The Metabotropic Glutamate Receptor Activates the Lipid Kinase PI3K in Drosophila Motor Neurons Through the Calcium/Calmodulin-Dependent Protein Kinase II and the Nonreceptor Tyrosine Protein Kinase DFak

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ABSTRACT Ligand activation of the metabotropic glutamate receptor (mGluR) activates the lipid kinase PI3K in both the mammalian central nervous system and Drosophila motor nerve terminal. In several subregions of the mammalian brain, mGluR-mediated PI3K activation is essential for a form of synaptic plasticity termed long-term depression (LTD), which is implicated in neurological diseases such as fragile X and autism. In Drosophila larval motor neurons, ligand activation of DmGluRA, the sole Drosophila mGluR, similarly mediates a PI3K-dependent downregulation of neuronal activity. The mechanism by which mGluR activates PI3K remains incompletely understood in either mammals or Drosophila. Here we identify CaMKII and the nonreceptor tyrosine kinase DFak as critical intermediates in the DmGluRA-dependent activation of PI3K at Drosophila motor nerve terminals. We find that transgene-induced CaMKII inhibition or the DFakCGT null mutation each block the ability of glutamate application to activate PI3K in larval motor nerve terminals, whereas transgene-induced CaMKII activation increases PI3K activity in motor nerve terminals in a DFak-dependent manner, even in the absence of glutamate application. We also find that CaMKII activation induces other PI3K-dependent effects, such as increased motor axon diameter and increased synapse number at the larval neuromuscular junction. CaMKII, but not PI3K, requires DFak activity for these increases. We conclude that the activation of PI3K by DmGluRA is mediated by CaMKII and DFak.

METABOTROPIC glutamate receptors (mGluRs), which are G protein-coupled receptors for which glutamate is ligand, mediate aspects of synaptic plasticity in several systems. In several regions of the mammalian brain, including the hippocampus, the cerebellum, the prefrontal cortex, and others, ligand activation of group I mGluRs induces a long-term depression of synaptic activity, termed mGluR-mediated long-term depression (LTD) (Luscher and Huber 2010). Induction of mGluR-mediated LTD both activates and requires the activation of the lipid kinase PI3 kinase (PI3K) and the downstream kinase Tor (Hou and Klann 2004). Several genetic diseases of the nervous system are predicted to increase sensitivity to activation of mGluR-mediated LTD. For example, increased sensitivity to induction of mGluR-mediated LTD has been observed in the mouse model for fragile X (Bear et al. 2004). Furthermore, the genes affected in tuberous sclerosis (Tsc1 and Tsc2) and neurofibromatosis (Nf1) encode proteins that downregulate Tor activity (Gao et al. 2002; Dasgupta et al. 2005). These observations raise the possibility that hyperactivation of mGluR-mediated LTD plays a causal role in the neurological phenotypes of fragile X, neurofibromatosis and tuberous sclerosis (Kelleher and Bear 2008). Because these diseases are each associated with an extremely high incidence of autism spectrum disorders (ASDs), and because several lines of evidence suggest that elevated PI3K activity is associated with ASDs (Serajee et al. 2003; Kwon et al. 2006; Mills et al. 2007; Cusco et al. 2009), it has been hypothesized that hyperactivation of this pathway might be responsible for ASDs as well. Thus it would be of interest to identify additional molecular components by which mGluR activation...
activates PI3K, and yet despite recent advances, this mechanism remains incompletely understood.

In Drosophila larval motor neurons, glutamate activation of the single mGluR, called DmGluRA, downregulates neuronal excitability (Bogdanik et al. 2004); glutamate both activates PI3K and requires PI3K activity for this downregulation (Howlett et al. 2008). Because glutamate is the excitatory neurotransmitter at the Drosophila neuromuscular junction (NMJ) (Jan and Jan 1976), it was hypothesized that this DmGluRA-mediated downregulation of neuronal excitability carried out a negative feedback on activity: glutamate released from motor nerve terminals would activate DmGluRA autoreceptors, which would then depress excitability.

Here we identify additional molecular components that mediate the activation of PI3K by DmGluRA in Drosophila larval motor nerve terminals. We find that activity of the calcium/calmodulin-dependent kinase II (CaMKII) is necessary for glutamate application to activate PI3K, and expression of the constitutively active CaMKII<sup>287D</sup> (Jin et al. 1998) is sufficient both to activate PI3K even in the absence of glutamate and to confer several other neuronal phenotypes consistent with PI3K hyperactivation. We also find that CaMKII<sup>287D</sup> requires the nonreceptor tyrosine kinase DFak for this PI3K activation: the DFak<sup>CG1</sup> null mutation (Grabbe et al. 2004) blocks the ability of glutamate application to activate PI3K and prevents CaMKII<sup>287D</sup> from hyperactivating PI3K. Finally, CaMKII<sup>287D</sup> expression completely suppresses the hyperexcitability conferred by the DmGluRA null mutation DmGluRA<sup>112b</sup>. We conclude that ligand activation of DmGluRA activates PI3K via CaMKII and DFak.

