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Ras-dependent and Ras-independent effects of PI3K in Drosophila motor neurons.

by

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Abstract

The lipid kinase PI3K plays key roles in cellular responses to activation of receptor tyrosine kinases or G protein coupled receptors such as the metabotropic glutamate receptor (mGluR). Activation of the PI3K catalytic subunit p110 occurs when the PI3K regulatory subunit p85 binds to phosphotyrosine residues present in upstream activating proteins. In addition, Ras is uniquely capable of activating PI3K in a p85-independent manner by binding to p110 at amino acids distinct from those recognized by p85. Because Ras, like p85, is activated by phosphotyrosines in upstream activators, it can be difficult to determine if particular PI3K-dependent processes require p85 or Ras. Here we ask if PI3K requires Ras activity for either of two different PI3K-regulated processes within Drosophila larval motor neurons. To address this question, we determined the effects on each process of transgenes and chromosomal mutations that decrease Ras activity, or mutations that eliminate the ability of PI3K to respond to activated Ras. We found that PI3K requires Ras activity to decrease motor neuron excitability, an effect mediated by ligand activation of the single Drosophila mGluR DmGluRA. In contrast, the ability of PI3K to increase synaptic bouton number is Ras independent. These results suggest that distinct regulatory mechanisms underlie the effects of PI3K on distinct phenotypic outputs. We additionally found that the glutamate-activation of DmGluRA initiates ERK signaling; however the signaling intermediates linking DmGluRA to this kinase cascade are unknown.

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Chapter 1: Introduction and Background

1.1 Significance

Cognitive function requires the formation and refinement of neural connections. The capacity of synapses to adapt in response to stimuli, called synaptic plasticity, which involves the strengthening or weakening of a synapse, is widely believed to be the mechanism underlying the retention of information in the brain, thus crucial for learning and memory (Davis, 2006; Martin et al., 2000; Bliss and Collingridge, 1993). Many neurological disorders, including the autism spectrum disorders (ASD)s, are thought to arise from imbalances in the excitatory and inhibitory processes of synaptic transmission that regulate synaptic plasticity (Rubenstein and Merzenich, 2003).

Recently, the phosphoinositide 3-kinase (PI3K) signaling pathway, most notable for its role in insulin-signaling and its effects on cellular growth and survivability (reviewed in Engelman et al., 2006), has emerged as a key mediator of the functionality and establishment of neural circuits. Hyperactivated PI3K signaling has recently been found to participate in the etiology of several neurological diseases that have co-morbidity with autism, including Neurofibromatosis 1 (NF1), tuberous sclerosis (TSC), and Fragile X Syndrome (FXS) (Kalkman, 2006; Levitt and Campbell, 2009; Serajee et al., 2003; Butler et al., 2005; Kwon et al., 2006; Mills et al., 2007). Therapeutics that have targeted the PI3K signaling pathway have proven useful in the treatment of cancer (Morgensztern and McLeod, 2005), therefore elucidating the molecular signaling

components that regulate PI3K activity in neural tissues may be additionally useful for the design of new treatments for neurological disease.

1.2 Phosphoinositide 3-kinase (PI3K) is involved in a multitude of cellular processes

1.2.1 The biochemistry of PI3K

PI3K functions by specifically phosphorylating the 3'-hydroxyl of the inositol ring of intracellular phosphatidylinositol (PtdIns), PtdIns 4-phosphate (PI4-P), and PtdIns(4,5)-biphosphate (PI(4,5)P_2 or PIP_2)(Panayotou,1998) (Fig.1). The phosphorylation of these inositol lipids into phosphoinositides (PI)s, particularly the second messenger PtdIns(3,4,5)P_3 (PIP_3), by PI3K is crucial for many cellular processes including cell growth and survival, the rearrangement of the cytoskeleton, membrane trafficking, and transcription (Lemmon, 2008; Hurley, 2006; Di Paolo and De Camilli, 2006; Roth, 2004; Brunet et al., 2001). The multiple isoforms of PI3K present in eukaryotic organisms are divided into three classes (class I-III) based on their structure, substrate specificity, and mode of regulation (Vanhaesebroeck et al., 1997). The class I PI3Ks are involved in cell-signaling downstream receptor tyrosine kinases and heterotrimeric G protein-coupled receptors (GPCRs) and are the focus of this study. The class II and class III PI3Ks are involved in G-protein beta-gamma subunit ($G_{B\gamma}$) activation and intracellular protein trafficking, respectively (MacDougall et al., 1995; Stephens et al., 1994; Schu et al., 1993). These classes of PI3K are beyond the scope of this work and will not be discussed here.

The vertebrate class I PI3Ks are heterodimers consisting of two subunits; a 1,068 AA catalytic subunit, called p110, and an adaptor protein, p85, which contains two Src homology domains (SH2) linked by an inter-SH2 region (Vanhaesebroeck et al., 2001; Panayotou, 1992). Three isoforms of p110 (p110α, p110β, p110γ) and seven associated adaptor proteins (generated from the alternate splicing of p85α, p85β, and p55γ) have been identified in mammals (Vanhaesebroeck and Alessi, 2000). Drosophila has only one class I PI3K, Dp110 (MacDougall et al., 1995), and a single, SH2-domain-containing adaptor protein, p60 (Weinkove et al., 1997).

The adaptor protein links the p110 catalytic subunit to different upstream signaling events via the binding of proteins that contain a phosphotyrosine in a Y(P)xxM motif (i.e. receptor tyrosine kinases) (Vanhaesebroeck et al., 2001; Vanhaesebroeck et al.,1997). The binding of the adaptor protein to the appropriate phosphotyrosine target subsequently recruits p110 to the membrane where interaction with the membrane-associated PtdIns substrates can occur (Vanhaesebroeck et al., 1997).

1.2.2 Overview of the PI3K signaling cascade

The specificity of PI3K in the activation of these various processes is mitigated by the engagement of a variety of effector molecules. Most notably is the serine/threonine kinase Akt (also known as protein kinase B, PKB). Akt contains an N-terminal pleckstrin homology (PH) domain that preferentially binds PIP_3 (James et al., 1996; Stephens et al., 1998). PIP_3 recruits Akt to the membrane, altering its conformation, enabling a second serine/threonine kinase,

phosphoinositide-dependent kinase-1 (PDK1), to phosphorylate and activate Akt (Thr308 in vertebrates and Thr505 in Drosophila) (Stephens et al., 1998; Alessi et al., 1997) (Fig.1.1). Activated Akt targets several substrates that are involved in growth and cell-cycle regulation including the forkhead box transcription factor (FOXO) (Puig et al., 2003) and the tuberous sclerosis complex (TSC1/2) (Potter et al., 2001). FOXO is a negative regulator of cellular growth and an activator of apoptosis (Junger et al., 2003; Paik et al., 2007). Phosphorylation of FOXO via Akt is inhibitory, thus PI3K signaling decreases FOXO activity, releasing the cell from FOXO-dependent growth inhibition. As a negative regulator of PI3K signaling, the phosphatase and tensin homologue (PTEN) opposes the activity of PI3K by preferentially dephosphorylating PIP_3 (Chu and Tarnawski, 2004) (Fig.1.1)

PI3K-Akt signaling regulates translation via mammalian target of rapamycin (mTOR) (Hay and Sonenberg, 2004; Inoki et al., 2002). The phosphorylation of TSC2 of the TSC1/2 complex by activated Akt inactivates the mTOR complex (Inoki et al., 2002). The TSC1/2 complex primarily functions as a GTPase-activating protein for Ras-homolog enriched in brain (Rheb) which directly activates mTOR; therefore, the inactivation of TSC1/2 allows Rheb to stimulate mTOR complex activity (Long et al., 2005; Sanack et al., 2007). The mTOR complex, consisting of two multi-protein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), is a regulator of cell growth, proliferation, cell survival, and translation (Laplante and Sabatini, 2009) mTORC1 promotes protein synthesis by phosphorylating the eukaryotic initiation factor 4E

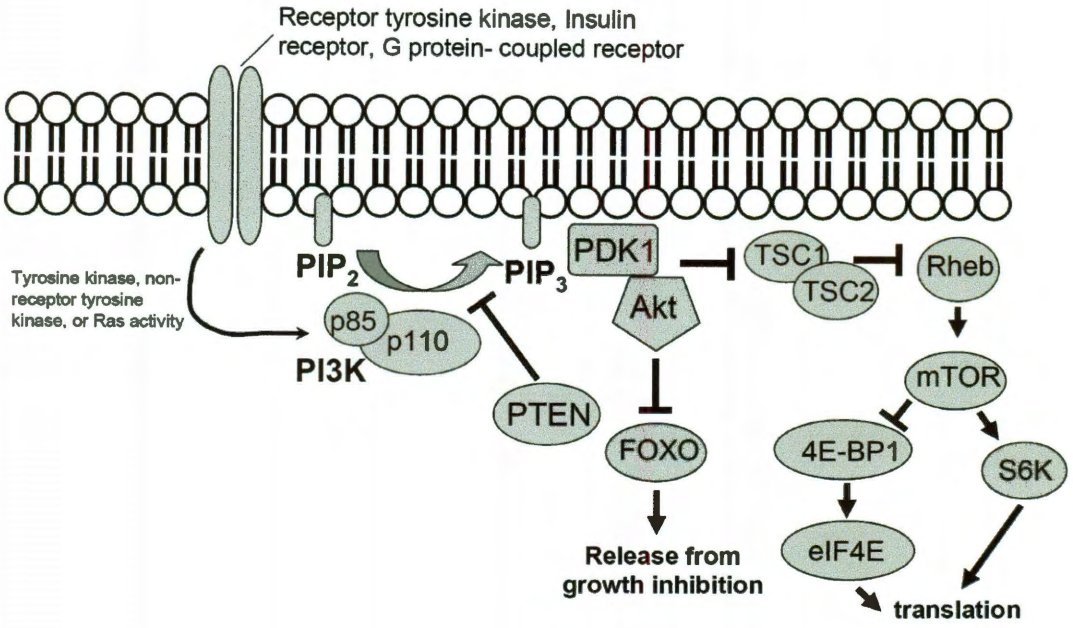


Figure 1.1: Overview of the PI3K signaling cascade. Class I PI3K molecules are typically activated by receptors that induce tyrosine kinase activity, insulin receptors, and G protein-coupled receptors. PI3K phosphorylates the inositol ring of phosphatidylinositol 4,5-biphosphate (PIP_2), producing the second messenger, phosphatidylinositol 3,4,5-triphosphate (PIP_3). The activity of PI3K is negatively regulated by phosphatase and tensin homolog (PTEN). PIP_3 recruits the serine/threonine kinase Akt to the membrane via the N-terminal pleckstrin homology domain of Akt, altering the conformation of Akt which enables a second kinase, phosphoinositide-dependent kinase-1 (PDK1), to phosphorylate and activate Akt. Activated Akt negatively regulates the activity of the transcription factor, forkhead box transcription factor (FOXO). Activated Akt additionally inactivates the tuberous sclerosis complex (TSC1/2), which functions as a GTPase for Ras-homolog enriched in brain (Rheb). Inactivation of TSC1/2 allows Rheb to activate the mammalian target of rapamycin (mTOR) complex. The mTOR complex promotes translation by phosphorylating and thus inhibiting the eukaryotic initiation factor 4E (eIF4E) binding protein (4E-BP1) complex, which allows the initiation factor eIF4E to bind and promote translation. mTOR additionally activates the ribosomal protein S6 kinase (S6K) which further enhances mRNA translation (reviewed in Vanhaesebroeck et al., 2001).

(eIF4E) binding protein (4E-BP1) and p70 S6 kinase (S6K). The phosphorylation of 4E-BP1 prevents it from binding eIF4E, enabling eIF4E to promote cap-dependent translation of ribosomal proteins (Hay and Sonnenberg, 2004). The phosphorylation of S6K by mTOR1 enhances the translation of 5' terminal oligopyrimidine tract-containing (5'-TOP) mRNAs (Dufner and Thomas, 1999; Jefferies et al., 1997). The regulation of 4E-BP1 as well as p70 S6K are crucial to neural processes, including those that govern synaptic plasticity (Antion et al., 2008; Gelinas et al., 2007; Banko et al., 2006), and the inappropriate regulation of mTOR signaling has been implicated in many human diseases including autism (Sabatini, 2006; Dann et al., 2007).

1.2.3 PI3K is involved in neural outgrowth processes

Changes in nervous system architecture have been linked to neurological dysfunction and are often hallmarks of mental retardation (Meredith and Mansvelder, 2010; Ess, 2006). PI3K is widely recognized as a principle intermediary of cellular growth and metabolism, and has recently emerged as a key mediator of synaptic growth, therefore influencing connectivity in the brain (Kwon et al., 2006). In cultured neurons, PI3K signaling is required for the outgrowth of neurites, which are neural protrusions that can differentiate into either axons or dendrites, in response to growth factors such as nerve growth factor (NGF) (Kimura et al., 1994). Neurite outgrowth can be blocked by the application of specific PI3K inhibitors and the overexpression of PI3K is sufficient to promote neurite outgrowth and elongation in the absence of NGF (Zheng et al., 2011; Kita et al., 1998).

PI3K signaling is additionally required for the outgrowth and morphogenesis of differentiated dendrites and axons. Factors that regulate dendritic growth, including Reelin, the epidermal growth factor CALEB/NGC, and Semaphorin-4D, have been found to influence dendritic complexity through the PI3K-Akt-mTOR pathway in the rat hippocampus (Jossin and Goffinet, 2007; Brandt et al., 2007; Vodrazka et al., 2009). Constitutively active PI3K, Akt, or the inhibition of PTEN have all been found to increase dendritic growth and to alter dendrite morphology (Jaworski et al., 2005; Kumar et al., 2005; Luikart et al., 2008). Axon growth in response to growth factors, including NGF and brain-derived neurotropic factor (BDNF), also requires PI3K signaling (Zhou et al., 2004; Tucker, 2002; Atwal et al., 2000). The PI3K effector Akt has also been found to be enriched in the growth cones of developing hippocampal axons (Shi et al., 2003). Pharmacological inhibition of PI3K or the expression of the PTEN in cultured hippocampal cells prevents axon elongation (Shi et al., 2003; Menager et al, 2004; Jiang et al., 2005; Yoshimura et al., 2006). PI3K mediates the effects on neurite outgrowth via the regulation of the Rho GTPases, Cdc42 and Rac1 (Aoki et al., 2004; Luo et al., 2002) and additionally regulates axon growth cones by influencing microtubule dynamics (Akiyama and Kamiguchi, 2010; Rodgers and Theibert, 2002).

1.2.4 PI3K is also involved in neural function via the regulation of two forms of synaptic plasticity: Long-term potentiation and long-term depression

As previously mentioned, synaptic plasticity is widely believed to be crucial for learning and memory (Davis, 2006; Martin et al., 2000; Bliss and Collingridge, 1993). There are two defined forms of plasticity: "short-term" and "long-term" plasticity that are based on the period of time that the change in activity modifies the function of the neuron. Short-term plastic phenomena, which include facilitation (an increase in the release of vesicles from a vesicle pool within the pre-synapse) and augmentation (when an action potential increases the efficiency at which neurotransmitters are released), are temporary changes in activity that are quickly rectified by the neuron returning back to its "resting state" (Stevens and Wesseling, 1999). Long-term plasticity results from extended periods of altered synaptic activity that generally occurs after the repeated stimulation of the neuron (Bliss and Lomo, 1973). Long-term potentiation (LTP) and long-term depression (LTD), forms of long-term synaptic plasticity that involve an increase or decrease in synaptic strength, respectively (Citri and Malenka, 2008), are triggered by synaptic activity in the brain (Bear, 1998) and require an increase in transcription (Kang and Schuman, 1996; Huber et al., 2000). These forms of plasticity generally occur at excitatory synapses and are thought to underlie the processes that govern cognitive function (Davis, 2006; Martin et al., 2000; Bear, 1998; Bliss and Collingridge, 1993).

PI3K signaling is involved in the establishment of both LTP and LTD which has been primarily studied in the excitatory synapses of the CA1 region of the vertebrate hippocampus (Bear and Malenka, 1994; Bliss and Collingridge, 1993).

LTP occurs when multiple synapses are simultaneously activated at a high frequency, resulting in the depolarization of the post-synaptic cell in conjunction with the release of the excitatory neurotransmitter, glutamate, from the pre-synaptic neuron (reviewed in Nicoll and Malenka, 1995). This combination results in the activation of the post-synaptic ionotropic glutamate receptors (iGluRs), α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) and N-methyl-D-aspartate (NMDA), which sensitizes the postsynaptic cell to further stimulation. PI3K has been found to co-localize with AMPA receptors and is required for the insertion of these receptors in to the post-synaptic membrane during the induction of LTP (Man et al., 2003). PI3K signaling is also required for the maintenance of LTP in the post-synapse by regulating translation via mTOR and p70 S6K (Karpova et al., 2006).

LTD is also mediated by the activation of synaptic NMDA receptors (Dudek and Bear, 1992) or by second type of glutamate receptor, the metabotropic glutamate receptor (mGluR) (Gladding et al., 2009) (Fig.1.2). Unlike LTP, LTD results in the de-sensitization of the post-synaptic cell occurring in response to a prolonged, low frequency stimulation. LTD can be initiated via the entry of Ca^{2+} through NMDA receptor channels or by the activation of mGluRs on the post-synaptic cell (discussed in more depth in the following section) (Malenka, 1995; Gladding et al., 2009). PI3K signaling couples the activation of mGluR to dendritic protein translation which is required to sustain LTD (Hou and Klann, 2004).

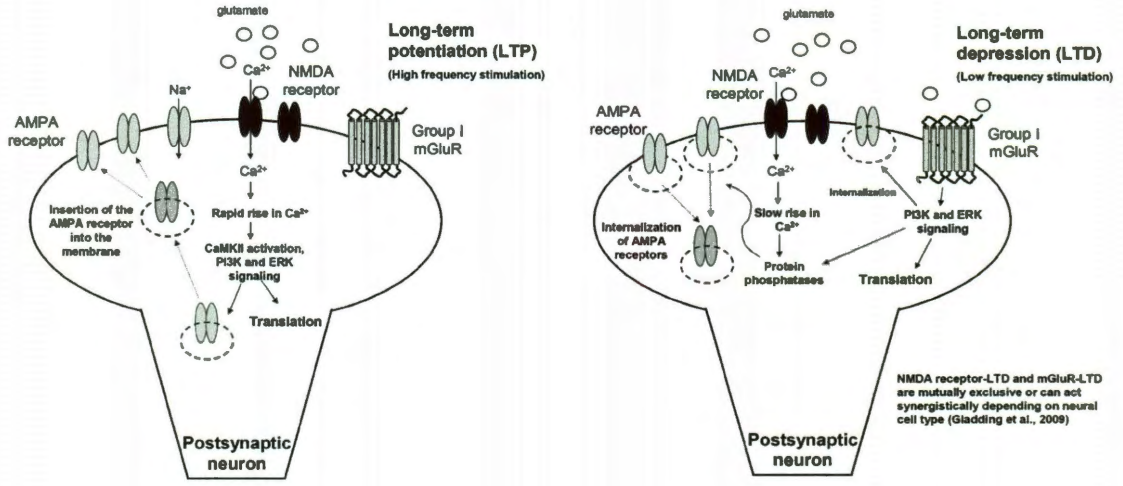


Figure 1.2: Long-term potentiation (LTP) and long-term depression (LTD) modify synaptic activity. (A) LTP is induced by high-frequency electrical stimulation resulting in postsynaptic depolarization. Depolarization removes Mg^{2+} from the NMDA receptor channel, which allows glutamate binding to permit Ca^{2+} to enter in to the cell through the channel. Ca^{2+} activates a series of signal transduction cascades that regulate the insertion of the AMPA receptor into the postsynaptic membrane via the activity of protein kinases. Membrane insertion of additional AMPA receptors increases the sensitivity of the postsynaptic cell to glutamate. The maintenance of later phases of LTP requires the translation of proteins involved in making new synaptic contacts. **(B)** LTD is induced by long periods of low-frequency electrical stimulation that also results in the activation of NMDA receptors, resulting in Ca^{2+} entry into the postsynaptic cell. The rise in $Ca^{2+}$is slower compared to the rise in Ca^{2+} during LTP and activates a series of phosphatases that are associated with the internalization of AMPA receptors from the membrane. Loss of the AMPA receptors decreases the sensitivity of the postsynaptic cell to glutamate. LTD is also mediated by the glutamate-activation of mGluRs, called mGluR-LTD. During this form of LTD, the activation of the group I mGluRs initiates a series of signal transduction cascades that regulate transcription. This form of LTD can be independent or work synergistically with NMDA receptor-LTD depending on the cell type. (Note: This overview reflects LTP/LTD in vertebrate hippocampal neurons. These processes differ in other neural cell types). (Reviewed in: Purves, D., et al., eds. Neuroscience, 4th ed. Massachusetts, Sinauer Assoc. Inc., 2008.)

1.2.5 Aberrant PI3K signaling is observed in neurological diseases that have co-morbidity with autism

Abnormalities in the regulation of excitatory synapses, altering the initiation and maintenance of LTP and LTD, have been linked to autism (Rubenstein and Merzenich, 2003). Aberrant PI3K signaling has been linked to a number of significant neurological diseases that have co-morbidity with autism, including Fragile X Syndrome (FXS), tuberous sclerosis (TSC), and Cowden/Lhermitte-Ducols syndrome (Bourgeois et al., 2009; Kelleher and Bear, 2008). The affected genes in FXS (*fragile X mental retardation protein*, *FMR1*), TSC (*tuberous sclerosis complex 1* and *2*, *TSC1/2*), and Cowden/Lhermitte-Ducols syndrome (*phosphatase and tensin homolog*, *PTEN*), encode negative regulators of the PI3K pathway (Gross et al., 2010; Levitt and Campbell, 2009; Kelleher and Bear, 2008; Williams et al., 2008; Kwon et al., 2006). Interestingly, gene copy number variants discovered in individuals with autism spectrum disorders (ASD) involve genes that affect the PI3K signaling pathway that are predicted to elevate PI3K activity (Cuscó et al., 2009). The mTOR inhibitor, rapamycin, is currently being used clinically as an immunosuppressant for organ transplants and for psoriasis (under the brand name Rapamune®, Wyeth Pharmaceuticals); however its use as for treatment for FXS and these other disorders that involve abnormal PI3K has been limited (reviewed in Ehninger and Silva, 2010). Rapamycin has been useful for the treatment of the tumors that form in association with TSC (Davies et al., 2008); however, the effect of this drug on cognition and neurological symptoms are still underway (de Vries, 2009).

Identifying additional components of the PI3K signaling pathway involved in the PI3K-mediated effects on synaptic plasticity may prove useful for the development of more effective therapeutics.

1.3 Metabotropic glutamate receptors are crucial for synaptic processes

1.3.1 Metabotropic glutamate receptors mediate synaptic plasticity by regulating neuronal excitability

In addition to the iGluR, another receptor type that is crucial to the regulation of excitatory synapses is the metabotropic glutamate receptor (mGluR). The mGluRs are G protein-coupled receptors and unlike the iGluRs are considered to be involved in "slow" synaptic transmission through the activation of second messengers (Ferraguti et al., 2008; Conn and Pin, 1997; Gerber et al., 2007). The mGluRs function to modulate neuronal excitability and are involved in the feedback regulation of neurotransmitter release (Schoepp, 2001; Ferraguti and Shigemoto, 2006).

The mGluRs can be categorized into three groups (group I-III) based on sequence homology and their associated downstream signaling components (Schoepp, 2001; Conn and Pin, 1997). In general, group I mGluRs (G_q-coupled) activate Ca^{2+} transients via a phospholipase-C (PLC) – inositol triphosphate (IP_3) receptor dependent pathway (Fig.1.3). The group II/III mGluRs (G_i/G_o coupled) are negatively coupled to adenylyl cyclase and inhibit the formation of cyclic

adenosine monophosphate (cAMP) (Fig.1.3) (Gerber et al., 2007; Conn and Pin, 1997). Group I mGluRs are commonly found on the postsynapse (Baude et al., 1993) and group II/III are localized both pre- and postsynaptically (Shigemoto et al., 1997). In particular, the group II mGluRs function as autoreceptors by detecting excess glutamate that has diffused out of the synapse during high-frequency synaptic activity (Bogdanik et al., 2004; Lujan et al., 1997; Shigemoto et al., 1997). The activation of group II mGluRs by glutamate initiates a negative-feedback signal that suppresses the additional release of glutamate by the neuron, thus controlling the strength of synaptic transmission and reducing the risk of glutamate toxicity (Vogt and Nicoll, 1999; Conn and Pin, 1997; Scanziani et al., 1997). This suppression is thought to occur via the inhibition of voltage-dependent Ca^{2+} channels, the activation of K^{+} channels, or by interfering with vesicle exocytosis (Takahashi et al., 1996; Scanziani et al., 1995).

Many neurological disorders, including anxiety disorders, epilepsy, and schizophrenia are considered to be disorders of glutamatergic synaptic transmission (Enz, 2007; Chapman et al., 1996). Coincidentally, alteration in mGluRs or mGluR signaling is implicated in a number of these same diseases (Enz, 2007). The implication that mGluRs may participate in these disorders makes investigating mGluR signaling an attractive area of study for the development of potential therapeutic targets (reviewed in Byrnes et al., 2009; Swanson et al., 2005).

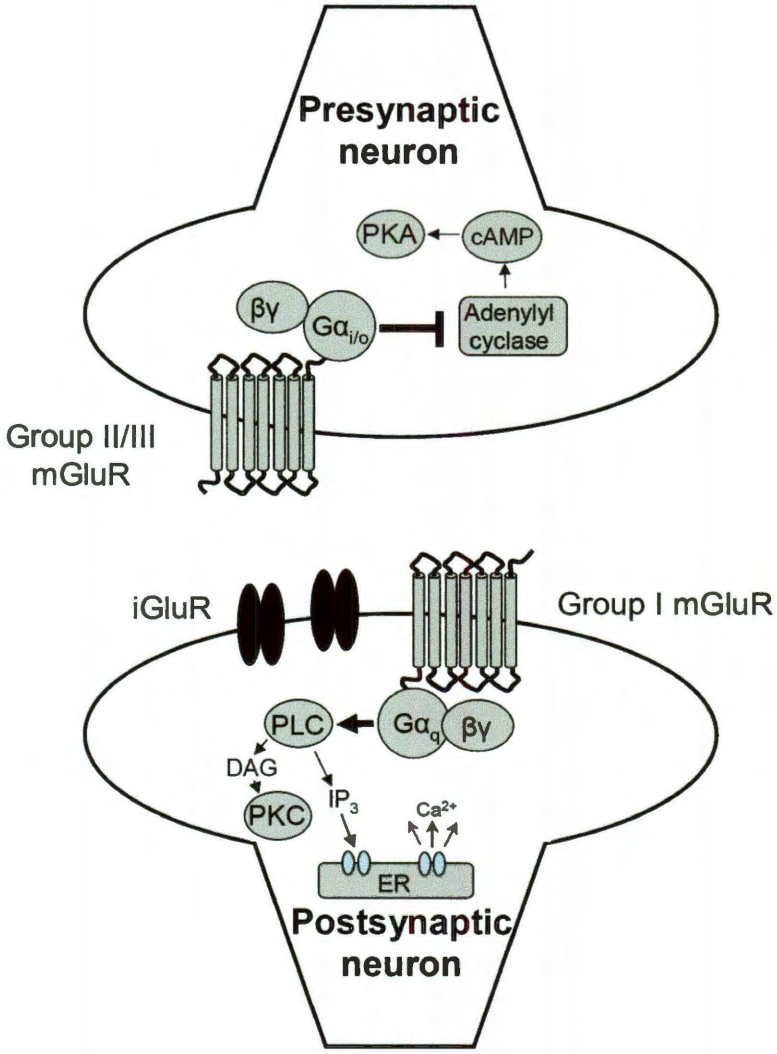


Figure 1.3: Overview of the group I/III metabotropic glutamate receptor (mGluR) signaling cascades. mGluR activation initiates G protein activity that increases or decreases the activation of downstream kinases. **(Top)** Group II/III mGluRs generally have pre-synaptic localization and are primarily coupled to $G\alpha_{i/o}$. Activation of group II/III mGluRs suppresses adenylyl cyclase activity, reducing cyclic AMP (cAMP) levels and protein kinase A (PKA) activity. **(Bottom)** Group I mGluRs are expressed primarily on postsynaptic sites and are linked to $G\alpha_q$. The activation of group I mGluRs activates phospholipase C (PLC) to generate diacylclycerol (DAG) and inositol 1.4.5-triphosphate (IP_3), leading to a release of Ca^{2+} from intracellular stores. DAG additionally simulates protein kinase C (PKC) activation. Another type of glutamate receptor, the ionotropic glutamate receptor (iGluR), is additionally found on postsynaptic sites and regulates fast-synaptic transmission. These receptors are comprised of the AMPA and NMDA receptors (reviewed in Kim et al., 2008; Schoepp, 2001).

