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Authors
Hauswirth, Anna G
Ford, Kevin J
Wang, Tingting
et al.

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A postsynaptic PI3K-cII dependent signaling controller for presynaptic homeostatic plasticity

Anna G Hauswirth1,3, Kevin J Ford1,3, Tingting Wang1,3, Richard D Fetter1,3, Amy Tong1,3, Graeme W Davis1,3*

1Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, United States; 3Kavli Institute for Fundamental Neuroscience, University of California, San Francisco, San Francisco, United States

Abstract Presynaptic homeostatic plasticity stabilizes information transfer at synaptic connections in organisms ranging from insect to human. By analogy with principles of engineering and control theory, the molecular implementation of PHP is thought to require postsynaptic signaling modules that encode homeostatic sensors, a set point, and a controller that regulates transsynaptic negative feedback. The molecular basis for these postsynaptic, homeostatic signaling elements remains unknown. Here, an electrophysiology-based screen of the Drosophila kinome and phosphatome defines a postsynaptic signaling platform that includes a required function for PI3K-cII, PI3K-cIII and the small GTPase Rab11 during the rapid and sustained expression of PHP. We present evidence that PI3K-cII localizes to Golgi-derived, clathrin-positive vesicles and is necessary to generate an endosomal pool of PI(3)P that recruits Rab11 to recycling endosomal membranes. A morphologically distinct subdivision of this platform concentrates postsynaptically where we propose it functions as a homeostatic controller for retrograde, trans-synaptic signaling.

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Introduction

Homeostatic signaling systems stabilize the functional properties of individual neurons and neural circuits through life (Marder and Prinz, 2002; Turrigiano et al., 1998; Burrone et al., 2002; Davis, 2006, 2013). Despite widespread documentation of neuronal homeostatic signaling, many fundamental questions remain unanswered. For example, given the potent action of homeostatic signaling systems, how can neural circuitry be modified during neural development, learning, and memory? Although seemingly contradictory, the homeostatic signaling systems that stabilize neural function throughout life may actually enable learning-related plasticity by creating a stable, predictable background upon which learning-related plasticity is layered (Davis, 2013; Keck et al., 2017). Therefore, defining the underlying molecular mechanisms of homeostatic plasticity may not only inform us about the mechanisms of neurological disease, these advances may inform us regarding how complex neural circuitry is able to accomplish an incredible diversity of behaviorally relevant tasks and, yet, retain the capacity for life-long, learning-related plasticity.

Neuronal homeostatic plasticity encompasses a range of compensatory signaling that can be subcategorized based upon the cellular processes that are controlled including ion channel gene expression, neuronal firing rate, postsynaptic neurotransmitter receptor abundance and presynaptic vesicle release (Anggono et al., 2011; Burrone et al., 2002; Davis, 2006, 2013; Haedo and Golowasch, 2006; Maffei and Fontanini, 2009; Marder and Prinz, 2002; Parrish et al., 2014; Stellwagen and Malenka, 2006; Turrigiano et al., 1998; Watt and Desai, 2010; Zhang et al., 2003). Presynaptic homeostatic potentiation (PHP) is an evolutionarily conserved form of neuronal

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homeostatic control that is expressed at the insect, rodent and human neuromuscular junctions (NMJ) (Cull-Candy et al., 1980; Davis and Müller, 2015; Frank et al., 2006; Plomp et al., 1995) and has been documented at mammalian central synapses (Burrone et al., 2002; Kim and Ryan, 2010; Liu and Tsien, 1995). PHP is initiated by the pharmacological inhibition of postsynaptic neurotransmitter receptors. The homeostatic enhancement of presynaptic vesicle release can be detected in a time frame of seconds to minutes, at both the insect and mouse NMJ (Frank et al., 2006; Wang et al., 2016). This implies the existence of postsynaptic signaling systems that can rapidly detect the disruption of neurotransmitter receptor function and convert this into retrograde, transsynaptic signals that accurately adjust presynaptic neurotransmitter release (Figure 1A; see also for review Davis, 2006, 2013; Müller et al., 2015). Notably, the rapid induction of PHP is transcription and translation independent (Goold and Davis, 2007), calcium-independent (Frank et al., 2009), and does not include a change in nerve terminal growth or active zone number (Frank et al., 2006, 2009; Harris et al., 2015; Younger et al., 2013; Wang et al., 2016).

There has been considerable progress identifying presynaptic effector molecules responsible for the expression of PHP (Dickman and Davis, 2009; Frank et al., 2009; Harris et al., 2015; Müller and Davis, 2012; Müller et al., 2015; Wang et al., 2016). There has also been progress identifying postsynaptic signaling molecules that control synaptic growth at the Drosophila NMJ (Ballard et al., 2014; Chen et al., 2012; DiAntonio et al., 2001; Harris et al., 2016; McCabe et al., 2004) as well as the long-term, translation-dependent maintenance of PHP (Goold and Davis, 2007; Kauwe et al., 2016; McCabe et al., 2004; Penney et al., 2012, 2016). However, to date, nothing is known about the postsynaptic signaling systems that initiate and control the rapid induction and expression of PHP.

Here, we report the completion of an unbiased, forward genetic screen of the Drosophila kinome and phosphatome, and the identification of a postsynaptic signaling system for the rapid expression of PHP that is based on the activity of postsynaptic Phosphoinoside-3-Kinase (PI3K) signaling. There are three classes of PI3-Kinases, all of which phosphorylate the 3 position of phosphatidylinositol (PtdIns). Class I PI3K catalyzes the conversion of P(4,5)P2 to P(3,4,5)P3 (PIP3) at the plasma membrane, enabling Akt-dependent control of cell growth and proliferation (Carracedo and Pandolfi, 2008; Vanhaesebroeck et al., 2012), and participating in the mechanisms of long-term potentiation (Knafo and Esteban, 2012). Class II and III PI3Ks (PI3K-cII and PI3K-cIII, respectively) both catalyze the conversion of PI to P(3)P, which is a major constituent of endosomal membranes. P(3)P itself may be a signaling molecule with switch like properties, functioning in the endosomal system as a signaling integrator (Zoncu et al., 2009). The majority of P(3)P is synthesized by PI3K-cII, which is involved in diverse cellular processes (Backer, 2008; Dall’Armi et al., 2013). By contrast, the cellular functions of PI3K-cII remain less well defined. PI3K-cII has been linked to the release of catecholamines (Meunier et al., 2005), immune mediators (Nigorikawa et al., 2014), insulin (Dominguez et al., 2011), surface expression and recycling of integrins (Ribeiro et al., 2011), and GLUT4 translocation to the plasma membrane, a mediator of metabolic homeostasis in muscle cells (Falasca et al., 2007). Here, we demonstrate that Class II and Class III PI3K-dependent signaling are necessary for the rapid expression of PHP, controlling signaling from Rab11-dependent, recycling endosomes. By doing so, we define a postsynaptic signaling platform for the rapid expression of PHP and define a novel action of PI3K-cII during neuronal homeostatic plasticity. To our knowledge, this is the first established postsynaptic function for PI3K-cII at a synapse in any organism.

Recently, it has become clear that the endosomal system has a profound influence on intracellular signaling and neural development. There is evidence that early and recycling endosomes can serve as sites of signaling intersection and may serve as signaling integrators and processors (Irannejad et al., 2015; Villaseñor et al., 2016). Furthermore, protein sorting within recycling endosomes, and novel routes of protein delivery to the plasma membrane, may specify the concentration of key signaling molecules at the cell surface (Choy et al., 2014; Hanus and Ehlers, 2016; Issman-Zecharya and Schuldiner, 2014; Solis et al., 2017). The essential role of endosomal protein trafficking is underscored by links to synapse development (Lloyd et al., 2002; Seto et al., 2002) and neurodegeneration (Pennetta et al., 2002; Sanhueza et al., 2014). Yet, connections to homeostatic plasticity remain to be established. Based upon the data presented here and building upon prior work on endosomal signaling in other systems, we speculate that postsynaptic PI3K-cII and Rab11-dependent recycling endosomes serve as as a postsynaptic ‘homeostatic controller’ that is essential for the specificity of retrograde, transsynaptic signaling.
**Genetic Screen:**

1. UAS-RNAi against the kinome and phosphatome
2. GAL4 expressed in neuron and muscle
3. Induce presynaptic homeostasis
4. Assay return to synaptic set point