Materials and Methods

**Drosophila stocks**

For all experiments, Drosophila larvae were reared on standard cornmeal/agar media at 22–23°C. The D42 Gal4 driver (Brand and Perrimon 1993; Parkes et al. 1998), which expresses in motor neurons, was provided by Tom Schwarz (Harvard Medical School, Boston, MA). Flies carrying the UAS–PI3K<sup>D954A</sup> (D954A) and UAS–PI3K–CAAX transgenes (Leevers et al. 1996), encoding dominant-negative and constitutively active PI3K, respectively, were provided by Sally Leevers (London Research Institute, London, UK). Flies carrying the UAS–ala, UAS–CaMKII<sup>287A</sup>, and UAS–CaMKII<sup>287D</sup> transgenes, which encode a CaMKII inhibitor peptide, calcium/calmodulin-dependent CaMKII, and calcium/calmodulin-independent CaMKII, respectively (Griffith et al. 1994; Park et al. 2002), were provided by Leslie C. Griffith (Brandeis University, Waltham, MA). Flies carrying the DFak<sup>CG1</sup> deletion mutation and UAS–DFak<sup>+</sup> transgene (Grabbe et al. 2004) were provided by Ruth Palmer (Umeå University, Umeå, Sweden). Flies carrying the DmGluRA<sup>112b</sup> deletion mutation (Bogdanik et al. 2004) were provided by Marie-Laure Parmentier (Unité Propre de Recherche Centre, Montpellier, France). All other fly stocks were provided by the Drosophila Stock Center (Bloomington, IN).

**Immunocytochemistry**

Larvae were grown to the wandering third instar stage in uncrowded half-pint bottles. Larvae were collected for experimentation only during the first and second days after the initial wandering third instar larvae appeared. For basal phosphorylated Akt (p-Akt) measurements, larvae were dissected in Schneider’s Drosophila media (Gibco) and fixed in Schneider’s Drosophila media containing 4% paraformaldehyde. For experiments involving glutamate application, larvae were dissected and then incubated for 1 min in Schneider’s Drosophila media containing 100 μM glutamate prior to the fixation procedure described above. Fixed larval tissues were incubated with a rabbit anti-Drosophila p-Akt (Ser505) polyclonal primary antibody (1:500 dilution; Cell Signaling Technologies). The antibody used was from a different lot from the antibody used in a previous report (Howlett et al. 2008) and thus might not have identical properties. Larvae were then incubated with Rhodamine Red conjugated goat antirabbit IgG (1:500 dilution; Jackson ImmunoResearch), and Cy-2 conjugated antibodies against horseradish peroxidase (1:200 dilution; Jackson ImmunoResearch). Immunolabeled larval tissues in standard phosphate buffered saline (PBS) (0.128 M NaCl, 2.0 mM KCl, 4.0 mM MgCl₂, 0.34 M sucrose, 5.0 mM Hepes, pH 7.1, and 0.15 mM CaCl₂) containing 50% glycerol were mounted onto slides. Care was taken to treat all samples identically during this procedure. NMJs from muscles 6 and 7 in segments A3, A4, A5, or A6 were imaged on a Zeiss LSM 510 confocal microscope system (Zeiss, Oberkochen, Germany) with a ×20 objective. Optical sections were 10 μm thick, which encompassed the entire NMJ. Optical parameters, including pinhole, gain, contrast, and brightness, were held constant for each experimental set. NMJs (marked by anti-HRP) were traced using the ImageJ (National Institutes of Health, Bethesda, MD) freehand selection tool and the selection was transferred to the anti-p-Akt image where pixel intensity value was quantified. Background was obtained by averaging p-Akt intensity from muscles 6 and 7 from the same abdominal hemisegment, excluding the muscle nuclei and the neuromuscular junction. This background was then subtracted from the mean motor nerve terminal p-Akt pixel intensity. This p-Akt pixel intensity was then normalized to the mean p-Akt pixel intensity of wild-type motor nerve terminals obtained in experiments performed in parallel.

To measure synaptic bouton number, larvae were grown, selected, and dissected as described above, fixed in standard PBS containing 4% paraformaldehyde and labeled with Cy-2 conjugated antibodies against horseradish peroxidase (1:200 dilution). Images were obtained as described above. The LSM Image Browser was used for quantifying bouton numbers and obtaining surface area of muscles 6 and 7 from abdominal segments A3 and A4. The number of boutons was counted manually.
Transmission electron microscopy

Transmission electron microscopy was performed as described previously (Lavery et al. 2007; Howlett et al. 2008). Briefly, larvae were grown, selected, and dissected as described above, fixed with glutaraldehyde and paraformaldehyde, stained with 0.5% OsO₄ and 2% uranyl acetate, and embedded in an eponate 12-araldite mixture. Ultrathin cross-sectional slices (pale gold, 120 nm thick) were cut with an ultramicrotome, poststained with uranyl acetate and Reynolds lead citrate, and analyzed with a JEOL 1230 (JEOL, Tokyo, Japan) transmission electron microscope at 80 kV. Carl Zeiss Axioversion software (Zeiss, Oberkochen, Germany) was used for analyzing axon diameter. Each experimental set was analyzed from the five largest axons (motor axons) from more than five different nerves from at least two different larvae.

