami and Sanyal laboratories (particularly Yoram Yerushalmi and Charles Hoeffer) for useful discussions and insights. We also thank the confocal microscopy facilities at the MCB department at the University of Arizona and at the Cell Biology department at Emory University. We are grateful to Daniel Eberl for pPTGAL and Scott Barolo for pStinger vectors. The anti-Syt (Kaushiki Menon and Kai Zinn) and anti-Elav (Gerald Rubin) antibodies were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. This work, initiated via NIDA grants DA15495 and DA17749 to MR, was supported by an NIH T32 post-doctoral training grant and grants from the URC, Emory University and a NARSAD Young Investigator Fellowship to SS, the FIRST fellowship to AF and an SFI Investigator grant to MR from the Science Foundation of Ireland.

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FIGURE LEGENDS

Figure 1. *comt; Kum* (CK) animals display activity-dependent plasticity of the neuromuscular synapse. (A) CK animals display dramatically increased locomotor activity as compared with age matched controls. When tested individually using the DAM (Drosophila Activity Monitor) assay, CK animals consistently show more beam breaks over a 24 hour period. Each column is an average of more than 50 flies for each 30 minute period beginning with 6:00 AM. Inset shows the mean cumulative number of beam breaks in the entire 24 hour period. CK animals show more than twice the number of beam crossings as compared to controls. (B, C) Larval neuromuscular synapses on muscle 6/7 in segment A2 are larger and release more neurotransmitter per action potential in CK animals as compared to controls. Representative synapse images, evoked excitatory junction current (EJCs) and miniature excitatory junction currents (mEJCs) are shown in panel B. Both *comt* and *Kum* single mutants show elevated transmitter release but not increased synaptic growth. Chronic inhibition of excitability in CK neurons using an open Shaker type potassium channel (EKO) abolishes increased synaptic growth and transmitter release observed in CK animals. Scale bar for synapse images = 50 μM. Vertical scale for EJC = 50 nA, for mEJC = 4 nA. Horizontal scale for EJC = 20 ms, for mEJC = 200 ms.

Figure 2. An *in vivo* reporter of Ras activation. (A) A new reporter is used to measure Ras activation *in vivo* and comprises the Ras binding domain (RBD) and Cysteine Rich Domain(CRD) from Drosophila Raf fused in frame to a FLAG tag (called RBD::FLAG). Expression of this reporter is controlled by UAS elements and can be directed to tissues

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of choice. RBD::FLAG localizes to regions where Ras is activated, usually on the surface of the cell (Ras-GTP). (B) RBD::FLAG trangene when expressed using the *elav*^{C155}-GAL4 line, results in a single fusion protein 18 KD in size when probed with anti-FLAG antibodies on a western blot of fly brain protein extract. (C, D) RBD::FLAG expression in developing eyes results in small, rough eyes presumably due to inhibition of Ras signaling. (E, F, G) RBD::FLAG localizes to membranous regions in ommatidial clusters in larval eye-discs when expressed from the *elav*^{C155}-GAL4 line. (H, I, J) This cellular localization is clustered and more intense in regions of high RTK signaling when constitutively activated Ras (Ras1V12S35) is co-expressed posterior to the morphogenetic furrow (compare insets in E and H). Scale bar in E is 50 µM.

Figure 3. Ras signaling is increased in CK animals and is required for synaptic plasticity. (A, D) The RBD::FLAG reporter localizes to neuronal cell membranes in wild type animals. (B, E) Membrane localization of RBD::FLAG intensifies significantly when activated Ras is co-expressed in neurons. (C, F) In CK brains, the RBD::FLAG reporter is similarly enriched on neuronal membranes (compare insets in A, B and C that show close-up of dorsal medial motor neurons in the nerve chord) suggesting elevated Ras signaling in CK animals. Anti-Zfh-1 staining is used to detect dorsal medial motor neurons in the ventral nerve chord. Dotted lines mark the dorsal midline in the VNC. Scale bar in A is 50 µM. (G) Increased synaptic size and strength in CK animals requires normal Ras/Raf signaling. Expression of dominant-negative Ras or Raf (N17 substitutions) in CK motor neurons prevents activity-dependent increase in synaptic size and strength. Expression of these dominant-negative transgenes in a wild type

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background do not influence synaptic properties (when animals are reared at 21°C, see materials and methods).