1.3.2 mGluRs mediate LTP and LTD via the regulation of translation

The mGluRs are involved in the establishment of both LTP and LTD (Holscher et al., 1999; Altinbilek and Manahan-Vaughan, 2009; Manahan-Vaughan, 1997). The induction of LTD by mGluRs, termed mGluR-LTD, requires the activation of the postsynaptic group I mGluRs as well as an increase in intracellular calcium (Ca^{2+}) (Kleppisch et al., 2001; Oliet et al., 1997; Shigemoto et al., 1997). mGluR-LTD results in a persistent decrease in post-synaptic AMPA receptors and the rapid translation of pre-existing dendritic mRNA, which increases protein synthesis in localized areas at or around dendritic spines (Huber et al., 2000; Job and Eberwine, 2001; Raymond et al., 2000; Karachot et al., 2001). Work from several groups has established that the Extracellular signal-related kinase/Mitogen-activated protein kinase (ERK/MAPK) and mTOR signaling pathways couple mGluR activation to translational machinery during both LTP and LTD (Gladding et al., 2009; Banko et al., 2006; Gallagher et al., 2004; Hou and Klann, 2004; Sweatt, 2004). The post-synaptic scaffolding protein, Homer, forms a signaling complex with group I mGluRs to couple these receptors to the ERK and PI3K signaling cascades (Rong et al., 2003; Ronesi and Huber, 2008) and is a specific requirement for the initiation of translation during mGluR-LTD (Ronesi and Huber, 2008).

Recently, a role for the presynaptic group II mGluRs in the establishment of LTD has also been identified. Pharmacological inhibition of group II mGluRs abolishes LTD in hippocampal neurons and results in spatial learning defects in rats (Altinbilek and Manahan-Vaughan, 2009). The group II mGluRs lack the

Homer-binding motif (Diagana et al., 2002) and the molecular mechanisms linking this receptor subtype to LTD are not clear. The altered expression of group II mGluRs have been suggested to contribute to the pathophysiology of schizophrenia and epilepsy (Gupta et al., 2005; Aronica et al., 1997); therefore analysis of the signaling intermediates is of interest to human health.

1.3.3 Aberrant mGluR and PI3K signaling appear to be involved in the etiology of Fragile X Syndrome

Overactive group I mGluR signaling, which results in abnormal LTD, has been implicated in epilepsy, cognitive impairment, and developmental delay (reviewed in Bear et al., 2004). Enhancement of mGluR-LTD has been observed in a mouse model of the human neurological disorder, Fragile X Syndrome (FXS) (Koekkoek et al., 2005; Huber et al., 2000). FXS is the most common form of inherited mental disability resulting from a loss-of function mutation in the gene encoding the Fragile X mental retardation protein (FMRP), which in humans is generally caused by the expansion of a CGG repeat sequence in the 5' untranslated region of the *fragile X mental retardation 1* (*FMR1*) gene (Garber et al., 2006; Bagni and Greenough, 2005; Feng et al., 1995). Patients exhibit a wide range of neurological deficits including cognitive impairment, seizures, attention deficits, and autism (Penagarikano et al., 2007). FMRP normally binds and represses the translation of mRNAs that are translated during mGluR-LTD (Banko et al., 2006; Weller et al., 1997), therefore loss of FMRP activity is most likely responsible for enhancement of mGluR-LTD observed in models of FXS

(Pfeiffer and Huber, 2009; Nosyreva and Huber, 2006; Muddashetty et al., 2007; Weiler et al., 2004).

New evidence indicates that loss of FMRP results in the dysregulation of the PI3K signaling pathway. It was previously demonstrated that the *de novo* protein synthesis required for the establishment of mGluR-LTD involves the mGluR initiation of the PI3K-Akt-mTOR cascade (Hou and Klann, 2004). *Fmr1* knockout (KO) mice, used as models for FXS, exhibit elevated S6K and 4E-BP activity, indicative of increased mTOR signaling (Sharma et al., 2010). Additionally, PI3K and the upstream activator PI3K enhancer (PIKE) are enhanced in *Fmr1* KO mice (Sharma et al., 2010). Loss of FMRP in KO mice also results in the excess mRNA translation of the catalytic subunit of PI3K, p110β, and that the basal enzymatic activity of PI3K was increased threefold in FXS synapses (Gross et al., 2010). Application of pharmacological inhibitors to cultured neurons that antagonize PI3K signaling corrects several phenotypes associated with FXS, including aberrant synaptic translation and excess spine density, suggesting that the PI3K signaling cascade may be a useful target for therapeutics (Gross et al., 2010).

1.4 The effects of PI3K signaling on synaptic plasticity may involve Ras

1.4.1 Ras, a GTPase, is an activator of PI3K

PI3K appears to be a critical regulator of both neural form and function and PI3K-pathway components may prove to be useful targets for the treatment of

neurological disorders such as ASD. Though it has been suggested that group I mGluRs may activate PI3K via a complex with the the scaffolding protein Homer (Ronesi and Huber, 2008) additional activators that link PI3K to group II mGluRs or to the synaptic growth-associated effects of PI3K activation have not been elucidated. One well-studied mechanism involves the receptor-tyrosine kinase activation of phosphotyrosines that activate PI3K via the regulatory subunit p85 (Pignataro and Ascoli, 1990). In addition to this route, PI3K can also be activated in a p85-independent manner by the monomeric guanosine triphosphatase (GTPase), Ras.

Ras, a member of a large family of membrane-associated GTPases, is an essential mediator of intracellular signaling, transducing extracellular messages from the cell membrane to within the cell (Giehl, 2005; Malumbres and Barbacid, 2003; Barbacid, 1987). Ras is well-known for its role in cell proliferation, differentiation, survival, and cell death and gain-in-function mutations in *ras* genes are closely associated with tumorigenesis (Giehl, 2005; Malumbes and Barbacid, 2003; Bos, 1989). Ras cycles between an active conformation (GTP binding) and an inactive conformation (GDP binding) and the switch between these two forms can be regulated by the activity of two families of proteins; the guanine exchange factors (GEFs), which enhance the activity of Ras, and the of GTPase-activating proteins (GAPs) that reduce Ras activity (Bernards and Settleman, 2004).

Ras functions through a series of downstream signaling cascades. The most notable effectors include the serine/threonine kinase Raf, Ral, and the class I

PI3K molecules (Fig.1.4). The Ras-Raf-Mitogen Activated Protein Kinase (MAPK; the MAPK family which also includes Extracellular signal-Regulated Kinase, ERK) signaling pathway is the best-characterized signaling cascade most-often involved in the cellular growth-related effects associated with Ras (Malumbres and Barbacid, 2003) (This pathway is discussed in more depth in Chapter 4). Perturbations in this effector pathway have also been implicated in numerous neurological disorders including Noonan, Costello, and cardio-facio syndromes (reviewed in Schubbert et al., 2007).

PI3K is also a well-studied Ras effector (Orme et al., 2006; Rodriguez-Viciana et al., 1994). Activated Ras binds to a specific Ras-binding domain (RBD) in the in the catalytic unit of PI3K, inducing a conformation change that modifies the phosphoinositide headgroup binding site that activates the kinase activity of PI3K (Rodriguez-Viciana et al., 1996). Activation by Ras has been suggested to be a requirement for the full activation of PI3K (Rodriguez-Viciana et al., 1996; Kodaki et al., 1994; Rodriguez-Viciana et al., 1994). Similar to PI3K, Ras is involved in synaptic plasticity and aberrant Ras activity results in alterations of synaptic outgrowth (Fivaz et al., 2008; Oinuma et al., 2007; Kumar et al., 2005; Jarwoski et al., 2005; Govek et al., 2005; Markus et al., 2002) LTD and LTP (Ye and Carrew, 2010; Hu et al., 2008; Arendt et al., 2004); however, it is unclear to what extent Ras and PI3K interact in these processes.

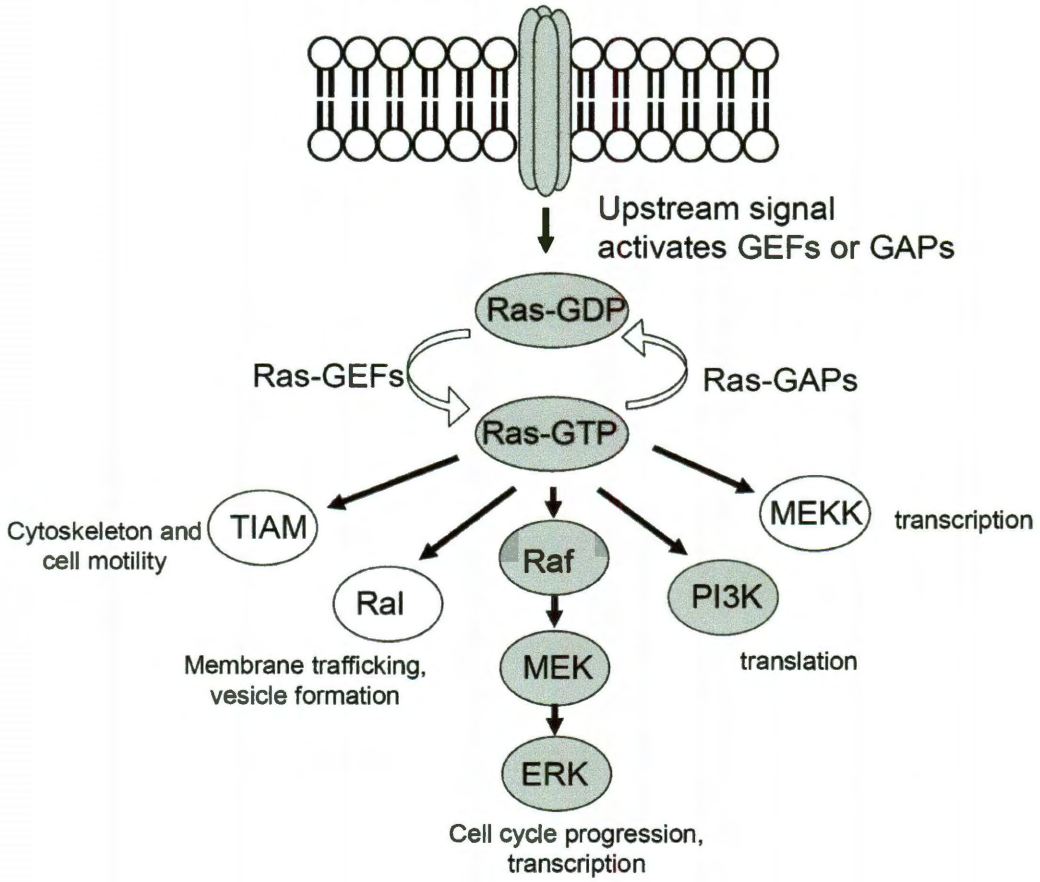


Figure 1.4: Overview of Ras effector cascades. Ras activation is initiated by the activation of several receptor types, including receptor-tyrosine kinases and G protein-coupled receptors. Activated Ras initiates multiple signal transduction cascades involved in a variety of cellular activities (note: the above list of effector molecules is not exhaustive). The two most critical in regards to this study are the PI3K and Raf effector pathways. Ras binds to a specific Ras-binding domain in the catalytic unit of PI3K, which activates PI3K. Activated Ras also activates the serine-threonine kinase Raf, that then activates the mitogen-activated protein kinase kinase (MEK). MEK phosphorylates and activates extracellular signal-related kinase (ERK), which is involved in the activation of transcription factors (reviewed in Malumbres and Barbacid, 2003).

1.4.2 Ras, like PI3K, is involved in synaptic plasticity and is associated with several significant neurological diseases

Ras signaling is involved in the modulation of synaptic structure. Ras has been identified in developing axons (Fivaz et al., 2008), and similar to PI3K, Ras overexpression has been demonstrated to increase both axon and dendrite outgrowth in both cultured cells and *in vivo* (Markus et al., 2002, Kumar et al., 2005; Jarwoski et al., 2005; Oinuma et al., 2007; Romero et al., 2007); whereas down-regulating Ras prevents dendritic spine maturation (Govek et al., 2005). Overexpression of Ras has additionally been found to modify axon thickness in the neurons of the vertebrate corpus callousm (Arendt et al., 2004) and Ras activity is additionally required for the growth and elongation of axons in response to growth factors such as nerve growth factor (NGF) (Markus et al., 2002; Zhou et al., 2004).

Similar to PI3K, Ras and the Ras family proteins appear to be critical for memory formation via the modification of LTP and LTD (reviewed in Ye and Carrow, 2010). A role for Ras in the establishment of LTP was found in mouse models of the human disease Neurofibromatosis-1 (NF1). NF1, which results from the mutation of the Ras-GAP encoding gene, *neurofibromatosis 1* (*NF1*) (Xu et al., 1990), is characterized by skeletal abnormalities, abnormal skin pigmentation, and tumors of the peripheral nerves (reviewed in McClatchey, 2007). These growth defects are suspected to be the result of hyperactivated Ras signaling (McClatchey et al., 2007; Gottfried et al., 2010). Patients with NF1 are also often diagnosed with learning disabilities and ASD that may be related to

the perturbations in LTP (Kelleher and Bear, 2008; Silva et al., 1997; Costa et al., 2001; Costa et al., 2002; Costa et al., 2005; Li et al, 2005; North et al., 2002). The injection of pharmacological inhibitors of Ras restores the abnormal LTP and memory defects observed in mouse models of NF1 indicating that in addition to the aberrant cellular growth effects, Ras is also facilitating changes in synaptic function (Costa et al., 2002; Li et al., 2005).

Though the hyperactivation of both Ras and PI3K have been observed in disorders related to autism, the possibility that they may be functioning in a similar pathway has not been carefully explored. One recent report suggests that Ras-PI3K signaling may be involved in LTP in mouse models of FXS (Hu et al., 2008). A significant reduction in LTP was observed in *FMR1* KO mice that correlated with the aberrant delivery of AMPA receptors to the post-synaptic membrane of hippocampal neurons (Hu et al., 2008). Similar to PI3K, Ras has been previously shown to be involved in the synaptic delivery of AMPA receptors to the post-synaptic membrane (Malinow and Malenka, 2002; Isaac et al., 2007), and it was found that the LTP defect could be restored by overexpressing wildtype Ras in these neurons (Hu et al., 2008). The rescue of LTP by Ras was blocked by the application of the PI3K pharmacological inhibitor, LY294002, indicating that Ras is acting on the AMPA receptor via PI3K (Hu et al., 2008).

1.4.3 Ras is positioned to activate both the ERK and PI3K signaling cascades: is Ras a requirement for PI3K-mediated effects on synaptic growth and function?

Ras is positioned to activate both the ERK and PI3K cascades and it is not always clear if the effects of Ras on cellular function are via the activation of only one or both of these effector pathways. Deciphering the downstream activity of Ras has been important for the treatment of human cancers. Interestingly, the activation of either the Ras-ERK or PI3K pathways appears to confer differing resistance to chemotherapeutic drugs in cancer cells. For example, in breast cancer cells, the activation of Ras-ERK provides resistance to doxorubicin and paclitaxel; whereas the activation of PI3K provides resistance to doxorubicin and 4-hydroxyl tamoxifin (reviewed in McCubrey et al., 2006). However, in prostate cancer cells, the PI3K pathway, but not the Ras-ERK pathway, is involved in drug resistance (McCubrey et al., 2006). These findings indicate that in different tumor types that these pathways may have distinct roles in the progression of oncogenesis. Understanding which of the Ras-effector pathways is altered under different oncogeneic conditions is critical for the application of effective cancer treatments. Therefore elucidating the specific activation of Ras-ERK or Ras-PI3K in the nervous system may also be useful for the treatment of nervous system disorders.

In the vertebrate nervous system, the ERK and PI3K cascades have been observed to function cooperatively to regulate neuron outgrowth; however, each pathway has also been found to have a differential effect on growth (Kumar et al., 2005; Jaworski et al., 2005; Arendt et al., 2004; Koh et al., 2002; Markus et al., 2002). The activation of Ras and the ERK and PI3K pathways is required for axon growth in cultures of dorsal root ganglion neurons; however, the activation

of Ras-ERK is primarily responsible for axon elongation whereas PI3K signaling is involved in the soma size and axon branching (Markus et al., 2002). Similarly, in cultured hippocampal cells, Ras activation facilitates dendrite outgrowth via the ERK and PI3K effector pathways and PI3K activation is also specifically involved in the size of the cell body and dendritic branching (Jaworski et al., 2005; Kumar et al., 2005). In the pyramidal neurons of the hippocampus, soma size depends on Ras-ERK signaling (Arendt et al., 2004), whereas Ras-ERK signaling is critical for dendrite outgrowth (Kumar et al., 2005). These studies clearly indicate that the ERK and PI3K pathways play distinct roles in the modification of neural and synaptic structure; however, pharmacological inhibitors that block PI3K signaling by targeting PI3K effectors were utilized to block PI3K signaling, so it is unclear to what extent Ras may be required for PI3K-mediated affects on neural growth.

Though it is well-established that PI3K participates in NMDA receptor-mediated LTP and mGluR-LTD, much less is known about the role of PI3K in pre-synaptic group II mGluR-mediated signaling. Our lab has recently found that the activation of the ortholog of the vertebrate group II mGluR in Drosophila initiates PI3K signaling in the larval motor neuron (Howlett et al., 2008); however it is unclear how these receptors activate PI3K. Numerous other G-coupled protein receptors are known to directly or indirectly activate Ras (reviewed in Goldsmith and Dhanasekaran, 2007), indicating that Ras may function as an mGluR-PI3K signaling intermediate. Deciphering the Ras-independent and Ras-dependent PI3K-mediated processes as they relate to synaptic outgrowth and

neural plasticity may prove useful for the treatment of significant neurological diseases.

1.5 Examination of Ras-dependent and Ras-independent activation of PI3K in neural processes in Drosophila

1.5.1 The Drosophila neuromuscular junction is an effective model to study neural function

The larval neuromuscular junction (nmj) of Drosophila has emerged as a useful model system for the study of synaptic function and development because of its relative simplicity, accessibility, and because it can be manipulated by a multitude of genetic techniques. The Drosophila nmj has been useful in the study of glutamatergic synapses (Bogdanik et al., 2004, Petersen et al., 1997; Schuster et al., 1991), neural development (Packard et al., 2003), and has also proven to be a useful system for electrophysiological studies (Peron et al., 2009; Jan and Jan 1976). Several forms of short-term synaptic plasticity, such as facilitation and post-tetanic potentiation and depression, as well as LTD have been demonstrated to occur at the neuromuscular junction (Guo and Zhong, 2006; Wu et al., 2005; Jan and Jan, 1978) making the Drosophila nmj a useful model to study the processes that govern synaptic plasticity.

1.5.2 Similar to what has been found in vertebrate systems, PI3K and Ras have been found to modify synaptic growth and activity in Drosophila

Our lab and others have previously examined the role of PI3K and Ras on various aspects of synaptic growth in Drosophila. The overexpression of Ras

and PI3K in the larval nmj was previously demonstrated to significantly increase axon arborization at the larval nmj (Koh et al., 2002; Martin-Peña et al., 2006; Howlett et al., 2008). The overexpression or knockdown of PI3K in the motor neuron also increases and decreases axon diameter, respectively (Howlett et al., 2008). Our lab recently found that the effects of PI3K on growth are via the mTOR/S6K effector pathway (Howlett et al., 2008). Koh et al., (2002) indicated that the Ras-ERK signaling cascade was principally responsible for synaptic outgrowth; however, it is unclear if Ras activation is additionally responsible for the PI3K-mediated effects on neural growth.

1.5.3 The activation of the Drosophila mGluR regulates excitability via PI3K in the motor neuron

Drosophila possesses one functional mGluR-family protein, Drosophila metabotropic receptor A (DmGluRA). Based on sequence analysis and its pharmacological profile, DmGluRA is most similar to the vertebrate group II mGluRs (Parmentier et al., 1996). DmGluRA has been identified presynpatically on the axons of the larval motor neurons in regions adjacent to the active zones of the neuron, suggesting that similar to the vertebrate group II mGluR, it may function like an autoreceptor (Bogdanik et al., 2004; Zhang et al., 1999). A null mutant of DmGluRA, *DmGluRA*^{112b}, previously generated via transposon excision, exhibits several neural defects (Bogdanik et al., 2004). The larval motor neurons exhibit a "hyperexcitable" electrophysiological phenotype when electrical stimulation is applied to the motor neuron. Additionally, the axons innervating larval nmjs have fewer branches and slightly enlarged synaptic

boutons (the boutons are round structures found on axon termini involved in the secretion of neurotransmitters) (Bogdanik et al., 2004). Our lab found that motor neuron excitability could be altered by the modulation of the PI3K signaling pathway and that the *DmGluRA*^{112b} hyperexcitability phenotype could be suppressed by expressing constitutively-active PI3K transgene (*PI3K-CAAX*) in the motor neuron (Howlett et al., 2008). We additionally found that the application of the mGluR ligand, glutamate, to Drosophila larval preparations results in a significant increase of phosphorylated-Akt (p-Akt), a product of PI3K signaling. The same effect was not observed when glutamate was applied to either *DmGluRA*^{112b} or to larvae expressing a DmGluRA RNA-interference (RNAi) transgene (Howlett et al., 2008). These data indicate that activated DmGluRA initiates PI3K signaling in the Drosophila motor neuron (Howlett et al., 2008).

To our knowledge, this is the first demonstration of the activation of PI3K by a group II mGluR. The group I mGluRs have been proposed to activate PI3K through the Homer scaffolding protein and the PI3K enhancer PIKE (Rong et al., 2003; Ronesi and Huber, 2008); however, the group II mGluRs, including DmGluRA, lack the Homer binding motifs (Digana et al., 2002) therefore are not predicted to activate PI3K via this mechanism.

1.5.4 The goal of this study is to delineate which neural processes are governed by the Ras-dependent/independent activation of PI3K in the Drosophila motor neuron

Because Ras is positioned to activate both the ERK and PI3K signaling cascades, it is not clear which PI3K-dependent neural processes require either

Ras or p85-mediated activation. Using the Drosophila nmj, we have examined if PI3K requires Ras activity in several PI3K-regulated processes: synaptic outgrowth, axon diameter size, the activation of PI3K by DmGluRA, and the modulation of motor neuron excitability. To this end we examined the effect of decreasing Ras activity using transgenes, chromosomal mutations, and a PI3K variant is not responsive to Ras-activation on each of these processes. We found that PI3K requires Ras activity to activate PI3K in response to the ligand-activation of DmGluRA. In addition, we found that Ras is required for the effects of PI3K on motor neuron excitability. Ras is however not required for the effects of PI3K on either synaptic growth or axon diameter. These results demonstrate that there are distinct regulatory mechanisms controlling the effects of PI3K on the growth and function of neurons which may have important implications to the neurological diseases that occur as a result of alterations to synaptic plasticity.

Chapter 2: The activation of PI3K by DmGluRA is a Ras-dependent process

2.1 Introduction

2.1.1 The Drosophila mGluR may function as an autoreceptor

Synaptic plasticity is governed by the "strengthening" and "weakening" of synaptic connections via changes in neural excitability (Davis, 2006; Katz and Shatz, 1996; Bliss and Collingridge, 1993). Neural excitability is in part mediated by the secretion of neurotransmitters that alter the generation of action potentials (Lipton and Kater, 1989). Glutamate is the principal excitatory neurotransmitter in the vertebrate brain and up to 40% of all synapses are glutamatergic (van den Pol et al., 1990). Glutamate can be highly toxic to neurons in a condition called excitotoxicity where excess synaptic glutamate can literally "over-stimulate" nerves to death (Sucher et al., 1997); therefore, the release and uptake of glutamate is tightly regulated (Meldrum, 2000).

The metabotropic glutamate receptors (mGluRs) are G protein coupled receptors primarily involved in the mediation of the neuron's response to and the additional release of glutamate (Schoepp, 2001; Ferraguti and Shigemoto, 2006). mGluRs are sub-divided into three groups based on their sequence homology and downstream signaling components (Schoepp, 2001). The postsynaptic group I mGluRs are involved in the establishment of both LTP and LTD (Holscher et al., 1999; Altinbilek and Manahan-Vaughan, 2009; Manahan-Vaughan, 1997). The role of this mGluR subtype in the facilitation of LTD has been the primary focus of a majority of studies examining mGluR-LTD (reviewed in Gladding et al.,

2009). The group II/III mGluRs, principally located on the presynaptic neuron and on the glia (in the case of group III), appear to primarily function as autoreceptors by negatively regulating glutamate or γ-aminobutyric acid (GABA) synaptic transmission (Schoepp, 2001). The group II mGluRs were recently found to regulate LTD in the hippocampus in response to both electrical and pharmacological-induced LTD (Poschel and Manahan-Vaughan et al., 2005; Altinbilek and Manahan-Vaughan, 2009), however the downstream signaling components involved their regulation of this process have not been identified.

Drosophila possesses one functional mGluR-family protein, Drosophila metabotropic glutamate receptor A (DmGluRA) that is most similar to the vertebrate group II mGluRs (Parmentier et al., 1996). Consistent with the vertebrate group II mGluRs, DmGluRA is located adjacent to the active zones of the pre-synapse where it is postulated to function as a glutamate autoreceptor (Bogdanik et al., 2004; Zhang et al., 1999). A null mutant of DmGluRA, *DmGluRA*^{112b}, exhibits normal, basal synaptic transmission; however when high frequency stimulation is applied, the motor neurons become hyperexcitable (Bogdanik et al., 2004; Howlett et al., 2008), indicating a failure in the capacity of these neurons to regulate their activity.