Electrophysiology

Larvae were grown and selected as described above and dissected in Jan's buffer (128 mM NaCl, 2.0 mM KCl, 4.0 mM MgCl₂, 34 mM sucrose, 4.8 mM Hepes, pH 7.1, and CaCl₂ concentration as specified in the text). Peripheral nerves were cut immediately posterior to their exit from the ventral ganglion and were stimulated with a suction electrode at a 5-V stimulus intensity. Muscle recordings were taken from muscle 6 in abdominal segments A3, A4, or A5. Stimulus duration, ~0.05 msec, was adjusted to 1.5 times threshold, which reproducibly stimulates both axons innervating muscles 6 and 7. Intracellular recording electrodes for muscle potentials were pulled with a Flaming/Brown micropipette puller to a tip resistance of 10–40 MΩ and filled with 3M KCl. Rate of onset of long-term facilitation (LTF) was reported as geometric means because the data show a positive skew.

For all LTF experiments, the bath solution contained 0.15 mM [Ca²⁺] and 100 μM quinidine, which is a potassium channel blocker that sensitizes the motor neuron and enables LTF to occur and be measured even in hypoexcitable neurons.

Results

CaMKII regulates PI3K activity in Drosophila motor nerve terminals

Metabotropic glutamate receptors (mGluRs) are G protein-coupled receptors for which glutamate is ligand. In Drosophila, the DmGluRA112b null mutation in the single mGluR increases motor neuron excitability by preventing activation of the lipid kinase PI3K (Bogdanik et al. 2004; Howlett et al. 2008). Given that glutamate is the major excitatory neurotransmitter at NMJs in insects such as Drosophila (Jan and Jan 1976), it was suggested that DmGluRA in motor neurons participate in a negative feedback: glutamate released from motor nerve terminals as a consequence of neuronal activity activates DmGluRA autoreceptors in motor nerve terminals. DmGluRA activation downregulates subsequent neuronal activity by activating PI3K. In this view, DmGluRA112b increases excitability by preventing PI3K activation and thus severing this negative feedback. This view is supported by the observation that transgene-induced PI3K inhibition increases neuronal excitability in a manner similar to DmGluRA112b, whereas transgene-induced PI3K activation decreases neuronal excitability (Howlett et al. 2008). However, the molecular mechanism by which glutamate/DmGluRA activates PI3K was not elucidated.

We initially hypothesized that CaMKII might be an essential intermediate in DmGluRA-mediated PI3K activation. Although the possibility that CaMKII can activate PI3K has only recently been reported (Ma et al. 2009), altered CaMKII activity alters certain motor neuron phenotypes in Drosophila in a manner similar to altered PI3K activity (Griffith et al. 1994; Park et al. 2002). One way that CaMKII activity can be altered is by expression of the constitutively active CaMKII²87D; this variant is rendered active even in the absence of calcium or calmodulin by the phosphomimetic T287D substitution in the autophosphorylation site (Fong et al. 1989). It was previously found that motor neuron expression of CaMKII²87D increased synapse number and decreased excitability in motor neurons (Park et al. 2002); both of these phenotypes are also exhibited in larvae expressing the constitutively active PI3K-CAAX (Martin-Pena et al. 2006; Howlett et al. 2008). Similarly, CaMKII inhibition accomplished by transgenic expression of the CaMKII inhibitory peptide “ala” (Joiner and Griffith, 1997) increases motor neuron excitability and decreases synapse number (Park et al. 2002); both of these phenotypes are also exhibited in transgenic larvae in which PI3K activity is blocked. Taken together, these results support the notion that levels of PI3K activity are specified, at least in part, by activity of CaMKII.

To test the prediction that altered CaMKII activity would alter PI3K activity in Drosophila larval motor nerve terminals, we used the D42 motor neuron Gal4 driver to express transgenes encoding either the nonactivated CaMKII²87A (which acts equivalently to wild type in all assays performed to date), the constitutively active CaMKII²87D, or the CaMKII inhibitory peptide ala in motor neurons, and then evaluated effects of these transgenes on PI3K activity. We monitored PI3K activity with an antibody against the phosphorylated form of the kinase Akt (p-Akt), which increases upon PI3K activation and is a well-established readout for PI3K activity (Colombani et al. 2005; Dionne et al. 2006; Palomero et al. 2007). We found that expressing the activated CaMKII²87D, but not the nonphosphorylatable CaMKII²87A, increased p-Akt levels in motor nerve terminals ~40% (Figure 1, P = 0.002, n = 29), which is similar to, but less extreme, than the twofold increase in p-Akt conferred by transgene-induced PI3K activation (Howlett et al. 2008). The magnitude of this response is similar to the magnitude of the p-Akt increase induced during mGluR-mediated LTD in the mouse hippocampus (Hou and Klann 2004), consistent with the possibility that the p-Akt increases in the Drosophila and mammalian systems occur via a common pathway.
The previous study in which this approach was used (Howlett et al. 2008) was not able to distinguish possible differences in basal p-Akt levels among genotypes because of high sample errors. However, by significantly increasing sample size and by including data normalization to accommodate signal variability, we were able to show that CaMKII inhibition via alma expression decreased basal p-Akt ~25% (Figure 1, \( P = 0.033, n = 16 \)). These results support the notion that CaMKII activity specifies PI3K activity in larval motor neurons.