**Figure 1.** Screen of *Drosophila* kinome and phosphatome in presynaptic homeostatic plasticity. (A) Schematic highlighting the trans-synaptic nature of presynaptic homeostatic plasticity. The ‘synaptic set point’ is operationally defined as the combined action of all pre- and postsynaptic parameters that reliably specify the transfer of information at the synapse. The synaptic set point is stably maintained by a trans-synaptic, homeostatic signaling circuit (red arrow) that includes postsynaptic sensors, retrograde feedback signaling, and presynaptic effectors that drive changes in presynaptic vesicle release. At right, the forward genetic screen of the *Drosophila* kinome and phosphatome is outlined. (B–C) Each point represents average data from a single NMJ. Purple points are in the presence of PhTX. Black points are in the absence of PhTX (baseline). The black hash marks on X and Y axes designate average mEPSP and EPSP amplitudes, respectively, without PhTX. The purple hash marks are averages in the presence of PhTX. The black line in (C) is a power curve fit, equation indicated on graph. The dotted blue lines encompass 95% of all wild-type data points. Recordings made at 0.35 mM [Ca$^{2+}$]_e. (D–E) Screen data of kinase and phosphatase UAS-RNAi driven by muscle and neuron GAL4 plotted as in (B–C) except that each point represent genotypic averages, yellow points (+PhTX) and black circles (-PhTX, baseline). The black line in (D) denotes two standard deviations below the population mean EPSP amplitude of control genotypes from (B). In (D) and (E) the red dot represents GAL4, UAS-RNAi for Pi3K68D. The black line in (E) is the same curve fit to the control data set in C, layered onto the experimental screen data for comparison. Blue dotted lines as in (C). Recordings made at 0.35 mM [Ca$^{2+}$]_e.

*Figure 1 continued on next page*
Results

There are at least 251 kinases and 86 phosphatases, either functionally annotated or predicted, in the Drosophila genome (Morrison et al., 2000). We used the GAL4-UAS system to drive available UAS-RNAi targeting 274 of the predicted 337 genes in the Drosophila kinome and phosphatome. UAS-RNAi were expressed in both neurons and muscle, and PHP was assessed at the third instar NMJ by direct measurement of synaptic transmission using intracellular recordings (Figure 1A). The rapid expression of PHP was assessed by application of sub-blocking concentrations of the glutamate receptor antagonist philanthotoxin-433 (PhTX; 15 μM), which causes a decrease in the amplitude of miniature excitatory postsynaptic potential amplitudes (mEPSPs) (Frank et al., 2006, Figure 1A). Excitatory postsynaptic potential amplitudes (EPSPs) initially decrease. However, within ten minutes, homeostatic signaling is engaged and drives an increase in presynaptic release (quantal content, calculated as EPSP/mEPSP). Increased presynaptic release precisely counteracts the decrease in receptor sensitivity and returns EPSP amplitudes back to the synaptic set point, baseline values. (Figure 1A; see Frank et al., 2006).

First, we generated a large data set to define the baseline parameters of PHP and quantify any effect caused by the heterozygous GAL4 lines used in our screen. We assessed PHP in heterozygous GAL4 lines (GAL4/+) crossed to a wild-type genetic background by recording mEPSP and EPSP amplitudes in the absence (baseline) or presence of PhTX. The data are plotted in Figure 1B and C, with each point representing average data from an individual NMJ recording. In Figure 1B, note that mEPSP amplitudes, across all recordings, decreased by 51.4% (see arrow, X-axis; p<0.001), while the average EPSP amplitude decreased by only 9.0% (p<0.001). When mEPSP amplitudes are plotted against quantal content, a significant correlation emerges (R^2 = 0.80; Figure 1C). This function (equation) defines the process of PHP whereby the magnitude of mEPSP decrease is offset by a corresponding increase in presynaptic release (quantal content). By plotting individual data, we can establish an interval that contains 95% of all data points within this control data set (blue lines). Average values and sample sizes can be found in Supplementary file 1, inclusive of data in all subsequent figures.

In our screen, we quantified average mEPSP, EPSP, quantal content, muscle input resistance and muscle resting membrane potential for each combination of UAS-RNAi and GAL4 driver. The screen data are plotted (Figure 1D–E), with each point representing the average of multiple NMJ recordings for an individual genotype. The majority of individual data points shown on the graphs (94%) represent averages of more than three individual recordings, with sample sizes ranging from 2 to 14 recordings per genotype, totaling 1150 NMJ recordings. Knockdown genotypes (UAS-RNAi/GAL4) were tested in the presence of PhTX (yellow circles). A subset of genotypes was also tested in the absence of PhTX (black circles, inclusive of ~100 muscle recordings). The average mEPSP amplitude across all genotypes decreased by 51.6% (arrow X-axis; p<0.001) and the average EPSP amplitude decreased by 11.2% (p<0.001). The similarity with the control data (Figure 1B) is indicative of robust homeostatic compensation when the majority of the kinases and phosphatases were knocked down in our screen. When mEPSP amplitudes are plotted against quantal content, the majority of data reside within the confidence interval created for our control data set (Figure 1E, blue lines). Two criteria were used to select candidate PHP genes: 1) those that reside below a cutoff of –2 standard deviations from the average EPSP amplitude of the control genotypes and 2) those that reside outside the blue lines defining 95% of control data (superimposed on the screen data) (Figure 1E, blue lines). Finally, our data demonstrate that there is no correlation observed between quantal content and either resting membrane potential or muscle input resistance (Figure 1—figure supplement 1).
Identification of class II PI3K as a homeostatic plasticity gene

We re-screened mEPSP and EPSP amplitudes in the presence and absence of PhTX, and identified 5 RNAi lines as verified hits from our screen (Table 1). These five lines target the PI3-Kinase Pi3K68D, the class III PI3-Kinase Vps34, the tyrosine-like kinase cd1, a putative kinase encoded by CG8726, and CamKII. In addition, we identified additional candidate plasticity genes with an apparent block of PHP (not yet independently verified) including PI4-Kinase (Pi4Killa) and CamKK (unpublished data KJF and GWD). The discovery of Pi3K68D as well as Vps34 and, potentially Pi4Killa, from a screen of more than 250 kinases and phosphatases, strongly implicates lipid kinase signaling in the homeostatic control of presynaptic neurotransmitter release.

Pi3K68D was one of the most robust hits from the screen with a very large change in mEPSP amplitude and a similarly large decrease in EPSP amplitude (see red line on 1D), indicative of a complete block of PHP (no difference in QC, p=0.17, Figure 1D,E). We chose to initially focus on the function of Pi3K68D, in part due the robustness of the phenotype and in part because very little is known about the function of class II PI3Ks within the nervous system of any organism. As such, we have the opportunity to expand the general knowledge of lipid kinase signaling pathways and define new mechanisms underlying PHP.

Pi3K68D encodes a class II PI3K that is necessary for PHP

Pi3K68D encodes a class II PI3K with homology to the three Class II PI3Ks encoded in the mammalian genome [Figure 2A; homology to PIK3C2A is 31% identical and 48% similar (Sievers et al., 2011). In order to pursue a formal genetic analysis of Pi3K68D we examined two existing transposon insertion mutations in the Pi3K68D locus (Pi3K68DGS residing in the 5’ UTR and Pi3K68DMB residing in an intron; Figure 2A). Since neither transposon resides in coding sequence, we also used the CRISPR-Cas9 system (Kondo and Ueda, 2013) to generate a new mutation Pi3K68DAlh. This mutation is a small insertion/deletion mutation resulting in a premature stop codon at amino acid 1440, in an intron; have the opportunity to expand the general knowledge of lipid kinase signaling pathways and define new mechanisms underlying PHP.