2.1.2 PI3K participates in glutamate-activated DmGluRA signaling in the Drosophila motor neruon

Our lab has previously shown that PI3K is important for the regulation of Drosophila motor neuron excitability (Howlett et al., 2008). We found that the expression of a constitutively-active PI3K transgene (*UAS-PI3K-CAAX*) in the

motor neuron causes a hypoexcitable, neural phenotype and that knocking down PI3K activity with either a dominant-negative PI3K transgene (*UAS-PI3K^{DN}*) or by expressing the PI3K inhibitor, *PTEN*, results in motor neuron hyperexcitability (Howlett et al., 2008). We determined that the hyperexcitable phenotype observed in the *DmGluRA^{112b}* mutant (Bogdanik et al., 2004) was the result of reduced PI3K signaling (Howlett et al., 2008). We demonstrated that the activation of DmGluRA in the larval motor neuron by the application of glutamate causes an increase in the levels of phosphorylated-Akt (p-Akt), a product of PI3K activation. This effect was not observed when glutamate was applied to either *DmGluRA^{112b}* or to larvae expressing a DmGluRA RNA interference (RNAi) transgene. We additionally found that we could rescue the *DmGluRA^{112b}* excitability defect by expressing *UAS-PI3K-CAAX* in motor neurons, indicating that PI3K is epistatic to DmGluRA (Howlett et al., 2008). The PI3K-mediated effect on neural excitability was determined to be the result of the downregulation of Drosophila FOXO (dFOXO) (Howlett et al., 2008) (Fig.2.1). Though the direct targets have yet to be identified, we suspect that dFOXO alters excitability by modulating the transcription of ion channel subunits or regulators (Howlett et al., 2008), or by targeting the translational repressor Pumilio (Schweers et al., 2002). To our knowledge this is the first demonstration of the activation of PI3K by a group II mGluR and it may prove to be relevant to mGluR signaling in vertebrates.

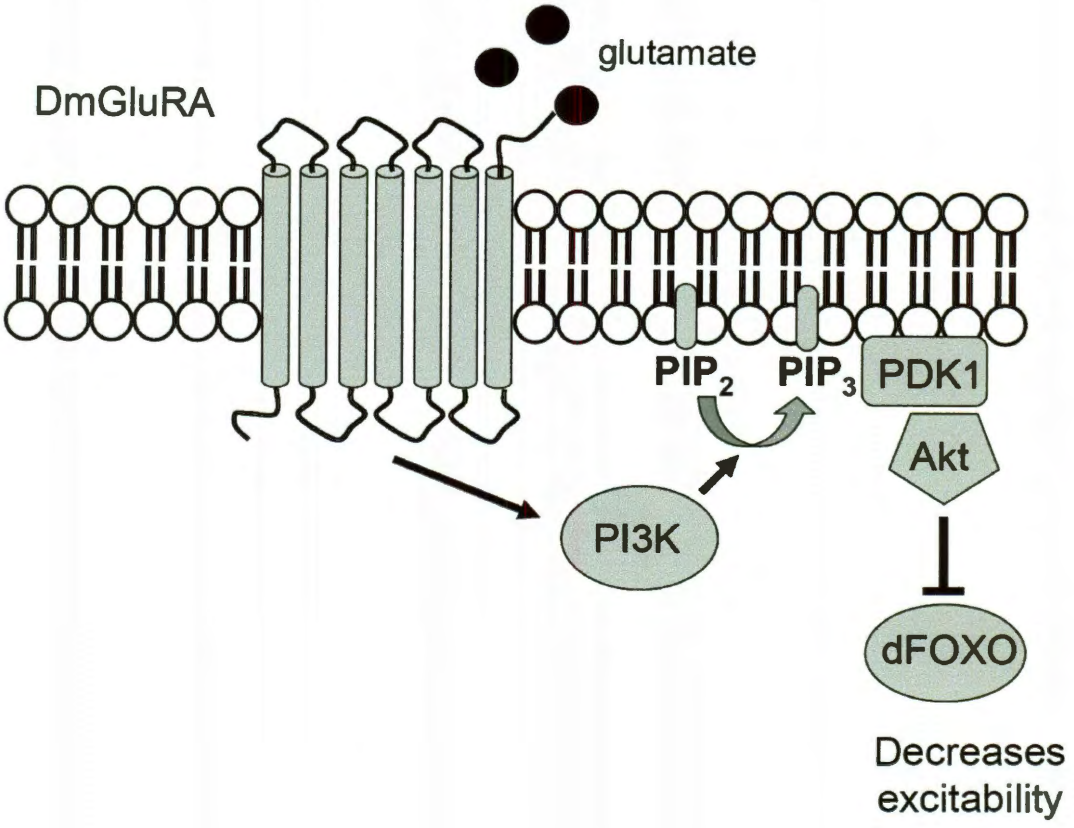


Figure 2.1: The activation of DmGluRA initiates PI3K the signaling cascade to downregulate neural excitability. Our lab previously determined that the ligand-activation of DmGluRA initiates PI3K signaling and that this activation is critical for the regulation of motor neuron excitability. PI3K phosphorylates and converts PIP_2 to PIP_3, recruiting the serine/threonine kinase Akt to the membrane via the N-terminal pleckstrin homology domain of Akt altering the conformation of Akt which enables the kinase PDK1 to further phosphorylate and activate Akt (reviewed in Vanhaesebroeck et al., 2001). Activated Akt negatively regulates the activity of the transcription factor dFOXO. The downregulation of dFOXO decreases neural excitability in the motor neuron (Howlett et al., 2008). The mechanisms by which dFOXO decreases excitability are not currently known; however, we postulate that the transcription of ion channel subunits or regulators may be involved.

2.1.3 The signaling pathway linking DmGluRA to PI3K has not been fully uncovered

As discussed in the Introduction, the group I mGluRs may interact with the scaffolding protein Homer to initiate the PI3K cascade (Rong et al., 2003; Ronesi and Huber, 2008). This interaction is critical for the regulation of translation during mGluR-LTD (Ronesi and Huber, 2008); however, it has not been conclusively demonstrated that the mGluR-Homer interaction activates PI3K. Additionally, the group II mGluRs, which are most similar to DmGluRA, lack the Homer binding motif (Diagana et al., 2002), therefore the mechanism by which activated DmGluRA is initiating the PI3K cascade is not immediately clear.

It was previously reported that the Calcium/calmodulin-dependent kinase II (CaMKII) and the Drosophila ortholog of the nonreceptor tyrosine kinase Focal adhesion kinase (DFak) are involved in the regulation of Drosophila neuronal excitability in the motor neuron (Griffith et al., 1994; Park et al., 2002; Ueda et al., 2008). Because DmGluRA additionally modifies motor neuron excitability suggests that CaMKII and DFak may function in a pathway similar to DmGluRA. In fact, our lab has now demonstrated that CaMKII and DFak are necessary for the activation of PI3K cascade downstream ligand-activated DmGluRA (Lin et al., 2011) (Fig.2.2). We found that the expression of a constitutively active $CaMKII^{T287D}$ transgene (Jin et al., 1998) is sufficient to activate PI3K in the absence of glutamate and that CaMKII can suppress the hyperexcitability phenotype of $DmGluRA^{112b}$. We also determined that DFak is required for this activation (Lin et al., 2011). A null mutation in DFak, $DFak^{CG1}$ (Grabbe et al.,

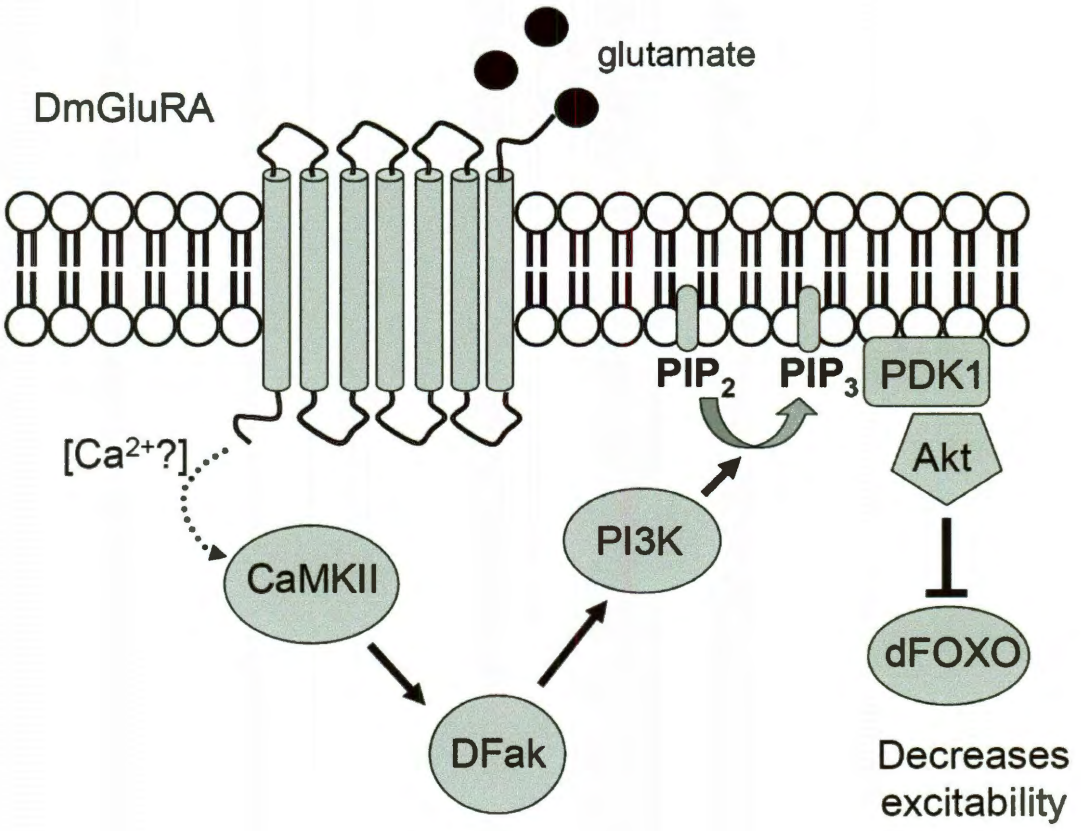


Figure 2.3: The activation of PI3K via DmGluRA requires CaMKII and DFak. The glutamate-activation of DmGluR most likely results in the production of intracellular Ca^{2+} intermediates, resulting in the activation of the Ca^{2+}/Calmodulin kinase, CaMKII. CaMKII subsequently activates Drosophila focal adhesion kinase (DFak) that then activates PI3K (Lin et al., 2011). Activation of PI3K regulates neural excitability via the modulation of the activity of dFOXO (Howlett et al., 2008)

2004), blocks the ability of glutamate-activated DmGluRA to initiate PI3K signaling and also prevents CaMKII from activating PI3K (Lin et al., 2011). Based on these results, we have concluded that the initiation of PI3K signaling by glutamate-activated DmGluRA requires both CaMKII and DFak (Fig.2.2).

2.1.4 The vertebrate Fak family member, Pyk2, can activate PI3K directly or via Ras: could Ras be a DFak-PI3K signaling intermediate in the Drosophila motor neuron?

The vertebrate nonreceptor tyrosine kinase, Fak, is involved in numerous cellular responses including cell adhesion, migration, differentiation, and cell survival (reviewed in Parsons, 2003). The closely related kinase, Pyk2, can be phosphorylated by CaMKII on multiple serines at the C-terminus which activates the phosphorylation of additional tyrosines (particularly Tyr402) by unknown mechanisms (Della Rocca et al., 1997; Zwick et al., 1999; Heidinger et al., 2002; Montiel et al., 2007). These phospho-tyrosines serve as binding sites for SH2 domain containing proteins such as the p85 regulatory subunit of PI3K and the Ras activator, growth factor receptor-bound protein 2 (GRB2). In fact, Pyk2, has been shown to activate both Ras and PI3K (Chen and Guan, 1994; Guinebault et al., 1995; Montiel et al., 2007). Because DFak is the only member of the Pyk2/Fak family in Drosophila, it may perform the functions of both vertebrate Fak and Pyk2, which raises the question if DFak directly activates or indirectly activates PI3K through Ras.

We suspect that Ras may be involved in PI3K activation in the motor nerve terminal because hyperactivated Ras confers several phenotypes that are

consistent with PI3K activation. As discussed in the Introduction, the overexpression of Ras in the Drosophila motor neuron increases synaptic outgrowth similar to what is observed with PI3K overexpression (Koh et al., 2002; Martin-Pena et al., 2006; Howlett et al., 2008). Additionally, examination of a null mutation in the Ras-GAP protein NF1, revealed a decrease in motor neuron excitability similar to the effects of *PI3K-CAAX* (Eric Howlett, unpublished data). These observations indicate that in addition to CaMKII and DFak, that Ras may additionally be involved in the signaling cascade initiated by DmGluRA activation.

2.1.5 The activation of PI3K by DmGluRA is Ras-dependent

To address the activation of PI3K by DmGluRA occurs in a Ras-independent or Ras-dependent manner we examined the levels of p-Akt in the motor neuron in a set of Ras transgenic lines, a Ras chromosomal loss-of-function mutant, and the PI3K variant, *gen-PI3K*^{RBD} that has a mutated Ras-binding domain before and after the application of glutamate. We additionally investigated the effect of inhibiting Ras activity on neural excitability. We found that the activation of PI3K by DmGluRA signaling requires Ras. Reducing Ras activity and eliminating the ability of Ras to bind PI3K, as is the case for the *gen-PI3K* mutant, blocks the glutamate-stimulated increase of p-Akt in the motor neurons and increases motor neuron excitability, consistent with reduced PI3K activity. The discovery that the effect of PI3K on the regulation of motor neuron excitability is a Ras-dependent process provides additional insight into the PI3K-mediated control of specific processes involved in neural plasticity.

2.2 Materials and Methods

2.2.1 *Gal4/UAS* transgene expression system

The *GAL4/UAS* transgene expression system is a robust methodology that allows for the temporal and spatial expression of genes *in vivo* in Drosophila (Brand and Perrimon, 1993). This system utilizes the specificity of the *Saccharomyces cerevisiae* transcription factor, Gal4, for the yeast promoter sequence *upstream activating sequence* (*UAS*). By conjugating *GAL4* to a specific promoter (either global or tissue-specific), Gal4 is effectively transcribed in the pattern of expression for that promoter. Gal4 specifically binds to and activates a *UAS*-conjugated transgene, thus expressing the *UAS*-conjugate in the pattern of the promoter expressing Gal4 (i.e., in muscle if the promoter is expressed in the muscle). This system allows for the expression of either reporter genes (such as green fluorescent protein, GFP) or modulatory transgenes (such as an RNA-interference transgene, RNAi) during development or in specific tissues of interest. Because *GAL4* and *UAS* are not endogenous fly genes, expression of Gal4 does not perturb the function of genes other than the *UAS*-conjugated transgenes.

2.2.2 Fly stocks and husbandry

All fly stocks were maintained on standard cornmeal/agar Drosophila media at 22°C in either half pint bottles or vials. General fly husbandry was carried out as described by Greenspan (1997). Experimental crosses/genotypes were kept in bottles and adult flies (10 male and 10 female) passaged to new bottles every four days to limit larval overcrowding.

Stocks: The principle *GAL4* driver stock utilized for these experiments, *D42-GAL4,* specifically expresses Gal4 in Drosophila motor neurons and was kindly provided to us by Tom Schwarz (Harvard Medical School, Boston, Massachusetts). To knockdown PI3K expression, we utilized a dominant-negative *UAS-PI3K^{DN}* transgene that has an aspartic acid to alanine substitution at residue 954 (D945A; in the ATP-binding site of PI3K) that renders the molecule inactive. We additionally used the transgene *UAS-PTEN*, which drives expression of the PI3K inhibitor *phosphatase and tensin homolog* (*PTEN*), to decrease PI3K activity. To increase PI3K activity, we utilized a constitutively-active *UAS-PI3K-CAAX* transgene that contains a COOH-terminal isoprenylation signal that increases the hydrophobicity of the C-terminus. This C-terminal modification helps to target the molecule to the membrane where it can interact with its substrates (Klippel et al., 1996; Leevers et al., 1996). We acquired the *PI3K^{DN}*, *PI3K-CAAX,* and *UAS-PTEN* stocks from the Bloomington Drosophila stock center (Bloomington, IN).

To modulate Ras activity in the motor neuron, we utilized a dominant-negative Ras transgene, *UAS-Ras^{N17}* that was provided to us by Denise Montell (John Hopkins Medical School, Baltimore, MD). This variant has an asparagine residue substitution at position 17 (N17) (Lee et al., 1996) that sequesters the guanine nucleotide exchange factor (GEF) and thus prevents the activation of wildtype Ras (Powers et al., 1989). To additionally knockdown Ras activity, we also acquired and used a RNA interference transgene, *UAS-RAS-RNAi,* (National Institute of Genetics, Mishima, Japan) and heteroallelic mutant larvae,

Ras^{e2F}/*Ras*^{12A}, which exhibit significantly lower levels of endogenous Ras (Zhong, 1995). *Ras*^{e2F} is a severe mutation that is homozygous lethal (Simon et al., 1991) and is kept as a balanced, heterozygous stock that was provided to us by Gerald Rubin (University of California, Berkeley, CA). *Ras*^{12A} is a hypomorphic mutation that reduces normal levels of Ras (Zhong, 1995) and was provided to us by Celeste Berg (University of Washington, Seattle, WA). The *Ras*^{e2F}/*Ras*^{12A} heteroallelic combination decreases Ras activity sufficiently to confer phenotypes but retains enough activity to maintain viability (Zhong et al., 1995). For Ras-PI3K epitasis experiments, we recombined the *Ras*^{N17} and *PI3K-CAAX* transgenes on to the same chromosome (Michael Stern) so they could be simultaneously expressed.

Ras activates PI3K by binding directly to the catalytic subunit of PI3K (Orme et al., 2006; Rodriguez-Viciana et al, 1994; Pacold et al., 2000). The Leevers lab (London, UK) designed and constructed a Ras-binding domain (RBD) PI3K mutant (*gen-PI3K*^{RBD}) by mutating four amino acids in the Ras-binding domain of the catalytic unit of PI3K (T231D, K250A, R253A, and K257A). This variant was produced as a genomic clone (so expression of this allele would be under the transcriptional control of the endogenous PI3K promoter) and was introduced into Drosophila as a transgene (Orme et al., 2006). A control line, *gen-PI3K*$^{+}$, with an unmodified Ras-binding domain, was similarly produced (Orme et al., 2006). Both of these stocks were provided to us by Sally Leevers (London Research Institute, London, UK). We crossed *gen-PI3K*$^{+}$ and *gen-PI3K*^{RBD} into a *PI3K*^{A} mutant allele background so that only the transgenic *PI3K*^{RBD} or *PI3K*$^{+}$ are

expressed in mutant larvae. *PI3K*^{A} was previously suggested to be a null allele of PI3K (Weinkove et al., 1999); however, we suspect that is in fact functioning like a dominant-negative for PI3K. *PI3K*^{A} is predicted to encode a 668 amino acid protein truncated at the C-terminus, which may confer partial dominant-negative properties.

2.2.3 Larval microdissection

The larval neuromuscular junction (nmj) has proven to be a powerful model for the study of synaptic properties and function (Peron et al., 2009; Guo and Zhong, 2006; Wu et al., 2005; Bogdanik et al., 2004; Packard et al., 2003; Petersen et al., 1997; Schuster et al., 1991; Jan and Jan, 1978); therefore it is the principle Drosophila tissue utilized for this study. For dissections, wandering, third instar larvae were collected from the sides of half pint bottles within 48 hours of their emergence. Larval dissections were performed as described by Jan and Jan (1978). In brief, an appropriately staged larva was placed into a dissection tray, and its head and tail pinned down with the dorsal side facing upwards using insect pins. The larva was then cut along the length of the dorsal midline, pinned open flat, and the guts and other major organs removed; however, the brain, ventral ganglion, and the peripheral nervous system were left intact (Fig.2.3).

2.2.4 Larval anti-phosphorylated Akt (p-Akt) analysis

Anti-p-Akt immunostaining: Akt is a downstream target of PI3K and is phosphorylated upon activation of PI3K, thus detection of phosphorylated-Akt can be used as a read-out of PI3K activity in the neuron (Dionne et al., 2006;

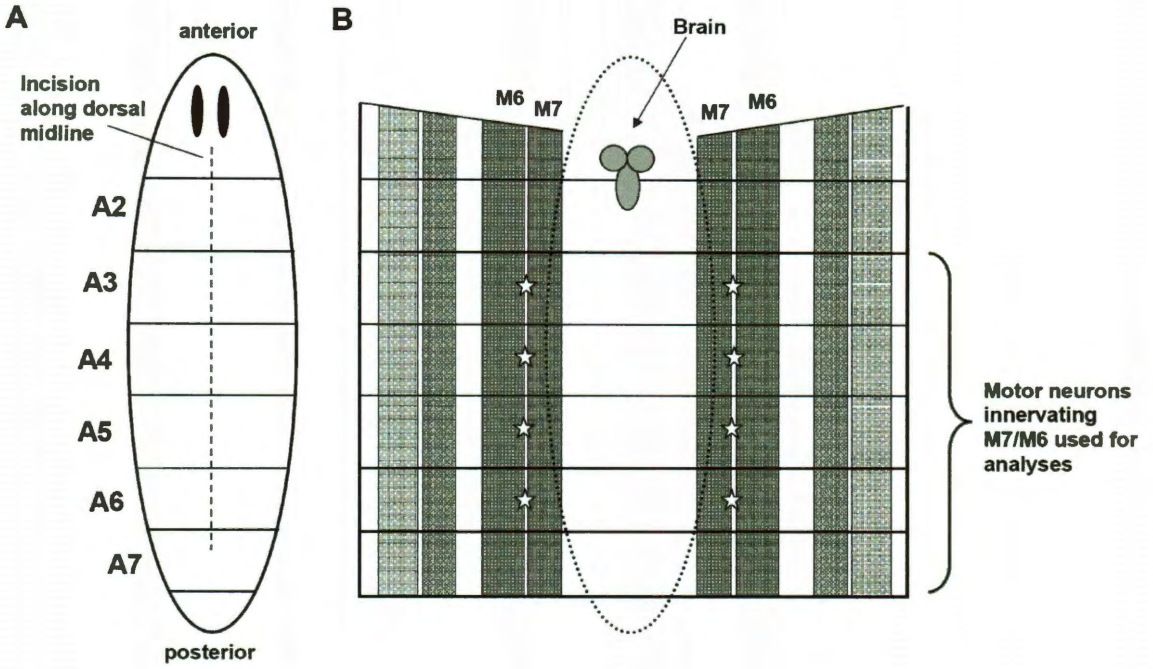


Figure 2.3: Overview of larval preparations used to analyze the Drosophila nmj. Larval dissections were performed using the standard protocol outlined by Jan and Jan (1978). **(A)** Diagram of a wandering, 3rd instar larva positioned dorsal side up. The larva has approximately 7 abdominal segments labeled from anterior to posterior as A2-A7, respectively. The most anterior segment, A1, is further subdivided; however, this region was not the focus of this study therefore these segments are not labeled here. The dashed line represents the position of the dorsal midline where primary incision for dissection was made. **(B)** Diagram of the larval fillet prep with the body wall of the larva pinned open. The brain and peripheral nervous system (not shown) were left intact; however, the guts and other major organs were removed. The neurons innervating the longitudinal muscles 7 and 6 (M7 and M6), were the focus of this study. The positions of the motor neurons used for the p-Akt analysis are noted with stars.

Colombani et al., 2005; Palomero et al., 2007; Howlett et al., 2008). To examine PI3K activity in the Drosophila nmj, wandering, third instar larvae were dissected in 1x Schneider's Drosophila Medium (S2 media) (Invitrogen, Carlsbad, CA). This media is formulated to grow Drosophila S2 cells in culture and is better suited than standard 1x phosphate buffered saline (1xPBS;137 mM NaCl, 2.7 mM KCl,

4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4) to keep dissected Drosophila tissues functionally active both during and (for a period of time) after dissection.

To investigate basal levels of p-Akt (levels of p-Akt without glutamate application) the dissected larvae were immediately fixed in 4% paraformaldehyde for 15 minutes, then washed 3x in 1x PBS-T (1xPBS with 0.1% Triton-X 100). The preps were incubated overnight at 4°C with antibodies against Drosophila p-Akt (monoclonal, directed against Ser505) raised in rabbit (Cell Signaling Technologies, Inc., Boston, MA) at a concentration of 1:500. For visualization, the preps were incubated overnight at 4°C with a Rhodamine Red-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch, Westgrove, PA) at a concentration of 1:1000. The neurons were also simultaneously counter-stained with Cy2-conjugated anti-horse radish perioxidase (HRP) (Jackson ImmunoResearch) at a concentration of 1:200. The anti-HRP antibody recognizes a carbohydrate on the surface of all Drosophila neurons, thus is a useful antibody for the visualization of neural structures in the fly (Jan and Jan, 1982).

Glutamate application: To investigate the activity of PI3K after the activation of DmGluRA by glutamate, larvae were dissected as described above; however, prior to fixation, 100 µM glutamate was added to the prep. To ensure that the glutamate would be applied evenly to the preps, 2 µL of a prepared 10 mM glutamate solution (glutamic acid monosodium salt monohydrate; Acros Organics) was added to 198 µL S2 media for a final concentration of 100 µM glutamate. The media used for the dissection was replaced with the glutamate

solution and the prep was incubated with glutamate for 1 minute. After incubation, the dissection plate was immediately submersed in two baths of 1xPBS to remove the glutamate solution. Fixation and the visualization of p-Akt and neural immunostaining were performed as described above.

Anti-p-Akt measurements: We used a Zeiss 510 laser scanning confocal microscope (LSM) collect images of a 10 μm thickness (corresponding to the depth of the neuron) using the 20X objective. Neurons between muscles 7 and 6 spanning the length of the larva (i.g. from segments A3-A7) were imaged (Fig. 2.3). To ensure that any observed changes in the anti-p-Akt staining intensity were due to transgenic manipulations affecting PI3K activity and not the result of image acquisition, the detector gain for the red channel (corresponding to the anti-p-Akt immunostaining) was set using the detector gain that established for the detection of the basal anti-p-Akt staining of the control preps. These same control gain settings were then used to acquire all of the subsequent neural images of the experimental preps. Because we observed day-to-day variation in anti-p-Akt fluorescent intensity, care was taken to always dissect an appropriate number of controls in parallel with the experimental larvae for every p-Akt experiment.

To measure the intensity of the anti-p-Akt staining, each neuron was traced by hand with the NIH ImageJ (http://rsbweb.nih.gov/ij) freehand selection tool using the anti-HRP staining as a guide. The traced outline of the neuron was then superimposed on to the image that corresponded to the anti-p-Akt staining of the same neuron. The pixel intensity inside the traced region was measured

using the Image J measurement tool. Because the localization of the anti-p-Akt often did not completely fill in the traced outline of the neuron, a background subtraction was performed to minimize the effect of any background staining on the pixel intensity measurement. The background subtraction was performed by averaging the fluorescent intensity of a random area (excluding regions containing nuclei) from muscles 6 and 7 from the image taken in the red channel. The muscle average was subtracted from the intensity measured from each traced neuron. The anti-p-Akt staining intensity from multiple neurons (~30 for each genotype) was measured and each of these individual measurements was normalized by dividing the measured intensity of each neuron by the overall average intensity of the basal p-Akt intensity of the controls. These normalized values were averaged for each data set and used to report the "average intensity" of the basal and glutamate-applied anti-p-Akt staining. Standard error was calculated using the normalized intensity values for both the experimental and control groups and significance was calculated using a one-way ANOVA and Fisher's LSD.