**Focal adhesion kinase (DFak) is required for CaMKII to activate PI3K**

Next we wanted to identify molecules that might serve as intermediates linking activated CaMKII with PI3K activation. We hypothesized that the nonreceptor tyrosine kinase focal adhesion kinase (DFak) might play such a role: DFak is the single *Drosophila* ortholog of the family which in mammals consists of both Fak and the closely related Pyk2. In mammals, CaMKII phosphorylates Pyk2 on multiple serine residues on the C terminus, which leads to Pyk2 tyrosine phosphorylation and activation by mechanisms that are incompletely understood (Della Rocca et al. 1997; Soltoff 1998; Zweck et al. 1999; Heidinger et al. 2002; Fan et al. 2005; Montiel et al. 2007). CaMKII also phosphorylates mammalian Fak on C-terminal serine residues, although the functional significance of these phosphorylation events remains unknown (Fan et al. 2005). Furthermore, both Pyk2 and Fak are capable of activating PI3K (Chen and Guan 1994; Schlaepfer et al. 1994; Guinebault et al. 1995; Chen et al. 1996; Dikic et al. 1996; Avraham et al. 2000; Rocic et al. 2001; Montiel et al. 2007). Two CaMKII phosphorylation sites on Fak, serines 843 and 910, are conserved in DFak, raising the possibility that CaMKII phosphorylates DFak at these serine positions, which leads to DFak and then PI3K activation (Grabbe et al. 2004).

If DFak acts downstream of CaMKII, then the DFak null mutation DFak\(^{-}\) is predicted to decrease motor nerve terminal PI3K activation, and prevent CaMKII\(^{287D}\)-dependent PI3K activation. As shown in Figure 1, PI3K activity in motor nerve terminals, as monitored by levels of p-Akt, was decreased in DFak\(^{-}\) to 80% of wild type (\( P = 0.008, n = 70 \)). Furthermore, this decrease was rescued by motor neuron expression of a DFak\(^{+}\) transgene (Figure 1), demonstrating that this effect of DFak\(^{-}\) resulted from loss of DFak specifically within motor neurons. Furthermore, DFak\(^{CGI}\) larvae expressing CaMKII\(^{287D}\) in motor neurons did not exhibit the increased p-Akt levels in motor nerve terminals observed when CaMKII\(^{287D}\) was expressed in an otherwise wild-type background (Figure 1, \( P < 0.001 \)). Rather, p-Akt levels were decreased to levels similar to those observed in DFak\(^{CGI}\) larvae in the absence of CaMKII\(^{287D}\) expression (\( P = 0.4 \)). Similarly, coexpression with the dominant-negative PI3K\(^{DN}\) (Leevers et al. 1996) blocked the ability of CaMKII\(^{287D}\) to increase p-Akt levels (Figure 1). Taken together, these results confirm that both DFak and PI3K activities are required for CaMKII to increase p-Akt levels.

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**Figure 1** CaMKII activity increases levels of p-Akt in larval motor nerve terminals in a DFak-dependent manner. (A) Representative confocal images of third instar larval neuromuscular junctions of wild type (WT), D42 \( > \) CaMKII\(^{287D}\), and DFak\(^{CGI}\) stained with anti-pAkt (top), anti-HRP (middle), and merged images (bottom). All images are taken from muscles 7 and 6 from abdominal segments A3, A4, A5, or A6. Bar, 20 \( \mu m \). (B) Mean normalized p-Akt intensity (y axis) ± SEM for the following genotypes (x axis): D42\(^{+}\), DFak\(^{CGI}\), D42 \( > \) CaMKII\(^{287D}\), D42 \( > \) CaMKII\(^{287D}\), D42 \( > \) P3K\(^{DN}\), and D42 \( > \) CaMKII\(^{287D}\), P3K\(^{DN}\). Anti-HRP was applied to outline nerve terminals. Pixel intensities were quantified using ImageJ software and background subtraction was performed as described in MATERIALS AND METHODS. The normalized p-Akt intensities are shown as a ratio change compared to wild type. From left to right, \( n = 65, 70, 29, 30, 16, 84, 35, \) and 18, respectively, for each genotype. One-way ANOVA and Fisher’s LSD were performed for statistical analysis and gave the following differences: For D42\(^{+}\) vs. DFak\(^{CGI}\), \( P = 0.008 \); for D42 \( > \) CaMKII\(^{287D}\), \( P = 0.002 \); for D42 \( > \) CaMKII\(^{287D}\), \( P = 0.062 \); vs. D42 \( > \) alma, \( P = 0.033 \); vs. DFak\(^{CGI}\), D42 \( > \) DFak\(^{+}\), \( P = 0.34 \). For DFak\(^{CGI}\), vs. DFak\(^{CGI}\), D42 \( > \) DFak\(^{+}\), \( P = 0.018 \); vs. DFak\(^{CGI}\), D42 \( > \) CaMKII\(^{287D}\), \( P = 0.40 \). For D42 \( > \) CaMKII\(^{287D}\) vs. DFak\(^{CGI}\), D42 \( > \) CaMKII\(^{287D}\), \( P < 0.001 \); vs. D42 \( > \) CaMKII\(^{287D}\), P3K\(^{DN}\), \( P = 0.001 \). P values <0.05 were considered statistically significant.
CaMKII and DFak are required for PI3K activation by glutamate application