Figure 4. The transcription factors Fos and CREB function downstream to Ras/MAPK signaling in CK animals to control synaptic plasticity. (A, B, C) Nuclear entry of dERK (*rolled*) in larval ventral nerve chord neurons is detected through a reporter of ERK nuclear entry, suggesting nuclear targets of MAPK signaling in these cells (see text for details, note co-localization of Elav and nls-GFP marked with an arrow). In the dorsal medial region of the VNC, ERK nuclear entry is detected in motor neurons that label with Elav and Zfh-1 (D, E, F). Note that nuclear entry of ERK is detected in several motor neurons (arrows in D, E and F), but not in some others (arrowheads in same panels). (G) Genetic experiments that demonstrate requirement of both Fos and CREB in CK or activated-Ras mediated synaptic plasticity. Motor neuron specific expression of an activated Ras transgene that preferentially activates ERK (UAS-Ras1V12S35) results in synapse growth (reported previously in Koh et. al., 2003) and increased transmitter release similar to that observed in CK animals. Both are strongly attenuated through either Fos (UAS-Fbz) or CREB (UAS-CREB2b) inhibition. These effects are context dependent since Ras1V12S35 mediated eye tumors (not seen in Ras1V12C40 or Ras1V12G37 transgenes that do not activate ERK) are not rescued through either Fos or CREB inhibition in the eye (H). (I) Schematic summarizing signaling events in that are operative in this new model of activity-dependent synaptic plasticity. Scale bar = 50 μM.

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Figure 5. A transcriptional reporter of Fos can be used to detect Fos transcription *in vivo*. (A, B, C) A reporter consisting of a 5 Kb Fos upstream enhancer driving GFP (or GAL4, see materials and methods) mirrors previously documented Fos protein expression in embryonic leading edge cells during dorsal closure (arrows). (D, E, F) Fos expression in the larval VNC is compared with anti-Elav staining (labels post-mitotic neuronal nuclei). Inset shows expression of the Fos transcriptional reporter in all dorsal medial motor neurons. (G, H, I) Fos-GAL4 expression in the VNC (visualized with nls-lacZ immunohistochemistry) correlates largely with anti-Fos staining using dFos specific antibodies. Inset shows that Fos protein and Fos reporter are both expressed in dorsal medial motor neurons. Scale bar = 50 µM.

Figure 6. Increased Fos transcription is detected *in vivo* in CK nerve chords under normal rearing conditions and following induction of seizures. (A, B, C, D) Fos transcription (nls-GFP) is detectable in ventral nerve chord neurons from control animals (compare with anti-Elav staining). (E, F, G, H) Neuronal Fos transcription is elevated as compared to controls in CK nerve chords. (I, J, K, L) An increased Fos transcriptional response is observed in control animals following a brief heat pulse at 40°C for 3 minutes (compare L with D). (M, N, O, P) A dramatic response from the Fos transcriptional reporter is seen following the same heat pulse delivered to CK animals (to induce seizures; compare P with H). Scale bar = 50 µM.

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Genotype	Mean Bouton Number	Mean EJC Amplitude (nAmps)
C380-OR	140	95
comatose	139	115
Ca-P60A (Kum)	137	120
comt; Kum (CK)	171	123
CK-EKO	110	69
EKO	106	67
CK-Ras[DN]	134	106
CK-Raf[DN]	133	98
Ras[DN]	147	100
Raf[DN]	138	86
CK-Fbz	110	55
CK-CREB2b	105	70
Fbz	100	75
CREB2b	99	65
Control (25°C)	199	82
Ras[act] (25°C)	261	114
Ras[act]-Fbz (25°C)	157	40
Ras[act]-CREB2b (25°C)	171	60
Fbz (25°C)	151	34
CREB2b (25°C)	162	56