2.2.5 Electrophysiology

To measure neuronal excitability, we measured the onset of a phenomenon termed "long term facilitation" (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 Ca^{2+}. Because Ca^{2+} is essential for neurotransmitter release to occur, the motor neuron initially responds to nerve stimulation at this low external Ca^{2+} with little neurotransmitter release, and thus

the muscle responds with depolarizations (termed excitatory junctional potential, or EJP) of a low amplitude. However, as repetitive stimulation continues, at a certain point during the stimulus train a threshold is reached and subsequent stimulations elicit EJPs of greatly increased amplitude, called LTF, resulting from a prolonged depolarization of the nerve terminal (Jan and Jan, 1978) (Fig.2.4). The number of stimulations required to reach this LTF threshold (LTF onset rate) is decreased by genetic conditions that increase neuronal excitability (Jan and Jan, 1978; Mallart et al., 1991; Poulain et al., 1994; Schweers et al., 2002; Stern and Ganetzky, 1989; Stern et al., 1990).

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 9 electrode at a 5V stimulus intensity. Muscle recordings were taken from muscle 6 in abdominal segments A3, A4 or A5 (Fig.2.4). Stimulus duration, approximately 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 LTF were reported as geometric means because the data show a positive skew. For all LTF experiments, the bath solution contained 0.15 mM Ca^{2+} 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. The electrophysiology experiments for this project were carried out by Chun-Jen (Curtis) Lin.

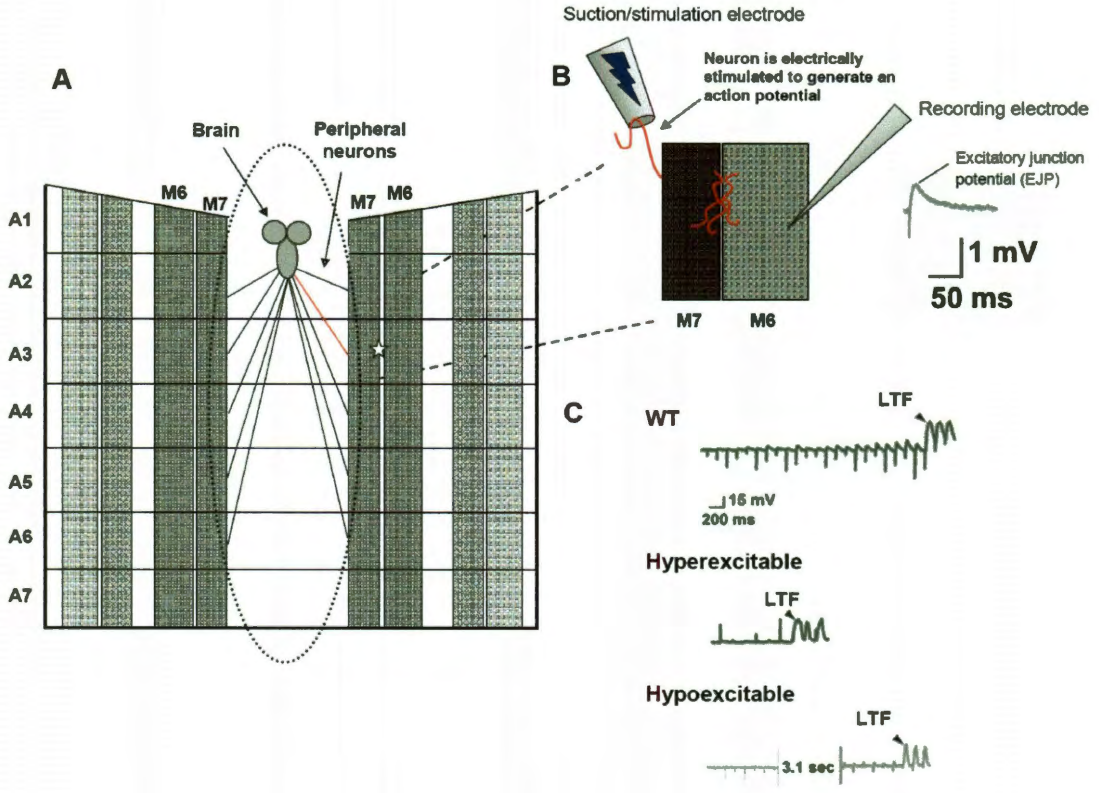


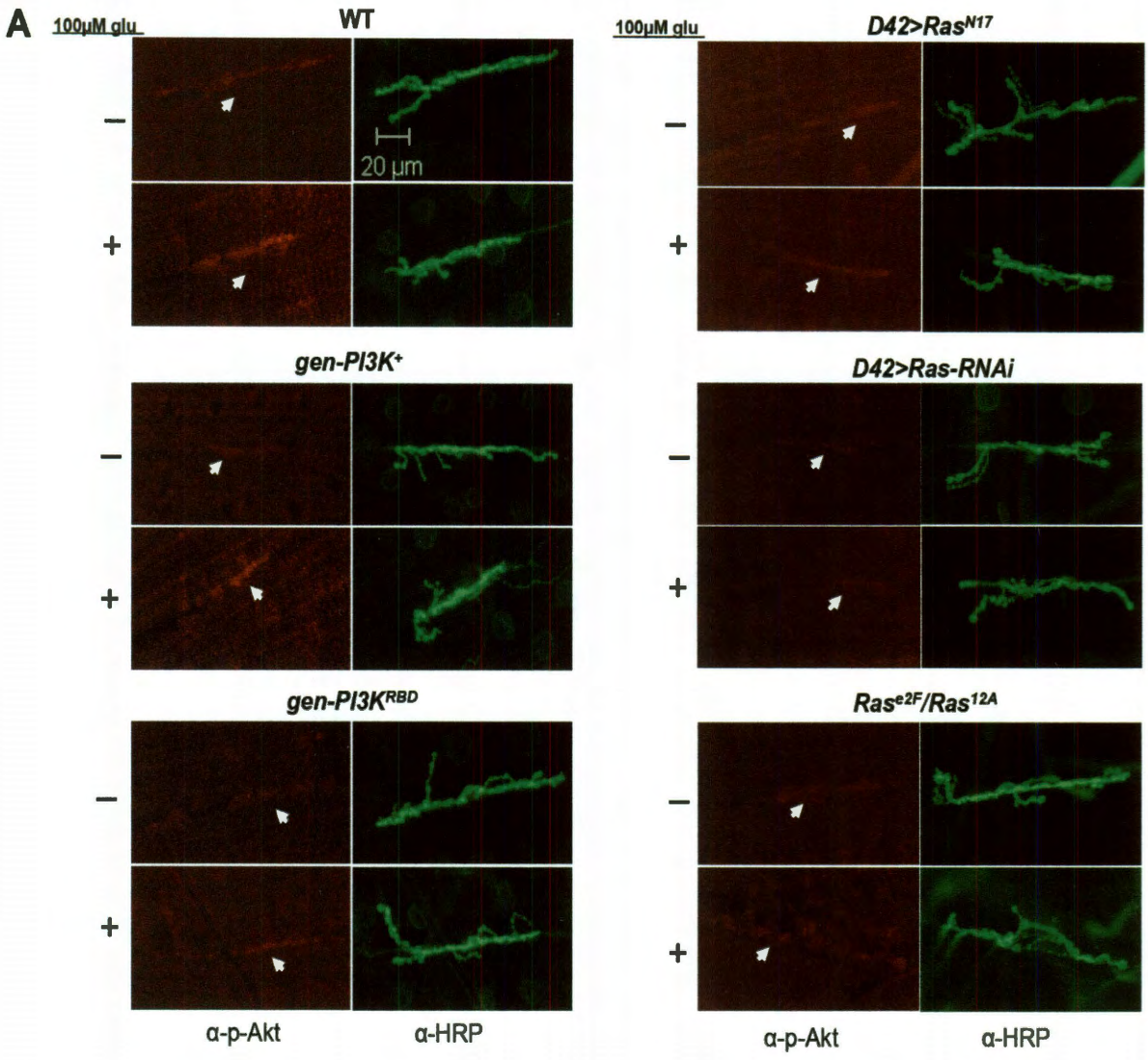
Figure 2.4: The larval motor neuron is useful for measuring neural excitability. (A) Larval fillet preps were used to access the motor neurons of a larval nmj. The neuron utilized for the electrophysiological recordings innervates muscles 7 and 6 (M7 and M6) from abdominal segments A3-A6. Only one neuron was stimulated per larvae. **(B)** A suction electrode draws up and stimulates a single neuron at a 5V stimulus intensity. A recoding electrode positioned in M6 of the selected segment measures the electric potential. Stimulations result in depolarizations (termed excitatory junctional potentials, EJPs) in the target muscle. **(C)** To measure excitability, long-term facilitation (LTF) is induced by subjecting the neuron to a train of repetitive stimulations. These repetitive stimulations initially result in EJPs of a low amplitude. As stimulation continues, a threshold is reached where subsequent stimulations result in EJPs of greatly increased amplitude, called LTF. Genetic conditions that increase neuronal excitability decrease the number of stimulations required to reach this LTF threshold (considered hyperexcitable) and genetic conditions that decrease neuronal excitability increase the number of stimulations to reach this LTF threshold (hypoexcitable) (Jan and Jan, 1978; Mallart et al., 1991; Poulain et al., 1994; Schweers et al., 2002; Stern and Ganetzky, 1989; Stern et al., 1990).

2.3 Results

2.3.1 Ras is required for glutamate-mediated activation of PI3K

Our lab previously demonstrated that glutamate application to the Drosophila larval nmj activates PI3K via the single metabotropic glutamate receptor DmGluRA (Howlett et al., 2008). PI3K is measured by monitoring levels of phosphorylated Akt (p-Akt) which increase with increasing PI3K activity (Dionne et al., 2006; Colombani et al., 2005; Palomero et al., 42007; Howlett et al., 2008). Increases in glutamate-evoked p-Akt are blocked in *DmGluRA*^{112b} mutants or when DmGluRA is knocked down by the motor neuron expression of *DmGluRA-RNAi* (Howlett et al., 2008). As shown in Figure 2.5, the application of glutamate to wildtype larvae induces a 30% increase in p-Akt levels (Fig. p=0.008).

To determine if Ras is involved in the activation of PI3K, we inhibited the activity of Ras using the *Gal4/UAS* system (Brand and Perrimon, 1993) to drive the expression of a dominant-negative Ras transgene (*Ras*^{N17}) and a *Ras-RNAi* transgene in the larval motor neuron and examined p-Akt activity using immunohistochemistry. We also examined p-Akt activity in the heterozygous chromosomal mutant *Ras*^{e2F}/*Ras*^{12A} that decreases Ras activity sufficiently to confer phenotypes but retains enough activity to maintain viability (Zhong et al., 1995). We found that *Ras*^{N17}, *Ras-RNAi, and Ras*^{e2F}/*Ras*^{12A} did not alter the basal levels of p-Akt (the level of p-Akt present without the application of glutamate); however, these mutants blocked the ability of glutamate to activate PI3K (Fig.2.5). These results indicate that Ras is a critical intermediate in the PI3K activation mediated by glutamate-liganded DmGluRA.


A
100μM glu
WT
20 μm
100μM glu
D42>Ras^N17
gen-PI3K+
D42>Ras-RNAi
gen-PI3K^RBD
Ras^e2F/Ras^12A
–
+
α-p-Akt
α-HRP
α-p-Akt
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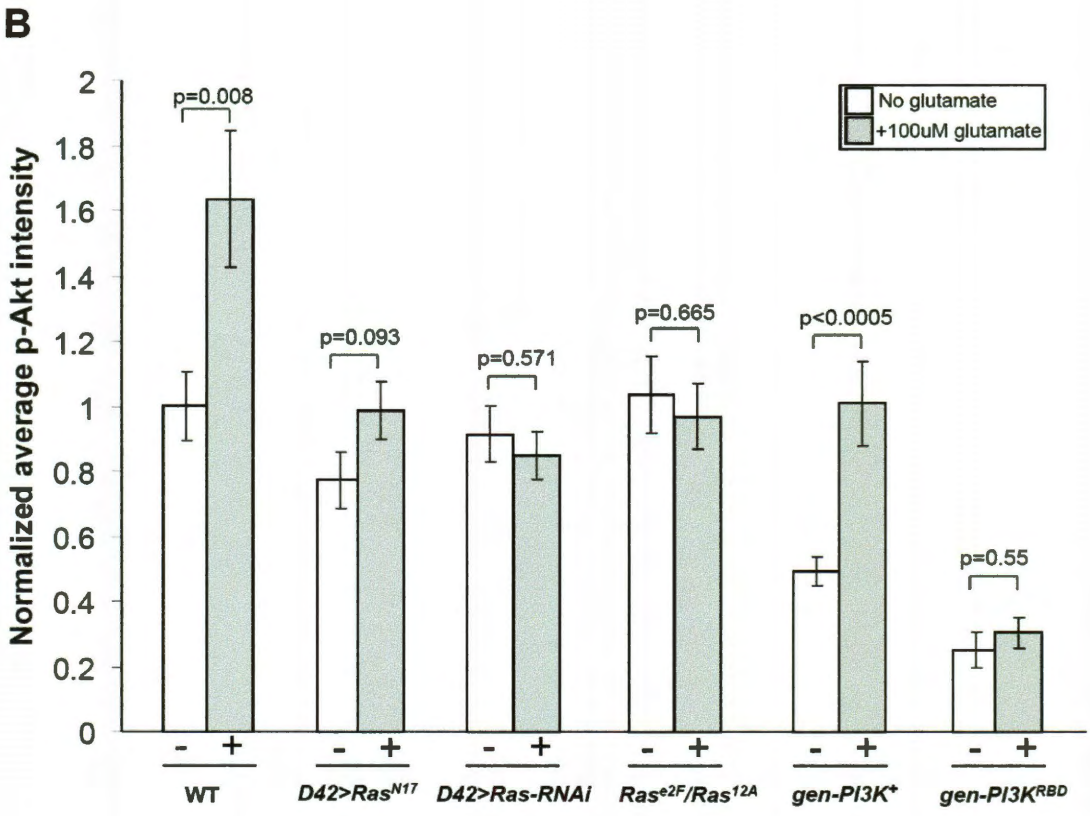


Figure 2.5: Ras is required for glutamate-induced p-Akt increases in larval motor nerve terminals (A) Representative images of larval neuromuscular junctions of wildtype, *D42>Ras^{N17}*, *D42>Ras-RNAi*, *Ras^{e2F}/Ras^{12A}*, *gen-PI3K^{+}*, and *gen-PI3K^{RBD}*. Neurons were labeled with anti-p-Akt (red) either without or following a 1 minute application of 100 µM glutamate. Preparations were also labeled with anti-HRP (green) to enable visualization of motor nerve terminals. White arrowheads indicate p-Akt immunoreactivity. Scale bar as indicated. **(B)** Means +/- SEMs of normalized pixel intensities (Y-axis) of the indicated genotypes (X-axis). The pixel intensity of the anti-p-Akt staining was averaged for each genotype and normalized to the average intensity of the control preparation: values from *Ras^{e2F}/Ras^{12A}*, *gen-PI3K^{+}* and *gen-PI3K^{RBD}* were normalized to WT, whereas values from *D42>Ras^{N17}* and *D42>Ras-RNAi* were normalized to *D42>+* (data not shown). For all genotypes, n=30. One-way ANOVA and Fisher's LSD were used to find the following significant differences: WT vs. *gen-PI3K^{+}*, $p<0.001$; vs. *gen-PI3K^{RBD}*, $p<0.001$. No significant differences in basal p-Akt were found for the following comparisons: *D42>+* vs. *D42>Ras^{N17}*, $p=0.936$; vs. *D42>Ras-RNAi*, $p=0.532$; WT vs. *Ras^{e2F}/Ras^{12A}*, $p=0.81$.

Ras has several effector pathways, including the Raf-ERK pathway, so to confirm that Ras inhibition was preventing increases in p-Akt by blocking PI3K activation, we examined the activation of p-Akt in a Ras-binding domain PI3K mutant, *gen-PI3K^{RBD}*. This PI3K variant carries a four amino acid substitution in the Ras binding domain of PI3K that prevents activation by Ras but does not effect the activation of Ras by p85-dependent mechanisms (Pacold et al., 2000; Orme et al., 2006). We found that the application of glutamate to the *gen-PI3K^{+}* expressing larvae resulted in a significant increase in the levels of p-Akt (Fig). This increase in p-Akt was not observed after glutamate was applied to the *gen-PI3K^{RBD}* larvae (Fig.2.5). These results confirm that Ras is a critical intermediate for the glutamate-activation of PI3K.

We also observed that the basal levels of p-Akt were decreased in the *gen-PI3K^{+}* larvae (Fig.2.5). These larvae only carry one copy of the *gen-PI3K^{+}* transgene and both copies of the endogenous *PI3K* have been replaced with the allele *PI3K^{A}*; therefore the basal decrease in p-Akt levels are most likely a consequence of decreased PI3K gene dosage. Likewise, the basal levels of p-Akt in the *gen-PI3K^{RBD}* larvae are lower than both wildtype and *gen-PI3K^{+}*. These larvae only have a single copy of the *gen-PI3K^{RBD}* transgene that is only responsive to p85-dependent activation which further limits PI3K activity. To further assess the effect of gene dosage on the levels of p-Akt, we compared the basal levels of wildtype p-Akt to the levels of *gen-PI3K^{+}* and *PI3K^{A}* crossed to wildtype. If the levels of p-Akt are sensitive to the amount of gene expression, we would expect to that the *gen-PI3K^{+}*/+ heterozygotes would have wildtype levels of

basal p-Akt and the *PI3K*^{A}/+ heterozygotes would have half as much p-Akt staining. As expected, the *PI3K*^{A}/+ heterozygotes had reduced p-Akt levels compared to wildtype (Fig.2.6). Interestingly, the *gen-PI3K*$^{+}$/+ heterozygotes additionally exhibited reduced p-Akt levels (Fig.2.6). We believe that this because *PI3K*^{A} is functioning like a dominant-negative rather than a null like previously reported (Weinkove et al., 1999). *PI3K*^{A} is predicted to encode a 668 AA protein truncated at the C-terminus (Weinkove et al., 1999), which may have dominant-negative properties.

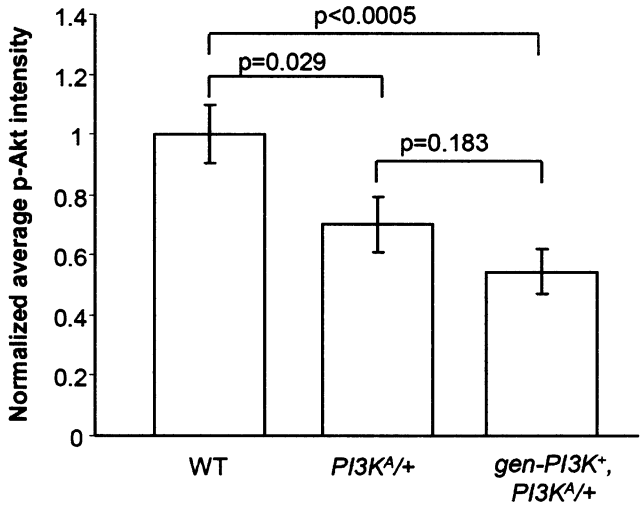


Figure 2.6: p-Akt in the motor neuron is affected by PI3K gene dosage. Means +/- SEMs of normalized pixel intensities (Y-axis) of the indicated genotypes (X-axis). The pixel intensity of the anti-p-Akt staining was averaged for each genotype and normalized to the average intensity of the wildtype preparation. For all genotypes, n=30. One-way ANOVA and Fisher's LSD were used to find the following significant differences: WT vs. PI3K^{+}/+, p=0.029; vs. *gen-PI3K*$^{+}$, *PI3K*^{A} /+, p<0.0005. No significant difference was found between *PI3K*^{A}/+ vs *gen-PI3K*$^{+}$, *PI3K*^{A}/+, p=0.183.

We have recently reported that CamKII and DFak are critical intermediates 0f DmGluRA-mediated PI3K signaling (Lin et al., 2011). Interestingly, we found that the inhibition of CaMKII and DFak in the motor neuron results in a decrease in

the basal levels of p-Akt, indicating that CamKII and DFak are regulating PI3K signaling even when DmGluRA is not activated (Lin et al., 2011). Inhibiting the activity of Ras does not similarly alter the basal levels of p-Akt (Fig.2.5), indicating that Ras is primarily engaged in the activation of PI3K in the motor neuron when DmGluRA is activated by glutamate.

2.3.2 The effect of PI3K on motor neuron hyperexcitability is Ras-dependent

We have previously demonstrated that increasing and decreasing PI3K activity increases and decreases neural excitability, respectively (Howlett et al., 2008). *DmGluRA*^{112b} exhibits increased motor neuron excitability (Bogdanik et al., 2004) and our lab has determined that this effect is the result of aberrant PI3K signaling. To explore the possibility that Ras is additionally involved in the regulation of excitably via DmGluRA-mediated PI3K signaling, we examined the neural excitability in a series of transgenic and chromosomal mutants that inhibit the activity of Ras. If Ras is required for this process, we would expect that inhibiting Ras would increase neural activity similar to what is observed when PI3K activity is decreased (Howlett et al., 2008).

To measure excitability, we used the rate of onset of a phenomenon called "long term facilitation" (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 Ca^{2+} (for a more detailed explanation, see Methods section above). At low Ca^{2+}, a repetitive stimulation eventually results in high amplitude depolarizations, termed excitatory junctional potentials (EJPs)

that are the result of asynchronous neurotransmitter release. The number of simulations required to reach this LTF threshold can be increased or decreased by changes in gene expression that alter neuronal excitability (Schweers et al., 2002; Poulain et al., 1994; Mallart et al., 1991; Stern et al., 1990; Stern and Ganetzky, 1989; Jan and Jan, 1978).

We measured the onset of LTF (the number of stimulations required to reach asynchronous neurotransmitter release) in larvae expressing the dominant-negative Ras^{N17} as well as the heterozygous chromosomal mutant, Ras^{e2F}/Ras^{12A}. We observed that both genotypes significantly increased the LTF onset rate (Fig.2.7), similar to what we observed when PI3K activity is inhibited. To confirm that Ras increases excitability by preventing PI3K activation, we also examined LTF onset rate in the PI3K variants, *gen-PI3K*$^{+}$ and *gen-PI3K*^{RBD}. We observed that gen-RBD has a LTF onset rate that was significantly greater than the *gen-PI3K*$^{+}$ or controls (Fig.2.7), indicating that *gen-PI3K*^{RBD} is hyperexcitable (Fig.2.7), suggesting that the capacity of PI3K to decrease neural activity requires Ras. This conclusion is consistent with the previous experiments suggesting that ligand-activated DmGluRA requires PI3K activity to decrease motor neuron excitability (Howlett et al., 2008) and that the ability of DmGluRA to activate PI3K requires Ras (Fig.2.5).

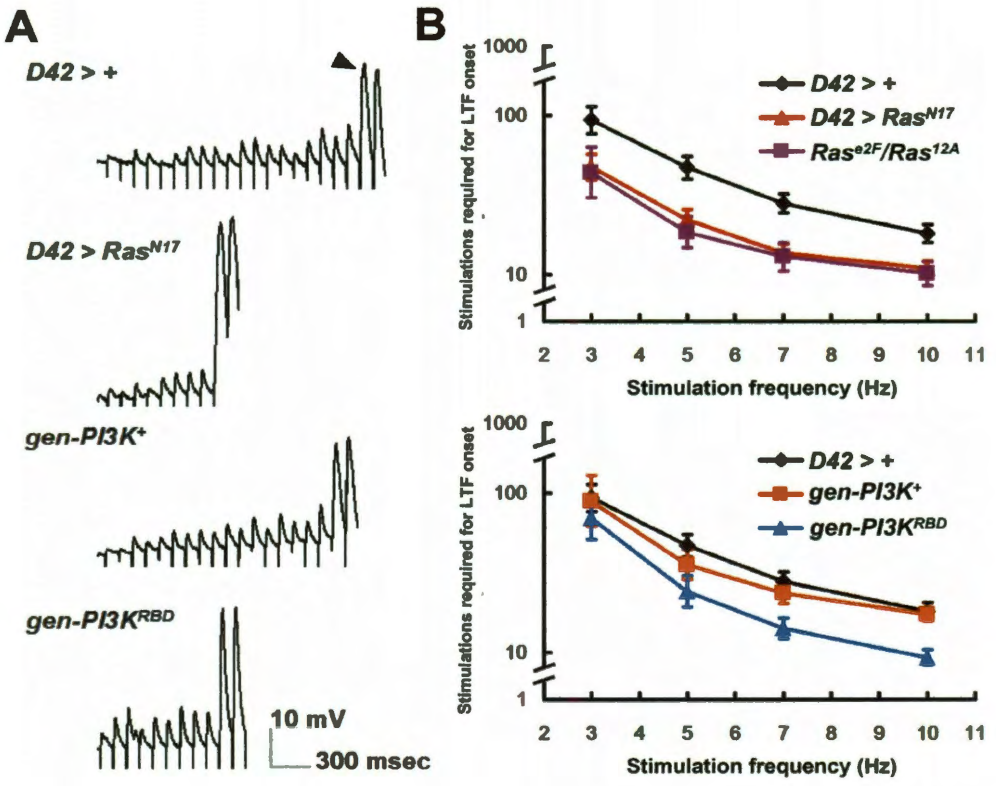


Figure 2.7: Preventing Ras-dependent PI3K activation increases neuronal excitability. The larval neuromuscular preparation (Jan and Jan, 1976) was used for all recordings. **(A)** Representative traces showing increased rate of onset of LTF in *D42>+*, *D42>Ras^{N17}*, *gen-PI3K^{+}*, and *gen-PI3K^{RBD}* in motor neurons. Arrowheads indicate the increased and asynchronous EJPs, indicative of onset of LTF. The bath solution contained 0.15 mM [Ca^{2+}] and 100 µM quinidine. Nerves were stimulated at a frequency of 10 Hz for each trace. **(B)** (Left panel) Geometric means +/- SEMs of the number of stimulations required to evoke LTF (Y-axis) at the indicated stimulus frequencies (X-axis) for the following genotypes: *D42>+*, *D42>Ras^{N17}*, *Ras^{e2F}/Ras^{12A}*. From left to right, n = 12, 13, 6. (Right panel) Geometric means +/- SEMs of the number of stimulations required to evoke LTF (Y-axis) at the indicated stimulus frequencies (X-axis) for the following genotypes: *D42>+*, *gen-PI3K^{+}*, *gen-PI3K^{RBD}*. From left to right, n= 12, 6, 7. One-way ANOVA and Fisher's LSD gave the following differences at 3 Hz, 5 Hz, 7 Hz, and 10 Hz, respectively: For *D42>+* vs. *D42>Ras^{N17}*, p=0.02, 0.002, 0.0008, 0.006; vs. *Ras^{e2F}/Ras^{12A}*, p=0.06, 0.005, 0.006, 0.016; vs. *gen-PI3K^{+}*, p=0.902, 0.355, 0.449, 0.827; vs. *gen-PI3K^{RBD}*, p=0.403, 0.29, 0.006, 0.002. For *gen-PI3K^{+}* vs. *gen-PI3K^{RBD}*, p=0.612, 0.245, 0.037, 0.002.

2.4 Discussion

2.4.1 DmGluRA-mediated activation of PI3K requires Ras

Our lab has previously shown that the ligand-activation of DmGluRA initiates a PI3K-mediated signaling cascade that negatively regulates neural excitability (Howlett et al., 2008). This study has found that Ras, an important modulator of cellular activity, is also an integral part of this signaling network. Inhibition of Ras via transgene expression and chromosomal loss-of-function blocks the ability of DmGluRA to activate the PI3K signaling cascade (Fig.2.5). Diminished PI3K signaling results in loss of negative feedback control and an abnormal increase in neural excitability (Fig.2.7) which we have previously shown is mediated by the inhibition of FOXO activity by PI3K-Akt signaling (Howlett et al., 2008). Furthermore, the observation that the Ras-insensitive PI3K variant, *gen-PI3K*^{RBD}, is hyperexcitable (Fig.2.7) additionally demonstrates the critical role of Ras in these feedback processes.