It was previously demonstrated that application of glutamate to larval NMJs in filleted third instar larval preparations activates PI3K, as monitored by glutamate-induced increases in p-Akt levels in motor nerve terminals (Howlett et al. 2008). Furthermore, these glutamate-evoked p-Akt increases were blocked by the null mutation DmGluRA112b or by presynaptic inhibition of either DmGluRA or PI3K (Howlett et al. 2008), which demonstrated that glutamate activated PI3K via DmGluRA autoreceptors. If CaMKII and DFak are critical intermediates in glutamate/DmGluRA-induced PI3K activation, then inhibition of either molecule is predicted to block this glutamate-induced PI3K activation. To test this possibility, we applied 0.1 mM glutamate to DFakCG1 larvae, or to larvae expressing the CaMKII inhibitory peptide ala in motor neurons. We found that wild-type larvae showed a robust glutamate-induced PI3K activation, as monitored by immunocytochemistry against p-Akt (Figure 2, P = 0.001), following 1 min of glutamate application. This percent increase is similar to the increase reported during mGluR-mediated LTD in the mouse hippocampus (Hou and Klann 2004) in which a 5-min application of a glutamate analog evoked an ~50% increase in p-Akt levels. In contrast, blocking either CaMKII or DFak blocked this increase (Figure 2, P = 0.64 and P = 0.77, respectively). Furthermore, the motor neuron expression of UAS-DFak + restored normal glutamate sensitivity to DFakCG1 (Figure 2).

CaMKII promotes synapse formation via DFak-dependent PI3K activation

CaMKII activity was previously shown to promote arborization and increase synapse number at the larval NMJ (Park et al. 2002) by phosphorylating discs large and thus decreasing levels of Fas2 (Koh et al. 1999). The observation that CaMKII acts downstream of integrins for this control of synapse structure (Beumer et al. 2002) is consistent with a role for DFak in this process as well. In addition, arborization and synapse number are regulated by PI3K activity: activating PI3K by expressing the constitutively active PI3K-CAAX increases synapse number ~2.5-fold, whereas blocking PI3K by expressing the dominant-negative PI3KDN decreases synapse number about twofold (Martin-Pena et al. 2006; Howlett et al. 2008). From these data and the results shown above, we hypothesized that CaMKII promotes synapse formation at least in part by activating PI3K in a DFak-dependent manner. We confirmed the previous findings of Park et al. (2002) that expressing CaMKII287D, but not CaMKII287A, in motor neurons increased bouton number ~30% (Figure 3, P = 0.001), an increase similar to, though less extreme, than the increase conferred by PI3K-CAAX.

We found that this CaMKII287D-dependent increase required PI3K activity: in larvae coexpressing PI3KDN and CaMKII287D, the CaMKII287D-dependent increase in bouton number was completely suppressed and in fact was decreased to a value indistinguishable from that conferred by PI3KDN alone (Figure 3).

To determine whether DFak is a required intermediate in this CaMKII-dependent PI3K activation, we measured synapse number in DFakCG1 larvae both in the presence and absence of CaMKII287D expression. We found that in the absence of CaMKII287D expression, synapse number in DFakCG1 larvae was highly variable and therefore differences with wild type failed to reach statistical significance (P = 0.051). However, there was a tendency for DFakCG1 synapse number to decrease, rather than increase, compared to wild type (Figure 3). Thus we were unable to confirm the finding of Tsai et al. (2008), who reported that DFak inhibition increases synapse number at the larval NMJ. However, DFakCG1 completely suppressed the increased synapse number conferred by CaMKII287D expression (Figure 3, P = 0.001); synapse number in DFakCG1 larvae expressing CaMKII287D was indistinguishable from synapse number in DFakCG1 alone (Figure 3, P = 0.89). These data support the notion that CaMKII activates PI3K in a DFak-dependent manner.

Figure 2. The glutamate-evoked increase in presynaptic p-Akt levels requires CaMKII and DFak. (A) Representative confocal images of third instar larval neuromuscular junctions of wild type (WT or +/-), DFakCG1 and DFakCG1; D42 > UAS-DFak + either prior to (--) or 1 min following (+) application of 100 µM glutamate. Larvae were stained with anti-p-Akt (left) and anti-HRP (right). All images are from muscles 7 and 6 of abdominal segments A3, A4, A5, or A6. Bar, 20 µm. (B) Mean normalized p-Akt intensity (y axis) ± SEM, both prior to and following 1-min glutamate application, for the following genotypes (x axis): D42--; DFakCG1; D42 > ala, and DFakCG1; D42 > DFak +. Anti-HRP was applied to outline nerve terminals. Pixel intensities were quantified using imageJ software and background subtraction was performed as described in Materials and Methods. The normalized p-Akt intensities are shown as a ratio change compared to wild type. From left to right, n = 65, 56, 70, 48, 84, 24, 32, and 36, respectively, for each genotype. One-way ANOVA and Fisher’s LSD were performed for statistical analysis. P values <0.05 were considered statistically significant.
We also found that blocking CaMKII in motor neurons by expressing the CaMKII-inhibitor peptide ala decreased synapse number about twofold (Figure 4, P < 0.001). If this decrease were a consequence of failure to activate PI3K, then expression of the constitutively active (and hence CaMKII independent) PI3K-CAAX would be predicted to suppress these effects of CaMKII inhibition. We confirmed this prediction: the ala-dependent decrease in synapse number was completely suppressed by coexpression with PI3K-CAAX: synapse number in larvae expressing both ala and PI3K-CAAX was significantly increased compared to synapse number in larvae expressing ala alone (Figure 4, P < 0.001) and was indistinguishable from synapse number in larvae expressing PI3K-CAAX alone (Figure 4, P = 0.53). In addition, we found that synapse number in larvae expressing PI3K-CAAX was similarly unaffected by the presence of DFakCG1 (Figure 4). Taken together, these results suggest that CaMKII increases synapse number by the DFak-dependent activation of PI3K.