To further define the extent to which loss of Pi3K68D affects baseline transmission and PHP, we plotted the data for each individual NMJ recording, comparing mEPSP amplitude and quantal content (Figure 2D). Sample size for baseline recordings from Pi3K68D in the absence of PhTX includes 75 individual recordings, and 52 individual recordings in the presence of PhTX. At wild-type synapses, as shown in Figure 1, quantal content increased homeostatically as average mEPSP amplitude decreased. Again, the data are fit with an exponential function indicative of homeostatic plasticity sustaining set point postsynaptic excitation ($R^2 = 0.62$; solid line Figure 2D, left). By contrast,
Figure 2. Mutations in Pi3K68D block the rapid expression of presynaptic homeostatic plasticity. (A) Top: Schematic of the Pi3K68D gene locus with mutations indicated. Below: Pi3K68D protein domains are indicated and compared to mouse class II PI3K proteins. (B) Representative EPSP and spontaneous mEPSP in wild-type (black) and mutant Pi3K68D (red), in the absence or presence of PhTX as indicated. Recordings made at 0.3 mM [Ca²⁺]. (C) Average mEPSP amplitude, EPSP amplitude, and presynaptic release (quantal content) in WT, Pi3K68D<sup>GS</sup>, Pi3K68D<sup>MB</sup>, and Pi3K68D<sup>AH1</sup>. Figure 2 continued on next page.
Figure 2 continued

Unfilled bars are in the absence of PhTX. Filled bars are in the presence of PhTX. Mean ± SEM, ns not significant, *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001; Student’s t-test. (D) Each point represents average data from an individual NMJ recording. For WT, recordings in absence of PhTX are filled black circles while those with PhTX are unfilled black circles. For Pi3K68D, recordings in absence of PhTX are grey circles while those with PhTX are red circles. The black or grey filled hash marks on the X and Y axes represent the average mEPSP amplitude and QC, respectively, without PhTX. The white and red hash marks on the X and Y axes represent average mEPSP amplitude and QC, respectively, with PhTX. The black line in the WT graph is a curve fit to this control data. The same wild-type curve-fit is overlaid on the Pi3K68D data for purposes of comparison. Dotted blue lies encompass 95% of wild-type data points. These same lines from wild-type are superimposed on the graph is a curve fit to this control data. The same wild-type curve-fit is overlaid on the Pi3K68D graph, at right.

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Pi3K68D mutant synapses do not adhere to the wild-type homeostatic function (Figure 2D, right, red). The fit from the wild-type data set, including the interval that contains 95% of the wild-type data (blue lines), is superimposed on the Pi3K68D data. A majority of all Pi3K68D data points lie outside our 95% control data interval (defined above). There is no statistically significant change in the average QC in the presence of PhTX despite a ~ 5 fold decrease in mEPSP amplitude in Pi3K68D, implying a failure of PHP (Figure 2). We also note that there is a small but significant decrease in baseline QC (minus PhTX) in the Pi3K68D mutant background (WT QC = 30.3 ± 1.2 and Pi3K68D QC = 26.1 ± 1.2; p=0.018; see below for further analysis and discussion of baseline transmission).

**Pi3K68D is necessary for the long-term maintenance of homeostatic potentiation**

PHP can be induced by genetic deletion of the muscle-specific glutamate receptor subunit GluRIIA (Petersen et al., 1997). This is an independent method to induce PHP, and it has been interpreted to reflect the long-term maintenance of PHP throughout lifespan (Mahoney et al., 2014; Marie et al., 2010). Genes involved in both the rapid expression and the long-term maintenance of PHP can be considered to be ‘core’ genes necessary for PHP. We found that GluRIIA; Pi3K68D<sup>AH1</sup> double mutant animals have decreased EPSP amplitude due to a failure to homeostatically increase quantal content (Figure 3A,B). Collectively these data show Pi3K68D is necessary for both the rapid expression and long-term maintenance of PHP and, as such, can be considered part of the core homeostatic machinery necessary for PHP.

**Pi3K68D mutants have normal morphology and glutamate receptor abundance**

We quantified various measures of synapse development and morphology to determine whether the disruption of homeostatic plasticity in Pi3K68D mutants might be secondary to changes in synaptic structure. There was no difference in bouton number or in active zone number (as quantified by Bruchpilot, BRP, a key component of the presynaptic active zone) comparing wild-type and Pi3K68D mutants (Figure 3—figure supplement 1). We found no significant difference between wild-type and Pi3K68D mutant larva in GluRIIA receptor subunit intensity (Figure 3—figure supplement 1). There was a small but significant increase in GluRIIB intensity (16% increase compared to wild-type, p=0.01, data not shown). We found no difference in average intensity of two additional synaptic markers including Clathrin Light Chain (CLC) and cysteine string protein (CSP, Figure 3—figure supplement 1). There was a small, significant increase in synaptic anti-synaptotagmin-1 intensity (27% increase, p=0.006. Figure 3—figure supplement 1). In conclusion, we find no evidence for a substantial decrease in key presynaptic proteins, postsynaptic neurotransmitter receptors, or bouton numbers in the Pi3K68D mutant.

**Pi3K68D is required postsynaptically for PHP**

To determine where Pi3K68D functions during PHP, pre- versus postsynaptically, we performed tissue-specific rescue experiments by expressing UAS-Pi3K68D-GFP (Velichkova et al., 2010) in the Pi3K68D mutant background. First, overexpression of UAS-Pi3K68D-GFP in motoneurons had no significant effect on baseline transmission and failed to rescue PHP when expressed in the Pi3K68D mutant background (Figure 4A). Next, muscle specific over-expression of UAS-Pi3K68D-GFP using MHC-GAL4 impaired muscle health and diminished baseline transmission (Figure 4B). Muscle-specific expression of UAS-Pi3K68D-GFP with the BG57-GAL4 driver was not viable (MacDougall et al., 2009).
However, over-expression of UAS-Pi3K68D-GFP with BG57-GAL4 in the Pi3K68D mutant background was viable and fully rescued PHP (Figure 4B). Muscle recordings from these rescue animals revealed impaired muscle resting membrane potentials (RMP) due to Pi3K68D overexpression (RMP = −60.1 ± 1.3 mV without PhTX and −59.3 ± 2.4 mV with PhTX compared to the Pi3K68D mutant: −68.2 ± 1.3 mV without PhTX and −68.0 ± 1.8 mV with PhTX). We find an associated decrease in mEPSP amplitude, likely due to diminished driving force (Figure 4B). None-the-less, postsynaptic expression of Pi3K68D fully restored PHP. Thus, Pi3K68D is necessary postsynaptically for PHP.

We next sought to determine whether Pi3K68D kinase activity is required for PHP. A previously published kinase dead transgene for Pi3K68D is no longer available (MacDougall et al., 2004). Therefore, we generated a new UAS-kinase-dead Pi3K68D by creating a small deletion (21 amino acids) within the kinase domain (Figure 4C), termed UAS-KD21-Pi3K68D. To confirm that this transgene inhibits kinase activity when expressed in a wild-type genetic background, we expressed UAS-KD21-Pi3K68D in the scutellar bristle lineage using ptc-GAL4. This produced supernumerary scutellar bristles, phenocopying the effects of the previously published kinase dead transgene (data not shown; MacDougall et al., 2004). When we over-expressed UAS-KD21-Pi3K68D in muscle, PHP was blocked (Figure 4C and D). A C-terminal m-Cherry tag allowed us to determine that the transgene was expressed in muscle (data not shown). In addition, muscle overexpression of UAS-KD21-Pi3K68D (BG57-GAL4) had no adverse effect on muscle health, unlike over-expression of wild-type Pi3K68D with BG57-GAL4, which causes lethality (see above). From these data, we conclude that the kinase activity of Pi3K68D is necessary for PHP. These data further confirm that Pi3K68D is necessary, in muscle, for PHP.

The N-terminal amino acids of mammalian PIK3C2A and PIK3C2B have a regulatory function, binding Clathrin and regulating the activity of the kinase (Gaidarov et al., 2005; Wheeler and Domin, 2006). Therefore, we sought to test whether disrupting this regulatory function would alter the rapid expression of PHP. We generated an N-terminal Pi3K68D deletion, removing the...
Figure 4. Postsynaptic Pi3K68D is necessary for presynaptic homeostatic plasticity. (A) Average data for measures as indicated, for conditions, as indicated. Genotypes tested: UAS-Pi3K68D-GFP driven by OK371-GAL4; (light grey), Pi3K68D^{AH1} (red), and Pi3K68D^{AH1} with UAS-Pi3K68D-GFP driven by OK371-GAL4; (blue). Recordings made at 0.3 mM [Ca^{2+}]_{o}. (B) Data as in (A). Genotypes tested WT (data re-plotted from A for direct comparison; black), UAS-Pi3K68D-GFP driven by MHC-GAL4 (grey), Pi3K68D^{AH1} (red), and Pi3K68D^{AH1} with UAS-Pi3K68D-GFP driven by BG57-GAL4 (blue).
orthologous first 363 amino acids, termed UAS-Pi3K68D-ΔN. When we over-expressed UAS-Pi3K68D-ΔN in muscle, PHP was blocked (Figure 4E). When Pi3K68D-ΔN protein localization was followed using an N-terminal mCherry tag, it was apparent that the transgene is more diffuse compared to the punctate distribution of a wild-type UAS-Pi3K68D-GFP transgene (see below and data not shown). We conclude that either proper kinase localization or regulation, potentially via Clathrin binding, is essential for robust homeostatic signaling. Collectively, these results demonstrate a required postsynaptic function of Pi3K68D for presynaptic homeostatic plasticity.