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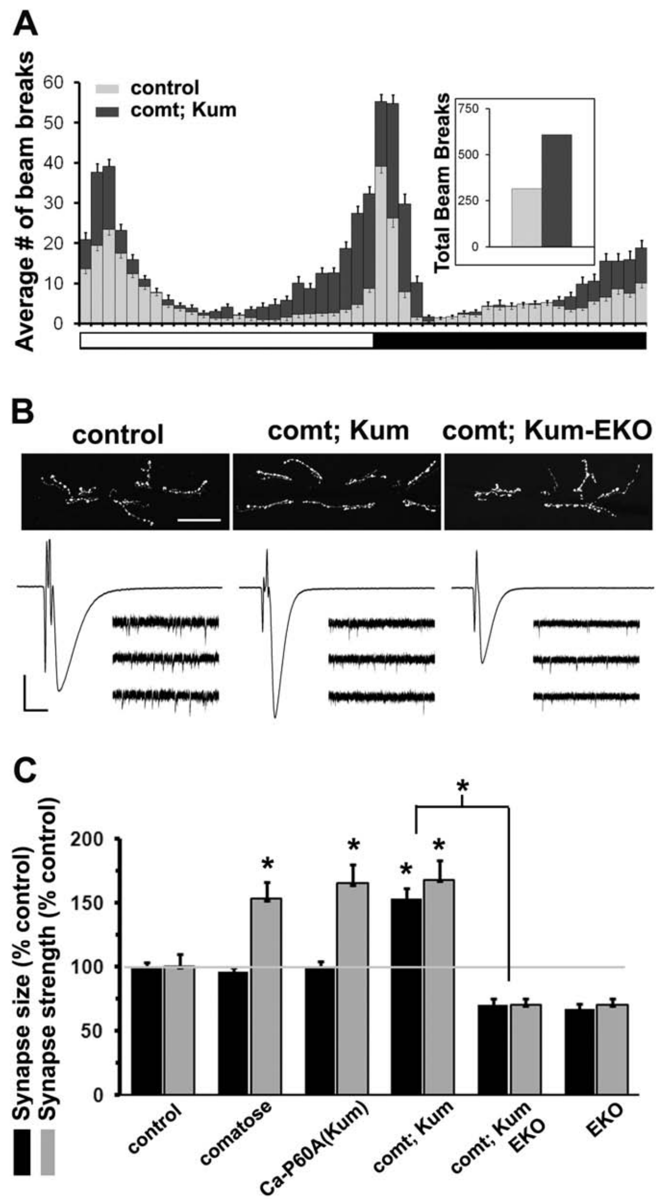
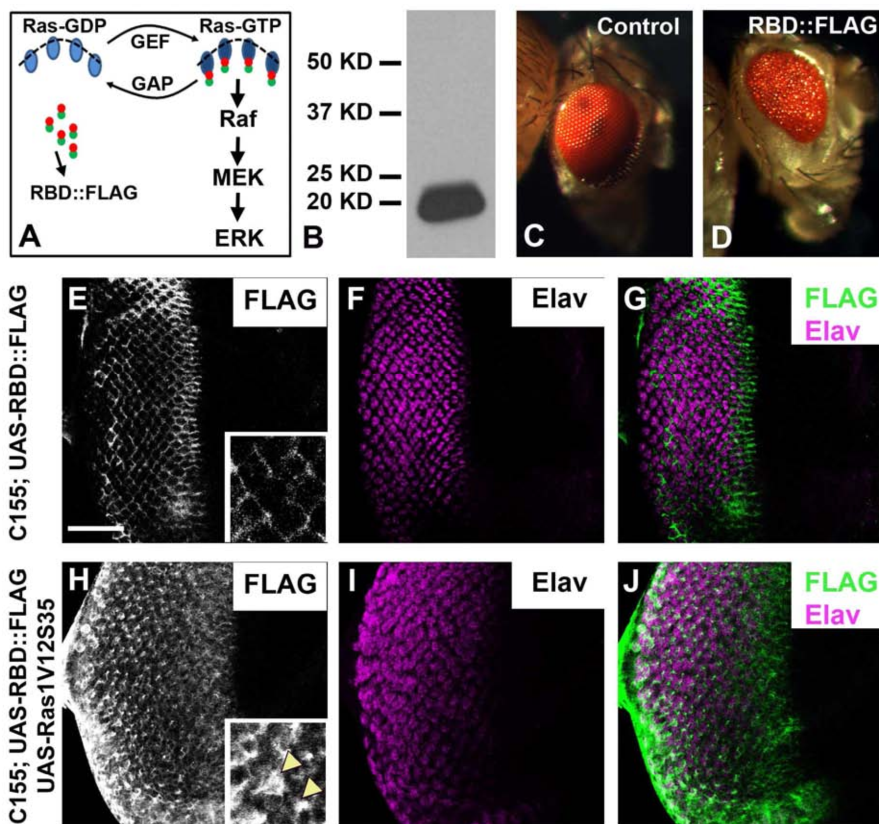