Certain genotypes exhibited altered bouton morphology in addition to altered bouton number. For example, boutons in DFakCG1 mutants often appear to be larger in size than wild type. The DmGluRA1126 mutation was similarly found to increase bouton size (Bogdanik et al. 2004). These observations are consistent with the possibility that DFak activation is attenuated in the absence of DmGluRA.

**CaMKII activity increases motor axon diameter by the DFak-dependent activation of PI3K**

Larval motor axon diameter is specified by the level of PI3K activity: expressing constitutively active PI3K-CAAX increases motor axon diameter ~75% (0.8–1.4 μm), whereas expressing the dominant-negative PI3KDNa decreases motor axon diameter (Howlett et al. 2008). To determine whether CaMKII similarly regulates motor axon diameter, we expressed CaMKII287D in motor axons and found as predicted significantly increased (~40%) motor axon diameters (Figure 5, P = 0.001), an increase similar to, but less extreme than, the increase conferred by PI3K-CAAX. In addition, the CaMKII287D-dependent increase, but not the PI3K-CAAX-dependent increase, in axon diameter was blocked by DFakCG1 (Figure 5, P = 0.004). Taken together, these results suggest that CaMKII increases motor axon diameter by the DFak-dependent activation of PI3K.

**Altered CaMKII and DFak activities alter motor neuron excitability**

PI3K activity regulates motor neuron excitability: transgene-induced PI3K activation decreases neuronal excitability, whereas transgene-induced PI3K inhibition increases neuronal excitability (Howlett et al. 2008). Additional reports have demonstrated that CaMKII and DFak also regulate Drosophila neuronal excitability (Griffith et al. 1994; Park et al. 2002; Ueda et al. 2008), raising the possibility that CaMKII, DFak, and PI3K might regulate excitability via a common pathway. However, in each previous report, different sets of excitability assays were used, which complicates attempts to assign the effects of these three genes to specific pathways.

To address this complication, we monitored motor neuron excitability in DFakCG1 and in larvae expressing the constitutively active CaMKII287D with the same assay previously employed to monitor excitability in larvae with altered PI3K activity. With this assay, the larval neuromuscular preparation (Jan and Jan 1976) transmitter release from motor neurons is evoked by nerve stimulation, and the
amount of transmitter released is inferred from the amplitude of the muscle depolarization (termed the excitatory junction potential, or EJP). Using this assay, we employed the same readouts to evaluate neuronal excitability in DFakCG1 larvae and in larvae expressing CaMKII1287D that were previously employed for altered PI3K (Howlett et al. 2008).

First, we measured rate of onset of LTF (Jan and Jan 1978). LTF is a form of synaptic plasticity induced when a larval motor neuron is subjected to a train of repetitive nerve stimulations at low external [Ca2+] at a certain point during the stimulus train, a threshold is reached, and subsequent stimulations elicit EJPs greatly increased in amplitude and duration. This long-term facilitation results from increased and asynchronous neurotransmitter release, which in turn results from increased duration of nerve terminal depolarization (Jan and Jan 1978). The number of stimulations required to reach this LTF threshold (LTF onset rate) is decreased by increased neuronal excitability (Jan and Jan 1978). The possibility that DFak is required in the motor neuron for this effect. To confirm that these effects on EJP amplitude reflected altered transmitter release rather than altered responsiveness of the muscle to transmitter, we compared the amplitude of miniature EJPs (mEJPs), which reflect the spontaneous release of individual vesicles of neurotransmitter, in wild-type larvae vs. DFakCG1 larvae and DFakCG1 larvae expressing DFak1 in motor neurons (Figure 6C). We found that mEJP amplitude was unaffected in these three genotypes. Thus the increased EJP amplitude observed in DFakCG1 reflects increased neurotransmitter release.