To our knowledge, a requirement for Ras during mGluR-mediated signaling has not been previously demonstrated. The activities of Ras and PI3K are known to modulate neural function, including LTP and LTD (reviewed in Ye and Carrow, 2010); however, a clear delineation between the Ras-dependent and Ras-independent PI3K-mediated mechanisms has not been established. This study indicates that Ras is required for the effects of PI3K on the modulation of neural function. It was previously shown that LTD can be reliably induced in the synapses of the Drosophila nmj and that Akt chromosomal mutants exhibit significant impairments in motor neuron LTD (Guo and Zhong, 2006). In

vertebrate systems, the PI3K-Akt-mTOR cascade is required for the initiation of group I mGluR-LTD (Hou and Klann, 2004) and though the group II mGluRs, of which DmGluRA is the most similar, have also been demonstrated to elicit LTD, it is currently unknown if there is a similar requirement for this pathway. We have previously established a role for DmGluRA-PI3K signaling in the regulation of neural excitability (Howlett et al., 2008) and the results of this study have now identified Ras as another key intermediate in the DmGluRA pathway mediating excitability. Downregulating excitability may be a key process in the establishment of LTD; therefore, these results indicate that Ras and PI3K may participate in LTD regulation in the Drosophila motor neuron. Because altered LTD is implicated in autism (reviewed in Bear et al., 2004) and altered group II mGluR expression is associated with (Gupta et al., 2005; Aronica et al., 1997; Pacheco et al., 2006), it may be of merit to investigate if this pathway is conserved in vertebrates.

2.4.2 Proposed model for the role of Ras in DmGluR-PI3K mediated negative feedback regulation

Based on several lines of evidence, our lab has proposed a model by which glutamate-activated DmGluRA engages PI3K in a negative feedback signal that downregulates neural excitability (Howlett et al., 2008). In this model, glutamate released from the motor nerve terminal as a consequence of neural activity, feeds back on DmGluRA located on the same nerve terminal. Glutamate binding to DmGluRA activates PI3K signaling which downregulates neural excitability via the inhibition of dFOXO (Fig.2.1) (Howlett et al., 2008).

We have previously shown that the activation of DmGluRA by glutamate increases levels of intracellular Ca^{2+} (Eric Howlett, unpublished data). We proposed that ligand-bound DmGluRA activates a phospholipase C (PLC) and inositol 1,4,5 trisphosphate receptor (IP_3R)-dependent Ca^{2+} transient that activates a signaling cascade via the Ca^{2+} /Calmodulin kinase CaMKII. Consistent with this hypothesis, we have recently demonstrated that CaMKII and DFak are critical signaling intermediates linking DmGluRA to PI3K activation (Fig) (Lin et al., 2011). This current study has now identified Ras as another important molecule essential for the activation of PI3K during DmGluRA-mediated intracellular signaling.

Because DFak is the only Fak/Pyk2 nontyrosine tyrosine kinase family member in Drosophila, it may perform the functions of both molecules. Pyk2 has been shown to activate Ras and PI3K (Chen and Guan, 1994; Chen et al., 1996; Guinebault et al., 1995; Montiel et al., 2007), therefore DFak may be activating PI3K indirectly via Ras during DmGluRA-initiated signaling (Fig.2.8). In this scenario, DmGluRA-mediated CamKII-DFak-Ras signaling would activate PI3K via the Ras-binding domain. Alternatively, activated DmGluRA may initiate two separate, parallel pathways, one involving CaMKII-DFak and the other involving Ras that converge on PI3K (Fig.2.8). In this second situation, Ras would activate PI3K via the Ras-binding domain and DFak would activate PI3K in a p85-dependent manner leading to the "full" activation of PI3K during DmGluRA-mediated signaling.

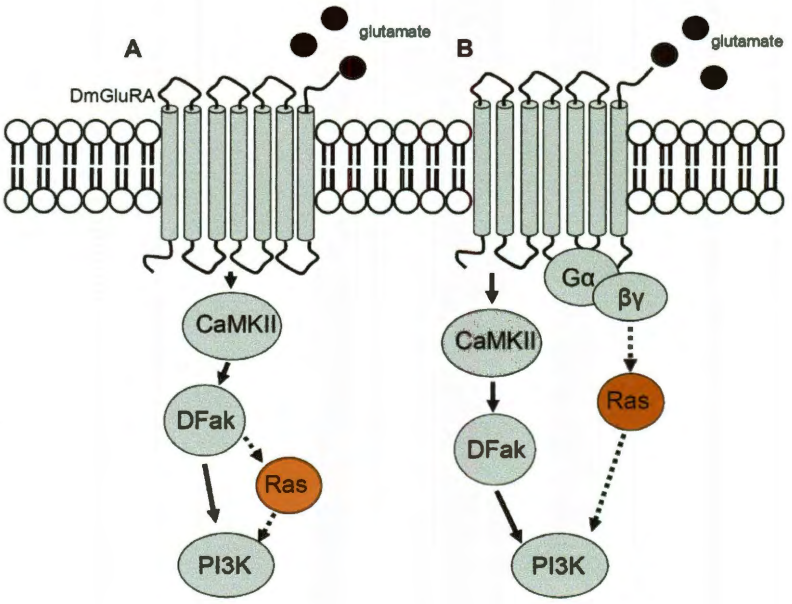


Figure 2.8: DmGluRA may activate Ras via DFak or through DFak-independent mechanisms. Ras is a critical signaling intermediate facilitating the initiation of PI3K in response to DmGluRA activation. **(A)** In response to DmGluRA activation, DFak may activate Ras, which then directly interacts with and activates PI3K via the Ras-binding domain of PI3K. This mechanism may be the exclusive activation step, or in concert with Ras, DFak may also interact and activate PI3K through the p60 subunit. **(B)** Activation of DmGluRA may initiate the CaMKII-DFak as well as a separate Ras-mediated pathway for the "full" activation of PI3K in response to a glutamate signal.

There are several potential mechanisms by which Ras may be activated by DmGluRA. Disassociated $G\alpha_i$ and $G\beta\gamma$-subunits of activated G proteins have been shown to modulate Ras in many different cell types (reviewed in Goldsmith and Dhanasekaran, 2007). DmGluRA has been shown to couple to G_i/G_o (Parmentier et al., 1996) and could potentially activate Ras via the disassociated α- or βγ-subunit. Additionally, increases in intracellular Ca^{2+} (from cellular influx or release of Ca^{2+} from intracellular stores) have been found to activate Ras (reviewed in Agell et al., 2002). In vertebrates, two neural specific Ras guanine

exchange factors (Ras-GEFs), Ras guanyl nucleotide-releasing protein (Ras-GRP) and Ras-guanine-nucleotide-releasing-factor (Ras-GRF), activate Ras in a Ca^{2+} dependent manner. Ras-GRP, coupled with diacylglyercol (DAG), activates Ras in neural cells (Ebinu et al., 1998). The second neural specific Ras-GEF molecule, Ras-GRF, activates Ras in a Ca^{2+} -calmodulin specific manner (Farnsworth et al., 1995). In neurons, CaMKII can additionally negatively regulate the Ras-inhibitor, synaptic GTPase-activating protein (SynGAP), via phosphorylation (Chen et al., 1998). These Ras-GEFs and SynGAP are known to participate in the modulation of LTP and structural plasticity (Vazquez et al., 2004; Ye et al., 2000; Kim et al., 1998) and therefore may be additional participants worth investigating in relation to mGluR-mediated signaling. We previously found that ligand-activated DmGluRA increases intracellular Ca^{2+} (Eric Howlett, unpublished data) and an ortholog of the vertebrate Ras-GRP (Son of sevenless; Sos) has been identified in Drosophila (Rogge et al., 1991). Additional experiments will need to be performed to determine if the DmGluRA-activation of Ras involves Ca^{2+} -related mechanisms in the motor neuron.

Chapter 3: The effect of PI3K on synaptic growth is Ras-independent

3.1 Introduction

3.1.1 Abnormal synaptic growth has been observed in several neurological disorders

Alterations in synaptic structure and morphology have been identified in several neurological disorders hallmarked by ASD, including Fragile X Syndrome (Kaufmann and Moser, 2000; Restivo et al., 2005). As previously discussed, PI3K signaling is involved in the processes that modify synaptic strength (Sharma et al., 2010; Gladding, 2009; Bourgeois, 2009; Hou and Klann, 2004; Man et al., 2003), axon guidance and growth during development (Akiyama and Kamiguchi, 2010; Vodrazka et al., 2009; Brandt et al., 2007; Jossin and Goffinet, 2007; Markus et al., 2002) as well as the regulation of the morphology and complexity of dendritic spines (Kumar et al., 2005; Jaworski et al., 2005). The fact that aberrant PI3K signaling has been identified in several neurological disorders that have co-morbidity for autistic traits, including FXS and tuberous sclerosis, illustrates the significant role that signaling pathways that link form and function play in the refinement of cognitive function. Investigating the initialization and regulation of the PI3K pathway components may elucidate novel strategies for the treatment of these disorders.

3.1.2 PI3K and Ras participate in neural growth processes

Current evidence points towards mTOR/S6K as the primary PI3K effector pathway mediating the growth effects of PI3K in the nervous system (Vodrazka et al., 2009; Howlett et al., 2008; Jossin and Goffinet, 2007; Kumar et al., 2005); however, the molecules facilitating the activation of PI3K in a majority of these processes have not been identified. The GTPase Ras has been recognized an important activator of PI3K (Orme et al., 2006; Rodriguez-Viciana et al., 1994). Ras binds to a specific Ras-binding domain (RBD) of the catalytic unit of PI3K, inducing a conformation change that activates the kinase activity of PI3K (Rodriguez-Viciana et al., 1996). The overexpression of Ras has been shown to increase dendrite size and complexity in transgenic mice (Arendt et al., 2004; Gartner et al., 2004; Alpar et al., 2004), similar to what has been observed when PI3K or components of the PI3K pathway are hyperactivated (Kumar et al., 2006; Jaworski et al., 2005; Markus et al., 2002), suggesting that Ras may participate in the activation of PI3K during PI3K-related neural outgrowth processes. However, the activation of the MAP kinase ERK via Ras has also been shown to modify neural outgrowth (Ye and Carew, 2010; Kumar et al., 2005; Jaworski et al., 2005; Arendt et al., 2004; Koh et al., 2002), therefore it is unclear if the growth phenotypes associated with Ras activity are due to the activation of one or perhaps both of these effector pathways.

The ERK and PI3K cascades have in fact been shown to function cooperatively in the outgrowth of axons and dendrites in cultured neurons, though each pathway appears to have a differential effect on growth (Kumar et

al., 2005; Jaworski et al., 2005; Markus et al., 2002). Both the ERK and PI3K cascades are required for outgrowth in cultured sensory neurons; however, ERK signaling is necessary for axon elongation whereas PI3K activation additionally modifies axon diameter and branching (Markus et al., 2002). Similarly, both the ERK and PI3K pathways act in concert to regulate dendrite formation in cultured hippocampal neurons, but PI3K activation also modifies dendrite size (Kumar et al., 2005; Jaworski et al., 2005). In both of these studies, the authors utilized pharmacological inhibitors of the ERK and PI3K cascades that target effectors downstream Ras (in the case of ERK, the pharmacological inhibitor U0126 inhibits the activity of MEK and in the case of PI3K, LY294002 competitively binds to and inactivates the PI3K ATP-binding site), therefore these studies did not specifically address if Ras is required for the effects of PI3K on dendrite growth.

Similar to vertebrates, the manipulation of the ERK and PI3K signaling pathways has also been found to alter synaptic outgrowth in Drosophila. Several studies have demonstrated that modulating PI3K activity in the motor neuron alters motor neuron arborization (Howlett et al., 2008; Martin-Peña et al., 2006). Our lab additionally found that PI3K activity modifies the diameter of the peripheral nerve axons (Howlett et al., 2008). Ras has additionally been reported to regulate motor neuron synaptic outgrowth through the Ras-ERK pathway (Koh et al., 2002). The activation of ERK was suggested to promote bouton formation by phosphorylating and thus downregulating the cell adhesion molecule Fasciclin II (FasII) (Koh et al., 2002; Koh et al., 1999). FasII, the Drosophila ortholog of the

vertebrate neural cell adhesion molecule (NCAM), forms a stabilization complex with Discs-Large (DLG) that must be removed to allow synaptic outgrowth to occur (Thomas et al., 1997; Davis et al., 1997; Zito et al., 1997; Tejedor et al., 1997).

3.1.3 The PI3K-mediated effects on synaptic growth are Ras-independent

Because the Drosophila larva provides a tractable system for the study of synaptic architecture, we decided to further explore a requirement for Ras during PI3K-mediated synaptic growth. To explore if the PI3K affects on neural growth are Ras-dependent or Ras-independent processes, we examined the effect of inhibiting Ras activity on arborization to determine if altering Ras activity could phenocopied the growth defects observed when PI3K activity is inhibited (Howlett et al., 2008; Martin-Peña et al., 2006). We additionally assayed the growth phenotypes associated with a Ras-insensitive PI3K mutant, *gen-PI3K*^{RBD}. We found that similar to reducing PI3K activity, knocking down Ras in the motor neuron diminishes arborization; however, the expression of a constitutively-active PI3K transgene did not completely block the Ras phenotype, suggesting that Ras is not epistatic to PI3K. Subsequent analysis of *gen-PI3K*^{RBD} additionally revealed no arborization defect, indicating that Ras and PI3K modulate arborization via separable pathways and that the effect of PI3K on synaptic growth is Ras-independent.

3.2 Materials and Methods

3.2.1 General fly husbandry and stocks

Husbandry: All fly stocks were maintained on standard cornmeal/agar Drosophila media at 22°C in either half pint bottles or vials (Genesse Scientific, San Diego, CA). General fly husbandry was carried out as described by Greenspan (1997). Experimental crosses/genotypes were kept in bottles and adult flies (10 male and 10 female) passaged to new bottles every four days to limit the overcrowding of offspring.

Stocks: The stocks utilized for these experiments were previously described in Chapter 2. In addition to these stocks, we also utilized the dominant-negative Raf transgenic stock, *UAS-Raf^{DN}*. This variant has a lysine to methionine substitution at position 497 (K497M) that renders the protein "kinase-dead" (Radke et al., 2001). For epitasis experiments, we also produced a *UAS-Raf^{DN}*, *UAS-PI3K-CAAX* double-mutant stock (Michael Stern).

3.2.2 Arborization analysis

To visualize the motor neurons innervating the Drosophila nmj, wandering, third instar larvae were collected and dissected as described in the Materials and Methods section of Chapter 2. Larvae were dissected in 1xPBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4). These larval preps were fixed in 4% paraformaldehyde for 15 minutes then washed 3x for 10 minutes in 1xPBS-T (1xPBS with 0.1% Triton-X 100). The larval preps were incubated overnight at 4°C with Cy2-conjugated antibodies against horseradish peroxidase (HRP) raised in goat (anti-HRP, Jackson ImmunoResearch) at a

concentration of 1:200. The larvae were mounted onto slides in 50% glycerol-1xPBS solution and visualized with a Zeiss 510 laser scanning confocal microscope (LSM) using the 20x objective. For the arborization analysis, the nmj of larval abdominal segments 3 and 4 (A3 and A4, respectively) between muscles 7 and 6 (Fig.3.1) were imaged. To quantify arborization, the total numbers of boutons (which are round protrusions extending from the axons that constitute the pre-synaptic region of a synapse) were counted for each of the

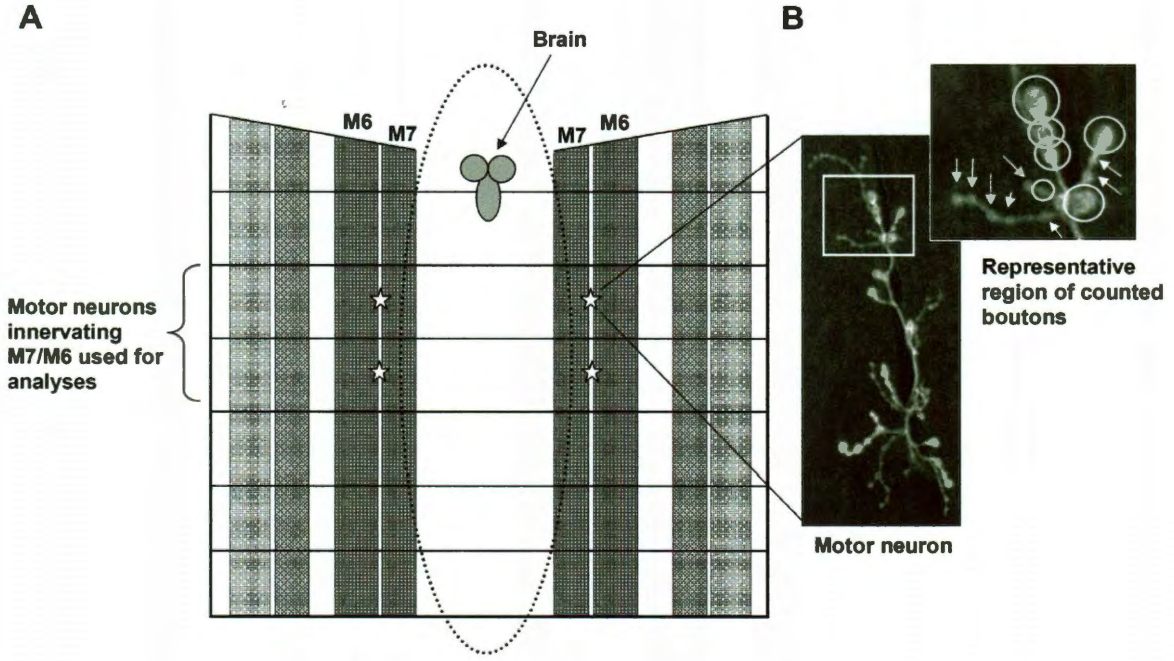


Figure 3.1 Location of motor neurons used for arborization analysis. Larval dissections were performed using the standard protocol outlined by Jan and Jan (1978). **(A)** Diagram of a fillet prep of a wandering, 3rd instar larva positioned dorsal side up. The motor neurons innervating muscles 7 and 6 (M7 and M6) of abdominal segments 3 and 4 (A3 and A4) were used for analysis. The locations of the neurons are denoted with stars. **(B)** Image of the neuron innervating M7 and M6. Neurons were visualized by immunostaining with an anti-HRP antibody. The insert shows an enlarged region of the neuron outlined with a white square. The circles and arrows indicate representative boutons that would have been counted as part of the arborization analysis

neurons in the areas defined above. The cell counter application in ImageJ was utilized to count the boutons from each image and an average number was calculated for each genotype. A standard error of the mean (SEM) was calculated for each genotype and an ANOVA and a Fisher's LSD used to evaluate significance.

3.3 Results

3.3.1 The effect of PI3K on synaptic growth is Ras-independent

The activity of Ras and PI3K have been shown to modify synaptic outgrowth (Akiyama and Kamiguchi, 2010; Ye and Carew, 2010; Howlett et al., 2008; Martin-Pena et al., 2006; Kumar et al., 2005; Jarwoski et al., 2005; Arendt et al., 2004; Koh et al., 2002). In Drosophila, the overexpression of Ras in the motor neuron was shown to significantly increase synaptic growth at the nmj (Koh et al., 2002). Similarly, the overexpression and inhibition of PI3K in the Drosophila motor neuron was demonstrated to increase and decrease synaptic growth, respectively (Howlett et al., 2008; Martin-Pena et al., 2006). Because Ras can activate PI3K (Rodriguez-Viciana et al., 1994; Orme et al., 2006), we wanted to investigate what PI3K-regulated growth processes may be Ras-dependent or Ras-independent in Drosophila motor neurons. To test if Ras and PI3K are functioning in the same pathway, we performed a series of experiments that altered the activity of Ras and PI3K via transgene expression in the motor neuron. Consistent with previous reports, we found that expression of the PI3K

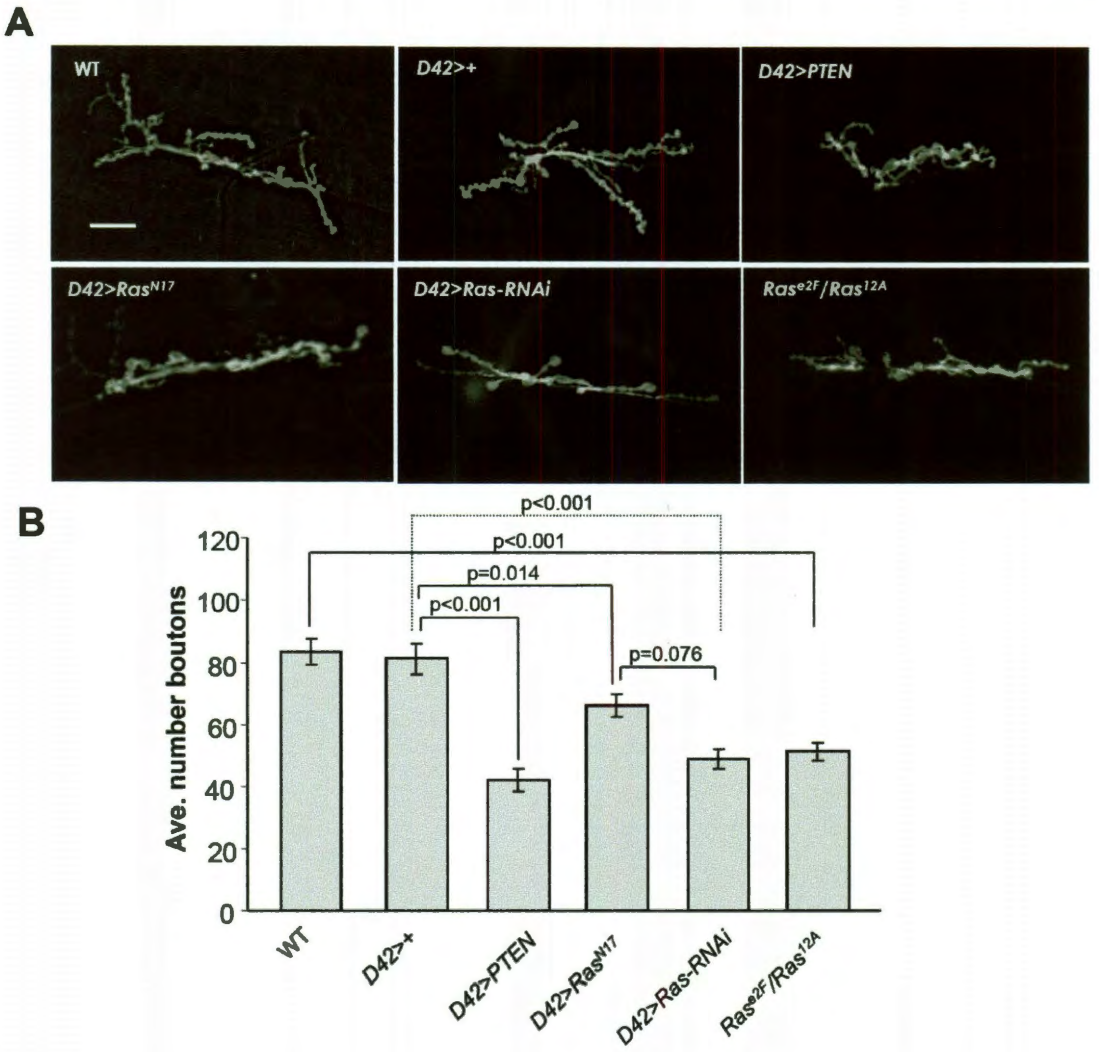


Figure 3.2: Ras activity promotes synaptic bouton formation. (A) Representative confocal images of neuromuscular junctions from larvae of the indicated genotypes labeled with an antibody against HRP. Motor neurons innervating muscles 6 and 7 from segments A3 or A4 are shown. Scale bar 20 µm. **(B)** Mean number +/- SEM of synaptic boutons (Y-axis) from neuromuscular junctions of the indicated genotypes (X-axis). Number of neuromuscular junctions analyzed: for WT, n=12; for *D42>+*, n=20; for *D42>PTEN*, n=17; for *D42>Ras^{N17}*, n=31; for *D42>Ras-RNAi*, n=11; for *Ras^{e2F}/Ras^{12A}*, n=27. Significant differences in bouton number among genotypes (from one way ANOVA and Fisher's LSD or Student's t test, as appropriate) are indicated on the figure.

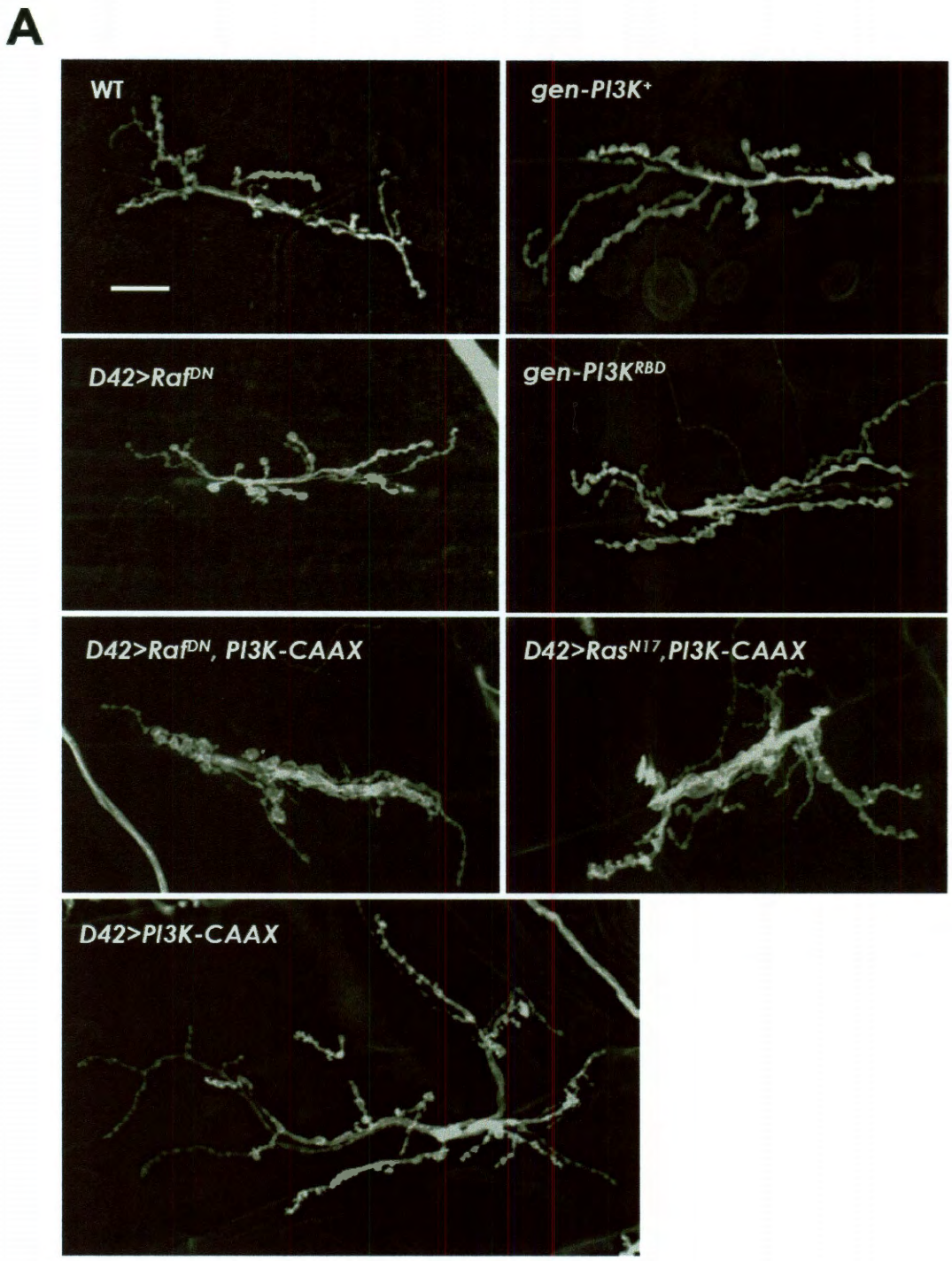
inhibitor, *PTEN*, significantly decreased arborization (Fig.3.2) whereas expression of a constitutively active PI3K transgene (*PI3K-CAAX*) increased arborization (Fig.3.3) as measured by the average total number of synaptic boutons. Similar to inhibiting PI3K, we found that the expression of a dominant-negative Ras transgene (Ras^{N17}) or a Ras RNA interference transgene (*Ras-RNAi*) significantly reduced motor neuron axon arborization (Fig.3.2). Synaptic outgrowth was also reduced in the Ras heteroalleic hypomorphic mutant, Ras^{e2F}/Ras^{12A} in which Ras activity is reduced in all tissues (Fig.3.2). These results indicate that Ras is required for synaptic outgrowth; however, it is unclear whether the effects of Ras are due to the inhibition of the Ras-ERK or the Ras-PI3K cascade. To address if PI3K is epistatic to Ras, we co-expressed constitutively-active *PI3K-CAAX* transgene with Ras^{N17} to determine if PI3K could block the Ras^{N17} growth defect. We found that expression of the constitutively active PI3K did in fact increase arborization compared to Ras^{N17}; however it did not completely block the Ras^{N17} arborization defect (Fig.3.3). This result suggests that Ras and PI3K may be affecting outgrowth via parallel pathways.