**Pi3K68D genetically interacts with previously characterized PHP genes**

The genes encoding Rab3 Interacting molecule (RIM; Müller et al., 2012), a central player in the presynaptic cytomatrix, and Multiplexin (DMP; Wang et al., 2014), a component of the extracellular matrix and precursor to Endostatin, have been shown to be required for PHP. Since homozygous mutations in each gene block PHP, we cannot perform standard double mutant genetic epistasis experiments. However, it has been possible to test double heterozygous mutant combinations for a block in PHP, thereby implicating genes in a common process, even if it is not possible to order them in single signaling pathway (Frank et al., 2009; Harris et al., 2015; Wang et al., 2014). Here we demonstrate that heterozygous mutations for Pi3K68D/+ as well as rim/+ and dmp/+ all express normal PHP (Figure 5A,B). However, trans-heterozygous animals for Pi3K68D and rim show a complete block in PHP (Figure 5A,B). In addition, trans-heterozygous animals for Pi3K68D and dmp show a statistically significant suppression of homeostatic plasticity (Figure 5A,B). These data are consistent with Pi3K68D functioning within a homeostatic signaling system inclusive of previously identified PHP genes. Since RIM is a presynaptic protein and Pi3K68D acts postsynaptically, these data further imply that these genes participate, at some level, in a trans-synaptic signaling system necessary for PHP.

Multiplexin is a extracellular matrix protein, and its cleavage product Endostatin is hypothesized to act as a retrograde signal during PHP (Wang et al., 2014). Expression of UAS-endostatin in neurons or muscles rescues dmp mutants during PHP, consistent with a requirement for Endostatin being secreted into the synaptic cleft. Because dmp and Pi3K68D showed a genetic interaction as trans-heterozygotes (strong suppression of PHP), we hypothesized that Pi3K68D may be necessary for the secretion of Endostatin. In this model, expression of UAS-endostatin would rescue PHP in Pi3K68D mutants. We expressed UAS-endostatin in the muscle of Pi3K68D mutants and found that homeostatic plasticity was still blocked (Figure 5—figure supplement 1). In this condition, we confirmed that Pi3K68D mutants do not inhibit secretion of Endostatin-GFP by labeling surface GFP under non-cell permeabilizing conditions (Figure 5—figure supplement 1). As a control for exclusive labeling of secreted protein, we demonstrate that a highly over-expressed cytoplasmic GFP shows not labeling under non-cell permeabilizing conditions (see S6K-GFP, an intracellular protein tagged with GFP). Therefore, we conclude that secreted Endostatin is not sufficient to rescue the defect in PHP in Pi3K68D mutants. As such, it is unlikely that impaired PHP is due to a failure to release Multiplexin or proteolytically process Multiplexin into Endostatin within the synaptic cleft.

**Loss of Pi3K68D disrupts a postsynaptic PI(3)P-Dependent Endosomal System**

The class II PI3 kinases (PI3K-cll) remain poorly characterized, particularly when compared to other components of lipid and endosomal signaling systems. It has been reported in other cell biological systems that PI3K-cll binds to Clathrin and is activated, in part, through this interaction (Domin et al., 2000; Gaidarov et al., 2005; Wheeler and Domin, 2006). In addition, PI3K-cll drives...
the generation of the lipid PI(3)P (MacDougall et al., 2004), which defines an early endosomal membrane system (Posor et al., 2015). A plausible scenario for the localization and function of PI3K-cII in the secretory pathway is diagrammed (Figure 6A,B). This model serves as a guide to our experiments (below).

To visualize the subcellular localization of Pi3K68D, we expressed UAS-Pi3K68D-GFP in muscle, co-stained with anti-Clathrin Light Chain (CLC) (Heerssen et al., 2008) and imaged the preparation using super-resolution structured illumination microscopy (Pielage et al., 2008). We demonstrate that Pi3K68D-GFP forms endosomal like structures that precisely co-localize with CLC throughout muscle, concentrating near the muscle surface (Figure 6C). The highly regular distribution of Pi3K68D puncta is reminiscent of the distributed Golgi system in skeletal muscle, observed in mammals (Ralston et al., 2001) and Drosophila (Johnson et al., 2015). Therefore, we attained YFP or GFP-tagged markers of the medial and trans-Golgi (Ye et al., 2007). We tested for co-localization of our Golgi markers with CLC antibody staining, since anti-CLC is co-localized with Pi3K68D-GFP. We find that CLC and trans-Golgi-YFP (GaIT-YFP) reside in closely associated vesicular compartments throughout muscle (Figure 6D). This finding is confirmed by use of a second medial Golgi-GFP marker (UAS-ManII-GFP) (Figure 6—figure supplement 1). We conclude that Pi3K68D is present on a Golgi-derived, Clathrin-positive membrane compartment, consistent with prior work in other systems (Domin et al., 2000; Wheeler and Domin, 2006).
Figure 6. The role of Pi3K68D in endosomal and trans-Golgi signaling. (A) A model of the Golgi and endosomal system illustrating proteins explored in this and subsequent figures. (B) A schematic of the NMJ, muscles 6 and 7. Large circles depict muscle nuclei. Synaptic boutons are outlined in black and filled gray. Dotted box indicates a region of interest (ROI). The ROI, expanded below, reveals the edge of a nucleus, and puncta defined by Pi3K68D (green) and CLC (magenta). This schematizes images presented in C–E. (C) Muscle ROI in WT and animals expressing UAS-Pi3K68D-GFP in muscle (MHC-GAL4). Staining as indicated; GFP (green) and CLC (magenta or white in WT image). Nuclei are outlined with a white dotted line. Insets at right. (D) ROI in muscle expressing GalT-YFP (trans-Golgi). Staining as indicated. Select puncta shown at higher magnification (right). (E) Muscle ROI in WT and Pi3K68DAH1 expressing UAS-2xFYVE-GFP. Staining as indicated. Nuclei as in (C). (F) FYVE-GFP puncta area and number per muscle ROI. Black Figure 6 continued on next page.
We next determined whether Pi3K68D is responsible for the generation of PI(3)P by quantifying the distribution of a UAS-2xFYVE-GFP transgene, encoding a FYVE-domain protein that directly binds to PI(3)P (Hammond and Balla, 2015). We found that Pi3K68D mutants have dramatically fewer, smaller FYVE-GFP puncta compared to wild-type larvae (Figure 6E,F). As a control, we quantified CLC levels in the same muscles and find that they are unaffected (Figure 6—figure supplement 2). Furthermore, FYVE-GFP puncta do not co-localize with CLC, consistent with the presence of Pi3K68D on an intermediate endosomal membrane that is required for the formation of PI(3)P-positive endosomes. Thus, Pi3K68D is required to generate a significant fraction of PI(3)P in postsynaptic muscle, a necessary step in the formation of early endosomes in other systems (Posor et al., 2015).

It has been established that PI(3)P production at the early endosome recruits effectors that sort proteins to late endosomes, autophagosomes, or recycling endosomes (Posor et al., 2015). The proteins Rab5, Rab7, and Rab11 mark early endosomes, late endosomes, and recycling endosomes respectively (Grant and Donaldson, 2009). We quantified endogenous protein levels of Rab5, Rab7, and Rab11 in the muscle of wild-type and Pi3K68D mutant larva. Rab5, 7, and 11 are all enriched at the synapse, but we cannot differentiate between pre-and postsynaptic proteins by antibody staining. Therefore, we focused our examinations in regions of interest in the muscle, adjacent to the synapse. We found that loss of Pi3K68D does not affect total muscle protein levels of Rab11, but there is a significant drop in the number of Rab11-positive puncta (124.7 in WT versus 33.14 in Pi3K68D mutant, p=0.01; Student’s t-test, two tailed; Figure 6G). We also examined levels of Rab5 in the Pi3K68D mutant and found that mean intensity in the muscle ROI also decreased (Figure 6H). The Rab5-positive puncta could not be sufficiently resolved to quantify puncta number. Finally, we find that total Rab7 protein increased in the Pi3K68D mutant (quantified by mean intensity), but the number of Rab7-positive puncta did not change (Figure 6—figure supplement 2). These data are consistent with the depletion of PI(3)P in the Pi3K68D mutant background causing impaired Rab11 recruitment to endosome derived vesicles that recycle to and from the plasma membrane. If so, this membrane trafficking system could be a signaling platform required for the rapid expression of PHP.