The possibility that CaMKII1287D constitutively activates PI3K would predict that CaMKII1287D expression would decrease LTF onset rate, which we also confirmed (Figure 6A). Second, we measured EJP amplitude evoked by single nerve stimulations at low external [Ca2+]. At these low [Ca2+], Ca2+ is limiting for transmitter release, and mutations that alter excitability, and hence Ca2+ influx, have significant effects on EJP amplitude (Jan and Jan 1978). In particular, transgene-induced PI3K activation decreases EJP amplitude, whereas transgene-induced PI3K inhibition increases EJP amplitude (Howlett et al. 2008). The possibility that DFak is required for DmGluRA-dependent PI3K activation predicted that DFakCG1 would prevent this PI3K activation and similarly increase EJP amplitude. We found that DFakCG1 significantly increased EJP amplitude at 0.15 mM and 0.2 mM [Ca2+] (Figure 6B), but was not significantly different from wild type at 0.1 mM [Ca2+], at which transmitter release is the smallest and errors are the greatest. This increased EJP amplitude was rescued by motor neuron-dependent expression of DFak+, confirming that DFak is required in the motor neuron for this effect. To confirm that these effects on EJP amplitude reflected altered transmitter release rather than altered responsiveness of the muscle to transmitter, we compared the amplitude of miniature EJPs (mEJPs), which reflect the spontaneous release of individual vesicles of neurotransmitter, in wild-type larvae vs. DFakCG1 larvae and DFakCG1 larvae expressing DFak1 in motor neurons (Figure 6C). We found that mEJP amplitude was unaffected in these three genotypes. Thus the increased EJP amplitude observed in DFakCG1 reflects increased neurotransmitter release.

The possibility that CaMKII1287D constitutively activates PI3K would predict that CaMKII1287D expression would decrease EJP amplitude. We found that as predicted, CaMKII1287D expression decreases EJP amplitude at each [Ca2+] tested.
(Figure 6B), while having no significant effect on mEJP amplitude (Figure 6C). This observation supports the conclusion that decreased neurotransmitter release underlies the decreased EJP amplitude conferred by CaMKII T287D.

**Activating CaMKII prevents hyperexcitability of the DmGluRA112b null mutant**

The DmGluRA112b null mutant exhibits extreme neuronal hyperexcitability, as shown by a greatly increased rate of onset of LTF (Bogdanik et al. 2004; Howlett et al. 2008). This hyperexcitability is completely suppressed by expression of the constitutively active PI3K-CAAX and thus reflects failure to activate PI3K (Howlett et al. 2008). If this failure to activate PI3K is a consequence of failure to activate CaMKII, then this hyperexcitability is predicted to be suppressed by transgene-induced expression of the constitutively active CaMKII T287D. To test this prediction, we compared rate of onset of LTF in DmGluRA112b mutants and in DmGluRA112b mutants expressing CaMKII T287D in motor neurons. We found that CaMKII T287D expression completely suppressed the hyperexcitability of DmGluRA112b, and decreased excitability to a level indistinguishable from the hypoexcitability in larvae in which CaMKII T287D was expressed in a DmGluRA + background (Figure 7). These results support the notion that CaMKII is a critical intermediate in the ability of ligand-activated DmGluRA to activate PI3K.

**Discussion**

In both mammalian central synapses and Drosophila larval motor neurons, activation by glutamate of the metabotropic glutamate receptor (mGluR) activates the lipid kinase PI3K, but the mechanism by which this activation occurs has not been elucidated. Here we identify CaMKII as a critical intermediate in the ability of the single Drosophila mGluR (DmGluRA) to activate PI3K and show that the ability of both activated DmGluRA and CaMKII to activate PI3K requires the nonreceptor tyrosine kinase, DFak (Figure 8). These results provide novel insights into the mechanism by which DmGluRA activation triggers the observed downregulation of subsequent neuronal activity in Drosophila motor neurons. These results might also be relevant to the mechanism by which mGluR activates PI3K in mammalian central synapses, a process implicated in fragile X, ASDs, neurofibromatosis, and tuberous sclerosis (Kelleher and Bear 2008).

How might CaMKII lead to the DFak-dependent activation of PI3K? Although the ability of CaMKII to activate PI3K has only recently been reported (Ma et al. 2009), it has been well established in mammals that CaMKII phosphorylates both Fak and Pyk2 on multiple serines on the C terminus. These phosphorylation events can activate Pyk2 by enabling subsequent tyrosine phosphorylations (particularly at Tyr402) via mechanisms that are incompletely understood (Della Rocca et al. 1997; Soltoff 1998; Zwick et al. 1999; Heidinger et al. 2002; Fan et al. 2005; Montiel et al. 2007). It has also been well established that Fak and Pyk2, when activated by tyrosine phosphorylation, are each able to activate PI3K: tyrosine-phosphorylated Fak binds p85, the PI3K regulatory subunit, via both the SH3 and SH2 domains (Chen and Guan 1994; Guinebault et al. 1995; Chen et al. 1996). In addition, both tyrosine-phosphorylated Fak and Pyk2 are capable of activating Ras via the conserved Grb2-SoS pathway (Schlaepfer et al. 1994; Dikic et al. 1996; Avraham et al. 2000; Rocic et al. 2001), which could in principle lead to the Ras-dependent, p85-independent activation of PI3K. These observations raise the possibility that Drosophila CaMKII...
might similarly activate PI3K by directly phosphorylating and activating DFak. Alternatively, DFak might function in a more indirect fashion, perhaps as a scaffold linking CaMKII and PI3K in a signaling complex. This alternative possibility would suggest that additional intermediates linking CaMKII and PI3K activation exist but are currently unidentified.