To further investigate a requirement for Ras, we additionally examined outgrowth in the Ras-insensitive PI3K variant, $gen\text{-}PI3K^{RBD}$, which has a four amino acid substitution in the Ras binding domain that prevents Ras from binding to and activating PI3K (Orme et al., 2006; Pacold et al., 2000). We expected that if Ras was required for the effect of PI3K on growth that arborization would be reduced in this mutant. We observed no significant difference in motor neuron arborization in the $PI3K^{RBD}$ variant compared to either the $gen\text{-}PI3K^{+}$ control or to

wildtype larvae (Fig.3.3). These results suggest that the effect of PI3K on axon arborization is Ras-independent.

3.3.2 Ras-ERK signaling is required for the synaptic outgrowth of the Drosophila motor neuron

Ras was previously shown to modify synaptic growth via the Ras-ERK-FasII in the Drosophila motor neuron (Koh et al., 2002). In vertebrate hippocampal neurons, both the Ras-ERK and PI3K pathways function cooperatively to regulate dendrite growth (Kumar et al., 2005; Jarwoksi et al., 2005). To investigate the possibility that Ras-ERK and PI3K are functioning in parallel, we additionally inhibited the activity of Raf, the principle Ras effector that mediates the Ras-ERK cascade, by expressing a dominant-negative Raf transgene (Raf^{DN}). Similar to inhibiting Ras and PI3K, expressing Raf^{DN} in the motor neuron significantly reduced axon arborization (Fig.3.3). To investigate if PI3K could promote synaptic growth in the absence of Ras-Raf-ERK activation, we co-expressed *PI3K-CAAX* with Raf^{DN}. We found that *PI3K-CAAX* could not block the Raf^{DN} arborization defect, indicating that the activity of PI3K is not sufficient to orchestrate synaptic outgrowth in the absence of Raf-ERK signaling. This data indicates that the Ras-ERK and PI3K pathways cooperate synergistically to facilitate synaptic outgrowth. Presumably, the quantitatively different outcomes on synaptic outgrowth conferred by Ras^{N17} and Raf^{DN} reflect differences in transgene expression which is an observation that is consistent in previous reports (Koh et al., 2002).


A
WT
gen-PI3K+
D42>RafDN
gen-PI3KRBD
D42>RafDN, PI3K-CAAX
D42>RasN17,PI3K-CAAX
D42>PI3K-CAAX

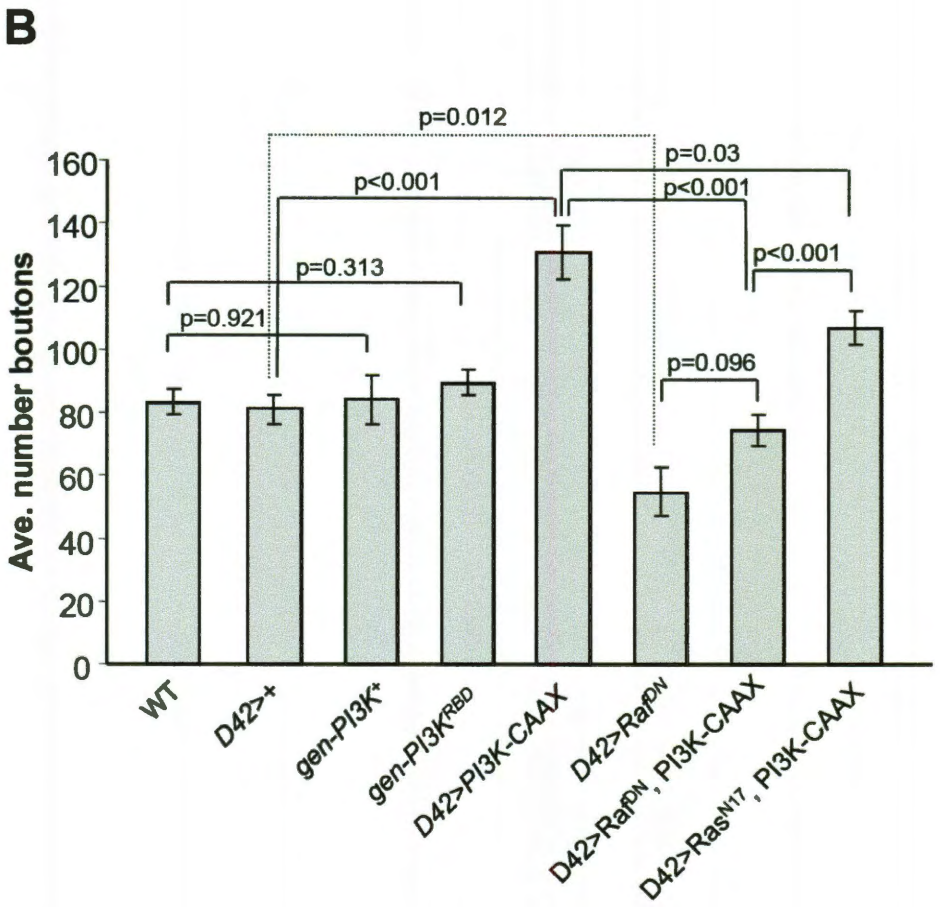


Figure 3.3: Ras regulates synaptic bouton number via Raf, not PI3K. **(A)** Representative images of larval neuromuscular junctions of wildtype (WT), *D42>Raf^{DN}*, *gen-PI3K^{+} gen-PI3K^{RBD}*, *D42>Raf^{DN}*, *PI3K-CAAX, D42>PI3K-CAAX.* Neurons were labeled with anti-HRP. Scale bar 20 µm. **(B)** Mean number +/- SEM of synaptic boutons (Y-axis) from neuromuscular junctions of the indicated genotypes (X-axis): WT, *D42>+*, *gen-PI3K^{+}*, *gen-PI3K^{RBD}*, *D42>PI3K-CAAX, D42>Raf^{DN}, PI3K-CAAX, D42>Ras^{N17}, PI3K-CAAX.* From left to right, n =12, 20, 10, 9, 26, 6, 28, 21. Significant differences in bouton number among genotypes (from one way ANOVA and Fisher's LSD or Student's t test, as appropriate) are indicated on the figure.

3.4 Discussion

3.4.1. The effect of PI3K on neural growth is Ras-independent

Previous reports by our lab and others have demonstrated that the activity of Ras and PI3K can alter Drosophila neural outgrowth (Howlett et al., 2008; Lavery et al., 2007; Martin-Peña, 2006; Koh et al., 2002). In this study we have found that the effect of PI3K on synaptic growth is Ras-independent. We found PI3K activity could not block the decrease in synaptic growth that occurred when Ras was inhibited in the motor neuron (Fig.3.3). This observation is consistent with a previous report that found that the expression of a constitutively-active Ras transgene that only activates the PI3K effector pathway (Ras^{V12C40}) failed to increase synaptic bouton number (Koh et al., 2002). We further demonstrated that the PI3K Ras-binding domain variant, $gen\text{-}PI3K^{RBD}$, did not exhibit a growth defect (Fig.3.3). These results indicate that Ras is not required for the effect of PI3K on synaptic growth.

3.4.2 CaMKII and Insulin growth factors may activate PI3K to mediate synaptic outgrowth

Subsequent signals activating PI3K to promote arborization are unknown; however, recent work by our lab suggests that the Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII) may act upstream PI3K to mediate arborization. We and others have demonstrated that CaMKII is both necessary and sufficient to promote motor neuron arborization (Lin et al., 2011; Beumer et al., 2002; Koh et al., 1999). We found that the expression of the CaMKII inhibitory peptide *ala2* decreases synaptic bouton number and that this decrease can be suppressed by

the expression of *PI3K-CAAX*, suggesting that CaMKII inhibition blocks arborization by preventing PI3K activation (Lin et al., 2011).

Alternatively, insulin growth factors may also function as potential PI3K activators during synaptic growth. The insulin receptor (INSR)-PI3K pathway is an important regulator of cell metabolism and it has been increasingly recognized that neuronal circuits are targets for insulin (Barsh and Schwartz, 2002; Konner et al., 2009). Insulin-like and Insulin Receptor-like immunoreactivity are present at the Drosophila larval nmj (Budnik et al., 1990) and both insulin and insulin growth factors are capable of activating PI3K in a Ras-independent manner (Backer et al., 1992).

3.4.3 The effect of PI3K on synaptic outgrowth requires ERK signaling

Similar to previous observations made in vertebrate systems, our data supports the notion that the Ras-ERK and PI3K pathways function in parallel to alter neural outgrowth (Kumar et al., 2005; Jaworski et al., 2005; Markus et al., 2002). Kumar et al., (2005) demonstrated that the application of either the PI3K pharmacological inhibitor LY294002 or the MEK inhibitor U0126 blocked the effect of constitutively active Ras on dendrite outgrowth, indicating that both pathways in concert were required to facilitate growth. We found that expression of *PI3K-CAAX* only partially suppressed the growth defect of Ras^{N17} and did not suppress the effect of Raf^{DN} on axon growth (Fig.3.3), suggesting that ERK signaling is required in concert for the effect of PI3K on synaptic outgrowth in the Drosophila motor neuron.

The Ras-ERK pathway was previously shown to participate in synapse formation in Drosophila via the modulation of the Discs-Large - Fascilin II (DLG-FasII) cell adhesion complex (Koh et al., 2002). FasII, the ortholog of the vertebrate neural cell adhesion molecule (NCAM) (Schuster et al., 1996), regulates presynaptic morphology and growth cone targeting (Thomas et al., 1997; Davis et al., 1997). DLG associates with synaptic cytoskeleton, clustering both FasII and Shaker K^+ channels, forming a stabilization complex that must be disassociated so that synaptic outgrowth can occur (Thomas et al., 1997; Zito et al., 1997; Tejedor et al., 1997). In *Aplysia,* it has been shown that FasII is directly phosphorylated by ERK/MAPK, which results in the internalization of FasII and the disassociation of the DLG-FasII stabilization complex (Bailey et al., 1997). Activated ERK has also been shown to displace FasII at the motor neuron terminus, resulting in increased synaptic growth (Koh et al., 2002). Our data indicate that both the Ras-ERK and PI3K cascades are cooperatively involved in motor neuron synaptic outgrowth. We hypothesize that Ras-ERK is required to downregulate FasII from the stabilization complex and that PI3K activation regulates protein translation via S6K (Howlett et al., 2008) to promote bouton formation (Fig.3.4)

CamKII also promotes synaptic outgrowth by phosphorylating and removing DLG from the DLG-FasII complex (Koh et al., 1999). Our lab has recently reported that we can rescue the growth defect associated with expression of the CamMKII inhibitory peptide *ala2* with *PI3K-CAAX* (Lin et al., 2011); however, this

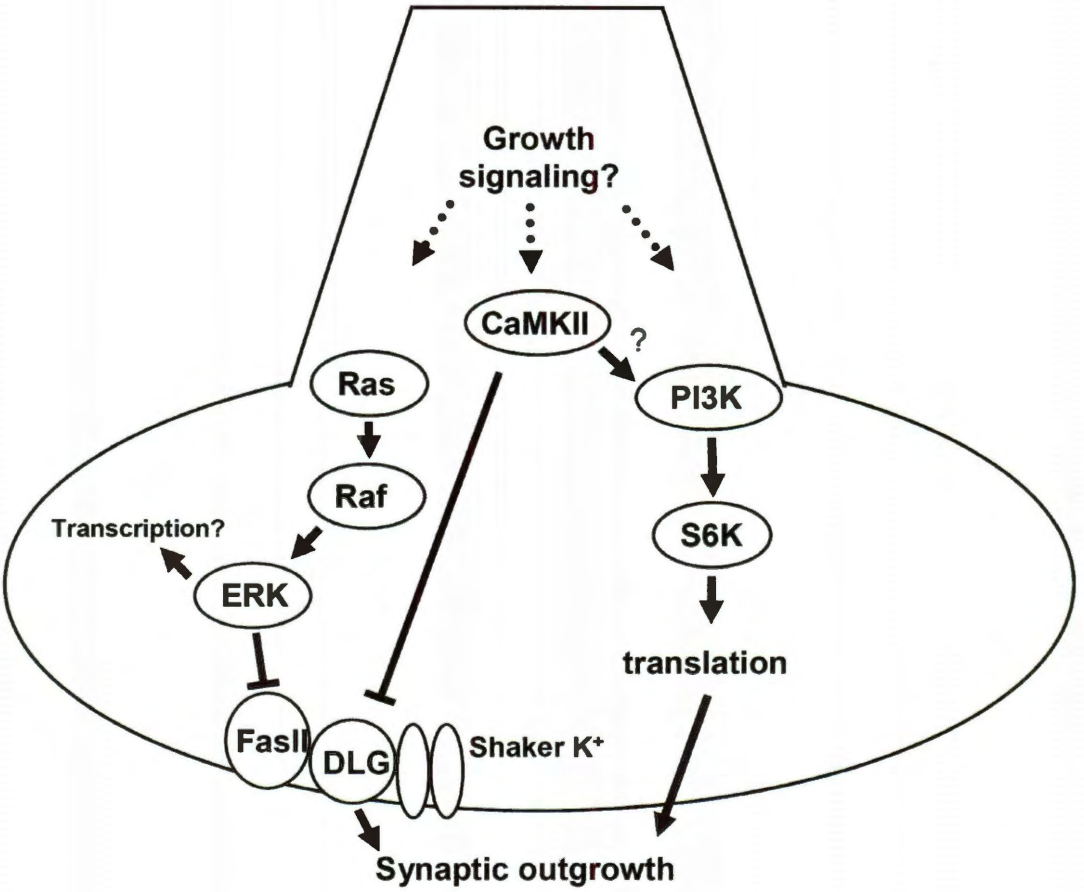


Figure 3.4: Synaptic outgrowth requires the activity of several pathways. Signaling pathways involved in the downregulation and the initiation of transcription/translation appear to be involved in the regulation of synaptic outgrowth. The Discs-Large (DLG) and Fascilin II (FasII) cell adhesion complex stabilize the synapse by clustering components of the cell's cytoskeleton and the Shaker K channel (Thomas et al., 1997; Zito et al., 1997; Tejedor et al., 1997). This complex must be destabilized in order for outgrowth to occur. CaMKII and ERK promote outgrowth by phosphorylating and thus downregulating DLG and FasII, respectively (Bailey et al., 1997; Koh et al., 2002; Koh et al., 1999). PI3K also regulates outgrowth, most likely through regulating transcription (Howlett et al., 2008). CaMKII and PI3K are not sufficient to promote outgrowth in the absence of ERK signaling (this study and preliminary, unpublished data); therefore ERK activation appears to be requirement for outgrowth. In addition to FasII, ERK may regulate additional targets that are required for outgrowth processes.

study found that the expression of *PI3K-CAAX* could not block the synaptic deficit associated with inhibiting Ras-ERK signaling (Fig.3.3), suggesting that intact Ras-ERK signaling is required for the effects of CaMKII and PI3K on synaptic growth. Because both CamKII and ERK modulate the DLG-FasII complex indicates that these molecules may be altering the complex in a different manner (i.e., ERK phosphorylation may more efficiently remove the complex). ERK may additionally have other targets distinct from CamKII that are involved in this process, including the transcription factor CREB, which has been shown to participate in neurite outgrowth in cultured sensory cells (Schmid et al., 1999; White et al., 2000).

Chapter 4: DmGluRA activation of ERK

4.1 Introduction

4.1.1 Overview of the biochemistry and function of the extracellular signal-related kinase (ERK)

The highly conserved mitogen-activated protein kinase (MAPK) families of signaling molecules are essential for the transmission of extracellular information into intracellular activities. Presently, four distinct MAPK cascades, which include extracellular signal-related kinase (ERK), c-Jun N-terminal kinase (JNK), p38, and Bruton's tyrosine kinase (BMK) have been indentified that participate in a wide-range of cellular function including the regulation of proliferation, differentiation, survival, apoptosis, and cellular migration (reviewed in Shaul and Seger, 2007). The ERK molecules and their related signaling cascades are of particular interest to neurobiology because they are highly expressed in the neurons of the central nervous system and appear to have a critical role in the processes of learning and memory (reviewed in Adams and Sweatt, 2002).

The transmission of signals through the related MAPK cascades occurs by the sequential activation of several sets of protein kinases (Fig.4.1). Signaling through the ERK cascade is typically initiated via the activation of Ras in response to growth factors and other extracellular stimuli. Ras activation recruits and activates a second protein kinase, Raf, at the plasma membrane (Wellbrock et al., 2004). Raf phosphorylates and activates the enzyme MAPK/ERK kinase (MEK), which in turn phosphorylates and activates ERK1 and ERK2 (also known as p44 and p45 kinase, respectively) (Nakielny et al., 1992) (Fig. 4.1). Activated

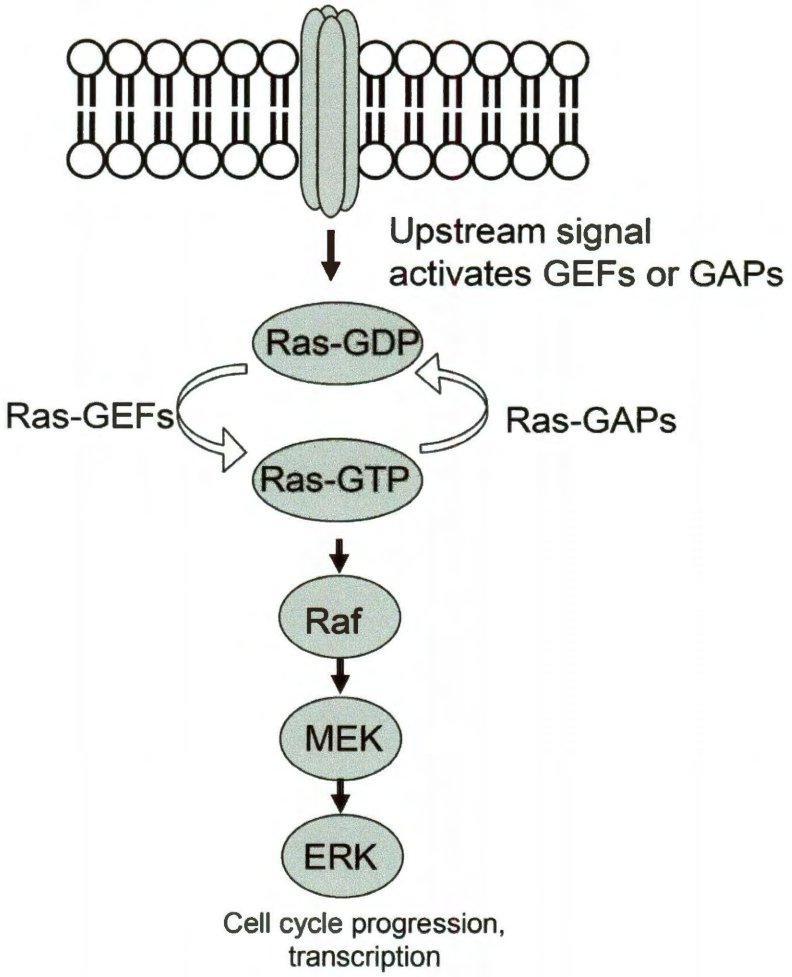


Figure 4.1: Overview of the Ras-ERK signal transduction pathway. The activation of extracellular signal-related kinase, ERK, a member of the mitogen-activated protein (MAP) kinase family, occurs through the sequential activation of several sets of kinases. The activation of Ras via receptor-tyrosine kinases or G protein-coupled receptors typically initiates the cascade. Activated Ras-GTP recruits and activates Raf at the plasma membrane. Raf phosphorylates and activates the enzyme MAP kinase kinase (MEK) which in turn phosphorylates and activates ERK1 and ERK2. ERK1/2 is involved in the regulation of a multitude of transcription factors, including cyclic-AMP response element binding protein (CREB) (Wellbrock et al., 2004; Nakielny et al., 1992; Shaul and Seger, 2007).

ERK phosphorylates a large number of substrates in the cell cytoplasm and in the nucleus including the transcription factors Elk1, c-Fos, p53, and c-Jun (reviewed in Shaul and Seger, 2007). ERK is also involved in the regulation of the activity of the transcription factor cyclic-AMP response element binding protein (CREB) via another set of kinases; the ribosomal S6 kinases (RSKs) and the mitogen- and stress-associated protein kinases (MSKs) (Hardingham et al., 2001; Xing et al., 1998; Deak et al., 1998). Many studies have indicated that CREB-dependent transcription is critical for learning and memory) (reviewed in Thomas and Huganir, 2004).

4.1.2 ERK signaling is involved in synaptic plasticity in vertebrates

Two forms of synaptic plasticity, LTD and LTP, which modify the strength of synaptic connections via changes in excitability, are widely believed to be integral for learning and memory by "encoding" distinct activity patterns within a neural network (Bruel-Jungerman et al., 2007; Davis, 2006; Katz and Shatz, 1996; Bliss and Collingridge, 1993). Research over the past decade has revealed that the regulation of the ERK signaling cascade plays a significant role in synaptic plasticity via the alteration of LTD and LTP in hippocampal and cortical neurons (Peng et al., 2010; Thomas and Huganir, 2004). Following LTP-inducing stimuli, phosphorylated ERK (p-ERK; activated) immunoreactivity develops rapidly in dendritic and somatic regions of hippocampal neurons, indicating that ERK substrates are found throughout the neuron (Davis et al., 2000; Dudek and Fields, 2001). Additional studies using rat hippocampal or cortex slices found that the application of the specific MEK pharmacological inhibitor PD98059 effectively

blocks LTP induction in these tissues (English and Sweatt, 1997; Dicristo et al., 2001). Injection of PD98059 into the brain also alters the memory performance of rats in several types of memory-related behavioral tests (Blum et al., 1999; Berman et al., 1998), further substantiating the role of LTP in memory formation. Numerous studies have demonstrated a requirement for ERK activity in several forms of synaptic plasticity including NMDA receptor independent forms of LTP, LTP induction in the dentate gyrus, and LTP in the amygdala, which is involved in fear conditioning (reviewed in Thomas and Huganir, 2004). Inhibiting ERK signaling also mediates LTD in Purkinje cells; however, this effect appears to be due the direct modulation of mGluR function by ERK rather than ERK functioning in a downstream signaling event related to LTD (Kawasaki et al., 1999).

The underlying mechanisms regarding ERK signaling and how it relates to the processes mediating LTP and LTD have been debated, though a great deal of evidence suggests it may occur via the modification in the number or activity of postsynaptic AMPA receptors or through the activity-dependent modification of dendritic spines (reviewed in Thomas and Huganir, 2004). These processes appear to be regulated by the effect ERK signaling on transcription and protein translation (reviewed in Peng et al., 2010). Transcription factors, such as Elk1 and CREB, may be important targets of ERK in the regulation of synaptic plasticity. Though many studies have found a requirement for ERK in the regulation of the processes that regulate synaptic plasticity, still little is known concerning the molecular components that activate this pathway. It is generally accepted that the ERK signaling cascade in neurons is initiated by activated Ras

(Margolis and Skolnk, 1994; Downward, 1996), in response to membrane depolarization (Rosen et al., 1994), or by G-coupled protein receptors (reviewed in Goldsmith and Dhanasekaran, 2007); however, which of these upstream components are modulating the activation of ERK in the processes related to synaptic plasticity are not clear.

4.1.3 mGluR activation initiates ERK signaling during LTP and LTD

As critical regulators neural excitatory transmission, mGluRs are important for the modulation of synaptic plasticity. mGluR mediated LTD, termed mGluR-LTD, results in a long-lasting decrease in synaptic strength and efficiency in response to low frequency simulation or to the application of group I mGluR agonists (reviewed in Gladding, 2009). The molecular mechanisms underlying induced mGluR-LTD have not been elucidated fully; however, many studies have demonstrated that protein synthesis is required to maintain LTD (reviewed in Gladding, 2009). Work from a number of groups has established that the ERK and the PI3K-mTOR signaling pathways are involved in coupling mGluR activation to translational machinery during LTD (Gladding et al., 2009; Antion et al., 2008; Banko et al., 2006; Gallagher et al., 2004; Hou and Klann, 2004; Sweatt, 2004). In the hippocampus, the activation of the initiation factor eIF4E and the S6 ribosomal proteins, key steps in the initiation of cap-dependent protein translation, are dependent on ERK phosphorylation (Antion et al., 2008; Kelleher et al., 2004; Banko et al., 2004). Blocking ERK signaling, thereby disrupting cap-dependent translation, inhibits mGluR-LTD (Gallagher et al., 2004; Huber et al., 2000).

The molecular mechanisms underlying the initiation of the ERK cascade via mGluR are not completely understood and appear to vary depending on the neural tissue type and the method of induction. As G protein-coupled receptors, the activation of group I mGluRs can initiate a Ca^{2+}-dependent signaling cascade via the activation of the effector phospholipase C (PLC), which results in an increase in inositol triphosphate (IP_3) and diacylglycerol (DAG) production that ultimately results in the release of Ca^{2+} from intracellular stores (Gladding et al., 2009; Pin and Duvoisin, 1995). This pathway has been previously demonstrated to activate ERK (Hermans and Challiss, 2001). The scaffolding protein Homer may also couple the group I mGluRs to ERK signaling in a Ca^{2+}-independent manner (Mao et al., 2005). The adaptor protein, caveolin-1, which associates with lipid rafts and negatively regulates endocytosis (Nabi and Le, 2003), has additionally been shown facilitate mGluR-dependent ERK signaling (Francesconi et al., 2009). The canonical ERK cascade activator, Ras, has also been suggested to play a role in mGluR-mediated ERK activation; however, this is not due to the direct activation of Ras by mGluRA and requires calcium influx (Walker et al., 2002).