**Postsynaptic Rab11 is necessary for PHP**

Rab11-positive endosomes are often referred to as recycling endosomes. These endosomes receive cargo, sorted within the PI(3)P positive endosome, and recycle the cargo back to the plasma membrane, thereby controlling the steady state concentration of important signaling molecules at the plasma membrane (Choy et al., 2014; Issman-Zecharya and Schuldiner, 2014; Zhang et al., 2011). We specifically knocked down Rab11 in muscle using a published UAS-Rab11-RNAi transgene (Beckett et al., 2013; Dietzl et al., 2007; Xiong et al., 2012) and a muscle-specific source of GAL4 (BG57-GAL4). We found that PHP was completely blocked (Figure 7A,B). It seems reasonable to conclude that the impaired Rab11 recruitment to early endosomes, and impaired generation/function of recycling endosomes, is responsible for the block of PHP in the Pi3K68D mutant.

**Postsynaptic Vps34 is necessary for PHP**

In most biological systems, the formation of the recycling endosomal compartment requires the action of a class III PI3K (PI3K-cIII, Figure 6A), referred to here as Vps34 for consistency with the yeast nomenclature. The Vps34 complex has been studied extensively and is thought to be the
Figure 7. Sub-synaptic specialization of a trans-Golgi network. (A) Representative traces for controls (BG57-GAL4/+; black) and UAS-Rab11-RNAi driven in muscle (red; BG57-GAL4) in the absence or presence of PhTX, as indicated. Recordings at 0.3 mM [Ca$^{2+}$]$_e$. (B) Genotypes tested are control (BG57-GAL4/+; grey) and UAS-Rab11-RNAi (red; muscle-expression via BG57-GAL4). (C) Average percent change in mEPSP amplitude (filled bars) and quantal content (open bars) in PhTX compared to baseline. UAS-Pi3K92E-RNAi (black; muscle-expression via MHC-GAL4), UAS-Pten over-expression (black, Figure 7 continued on next page...
primary mechanism for generating intracellular pools of PI(3)P that are required for recycling endosome transition to and from the plasma membrane (Backer, 2008; Dall’Armi et al., 2013; Juhász et al., 2008). Importantly, in our unbiased forward genetic screen, we also identified the Drosophila orthologue of a class III PI3K (Vps34, Table 1). This is the only gene in Drosophila that encodes a class III PI3K and should, therefore, be required in muscle for recycling endosome formation and function. We asked whether Drosophila Vps34 is required in muscle for PHP. Vps34 null mutations are third-instar lethal (Juhász et al., 2008). Never-the-less, we confirmed the results of our genetic screen that muscle specific knockdown of Vps34 blocks PHP (Figure 7C). Thus, three independent genes, two of which were derived from the results of a forward genetic screen, highlight an essential role for postsynaptic recycling endosomes in the rapid expression of PHP.

Finally, we thought it important to test whether a generalized disruption of the muscle lipid kinase signaling system might interfere with PHP. Therefore, to complete our analysis, we assessed whether the Drosophila class I PI3K might also be necessary, in muscle, for PHP. We found that PHP was normal when we expressed UAS-RNAi to knock down class I PI3K (Pi3K92E) in muscle (Figure 7C). To further address this possibility, we over-expressed the Pten phosphatase (Huang et al., 1999), which acts to oppose the kinase activity of class I PI3K. This also had no effect on the induction of PHP (Figure 7C). Functionally, the class I PI3K generates PI(3,4,5)P3 and is linked to insulin and TOR signaling (Knafo and Esteban, 2012). The lack of an observed effect on the rapid expression of PHP is consistent with prior observations that TOR and S6K act in muscle in a translation-dependent manner to consolidate or maintain expression of PHP, but are dispensable for the rapid expression process (Penney et al., 2012).

A postsynaptic Golgi compartment at the Drosophila NMJ

The time-course of PHP induction, occurring in seconds to minutes, implies the existence of mechanisms to control the secretion of retrograde signaling molecules at the postsynaptic side of the active zone. Recent work has demonstrated that Synaptotagmin-4 and Syntaxin-4 control the secretion of growth factors at the postsynaptic side of the NMJ (Akbergenova and Littleton, 2017; Harris et al., 2016; Rodal et al., 2011). However, synt4 mutations were found to have normal PHP (Dickman and Davis, 2009). Thus, a separate secretory system must be involved in the rapid expression of PHP.

When examining the distributed Golgi system in Drosophila muscle, we discovered that Golgi adopt a distinct morphology within the postsynaptic membranes at the NMJ. In this region, Golgi are statistically significantly smaller and appear more dense than in the surrounding muscle (Figure 7D,G). Similarly, puncta of FYVE-GFP and Pi3K68D-GFP are significantly smaller and more densely packed in the subsynaptic region (Figure 7E–G). While the functional relevance of this distinction remains unclear, it is clear that the entire secretory system, inclusive of Golgi, and endosomes are concentrated to the postsynaptic membrane system where it is poised to participate in retrograde, homeostatic signaling, among other synaptic functions.

Finally, we have employed 3D TEM tomography to examine the architecture of the SSR to explore the interface of the neuronal and endosomal membrane systems at the NMJ. The postsynaptic membrane membranes at the Drosophila NMJ are termed the sub-synaptic reticulum (SSR). The SSR is a complex, multi-layered membrane structure that envelopes the nerve terminal. It is well established that endosomal and secretory proteins localize to the SSR and, in some cases, concentrate at this structure (Akbergenova and Littleton, 2017). But, because the SSR membrane architecture is so complex (~1 μM thick), it remains unclear how the pre- and postsynaptic membranes of...
the nerve terminal interface with the SSR membrane system. Employing 3D TEM tomography (see Materials and methods), we reveal that the postsynaptic plasma membrane often invaginates into the SSR membrane system at sites that are directly adjacent to the active zone. Active zones are defined as sites of electron density between the pre- and postsynaptic plasma membranes, clustered presynaptic vesicles and the presence of a presynaptic T-bar (Figure 7H; Video 1). The sites of postsynaptic membrane invagination occur directly adjacent to the tightly opposed membranes of the synaptic cleft, creating a postsynaptic membrane that is in direct proximity to the SSR membranes and, by extension, in close proximity to the muscle endosomal and secretory signaling systems. Finally, the synaptic cleft also becomes continuous with the inter-cellular spaces within the SSR. This organization would facilitate the exchange of signaling information between the muscle and synapse, perhaps enabling the type of rapid, homeostatic signaling that is characteristic of PHP.

Loss of Pi3K68D renders the expression of PHP sensitive to changes in extracellular calcium

In order to explore why PHP fails following loss of Pi3K68D, we examined the rapid expression of PHP across a range of extracellular calcium concentrations, from 0.3 to 1.5 mM [Ca\(^{2+}\)]\(_o\). Remarkably, while PHP remained completely blocked at 0.5 mM [Ca\(^{2+}\)]\(_o\), PHP was restored in the range of 0.7–1.5 mM [Ca\(^{2+}\)]\(_o\). Indeed, there is a switch-like transition in the expression of PHP between 0.5 and 0.7 mM [Ca\(^{2+}\)]\(_o\) (Figure 8A,B). This is clearly observed by plotting the percent reduction in mEPSP amplitude caused by the application of PhTX versus the percent change in presynaptic release (quantal content), which defines the homeostatic response to PhTX application (Figure 8B).