A
Average # of beam breaks
control
comt; Kum
Total Beam Breaks
B
control
comt; Kum
comt; Kum-EKO
C
Synapse size (% control)
Synapse strength (% control)
control
comatose
Ca-P60A(Kum)
comt; Kum
comt; Kum EKO
EKO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GEF
Ras-GTP
GAP
Raf
MEK
ERK
RBD::FLAG
A
50 KD
37 KD
25 KD
20 KD
B
Control
C
RBD::FLAG
D
C155; UAS-RBD::FLAG
E
FLAG
F
Elav
G
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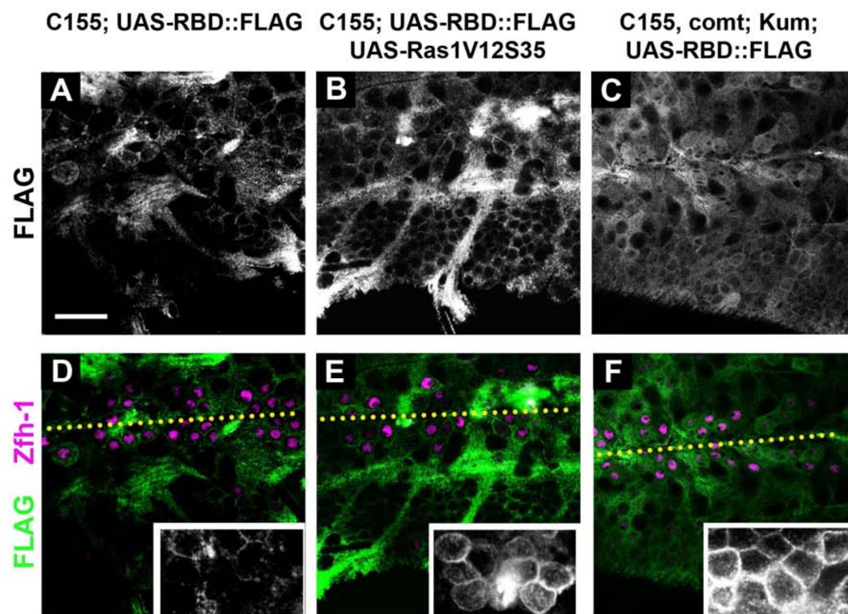