LTD can be also be induced by the electrical and chemical stimulation of the presynaptic group II mGluRs (Altinbilek and Manahan-Vaughan, 2009; Poschel and Manahan-Vaughan, 2005; Manahan-Vaughan, 1997; Huang et al., 1997); however, much less is known regarding the molecular mechanisms governing the effect of the presynaptic group II mGluRs on LTD. Agonist-activation of group II mGluRs in the vertebrate hippocampus was previously demonstrated to

activate ERK in the glia and interneurons (Berkeley and Levey, 2003) indicating that the ERK cascade may also be involved in the effects of the group II mGluRs on synaptic plasticity.

4.1.4 Glutamate-activated DmGluRA initiates ERK signaling

The Drosophila larval nmj is a tractable system to investigate the molecular mechanisms governing synaptic plasticity due to the ease of accessing identifiable synapses (Keshishian et al., 1996) and the fact that both short and long terms of plasticity have been demonstrated to occur at the nmj (Guo and Zhong, 2006; Broadie et al., 1997; Jan and Jan, 1978). As discussed in previous chapters, Drosophila has a single metabotropic glutamate receptor, DmGluRA, a G protein-coupled receptor that is most similar to the vertebrate group II mGluRs (Bogdanik et al., 2004; Parmentier et al., 1996). In vertebrates, the activation of both the group I and group II mGluRs during the induction of either LTP or LTD, appears to involve the ERK cascade; however, to our knowledge, it is unknown if ERK signaling is similarly initiated by DmGluRA activation.

The ERK cascade is involved in neural signaling and has been demonstrated to participate in mGluR-mediated effects on synaptic plasticity (Peng et al., 2010; Thomas and Huganir, 2004; Lonze et al., 2002; Davis et al., 2000; Dudek and Fields, 2001). The presynaptic group II mGluRs also regulate LTD; however, the mechanisms governing these processes are relatively unexplored. It was previously demonstrated that agonist-activation of the group II mGluRs in the hippocampus results in an increase in ERK phosphorylation in the glia and

interneurons (Berkeley and Levey, 2002); therefore we wanted to investigate if the activation of DmGluRA initiates ERK in the Drosophila motor neuron. Elucidating a connection between DmGluRA activation and ERK signaling in Drosophila may uncover a similar mechanism related to the group II mGluRs in vertebrates.

To assess if the activation of DmGluRA via glutamate initiates the ERK cascade, we first examined if the application of glutamate increased the level of phosphorylated ERK (p-ERK) in the motor neuron and confirmed that the increase in p-ERK required DmGluRA activity. We found that the application of glutamate to the larval nmj increases p-ERK and that this increase is dependent on DmGluRA. We then used a series of transgenes to alter the activity of several molecules potentially involved in the activation of ERK in the neuron to investigate potential signaling intermediates. We determined that unlike the initiation of the PI3K cascade via DmGluRA, that CaMKII and DFak are not involved in the activation of ERK. We also unexpectedly found that inhibiting Ras, the principle activator of the ERK signaling cascade, resulted in a significant increase in the basal levels of p-ERK; whereas knocking down the Ras effector Raf decreased ERK activity. These results suggest that DmGluRA activates ERK and PI3K via separate pathways; however, the signaling components involved in DmGluRA-mediated ERK activation remain undefined.

4.2 Materials and Methods

4.2.1 Fly husbandry and stocks

All fly stocks were maintained as described in previous chapters and experimental crosses (10 male and 10 female) were prepared in bottles and adults were passaged every four days to limit the overcrowding of larvae. The *GAL4* driver stock utilized for these experiments was the motor neuron driver, *D42-GAL4.*

To evaluate the DmGluRA activation of ERK, we used the DmGluRA null mutant, $DmGluRA^{112b}$ that was produced via the transponson excision of the start codon and a portion of the extracellular ligand binding domain of DmGluRA (Bogdanik et al., 2004) (Bloomington). In addition to the *Ras-RNAi* transgene previously described, also we used a dominant-negative Raf transgene, $UAS\text{-}Raf^{DN}$, which has a lysine to methionine substitution at residue 497 (K497M) (Bloomington Stock Center). We additionally utilized an inositol triphosphate receptor RNAi transgene, $UAS\text{-}IP_3R\text{-}RNAi$ (Drosophila Genetic Resource Center, Kyoto, Japan) and a constitutively active CaMKII transgene, $UAS\text{-}CamKII^{T287D}$ that has a phosphomimetic threonine to aspartate substitution in the autophosphorylation site (T287D) (Fong et al., 1989) provided by Leslie Griffith (Brandeis University). A null mutation in Drosophila focal adhesion kinase, $DFak^{CG1}$ was provided to us by Ruth Palmer, Umeå University, Sweden.

4.2.1 Larval anti-phosphorylated ERK (p-ERK) analysis

These experiments were performed similarly to the anti-p-Akt experiments previously described in Chapter 3. Wandering, third instar larvae were collected

from the sides of bottles within an appropriate window of time (48 hours) and dissected for immunohistochemistry using Drosophila S2 media as described in Chapter 2. To detect levels of activated ERK (phosphorylated ERK, p-ERK) we utilized a monoclonal, anti-ERK/MAP Kinase antibody (Sigma) (1:20) that recognizes the two phosphorylated Thr202 and Tyr204 residues in the regulatory site of ERK that are required for the full activation of the kinase. This antibody does not recognize the monophosphorylated or non-phosphorylated forms of ERK. This anti-p-ERK antibody has been previously used in the Drosophila nmj (Koh et al., 2002; Tsai et al., 2008). The confocal microscopy and data analysis were performed as described for the p-Akt experiments described previously in Chapter 2.

4.3 Results for the anti-p-ERK analysis

4.3.1 Glutamate-activated DmGluRA increases p-ERK in the motor neuron

In vertebrates, the neural modulatory events involved in synaptic plasticity appear to involve the activation of mGluRs and ERK. It is widely accepted that mGluR-LTD involves ERK signaling (Peng et al., 2010; Gladding, 2009; Antion et al., 2008; Banko et al., 2006; Gallagher et al., 2004; Thomas and Huganir, 2004). The pharmacological activation of group II mGluRs in the hippocampus have additonally been shown elevate levels of p-ERK in the glia and interneurons (Berkeley and Levey, 2003). To investigate if the activation of DmGluRA involves ERK signaling in Drosophila, we applied 100 μM of glutamate to the larval nmj

and examined the induction of ERK signaling via anti-p-ERK immunostaining. We found that the application of glutamate to wildtype larval nmjs resulted in a significant increase in the levels of p-ERK (Fig. 4.2). Analysis of the null mutant *DmGluRA*^{112b} revealed significantly lower, basal levels of p-ERK (Fig.4.2). We also found that the application of glutamate did not result in an increase in p-ERK immunoreactivity (Fig.4.2). These results indicate that activated DmGluRA initiates ERK signaling in the Drosophila motor neuron.

4.3.2 Blocking the release of intracellular Ca^{2+} from the endoplasmic reticulum has no effect DmGluRA-ERK signaling

The ERK pathway has been demonstrated to be a target of neuronal Ca^{2+} signaling (Wiegert and Bading, 2010; Grewal et al., 2000). Our lab previously found that the activation of DmGluRA increases intracellular Ca^{2+} (unpublished data). We have also recently demonstrated that the Ca^{2+}-Calmodulin dependent kinase, CaMKII, is an integral component of activated DmGluRA signaling (Lin et al., 2011). To investigate if DmGluRA activates the ERK cascade via the release of intracellular Ca^{2+} from the endoplasmic reticulum (ER), we expressed an inositol 1,4,5-triphospate receptor-RNAi (IP_3R-RNAi) transgene in the motor neuron. Expression of this transgene decreased the basal levels of p-ERK (Fig.4.2); however, we found that the inhibition of the IP_3R did not block the glutamate-induced increase in p-ERK, suggesting that DmGluRA activation of ERK may not involve the release of Ca^{2+} from the ER.

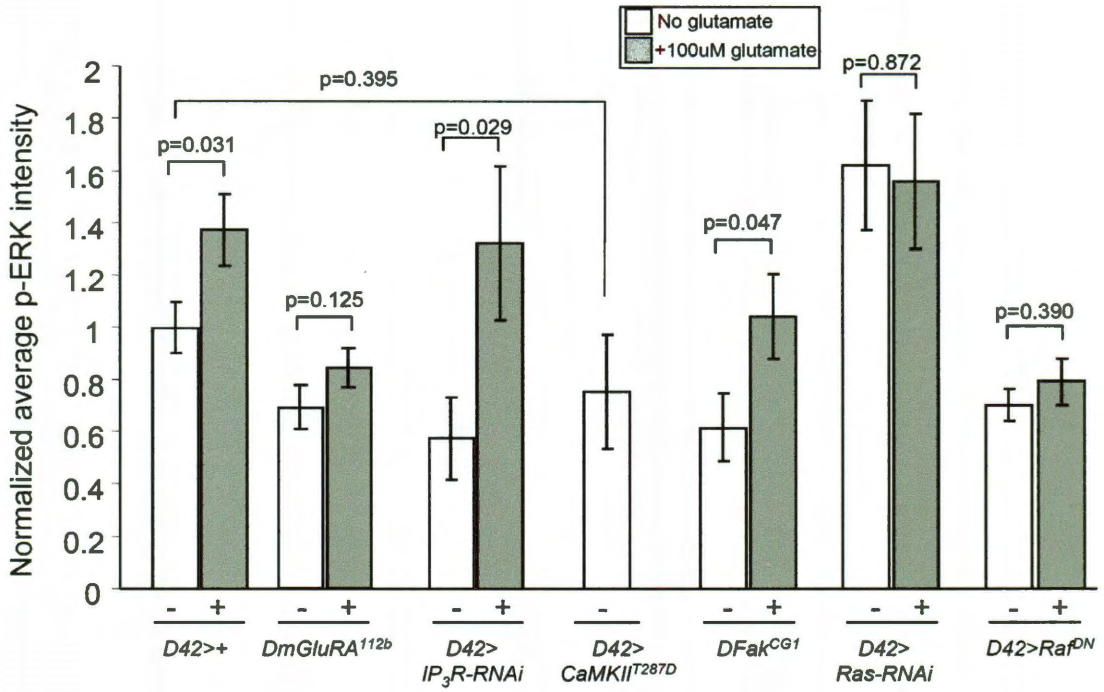


Figure 4.2: ERK activation occurs via a distinct DmGluRA-mediated pathway. Anti-p-ERK immunoreactivity was measured before and after the application of 100 µM glutamate for *D42>+* (control), *DmGluRA*^{112b}, *D42>IP*$_3$*R-RNAi*, *D42>CaMKII*^{T287D} (basal only), *DFak*^{CG1}, *D42>Ras-RNAi*, *D42>Raf*^{DN}. Means +/- SEMs of normalized pixel intensities (Y-axis) of the indicated genotypes (X-axis). The pixel intensity of the anti-p-ERK staining was averaged for each genotype and normalized to the average intensity of the control preparation: values from *DmGluRA*^{112b}, *DFak*^{CG1} were normalized to WT (not shown), whereas values from *D42>IP*$_3$*R-RNAi*, *D42>CaMKII*^{T287D}, *D42>Ras-RNAi*, and *D42>Raf*^{DN} were normalized to *D42>+*. For all genotypes, n=30. One-way ANOVA and Fisher's LSD were used to find the following significant differences: WT vs. *DmGluRA*^{112b}, p=0.005; D42>+ vs. *D42>IP*$_3$*R-RNAi*, p=0.036, vs. *D42>Ras-RNAi*, p=0.028; vs. *D42>Raf*^{DN}, p=0.008. No significant differences were found for the following comparisons: WT vs. *DFak*^{CG1}, p=0.069; *D42>+* vs. *D42>CaMKII*^{T287D}, p=0.395.

4.3.3 CaMKII is not involved in DmGluRA-ERK signaling

The activation of ERK during group I mGluR- LTD was previously shown to involve CaMKII (Zhu et al., 2002; Choe and Wang, 2001). We have also shown that CaMKII is an important signaling intermediate for DmGluRA-mediated PI3K activation (Lin et al., 2011). To investigate if DmGluRA may also activate ERK via CaMKII, we expressed the constitutively-active *CaMKII*^{T287D} transgene in the Drosophila motor neuron. If CaMKII was involved in the activation of the ERK cascade, we expected to observe an increase in the level of p-ERK in the neuron; however, we observed no difference in the basal levels of p-ERK compared to wildtype (Fig.4.2) which suggests that CaMKII does not participate in the activation of ERK.

4.3.4 Drosophila Focal adhesion kinase (DFak) is not involved in the activation of ERK by DmGluRA

The non-receptor tyrosine kinase Fak is involved in numerous cellular responses including cell adhesion, migration, differentiation (reviewed in Parsons, 2003) and can be activated by G-coupled protein receptors via CamKII; (Fan et al., 2005). We have recently demonstrated that DFak is epistatic to CaMKII during glutamate-activated DmGluRA-PI3K signaling (Lin et al., 2011); therefore we wanted to investigate if DFak also participates in ERK activation. We examined the levels of p-ERK immunoreactivity in the null mutant, *DFak*^{CG1} and found that the basal levels of p-ERK were not significantly different than wildtype. We also found that the glutamate-evoked increase in p-ERK is not

blocked in this mutant (Fig.4.2), indicating that DFak is not involved in DmGluRA-mediated ERK signaling.

4.3.3 Inhibiting Ras activity increases basal levels of p-ERK in the motor neuron

The canonical ERK activation pathway involves the activation of the Ras-Raf-MEK-ERK cascade (Wellbrock et al., 2004; Nakielny et al., 1992). During the course of this study, we have found that Ras is a critical intermediate in the DmGluRA-PI3K regulation of neural excitability. To investigate if Ras is additionally involved in the activation of ERK via DmGluRA, we expressed *Ras-RNAi* in the motor neuron and examined levels of p-ERK. Unexpectedly, expression of *Ras-RNAi* resulted in a significantly higher, basal level of p-ERK (Fig 4.2). The application of glutamate to the nmj did not further increase p-ERK immunoreactivity in the neuron (Fig.4.2), indicating that either *Ras-RNAi* blocks an additional increase in p-ERK, or that the maximum levels of detectable p-ERK have been reached.

To additionally explore the role of the canonical Ras-Raf pathway on ERK activation, we also examined the modulation of the kinase Raf. We found that expression of a dominant-negative Raf transgene (Raf^{DN}) in the motor neuron decreased the basal levels of p-ERK (Fig.4.2). Expression of this transgene also blocked the glutamate-induced increase in p-ERK (Fig.4.2). This result tentatively indicates that Raf is involved in the DmGluRA activation of ERK; however, the discrepancies between this result and the *Ras-RNAi* result make it

difficult to conclude if the Ras-Raf pathway is involved in ERK activation via DmGluRA.

4.4 Discussion

4.4.1 Activated DmGluRA initiates ERK signaling

In vertebrates, the ERK cascade has been shown to be important for group I mGluR-LTD by regulating gene transcription and translation (Francesconi et al., 2009; Antion et al., 2008; Banko et al., 2006; Gallagher et al., 2004; Hou and Klann, 2004). The group II mGluRs also modulate LTD (Altinbilek and Manahan-Vaughan, 2009); and agonist-activation of these receptors has been found to increase p-ERK in the glia and the interneruons (Berkeley and Levey, 2003); however, little else is known concerning the molecular components that may be involved in the regulation of LTD by this receptor. We have found that the application of glutamate to the larval nmj increases the levels of p-ERK in the motor neuron (Fig.4.2). This increase was blocked by the DmGluRA null mutant, indicating that DmGluRA is required for glutamate-induced ERK activation. To our knowledge, this is the first demonstration that glutamate-liganded DmGluRA activates ERK signaling in the Drosophila motor neuron and may prove to be a conserved signaling mechanism for the presynpatic group II mGluRs in vertebrates.

4.4.2 Inhibiting the IP_3 receptor does not block DmGluRA activation of ERK

The group I mGluRs activate ERK during mGluR-LTD (Peng et al., 2010; Thomas and Huganir, 2004; Lonze et al., 2002; Davis et al., 2001; Dudek and

Fields, 2001). This activation may occur via the activation of PLC-IP_3 (Gladding et al., 2009; Hermans and Challiss, 2001; Pin and Duvoisin, 1995). Though sequence analysis has indicated that DmGluRA is most similar to the group II, which primarily inhibit adenylyl cyclase (Schoepp, 2001), because DmGluRA is the only mGluR in Drosophila, it has been proposed to function similarly to the group I (Bogdanik et al., 2004; Parmentier et al., 1996). Activated PLC cleaves the PIP_2 into inositol 1,4,5-triphospate (IP_3) which binds to the IP_3 receptors (IP_3R) of the ER, releasing Ca^{2+} into the cell. Our lab additionally found that the activation of DmGluRA via glutamate increases the level of intracellular Ca^{2+} in the motor neuron (unpublished data) further suggesting that PLC- IP_3 may be activated. We wanted to investigate the possibility that DmGluRA was activating ERK via the release of Ca^{2+} from the intracellular stores of the endoplasmic reticulum (ER). We found that the expression of an *IP_3R-RNAi* transgene lowered the basal levels of p-ERK; however this transgene did not block the glutamate-stimulated increase in p-ERK (Fig.4.2). This result suggests that either DmGluRA does not activate the PLC-IP_3 pathway releasing Ca^{2+} from the ER, or that ERK is not activated by DmGluRA via Ca^{2+}.

We have recently shown that CaMKII, a kinase whose activity relies on Ca^{2+}, is a critical signaling intermediate for DmGluRA-mediate PI3K activation (Lin et al., 2011). We hypothesized that DmGluRA activation is a Ca^{2+} –dependent process. Activated DmGluRA could be activating ERK via a process that does not involve the IP_3R- mediated release of Ca^{2+} from the ER. One of these possibilities could be the activation of the ryanodine receptor (RyR), which has

been found to play a role in supplying Ca^{2+} in mammalian nerve terminals (Collin et al., 2005; Verkhratsky, 2002). It is also possible that glutamate stimulates an increase in Ca^{2+} by triggering extracellular Ca^{2+} influx via voltage gated channels. It has been well-established that Ca^{2+}channels are regulated by the binding of the Gβγ-subunit, though this interaction is typically inhibitory (de Waard et al., 2005; Tedford and Zamponi, 2006) it still might be possible that DmGluRA activates Ca^{2+} influx though these channels.

4.4.3 DmGluRA activates PI3K and ERK via distinct pathways

In the vertebrate hippocampus, the activation of ERK was demonstrated occur via CaMKII during mGluR-LTD (Zhu et al., 2002). Our lab has recently shown that both CaMKII and DFak are critical signaling intermediates during the activation PI3K by DmGluRA in the Drosophila motor neuron (Lin et al., 2011). Because the activation of DmGluRA by glutamate increases p-ERK in the neuron, we wanted to additionally investigate if CaMKII or DFak are required for DmGluRA-mediated ERK signaling. We examined the basal levels of p-ERK in neurons expressing a constitutively-active CamKII transgene and found no increase in p-ERK. In the null mutant Fak^{CG1}, the basal levels of p-ERK were slightly reduced; however, the increase in p-ERK after glutamate application was not blocked in this mutant. These results indicate that CaMKII and DFak are not involved in the DmGluR-mediated activation of ERK.

4.4.4 Additional molecules involved in Raf activation may be responsible for increasing basal levels of p-ERK in response to diminished Ras activity

Canonical ERK signaling occurs via the activation of the Ras-Raf-MEK-ERK cascade in response to growth factors and other stimuli. We have now also demonstrated that Ras is required for the glutamate-activation of PI3K via DmGluRA. Because Ras is a widely-accepted activator of the ERK pathway, we investigated the if Ras and Raf are involved in DmGluRA-mediated ERK signaling.

Unexpectedly, we found that inhibiting Ras in the motor neuron with *Ras-RNAi* increased the basal level p-ERK compared to wildtype (Fig.4.2). The application of glutamate did not further increase p-ERK levels; however, we do not know if this is because the RNAi is blocking an additional increase in ERK or if we reached the maximum detectible limits of p-ERK in the neuron. Though the inhibition of Ras produced a highly unusual result, knocking down the activity of Raf more predictably reduced the basal levels of p-ERK and blocked the glutamate-induced increase in p-ERK in the motor neuron (Fig). This observation agrees with the canonical signaling pathway and tentatively suggests that Raf is a DmGluRA-ERK signaling intermediate.

It is unclear why knocking down Ras in the Drosophila motor neuron resulted in a significant increase in p-ERK immunoreactivity. One hypothesis is that the inhibition of Ras unbalanced a complex set of regulatory mechanisms that normally modulate the activity of the Ras-ERK pathway. There are a multitude of additional proteins that are known to participate in the regulation of the Ras-ERK pathway; however, their exact modes of action, especially in the Drosophila motor neuron, are not well-understood. One of these molecules is the tyrosine

protein kinase Src homolog at 64B (Src64B) that has been proposed to function in a pathway similar to Ras during neural development and alters synaptic outgrowth in Drosophila (Kussick et al., 1993; Tsai et al., 2008). Src64B can bind to and activate Raf in a Ras-independent manner indicating that like Ras, Src64B can also activate ERK signaling (Xia et al., 2008). Src64B is also predicted to interact with Ras (String Database, http://string-db.org and BioGRID version 3.1, http://thebiogrid.org), suggesting that in addition to activating Raf, Src64B may also effect Ras activity.

Other members of the Ras superfamily that have been identified at the synapse that might be involved in the activation of ERK are the Rap GTPases, Rap1 and Rap2 (Peng et al., 2004; Pizon et al., 1988). Rap1 has been shown to alter excitability via the activation of ERK downstream group I mGluR in mammalian pyramidal cells (Morozov et al., 2003). Conversely, Rap2 is a negative regulator of dendritic spine growth and ERK signaling (Ryu et al., 2008). It has been proposed that Rap1 and Rap2 negatively regulate synaptic strength by opposing the activity of Ras (Fu et al., 2007; Zhu et al., 2005; Pak et al., 2001); however their exact function in neurons remain undefined. Drosophila Rap1 (Dras3) is 88% identical to human Rap1 (Hariharan et al., 1991), and therefore may similarly activate ERK like its vertebrate ortholog. The activity of the Rap molecules could potentially alter basal ERK activity in the absence of Ras in the motor neuron; however, these interactions remain to be explored.

Chapter 5: Synopsis

5.1 Ras dependent and independent activation of PI3K in the Drosophila motor neuron

5.1.1 The effects of PI3K on neural excitability requires Ras

The signal transducer, PI3K, participates in the mediation of a myriad of cellular processes in response to insulin-signaling, growth factors, and signals related to cell survival (reviewed in Engleman et al., 2006). PI3K is also recognized as an intermediary of synaptic growth and neural function (Iwanami et al., 2009; Endersby and Baker, 2008). Two forms of synaptic plasticity, long-term depression (LTD) and long-term potentiation (LTP), which involve changes in neural excitability that are believed to "pattern" learning and memory in the animal brain (Davis, 2006; Martin et al., 2000; Bear, 1994; Bliss and Collingridge, 1993), are governed in part by PI3K-mediated signaling (Sharma et al., 2010; Gladding, 2009; Bourgeois, 2009; Hou and Klann, 2004; Man et al., 2003). Abnormalities in LTP and LTD have been linked to autism (Rubenstein and Merzenich, 2003) and several significant neurological diseases that have co-morbidity with autism, including Fragile X Syndrome (FXS), Neurofibromatosis-1 (NF1), and Tuberous sclerosis (TSC), exhibit altered PI3K signaling (Bourgeois, 2009; Cuscó et al., 2009; Kelleher and Bear, 2008; Gross et al., 2010; Levitt and Campbell, 2009; Williams et al., 2008; Kwon et al., 2006). Defining the molecular elements that regulate PI3K may identify useful therapeutic targets for these and other related neurological disorders.

The metabotropic glutamate receptors (mGluRs) regulate neural excitability and have been implicated in a number of diseases including anxiety, epilepsy, and schizophrenia (Enz, 2007; Chapman et al., 1996). The group I mGluRs are involved in the establishment of LTP and LTD (Altinbilek and Manahan-Vaughan, 2009; Holscher et al., 1999; Manahan-Vaughan, 1997) and an abnormally enhanced LTD, believed to be the result of aberrant mGluR signaling, has been found in mouse models of FXS (Pfeiffer and Huber, 2009; Ronesi and Huber, 2008; Muddashetty et al., 2007; Banko et al., 2006; Koekkoek et al., 2005; Huber et al., 2002). Elevated PI3K activity, suspected to be the result of abnormal mGluR-LTD, has additionally been observed in FXS, resulting in aberrant synaptic translation and excess dendritic spine growth (Sharma et al., 2010; Gross et al., 2010; Hou and Klann, 2004). The group I mGluRs have been suggested to activate PI3K during mGluR-LTD via the scaffolding protein Homer (Ronesi and Huber, 2008), although the activation of PI3K via this mechanism has not been demonstrated. The presynaptic group II mGluRs additionally appear to be involved in the establishment of LTD (Altinbilek and Manahan-Vaughan, 2009; Poschel and Manahan-Vaughan, 2005). Our lab has shown that the activation of the Drosophila mGluR ortholog, DmGluRA, which is most similar to the vertebrate group II mGluRs, initiates PI3K signaling in the motor neuron (Howlett et al., 2008). The group II receptors lack the Homer-binding motif found in the group I (Diagana et al., 2002), therefore the mechanisms by which DmGluRA may be facilitating the activation of PI3K is not clear.

Similar to PI3K, the GTPase Ras has been demonstrated to modulate synaptic structure and function, as well as participate in the establishment of LTP and LTD (Ye and Carrow, 2010; Kumar et al., 2005; Jarwoski et al., 2005; Arendt et al., 2004; Markus et al., 2002). PI3K is a known effector of Ras (Orme et al., 2006; Rodriguez-Viciana et al., 1994); however, which PI3K-mediated neural processes require Ras has not been clearly defined. We have now demonstrated that the activation of PI3K via glutamate-liganded DmGluRA requires Ras and that the activity of Ras alters the effect of PI3K on neural excitability in the Drosophila motor neuron.

We have shown that the inhibition of Ras via transgene expression and chromosomal loss-of-function blocks the ability of DmGluRA to activate the PI3K signaling cascade as measured by the levels of p-Akt (Fig.2.5). PI3K is crucial for the regulation of neural excitability by DmGluRA and diminished PI3K signaling results in an abnormal increase in neural excitability that we have previously shown is mediated by the inhibition of dFOXO by PI3K-Akt signaling (Howlett et al., 2008). We found that inhibiting Ras results in a hyperexictable, neural phenotype (Fig.2.7) consistent with decreased PI3K signaling (Howlett et al., 2008). Furthermore, the observation that the Ras-insensitive PI3K variant, *gen-PI3K*^{RBD}, is hyperexcitable (Fig.2.7) additionally indicates that Ras is a critical component in the DmGluRA-PI3K mediated regulation of motor neuron excitability.