We performed several additional experiments to explore the switch-like calcium sensitivity of PHP in Pi3K68D mutants. First, PHP is blocked at 0.3 mM [Ca\(^{2+}\)]\(_o\) in the Pi3K68D-GluRIIA double mutant (see above), but normally expressed at 1.5 mM [Ca\(^{2+}\)]\(_o\) (Figure 8C). Since the Pi3K68D-GluRIIA double mutants develop at elevated calcium in vivo (1.5 mM [Ca\(^{2+}\)]\(_o\) is the assumed average physiological calcium concentration), PHP must have been induced and fully expressed throughout larval life. Thus, loss of postsynaptic Pi3K68D must render the presynaptic expression mechanism sensitive to lower concentrations of extracellular calcium. We confirmed this conclusion by incubating the Pi3K68D mutant in PhTX at 0.3 mM [Ca\(^{2+}\)]\(_o\). Then, we immediately switched the preparation to 1.5 mM [Ca\(^{2+}\)]\(_o\) and found that PHP is fully expressed. Thus, the presynaptic expression of PHP has been rendered acutely calcium sensitive in Pi3K68D.

One possible explanation for the switch-like, calcium-dependence of PHP is that loss of Pi3K68D is somehow compensated by changes in Vp34 expression or activity, partially substituting for loss of Pi3K68D (note, however, that PI(3)P levels are substantially diminished in Pi3K68D, see above). To address this, we generated double heterozygous animals of Pi3K68D/+, but find that PHP is normally expressed (data not shown). Next, we removed one copy of Vps34/+ from the Pi3K68D homozygous mutant and, again, we find that PHP is fully expressed at 1.0 mM [Ca\(^{2+}\)]\(_o\) (Figure 8—figure supplement 1). It remains impossible to completely eliminate possible compensation by Vps34, since this gene is essential for viability. However, it does not appear that enhanced Vps34 expression is substituting for the loss of Pi3K68D.

Finally, we note that baseline neurotransmitter release appears to be differentially affected by changing extracellular calcium in Pi3K68D. Therefore, we explored the possibility that altered release is related to the switch-like effect of external calcium on PHP expression. Presynaptic release is wild-type when recordings are made at 0.5 mM [Ca\(^{2+}\)]\(_o\) (Figure 8D). However, in the range of 0.7–1.5 mM [Ca\(^{2+}\)]\(_o\), we find a significant deficit in average EPSC amplitude (Figure 8D), without a change in mEPSP amplitude (Supplementary file 1). Consistent with a
change in presynaptic release, we observe a decrease in presynaptic release probability during short, high-frequency stimulus trains ($P_{\text{train}}$; see Materials and methods) and decreased short-term synaptic depression (Figure 8E–F). However, the observed decrease in presynaptic release cannot be causally linked to impaired PHP. Three independent postsynaptic manipulations cause impaired PHP at low extracellular calcium without altering PHP at elevated calcium and without affecting presynaptic release at elevated calcium (Supplementary file 1). These manipulations include: (1) postsynaptic expression of the $\text{PI3K}_{68\text{D}}$ kinase dead transgene, (2) postsynaptic knockdown of $\text{Rab11}$ and (3) postsynaptic knockdown of $\text{Vps34}$. In all manipulations, PHP is impaired at 0.3 mM $[\text{Ca}^{2+}]_{\text{e}}$ (Figures 4E and 7A) but restored at 1.5 mM $[\text{Ca}^{2+}]_{\text{e}}$ with no defect in baseline transmission (Supplementary file 1). Note that impaired muscle health caused by muscle over-expression of wild-type $\text{PI3K}_{68\text{D}}$ precludes two-electrode voltage clamp experiments and, therefore, analysis of postsynaptic rescue of baseline neurotransmission.
Discussion

We have screened the Drosophila kinome and phosphatome for genes that control the rapid expression of PHP. This screen identified three components of a conserved, postsynaptic lipid signaling pathway that is essential for the robust expression of PHP including: (1) class II PI3K, (2) class III PI3K (Vps34) and a gene encoding the Drosophila orthologue of Pi4K (not examined in detail in this study). We go on to demonstrate that Pi3K68D is essential, postsynaptically for PHP. Pi3K68D resides on a Clathrin-positive membrane compartment that is positioned directly adjacent to Golgi membranes, throughout muscle and concentrated at the postsynaptic side of the synapse. Pi3K68D is necessary for the maintenance of postsynaptic PI(3)P levels and the recruitment of Rab11 to intracellular membranes, likely PI(3)P-positive recycling endosomes. Postsynaptic Rab11 and Vps34 knockdown block PHP in an unusual, calcium-dependent manner that phenocopies (membranes, throughout muscle and concentrated at the postsynaptic side of the synapse. Rab11-positive recycling endosomes, that is essential for PHP.

First, we consider the possibility that the absence of postsynaptic PI3K and Rab11 signaling could alter the molecular composition or development of the presynaptic terminal due to the persistent absence of a retrograde signal that controls generalized synapse development or growth. Several observations demonstrate that impaired PHP is not a secondary consequence of a general defect in synapse development. We report three independent postsynaptic manipulations (postsynaptic expression of kinase dead Pi3K68D, postsynaptic knockdown of Rab11, and postsynaptic knockdown of Vps34) that have no effect on presynaptic release at any [Ca^{2+}]_o, yet block PHP at low [Ca^{2+}]_o. In addition, we find no obvious defect in anatomical synapse development (Figure 3—figure supplement 1).

Next, we consider the possibility that postsynaptic PI3K and Rab11 signaling eliminate a retrograde signal that is specific for PHP. We recently demonstrated that Semaphorin2b (Sema2b) and...
PlexinB (PlexB) define a retrograde signal at the Drosophila NMJ that is necessary for PHP (Orr et al., 2017). However, both Sema2b and PlexB are essential for the rapid induction of PHP, inclusive of experiments at low and elevated extracellular calcium. Further, acute application of recombinant Sema2b is sufficient to fully induce PHP. Since the induction of PHP remains fully intact in the Pi3K68D mutant, and since PHP is rendered calcium sensitive, it suggests that altered Sema2b secretion is not the cause of impaired PHP in the Pi3K68D mutant. Never-the-less, this possibility will be directly tested in the future.

Altered retrograde signaling specificity

Next, we consider the possibility that the loss of PI3K and Rab11 signaling causes aberrant or inappropriate retrograde signaling, thereby impairing the expression of PHP. This is a plausible scenario because the induction of presynaptic homeostatic plasticity suffers from a common problem inherent to many intracellular signaling systems: two incompatible outcomes (1. presynaptic homeostatic potentiation and 2. presynaptic homeostatic depression - PHD) are produced from a common input, and it remains unclear how signaling specificity is achieved. The topic of signaling specificity has been studied in several systems. One system, budding yeast, is a good example. Different pheromone concentrations can induce several distinct behaviors in budding yeast despite having a common input (pheromone concentration) and underlying signaling systems (Saito, 2010; Schwartz and Madhani, 2004). Signaling specificity degrades in the background of mutations that affect Map Kinase scaffolding proteins (Schwartz and Madhani, 2004). In a similar fashion, presynaptic homeostatic plasticity is induced by a change in mEPSP amplitude. A decrease in mEPSP amplitude causes the induction of PHP, whereas an increase in mEPSP amplitude causes the induction of presynaptic homeostatic plasticity (PHD) (Daniels et al., 2004; Gavinó et al., 2015). If a common sensor is employed to detect deviations in average mEPSP amplitude, how is this converted into the specific induction of either PHP or PHD? It has been shown that PHD and PHP can be sequentially induced (Gavinó et al., 2015). But, it remains unknown what would happen if the mechanisms of PHP and PHD were simultaneously induced. Under normal conditions this would never occur because mEPSP amplitudes cannot be simultaneously increased and decreased. But, if signaling specificity were degraded in animals lacking postsynaptic PI3K or Rab11, then the expression of PHP and PHD might coincide and create a mechanistic clash in the presynaptic terminal (Figure 9).

Signaling and recycling endosomes are, in many respects, ideally suited to achieve signaling specificity during homeostatic plasticity. Signaling specificity can be achieved by mechanisms including sub-cellular compartmentalization of pathways, physically separating signaling elements with protein scaffolds, or through mechanisms of cross-pathway inhibition (Bardwell et al., 2007; Haney et al., 2010; Schwartz and Madhani, 2004). Well-established mechanisms of protein sorting within recycling endosomes could physically compartmentalize signaling underlying PHP versus PHD (Cullen, 2008; Grant and Donaldson, 2009). Alternatively, recycling endosomes can serve as a focal point for signal digitization, integration, and, perhaps, cross-pathway inhibition (Irannejad et al., 2013, 2015; Villaseñor et al., 2015; Villaseñor et al., 2016). Thus, we propose that the loss of postsynaptic PI3K and Rab11 compromises the function of the postsynaptic endosomal platform that we have identified, thereby degrading homeostatic signaling specificity. As such, this platform could be considered a ‘homeostatic controller’ that converts homeostatic error signaling into specific, homeostatic, retrograde signaling for either PHP or PHD. One such scenario is proposed in Figure 9.