The observation that DmGluRA-mediated activation of PI3K requires CaMKII implies that DmGluRA activation increases intracellular Ca$^{2+}$ levels in Drosophila motor nerve terminals as a necessary step in PI3K activation. The source of Ca$^{2+}$ for this activation is not known. However in mammals, activation of group I mGluRs, which are responsible for mGluR-mediated LTD in the hippocampus and cerebellum, induce phospholipase C and IP3-mediated Ca$^{2+}$ transients (Pin et al. 1994), which are essential intermediates in cerebellar mGluR-mediated LTD (Ito 2001). Although the Drosophila DmGluRA is most similar to mammalian group I mGluRs, which are not known to activate Ca$^{2+}$ transients, given that DmGluRA is the sole mGluR in Drosophila, it seems possible that DmGluRA might carry out many of the functions carried out by each of the three groups of mGluRs in mammals, as suggested previously (Pan et al. 2008). Alternatively, it is possible that DmGluRA activation might...
increase intracellular Ca\(^{2+}\) via the ryanodine receptor, which was previously shown to be an essential activator of CaMKII in *Drosophila* larval motor nerve terminals (Shakiryanova et al. 2007).

The ability of CaMKII to activate PI3K requires the nonreceptor tyrosine kinase DFak; the DFak\(^{CG1}\) null mutation completely blocks the ability of glutamate applied to motor nerve terminals to activate PI3K, completely suppresses the increase in basal p-Akt levels conferred by CaMKII\(^{T287D}\), and blocks the ability of CaMKII\(^{T287D}\) to confer two additional PI3K-dependent phenotypes: increased synapse number at the NMJ and increased motor axon diameter. These results identify DFak as an essential intermediate in PI3K activation by DmGluRA and CaMKII. However, DFak\(^{CG1}\) mutants fail to exhibit other phenotypes conferred by decreased PI3K activity: in an otherwise wild-type background, DFak\(^{CG1}\) larvae exhibit only minor effects on NMJ synapse number or motor axon diameters, which are each significantly decreased by decreased PI3K (Martin-Pena et al. 2006; Howlett et al. 2008). These results raise the possibility that, whereas PI3K activation by DmGluRA and CaMKII is blocked in DFak\(^{CG1}\), total PI3K activity is not strongly decreased because other significant routes to PI3K activation are DFak independent. Alternatively, DFak might participate in signaling pathways distinct from the CaMKII-DFak-PI3K pathway we have identified, which would oppose the effects of PI3K on synapse number and axon diameter. In this view, CaMKII would preferentially promote the ability of DFak to activate PI3K, rather than other DFak-dependent pathways.

In several subregions of the mammalian brain, ligand activation of group I mGluRs induces LTD, a type of synaptic plasticity. This induction both activates and requires the activity of PI3K as well as the PI3K-activated kinase Tor (Hou and Klann 2004; Ronesi and Huber 2008). Several lines of evidence have led to the proposal that increased sensitivity to mGluR-mediated LTD induction might underlie specific neurogenetic disorders. In particular, mice null for the gene affected in fragile X, which is associated with an extremely high incidence of autism as well as other cognitive deficits, exhibit increased sensitivity to mGluR-mediated LTD induction in both the hippocampus (Huber et al. 2002) and cerebellum (Koekkkoek et al. 2005). Furthermore, the genes identified in two additional diseases associated with a high incidence of autism, neurofibromatosis (*Nf1*) and tuberous sclerosis (*Tsc1* and *Tsc2*), each encode negative regulators of the PI3K pathway: *Nf1* encodes a Ras GTPase activator protein (*Xu et al.* 1990), which inhibits the PI3K activator Ras (*Rodriguez-Viciana et al.* 1994), whereas the Tsc proteins are Tor inhibitors that are in turn inhibited by PI3K activity (*Gao et al.* 2002; *Inoki et al.* 2002). Thus loss of *Nf1* or *Tsc* might also increase sensitivity to mGluR-mediated LTD. Finally, several lines of direct evidence suggest that PI3K activation may also be involved in autistic behaviors, but these relationships remain to be explored in the context of the DFak-PI3K pathway.
evidence suggest that PI3K hyperactivation plays a causal role in autism (Serajee et al. 2003; Butler et al. 2005; Kwon et al. 2006; Mills et al. 2007). For example, DNA copy number variants observed in individuals with autism but not unaffected individuals identify at high frequency PI3K subunits or regulators, and each genetic change is predicted to elevate PI3K activity (Cusco et al. 2009). In addition, a translocation that increases expression of the translation factor eIF-4E, which is known to be activated by the PI3K pathway (Miron et al. 2003; Puig et al. 2003), plays a direct, causal role in autism (Neves-Pereira et al. 2009). The potential involvement of mGluR-mediated LTD in these neurogenetic disorders increases interest in identifying the molecular intermediates that participate in this pathway, but these intermediates are for the most part unidentified. Thus, the possibility that CaMKII and Fak might participate in mGluR-mediated PI3K activation in mammals as well as Drosophila might have significant medical interest.

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Literature Cited


Poulin, C., A. Ferrus, and A. Mallart, 1994 Modulation of type A K+ current in Drosophila larval muscle by internal Ca2++; effects of the overexpression of frequenin. Pflugers Arch. 427: 71–79.


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