5.1.2 The effect of PI3K on synaptic outgrowth is Ras-independent

Alterations in synaptic structure and morphology have been identified in several neurological disorders hallmarked by ASD, including FXS (Kaufmann and Moser, 2000; Restivo et al., 2005). As previously discussed, PI3K signaling is involved in the processes that modify synaptic strength (Sharma et al., 2010; Gladding, 2009; Bourgeois, 2009; Hou and Klann, 2004; Man et al., 2003) and outgrowth (Akiyama and Kamiguchi, 2010; Vodrazka et al., 2009; Brandt et al., 2007; Jossin and Goffinet, 2007; Kumar et al., 2005; Jaworski et al., 2005; Markus et al., 2002). Aberrant PI3K signaling is implicated in several neurological disorders (Gross et al., 2010; Bourgen, 2009; Cuscó et al., 2009; Kelleher and Bear, 2008; Levitt and Campbell, 2009; Williams et al., 2008; Kwon et al., 2006), underlining the importance of understanding the signaling pathways that regulate neural form and function.

Both activated Ras and PI3K have been demonstrated to participate in synaptic outgrowth (Akiyama and Kamiguchi, 2010; Vodrazka et al., 2009; Fivaz et al., 2008; Brandt et al., 2007; Oinuma et al., 2007; Jossin and Goffinet, 2007; Romero et al., 2007; Kumar et al., 2005; Jaworski et al., 2005; Markus et al., 2002); however, in addition to PI3K, Ras is positioned to activate a second signaling cascade, the ERK cascade, that has also been demonstrated to alter synaptic outgrowth (Kumar et al., 2005; Jaworski et al., 2005; Arendt et al., 2004; Markus et al., 2002; Koh et al., 2002). Deciphering the downstream specific activity of Ras has been important for the effective treatment of human cancers

(McCubrey et al., 2006) and therefore may also be important for the development of neurological disease therapies.

We found that effect of PI3K on synaptic growth is Ras-independent. Though inhibiting Ras activity diminished outgrowth (Fig.3.2), similar to what is observed when PI3K activity is decreased (Howlett et al., 2008; Martin-Peña et al., 2006), we found that expressing PI3K did not block the effect of Ras inhibition on growth (Fig.3.3). This observation is consistent with a previous report that found that the expression of a constitutively-active Ras transgene that only activates the PI3K effector pathway (Ras^{V12C40}) failed to increase synaptic bouton number (Koh et al., 2002). We additionally did not observe any growth defects in $gen\text{-}PI3K^{RBD}$ (Fig.3.3). These results indicate that Ras is not required for the effects of PI3K on synaptic growth.

Recent work by our lab suggests that the Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII) may act upstream PI3K to mediate arborization (Lin et al., 2011). We and others have demonstrated that CaMKII promotes motor neuron arborization (Lin et al., 2011; Beumer et al., 2002; Koh et al., 1999). We also found the expression of an active form of PI3K can block the decrease synaptic outgrowth conferred by the expression of the CaMKII inhibitory peptide, *ala2*, suggesting that CaMKII inhibition blocks arborization by preventing PI3K activation (Lin et al., 2011). Alternatively, the activation of PI3K signaling by insulin growth factors, which occurs in a Ras-independent manner (Backer et al., 1992) may also regulate synaptic growth. Neuronal circuits are targets for insulin (Barsh and Schwartz, 2002; Konner et al., 2009) and Insulin-like and Insulin

Receptor-like immunoreactivity are present at the Drosophila larval nmj (Budnik et al., 1990).

The Ras-ERK pathway was previously shown to participate in synapse formation in Drosophila via the modulation of the Discs-Large (DLG)-Fascilin II (FasII) cell adhesion complex (Koh et al., 2002). FasII, the Drosophila ortholog of NCAM (Schuster et al., 1996), and DLG cluster Shaker K^+ channels and the cytoskeleton, forming a complex that must be disassociated for outgrowth to occur (Thomas et al., 1997; Davis et al., 1997; Zito et al., 1997; Tejedor et al., 1997). In *Aplysia,* it has been shown that FasII is phosphorylated by ERK, resulting in the internalization of FasII and the disassociation of the DLG-FasII stabilization complex (Bailey et al., 1997). Similarly in Drosophila, activated ERK was observed to displace FasII at the motor neuron terminus, increasing synaptic growth (Koh et al., 2002). Our data also supports that Ras-ERK signaling is required for synaptic outgrowth. Similar to what was observed when we knocked down Ras, we found that we could significantly reduce motor neuron arborization by inhibiting the activity of Raf (Fig.3.3). Interestingly, though we could block the synaptic growth defects caused by the expression of *ala2* with an active form of PI3K (Lin et al., 2011), we could not similarly block the growth defects conferred by the inhibition of Ras and Raf (Fig.3.3). These results indicate that ERK signaling is necessary for the effects of both CamKII and PI3K on synaptic growth.

CaMKII has been previously demonstrated to promote synaptic outgrowth removing DLG from the DLG-FasII complex (Koh et al., 1999), therefore the

effect of CaMKII on growth appears to be both direct and indirect via PI3K. The inhibition of PI3K and CaMKII in the motor neuron reduces synaptic outgrowth (Lin et al., 2011; Howlett et al., 2008; Martin-Pena et al., 2006). We have found that these defects can be blocked by the expression of an active form of PI3K (Lin et al., 2011; Howlett et al., 2008); however, in this study it was observed that the synaptic growth defects that occur as a result of inhibiting components of the ERK signaling pathway (i.e. Ras and Raf) cannot be similarly rescued by PI3K (Fig.3.3). This suggests that Ras-ERK signaling is required for synaptic outgrowth. Intact Ras-ERK signaling is also appears to be required for the effects of CaMKII and PI3K on synaptic outgrowth.

These data indicate that parallel pathways cooperate to orchestrate synaptic outgrowth. We hypothesize that for bouton growth to occur, the CaMKII and Ras-ERK signaling pathways are required to disable the DLG-FasII complex, reducing bouton stabilization, and that the PI3K cascade is necessary to initiate protein translation via S6K/mTOR (Howlett et al., 2008) (Fig.3.4). The observation that Ras-ERK signaling is a requirement for the effects of either CaMKII or PI3K on growth indicates that ERK may act on the DLG-FasII stabilization complex in a manner distinct from the effect of activated CaMKII (i.e., ERK phosphorylation may more efficiently remove the complex). ERK may additionally have other targets distinct from CamKII that are required for this process, including the transcription factor CREB, which has been shown to participate in neurite outgrowth in cultured sensory cells (Schmid et al., 2001; White et al., 2000).

5.1.3 Functionally distinct pools of PI3K may exist in the Drosophila motor neuron

The motor neurons of the DmGluRA null mutant, *DmGluRA*^{112b}, are significantly hyperexcitable; however, though these mutants have aberrant PI3K signaling (Howlett et al., 2008) they exhibit only a slightly reduced number of enlarged, synaptic boutons (Bogdanik et al., 2004). Furthermore, we observed that *gen-PI3K*^{RBD}, which cannot be activated by Ras, increases neuronal excitability (indicative of decreased PI3K activity) but elicits wildtype synaptic bouton number (indicating wildtype PI3K activity). These observations suggest that the PI3K activity that is available to regulate excitability is not available to influence synaptic growth, raising the possibility that there are pools of PI3K in the motor neuron mediating distinct neural processes (Fig.5.1).

Functionally discrete pools of non-diffusible PI3K have been previously reported to occur in the dendritic spines of the hippocampus (Man et al., 2003). These pools of PI3K are compartmentalized via their association with AMPA receptors (Man et al., 2003). Other molecules, including Ca^{2+}, the MAP kinases JNK and ERK, and the translational regulator eIF4E have additionally been shown to have localized activity in neurons (Asaki et al., 2003; Luttrell, 2002; Mulvaney and Roberson, 2000; Mulvaney et al., 1999). These functional pools are at least in part due to the spatial localization of these molecules in the cell; therefore the pool of PI3K that is activated by DmGluRA may be spatially separated from the PI3K that is activated to promote synaptic growth. The fact

that DmGluRA is not located in the active zones in motor neurons where synaptic growth would occur is consistent with this possibility (Bogdanik et al., 2004).

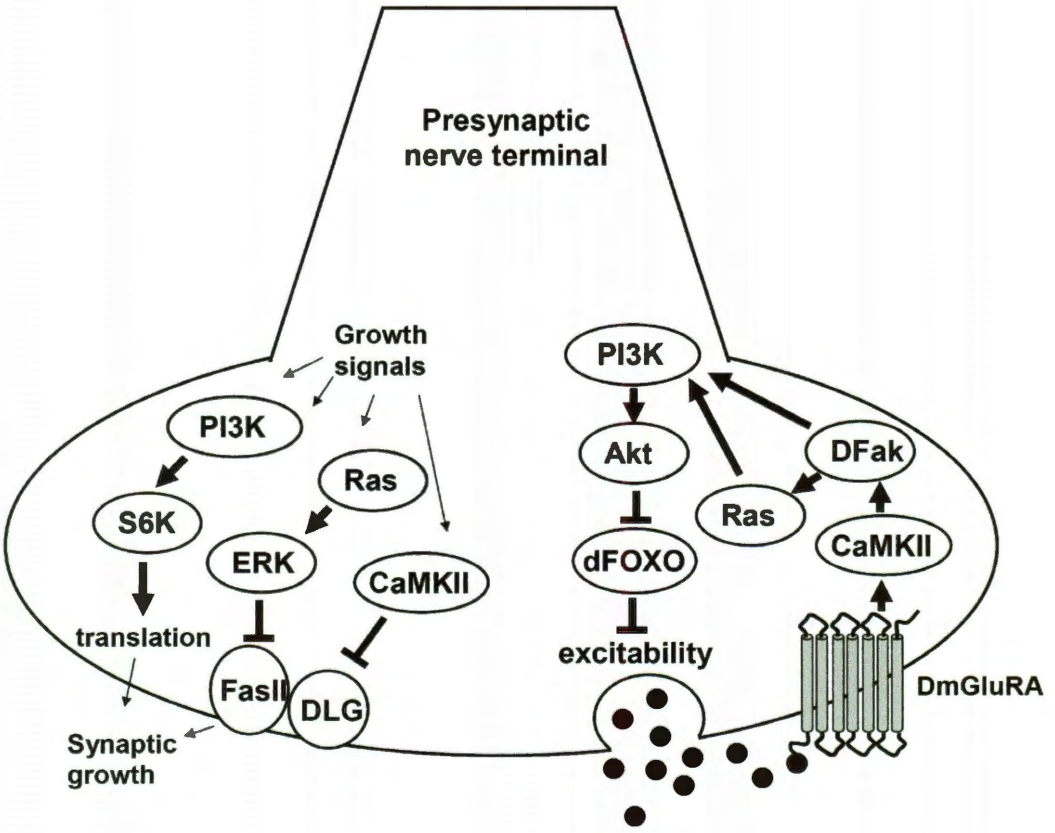


Figure 5.1: Functionally discrete pools of PI3K regulate distinct neuronal processes. Two functionally discrete pools of PI3K function in Drosophila motor nerve terminals. In the first pool, PI3K is activated by glutamate, presumably released from motor nerve terminals and acting via DmGluRA in an autocrine pathway. This PI3K activation is Ras dependent and downregulates excitability via the Akt-dependent phosphorylation and inhibition of the transcription factor dFOXO. CaMKII and DFak activities are also required for this PI3K activation. In the second pool, PI3K is activated by unknown means and promotes synaptic growth and bouton formation via the S6K-dependent activation of translation. This PI3K activation also requires CaMKII and DFak, but not Ras. However, Ras participates in synaptic growth and bouton formation via the Raf/Erk-dependent phosphorylation and downregulation of FasII and DLG. The active PI3K found in each pool do not appear to be easily interchangeable: Blocking DmGluRA increases neuronal excitability but has only mild effects on synaptic bouton number (Bogdanik et al., 2004), whereas blocking the ability of Ras to activate PI3K increases neuronal excitability but has not effect on synaptic bouton number.

5.1.4 Proposed model for role of Ras and PI3K in excitability and synaptic outgrowth in the Drosophila motor neuron

The observations described in this document, taken together with our recent, published findings by Howlett et al. (2008) and Lin et al. (2011), have led us to suggest the following mechanism for the activation of PI3K by glutamate-liganded DmGluRA at the Drosophila motor neuron nerve terminal (Fig.5.2). In this proposed mechanism, DmGluRA signaling triggers an increase in intracellular Ca^{2+}, most likely through PLC and IP_3 receptor activation. This Ca^{2+} transient activates the Ca^{2+}/calmodulin-dependent kinase II (CaMKII) which in turn, phosphorylates Drosophila Focal adhesion kinase (DFak). Phosphorylated DFak then activates PI3K, which downregulates neuronal excitability by the inhibition of the Drosophila Forkhead box transcription factor (dFOXO). dFOXO might regulate excitability via the transcription of ion channels subunits or additional regulators. We have additionally shown that the activation of PI3K increases synaptic growth by regulating translation via S6K/mTOR signaling.

The results of this current study now indicate that Ras is additionally involved in the activation of PI3K via DmGluRA-mediated signaling. In vertebrates, both Fak and the other Fak family member, Pyk2, have been shown to activate PI3K via the p85-regulatory subunit (Guinebault et al., 1995; Chen and Guan, 1994; Chen et al, 1996). Activated Fak and Pyk2 are also capable of activating Ras via the conserved Grb2-SoS pathway (Rocic et al., 2001; Dikic et al., 1996; Schlaepfer et al., 1994); which could lead to the Ras-dependent activation of PI3K. These data suggest activated DmGluRA-mediated signaling could activate

Ras via DFak, or that the activation of both DFak and Ras cooperatively bind to and activate PI3K (Fig.5.2). Additional experiments need to be performed to further examine these possibilities.

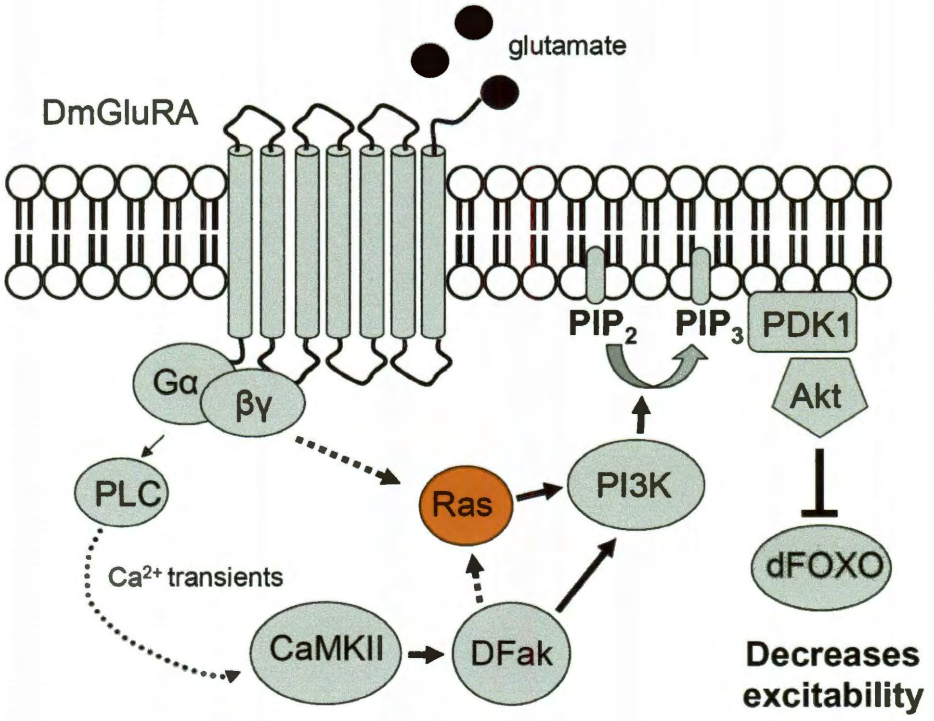


Figure 5.2: CaMKII, DFak, and Ras are critical signaling intermediates in the activation of PI3K downstream DmGluRA. Based on the findings of this study and previous results from our lab (Lin et al., 2011; Howlett et al., 2008), we have proposed a model for the initiation of PI3K via glutamate-activated DmGluRA. Glutamate-activation of DmGluR results in the production of intracellular Ca^{2+} intermediates, most likely through PLC-IP_3 signaling. The subsequent increase in internal Ca^{2+} in turn activates the Ca^{2+}/Calmodulin kinase, CaMKII. CaMKII activates Drosophila focal adhesion kinase (DFak) which then activates PI3K (Lin et al., 2011). The activation of PI3K by DFak occurs via Ras or in concert with Ras. PI3K phosphorylates and activate Akt (reviewed in Vanhaesebroeck et al., 2001). Activated Akt negatively regulates the activity of the transcription factor dFOXO. The downregulation of dFOXO decreases neural excitability in the motor neuron (Howlett et al., 2008). The mechanisms by which dFOXO decreases excitability are not currently known; however, we postulate that the transcription of ion channel subunits or regulators may be involved. The dashed lines indicate putative activatory pathways.

5.2 Role for Ras-PI3K in Drosophila LTD

5.2.1 PI3K and Ras are involved in LTP

Abnormalities in the processes of long-term depression (LTD) and long-term potentiation (LTP), which modify the strength and efficacy of synaptic connections, are linked to significant neurological diseases that have co-morbidity with autism, including Fragile X Syndrome (FXS) and Neurofibromatosis-1 (NF1) (Bourgeois, 2009; Kelleher and Bear, 2008; Li et al., 2005; Rubenstein and Merzenich, 2003; Costa et al., 2002). LTP is initiated by the glutamate-liganded activation and Ca^{2+} influx through the NMDA subset of iGluRs, triggering several intracellular signaling cascades that potentiate the activity of a second iGluR, AMPA (Bear and Malenka, 1994; Bliss and Collingridge, 1993). LTP involves the insertion of the AMPA receptors into the postsynaptic membrane, which is believed to sensitize the postsynaptic cell to further signaling (Lu et al., 2001). Though the exact mechanisms regulating AMPA receptor insertion are still debated, it has been found that the p85-subunit of PI3K co-localizes with the glutamate receptor 2 (GluR2) subunit of the AMPA, helping to direct the insertion of the receptor into the membrane (Man et al., 2003). PI3K signaling is additionally required to maintain the duration of LTP by modifying translation via mTOR and p70 S6K (Cammalleri et al., 2003; Karpova et al., 2006). A role for PI3K in LTD, defined as the desensitization of a neuron to stimuli, has also been established. PI3K signaling, in cooperation with the ERK pathway, is activated by the agonist-activation of postsynpatic mGluRs, coupling the activation of the receptor to protein translation during mGluR-LTD (Antion et

al., 2008; Banko et al., 2006; Hou and Klann, 2004). The scaffolding protein Homer has been proposed to form a complex with mGluR and the PI3K-enhancer, PIKE, during mGluR-LTD, potentially linking PI3K to mGluR activation (Rong et al., 2003; Ronesi and Huber, 2008). Insulin-induced LTD via NMDA receptor activation has additionally been found to require PI3K signaling (van der Heide et al., 2005).

The role of Ras in the modulation of LTP and LTD is much less defined, though there is evidence that Ras participates in LTP. Patients with NF1, resulting from a mutation in the Ras-GAP encoding gene, *NF1* (Xu et al., 1990), are frequently diagnosed with autism which may be a result of perturbations in LTP that are observed in mouse models of the disease (Kelleher and Bear, 2008; Silva et al., 1997; Costa et al., 2001; Costa et al., 2002; Costa et al., 2005; Li et al, 2005; North et al., 2002). Ras may participate in LTP similar to PI3K by mediating the insertion of AMPA receptor subunits into the postsynaptic membrane (Qin et al., 2005; Zhu et al., 2002). Spontaneous neural activity or low levels of induced Ras activity has been observed to potentiate the delivery of the GluR2-long (GluR2L) splice variant subunit-containing AMPA receptors to the membrane via the Ras-ERK pathway (Qin et al., 2005; Zhu et al., 2002). Increased neural activity additionally stimulates Ras-PI3K signaling, mediating glutamate receptor 1 (GluR1) subunit AMPA receptor potentiation (Qin et al., 2005). The overexpression of Ras was additionally found to block the LTP defect in mouse models of FXS via the rescue of AMPA receptor defects (Hu et al.,

2008). These data indicate a role for Ras in synaptic processes related to LTP; however, could Ras also be involved in LTD?

5.2.2 Ras may be involved in LTD in the Drosophila motor neuron

The activation of cap-dependent translation during mGluR-LTD has been shown to require the coordinated signaling between two cascades that are activated by Ras; the PI3K-mTOR and ERK signaling pathways (Banko et al., 2006; Gallagher et al., 2004; Antion et al., 2008). G protein-coupled receptors have been previously shown to modulate Ras in a variety of cell types (reviewed in Goldsmith and Dhanasekaran, 2007). Though it is postulated that Homer facilitates the interaction between the group I mGluRs and PI3K (Rong et al., 2003; Ronesi and Huber, 2008), it is unclear how this complex activates PI3K; therefore a role for Ras in the activation of PI3K can not been entirely ruled out. Because the group II mGluRs do not have a Homer-binding domain (Diagana et al., 2002) further raises the possibility that Ras may function as a signaling intermediate especially in regards to this mGluR subtype. In this study we have shown that the activation of PI3K by the vertebrate group II ortholog in Drosophila, DmGluRA, requires the activity of Ras. Because the altered expression of group II mGluRs have been suggested to contribute to the pathophysiology of schizophrenia and epilepsy (Gupta et al., 2005; Aronica et al., 1997) it is of interest to human health to determine if the pathway components related to group II mGluR-LTD are conserved in vertebrates.

Several lines of evidence indicate that Ras may be involved in synaptic LTD. Firstly, it is important to note that both the electrical and chemical stimulation of

the presynaptic group II mGluRs has been demonstrated to elicit LTD in vertebrates (Altinbilek and Manahan-Vaughan, 2009; Poschel and Manahan-Vaughan, 2005; Manahan-Vaughan, 1997; Huang et al., 1997). Additionally, the activation of group II mGluR-LTD in the vertebrate hippocampus was additionally found to activate ERK, a downstream target of Ras signaling, in the surrounding glia and interneurons of the vertebrate hippocampus (Berkeley and Levey, 2003). LTD has been demonstrated to be reliably induced in the presynaptic motor neuron of the Drosophila nmj and it was further discovered that a series of Akt hypomorphs show impaired LTD (Guo and Zhong, 2006), indicating that PI3K signaling may be involved. We have previously demonstrated that neural excitability is regulated by the DmGluRA activation of PI3K-Akt (Howlett et al., 2008), which we would expect is critical for establishing LTD. We have now determined that Ras activity is critical for the effect of PI3K on excitability; therefore we proposed that Ras may act upstream PI3K-Akt during LTD in the Drosophila motor neuron.

5.2.3 Ras activity as a target for autism therapeutics

Hyperactivated PI3K signaling has been linked to many significant neurological diseases including FXS, tuberous sclerosis (TSC), and Cowden/Lhermitte-Ducols syndrome (Bourgeois, 2009; Kelleher and Bear, 2008; Gross et al., 2010; Levitt and Campbell, 2009; Kelleher and Bear, 2008; Williams, et al., 2008; Kwon et al., 2006). The use of pharmacological pathway inhibitors, including rapamycin, have proven useful for the treatment of the tumors that form in conjunction with TSC (Davies et al., 2008) however, the effect of this drug on

the cognitive defects associated with these diseases is limited and still under review (de Vries, 2010). Because PI3K is a ubiquitous signaling molecule involved in a multitude of cellular processes throughout the body, it is important that future therapeutics can more specifically target aspects of the PI3K pathway that are dysregulated in neurons to prevent potential side effects.

Ras is additionally a ubiquitous molecule; however, in vertebrates there are two neural specific Ras guanine exchange factors (Ras-GEFs), Ras guanyl nucleotide-releasing protein (Ras-GRP) and the Ras-guanine-nucleotide-releasing-factor (Ras-GRF), that activate Ras in response to neural activity (Ebinu et al., 1998; Farnsworth et al., 1995). These factors are present at synapses and appear to be involved in synaptic plasticity (Tian et al., 2004). Additionally, the Ras-inhibitor synaptic GTPase-activating protein (SynGAP) is also found specifically in vertebrate neurons (Chen et al., 1998). Because we have shown that Ras activity is critical for the effect of PI3K on neural excitability, drugs that alter the effects of these molecules that are specially expressed in the nervous system may be effective in the modulation of the aberrant PI3K signaling observed in many neurological disorders related to autism.

5.3 Future work

In this study we have demonstrated that the effect of PI3K in the regulation of neural excitability is a Ras-dependent process; however, several important questions remain that warrant additional investigation. The mechanism by which glutamate-liganded DmGluRA initiates the activation of Ras is not entirely clear.

We have shown that CaMKII and DFak are critical DmGluRA-PI3K signaling intermediates (Lin et al., 2011). We suspect that DFak may be responsible for the activation of Ras; however, we have not clearly demonstrated that Ras is epistatic to DFak. The disassociated Gα and Gβγ subunits of activated G protein-coupled receptors have been found to activate Ras in a variety of cell types (reviewed in Goldsmith and Dhanasekaran, 2007); therefore Ras may be activated independently and in parallel with DFak during DmGluRA-mediated signaling. To investigate this possibility, we need to examine the level of p-Akt after glutamate application in a series of G protein subunit –RNAi transgenic lines that would inhibit the activity of the Gα and Gβγ subunits in the Drosophila motor neuron. In addition to potentially disrupting Ras activation, knocking down the activity of these subunits may also alter DmGluRA-CaMKII-DFak signaling; therefore we would need to additionally explore the effect of G protein subunit RNA-knockdown in the presence of a *UAS-DFak* construct that would rescue the potential effect on this pathway. If the expression of the G protein subunit RNAi in the presence of UAS-DFak blocks the glutamate-induced increase in p-Akt then we could conclude that Ras is activated via a separate, G protein-receptor-mediated pathway and that both DFak and Ras are required for the full activation of PI3K in response to DmGluRA activation.

Ras interacts and activates PI3K via the Ras-binding domain of the catalytic unit of PI3K (Rodriguez-Viciana et al., 1994; Rodriguez-Viciana et al., 1996), whereas DFak activates PI3K via the p85 (p60 in Drosophila) subunit (Pignataro and Ascoli, 1990). To further investigate if DFak is activating PI3K directly or

indirectly though Ras, a *p60-RNAi* could be generated and expressed in the motor neuron and the level of p-Akt analyzed. If the expression of *p60-RNAi* does not block the glutamate-induced increase of p-Akt then this would indicate that DFak activates PI3K indirectly via Ras. However, if inhibiting p60 does block the induction of p-Akt, this would suggest that Ras and DFak independently activate PI3K and that both are required for the full activation of PI3K by DmGluRA. We have recently found that the increase in p-Akt after glutamate application is blocked in the DFak null mutant, $DFak^{CG1}$ (Lin et al., 2011). To additionally determine if Ras is required for the activation of PI3K via DFak, we could additionally express $UAS\text{-}Ras^{+}$ in the $DFak^{CG1}$ background to investigate if Ras blocks the $DFak^{CG1}$ phenotype. If expression of Ras cannot block the $DFak^{CG1}$ p-Akt phenotype, then this would suggest that both DFak and Ras are required for the activation of PI3K; however, if Ras expression can in fact block the $DFak^{CG1}$ phenotype, this would indicate that Ras is required for the activation of PI3K by DFak and that Ras is epistatic to DFak during DmGluRA-mediated signaling.

Additional experiments that would be of merit to pursue would be an investigation of LTD in the Drosophila motor neuron using a series of PI3K and Ras transgenic mutants. These studies may validate the role of PI3K and Ras in the group II mGluR-mediated LTD in Drosophila, which may help to elucidate a similar pathway in vertebrates.

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