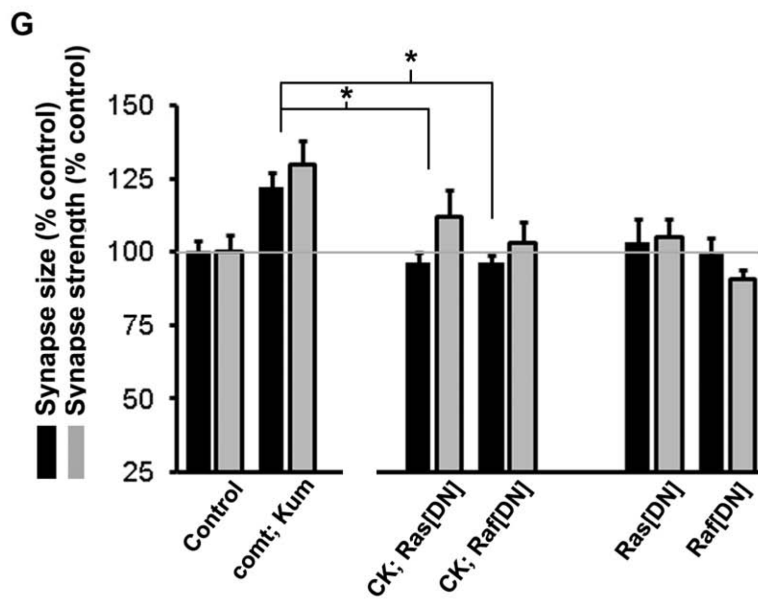

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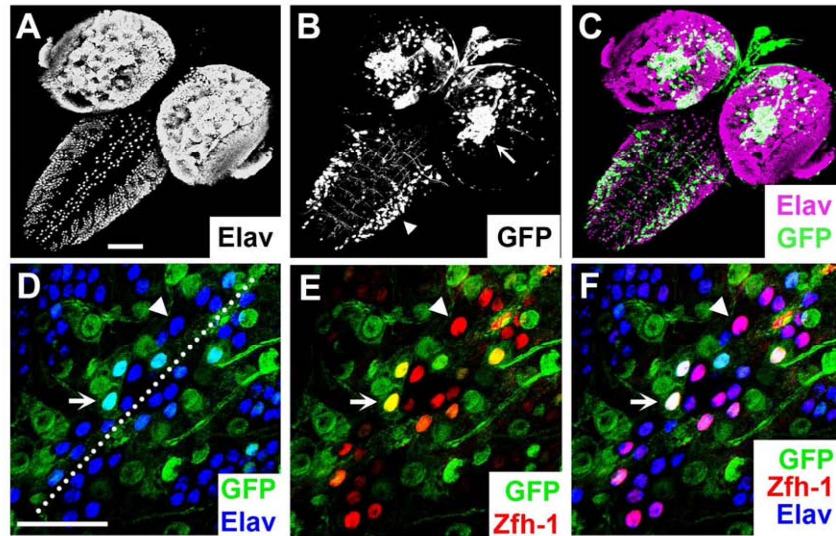