We have also considered other models, but do not favor them. It remains formally possible that the calcium-sensitivity of PHP expression could be explained by a partially functioning PHP signaling system. This seems unlikely given that the same phenotype is observed in four independent genetic manipulations including a null mutation in Pi3K68D, postsynaptic expression of kinase dead Pi3K68D, postsynaptic knockdown of Rab11, and postsynaptic knockdown of Vps34. Furthermore, prior experiments examining hypomorphic and trans-heterozygous genetic interactions among essential PHP genes suggest that PHP is either diminished across the entire calcium spectrum or fully functional (Davis and Müller, 2015; Genç et al., 2017; Harris et al., 2015; Orr et al., 2017; Wang et al., 2016; Younger et al., 2013). So, there is no evidence that partial disruption of PHP could account for calcium-sensitive expression of PHP. Finally, our experiments argue against the possibility that compensatory changes in Vps34 expression partially rescue the Pi3K68D mutant phenotype (Figure 8-figure supplement 1).
We also note another common signaling module that emerged from our genetic screen. Both CamKII and CamKK were identified as potential hits. The identification of CamKII is supported by prior work showing the expression of dominant negative CamKII transgenes disrupt the long-term maintenance of PHP in the GluRIIA mutant background (Haghighi et al., 2003). It has been assumed that postsynaptic calcium is used to detect the PhTX or GluRIIA-dependent perturbation. But, the logic remains unclear. PHP is induced by diminished GluR function and, therefore, diminished postsynaptic calcium influx (Newman et al., 2017). This should diminish activation of CamKII and yet, loss of CamKII blocks PHP. An interesting alternative model is that calcium and calmodulin-dependent kinase activity facilitate the function of the postsynaptic endosomal membrane system. Both calcium and calmodulin are necessary for endosomal membrane fusion (Colombo et al., 1997; Lawe et al., 2003). In this manner, the action of CamKK and CamKII would be entirely consistent with the identification of Class II/III PI3K and Rab11 as homeostatic plasticity genes.

**Conclusion**

We have uncovered novel postsynaptic mechanisms that drive homeostatic plasticity. Eventually, continued progress in this direction may make it possible to not only reveal how stable neural function is achieved throughout life, but to uncover new rules that are essential for the processing of information throughout the nervous system. In particular, PHP has a very large dynamic range, whether one considers data from Drosophila or human NMJ or mammalian central synapses. The homeostatic control of presynaptic release can achieve a 7-fold change in synaptic gain, and yet retains the ability to offset even small changes in postsynaptic neurotransmitter receptor function (Cull-Candy et al., 1980; Müller et al., 2015). Thus, we expect that the regulatory systems that achieve PHP will be complex and have a profound impact on brain function. Here, we have defined

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**Figure 9.** Model for the control of PHP and PHD from an endosomal ‘controller’. (A) A model of homeostatic feedback control inclusive of feedback for both PHP and PHD. The sign for modulation of presynaptic release is indicated. We speculate that signaling pathway cross-inhibition allows for either PHP (red) or PHD (blue) to be selectively induced. When mEPSP amplitudes are decreased (PhTX), this is detected by the sensor and differs from the synaptic set point, causing an error signal to be generated. The error is then relayed to a homeostatic ‘controller’ where the error is integrated and signaling is induced corresponding to the specific induction of either PHP (red) or PHD (blue). We propose that cross pathway inhibition at the level of the controller allows for the specific induction of either PHP or PHD. We propose that the ‘controller’ is organized within the PI3K and Rab11-dependent recycling endosomal signaling platform and that loss of this signaling platform leads to inappropriate induction of PHD in the presence of PhTX, causing a mechanistic clash at the level of the presynaptic terminal. (B) A model of the neuromuscular junction, highlighting mechanisms of PHP in red (increased calcium influx, increased RRP, and increased vesicle coupling) and PHD in blue, for which very little is understood mechanistically.

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**Postsynaptic Calcium-dependent kinase signaling**

We also note another common signaling module that emerged from our genetic screen. Both CamKII and CamKK were identified as potential hits. The identification of CamKII is supported by prior work showing the expression of dominant negative CamKII transgenes disrupt the long-term maintenance of PHP in the GluRIIA mutant background (Haghighi et al., 2003). It has been assumed that postsynaptic calcium is used to detect the PhTX or GluRIIA-dependent perturbation. But, the logic remains unclear. PHP is induced by diminished GluR function and, therefore, diminished postsynaptic calcium influx (Newman et al., 2017). This should diminish activation of CamKII and yet, loss of CamKII blocks PHP. An interesting alternative model is that calcium and calmodulin-dependent kinase activity facilitate the function of the postsynaptic endosomal membrane system. Both calcium and calmodulin are necessary for endosomal membrane fusion (Colombo et al., 1997; Lawe et al., 2003). In this manner, the action of CamKK and CamKII would be entirely consistent with the identification of Class II/III PI3K and Rab11 as homeostatic plasticity genes.
a postsynaptic signaling system responsible for the rapid expression of PHP and propose a novel, albeit speculative, model for the postsynaptic control of PHP, taking into account the need for signaling specificity for the first time. Whether or not we are absolutely correct in proposing how these molecules function within a homeostatic signaling system, their identification paves the way for future advances in understanding how homeostatic signaling is designed and implemented at a cellular and molecular level.

### Materials and methods

#### Key resources table

| Reagent type (species) or resource | Designation |
|-----------------------------------|-------------|
| Gene (Drosophila melanogaster)    | Pi3K68D     |
| Gene (D. melanogaster)            | Rab11       |
| Gene (D. melanogaster)            | Vps34       |
| Strain/strain background          | WT; w1118   |
| Genetic reagent (D. melanogaster) | GluRIIA<sup>W;16</sup>; GluRIIA |
| Genetic reagent (D. melanogaster) | elav<sup>C155</sup>-GAL4 |
| Genetic reagent (D. melanogaster) | OK371-GAL4 |
| Genetic reagent (D. melanogaster) | MHC-GAL4    |
| Genetic reagent (D. melanogaster) | BG57-GAL4   |
| Genetic reagent (D. melanogaster) | rim<sup>103</sup>; rim |
| Genetic reagent (D. melanogaster) | dmp<sup>07253</sup>; dmp |
| Genetic reagent (D. melanogaster) | Pi3K68D-RNAi |
| Genetic reagent (D. melanogaster) | UAS-Pi3K68D: GFP |
| Genetic reagent (D. melanogaster) | Vps34<sup>Mc22</sup> |
| Genetic reagent (D. melanogaster) | Pi3K68D-MB  |
| Genetic reagent (D. melanogaster) | Pi3K68D-GS  |
| Genetic reagent (D. melanogaster) | nos-GAL4VP14, UAS-cas9 |
| Genetic reagent (D. melanogaster) | Pi3K Class I Pi3K92E RNAi |
| Genetic reagent (D. melanogaster) | Pi3K Class III RNAi; Vps34 RNAi |

#### Details

- **Pi3K68D**
  - Source: NA
  - Identifier: FLYB: FBgn0015278

- **Rab11**
  - Source: NA
  - Identifier: FLYB: FBgn0015790

- **Vps34**
  - Source: NA
  - Identifier: FLYB: FBgn0015277

- **WT; w1118**
  - Source: NA
  - Identifier: w<sup>1118</sup>

- **GluRIIA<sup>W;16</sup>; GluRIIA**
  - Source: (Petersen et al., 1997)
  - PMID: 9427247
  - Identifier: FLYB: FBal0085982

- **elav<sup>C155</sup>-GAL4**
  - Source: BDSC: 458
  - Identifier: FLYB: FBst0000458

- **OK371-GAL4**
  - Source: (Mahr and Aberle, 2006)
  - PMID: 16378756

- **MHC-GAL4**
  - Source: (Petersen et al., 1997)
  - PMID: 9427247

- **BG57-GAL4**
  - Source: (Budnik et al., 1996)
  - PMID: 8893021

- **rim<sup>103</sup>; rim**
  - Source: (Müller et al., 2012)
  - PMID: 23175813

- **dmp<sup>07253</sup>; dmp**
  - Source: Bloomington Stock Center
  - Identifier: BDSC: 19062; FLYB: FBst0019062