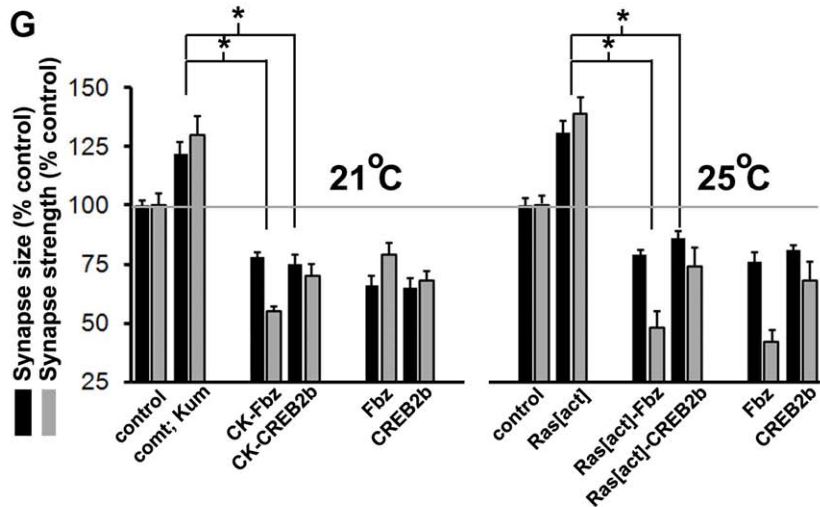

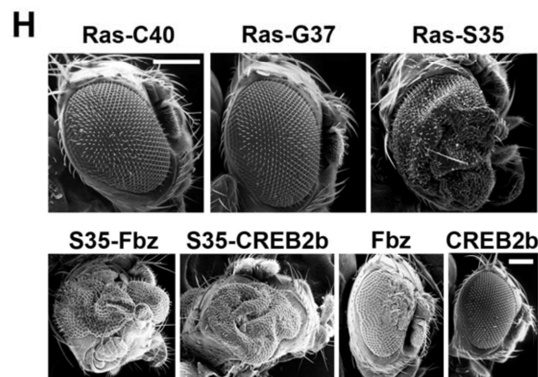

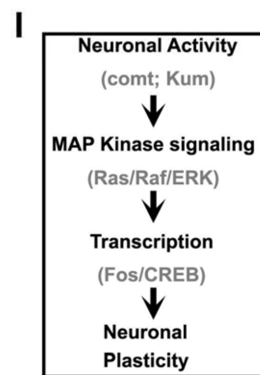

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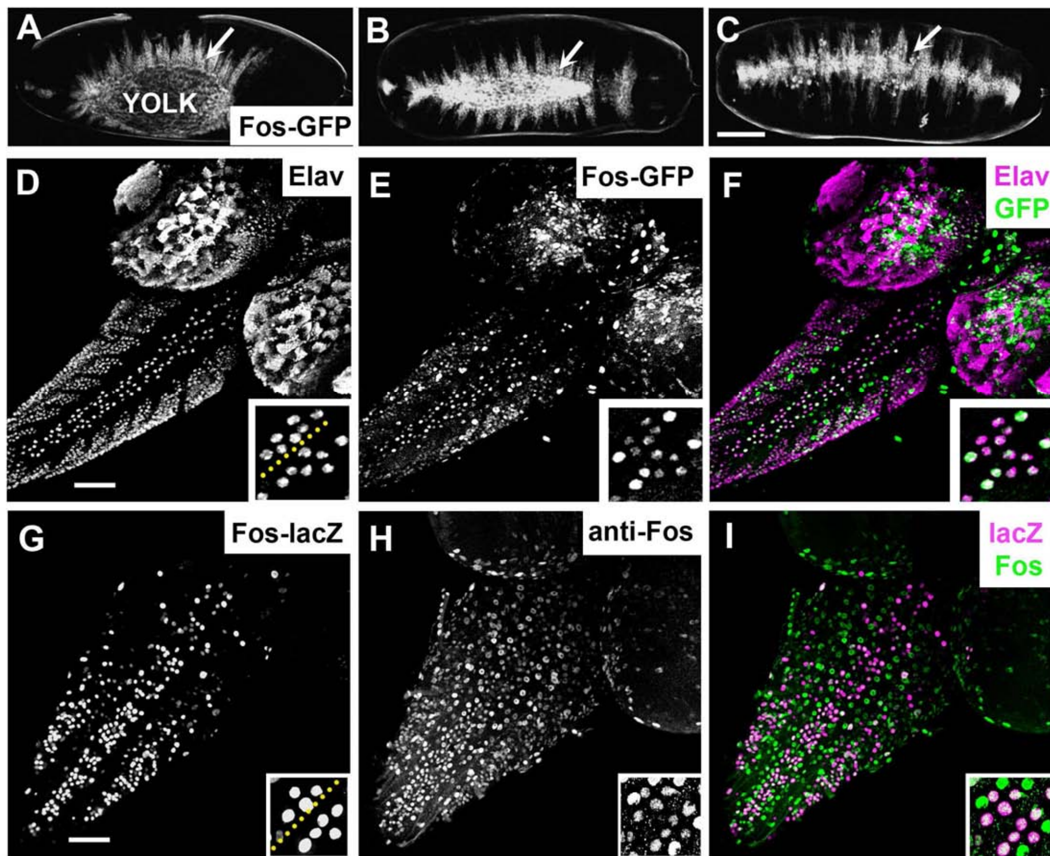

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