- **Pi3K68D-RNAi**
  - Source: Exelixis Collection
  - Identifier: HMS.01296

- **UAS-Pi3K68D: GFP**
  - Source: (Velichkova et al., 2010)
  - PMID: 20696708

- **Vps34<sup>Mc22</sup>**
  - Source: (Juhasz et al., 2008)
  - PMID: 18474623

- **Pi3K68D-MB**
  - Source: Bloomington Drosophila Stock Center
  - Identifier: BDSC: 26363; FLYB: FBst0026363

- **Pi3K68D-GS**
  - Source: Kyoto Stock Center
  - Identifier: KSC: 203158

- **nos-GAL4VP14, UAS-cas9**
  - Source: (Port et al., 2014)
  - PMID: 25002478

- **Pi3K Class I Pi3K92E RNAi**
  - Source: Bloomington Drosophila Stock Center
  - Identifier: BDSC: 27690; FLYB: FBst0027690

- **Pi3K Class III RNAi; Vps34 RNAi**
  - Source: Bloomington Drosophila Stock Center
  - Identifier: BDSC: 33384; FLYB: FBst0033384

*Continued on next page*
| Reagent type (species) or resource | Designation | Source or reference | Identifier | Additional information |
|-----------------------------------|-------------|---------------------|------------|------------------------|
| Genetic reagent (D. melanogaster) | Pten-RNAi   | Bloomington Drosophila Stock Center | BDSC: 33643, FLYB: FBst0033643 | Flybase symbol: y[1] v[1]; P[y+[t7.7]+] v+[t1.8]=TRiP.HMS00044 attP2TRiP.HMS00044 attP2 |
| Genetic reagent (D. melanogaster) | UAS-GFP-myc-2xFYVE | Bloomington Drosophila Stock Center | BDSC: 42712, FLYB FBst0042712 | Flybase symbol: w[+]; P[w[+]mC]=UAS-GFP-myc-2xFYVE |
| Genetic reagent (D. melanogaster) | UAS-Rab11 RNAi | Vienna Drosophila RNAi Center | VDRC: 22198; FLYB FBst0454467 | Flybase symbol: w[1118]; P{GD11761}v22198 |
| Genetic reagent (D. melanogaster) | UAS-Man1-GFP | (Ye et al., 2007) PMID: 17719548 | | |
| Genetic reagent (D. melanogaster) | UAS-GalT-YFP | (Ye et al., 2007) PMID: 17719548 | | |
| Genetic reagent (D. melanogaster) | UAS-endostatin | (Meyer and Moussian, 2009) PMID: 19469789 | | |
| Genetic reagent (D. melanogaster) | UAS-endostatin-GFP | (Meyer and Moussian, 2009) PMID: 19469789 | | |
| Genetic reagent (D. melanogaster) | Pi3K68DΔH1 | This paper | Indel mutation, premature stop codon at amino acid 1440, made with CRISPR-CAS9 |
| Genetic reagent (D. melanogaster) | UAS-Pi3K68D-D21; UAS-Pi3K68D-KD | This paper | Generated using site-directed mutagenesis with primers TTGGAAA CTITTAAGAGAGATC and CATGA TGTTGTGCTATGTGG then subsequently cloned into 1100 mCherry. |
| Genetic reagent (D. melanogaster) | UAS-Pi3K68D-DN | This paper | Primers CACCATGAACGACACC GCCTCCGAC and GTTCCTGGACACC GCGCC were used to amplify Pi3K68D-DN, which was then cloned into destination vector 1100 mCherry |
| recombinant DNA reagent | Pi3K68D gRNA | This paper | ACAGCACTCTGGTACTCGAG for generation of Pi3K68DΔH1 |
| recombinant DNA reagent | pCDF3-dU6:3gRNA vector | Addgene | Addgene plasmid #49410 |
| recombinant DNA reagent | pENTR/D-TOPO | Invitrogen | K240020 |
| recombinant DNA reagent | destination vector 1100 mCherry | NA | Gift from Dion Dickman |
| Antibody | anti-BRP (mouse monoclonal) | Developmental Studies Hybridoma Bank | DSHB: nc82 | 1:100, Bouin’s fixative |
| Antibody | anti-Discs large; anti-DLG (rabbit) | (Budnik et al., 1996) PMID 8893021 | 1:1,000, Bouin’s fixative |
| Antibody | anti-GFP (mouse monoclonal) | Invitrogen | Invitrogen clone 3E6; A-11120 | 1:500, Bouin’s fixative |
| Antibody | anti-GluRIIA (mouse monoclonal) | Developmental Studies Hybridoma Bank | DSHB: 884D2 (MH2B) | 1:100, Bouin’s fixative |
| Antibody | anti-GluRIIB (rabbit polyclonal) | (Marrus et al., 2004) PMID 14960613 | 1:2500, Bouin’s fixative |
| Antibody | anti-CLC (rabbit polyclonal) | (Heerssen et al., 2008) PMID: 18366056 | 1:1000, 4% PFA |
| Antibody | Anti-CSP (mouse monoclonal) | (Zinsmaier et al., 1990) PMID 2129171 | 1:250, 4% PFA |
| Antibody | Anti-Syt1 (rabbit polyclonal) | Other | 1:1000, 4% PFA, gift from Troy Littleton |
| Reagent type (species) or resource | Designation | Source or reference | Identifier | Additional information |
|------------------------------------|-------------|---------------------|------------|------------------------|
| Antibody                          | Anti-Rab5   | (Tanaka and Nakamura, 2008) PMID: 18272590 | 1:1000, 4% PFA, gift from Tsubasa Tanaka |
| Antibody                          | Anti-Rab7   | (Tanaka and Nakamura, 2008) PMID: 18272590 | 1:1000, 4% PFA, gift from Tsubasa Tanaka |
| Antibody                          | Anti-Rab11  | (Tanaka and Nakamura, 2008) PMID: 18272590 | 1:1000, 4% PFA, gift from Tsubasa Tanaka |
| Antibody                          | Alexa conjugated secondary antibodies (488, 555, 647) | Jackson Immuno-research laboratories | 1:500 |

**Sequence based reagent**

Primer set: Primers for sequencing Pi3K68D CRISPR mutation

**Software, algorithm**

mEPSP analysis: Synaptosoft

mEPSC, Pr, RRP, train analysis: (Müller et al., 2015)

**Generation of CRISPR mutant for Pi3K68D**

The Pi3K68D premature stop mutation was generated following the protocol of (Kondo and Ueda, 2013). Pi3K68D gRNA was selected using the CRISPR optimal target finder website (http://tools.fly-crispr.molbio.wisc.edu/targetFinder). The gRNA sequence ACAGCACTCTGGTACTCGAG was cloned into the pCDF3-dU6:3gRNA vector (Addgene plasmid #49410, Simon Bullock). Flies expressing the UAS-gRNA were crossed with flies expressing UAS-Cas9 in the germline (nos-GAL4VP14, UAS-cas9). Male offspring were used to create unique stable lines after removing the UAS-Cas9 and removing in the next generation the UAS-gRNA. Putative Pi3K68D mutants were sequenced to identify the nature of the Cas9 mediated mutation using the primers GTTTCCAAAAACATCTGAGCATCG and ATGACTTGCAAGGATCG.

**Electrophysiology**

Sharp-electrode recordings and two-electrode voltage clamp recordings were made from muscle six in abdominal segments 2 and 3 from third-instar larvae using an Axoclamp 900A amplifier (Molecular Devices), as described previously (Frank et al., 2006; Müller et al., 2012). Recordings were made in HL3 saline containing the following components: NaCl (70 mM), KCl (5 mM), MgCl₂ (10 mM),
NaHCO$_3$ (10 mM), sucrose (115 mM), trehalose (5 mM), HEPES (5 mM), and CaCl$_2$ (as indicated in figures). For acute pharmacological homeostatic challenge, unstretched larva were incubated in Phlanthotoxin-433 (PhTX; 15 μM; Sigma-Aldrich) for 10 min. (Frank et al., 2006). Recordings were excluded if the resting membrane potential (RMP) was more depolarized than −60 mV, except for over-expression of UAS-PTEN and UAS-Pi3K68D which uniformly compromised RMP. A threshold 40% decrease in mEPSP amplitude, below average baseline, was used to confirm the activity of PhTX. For UAS-Rab11-RNAi expression a 15% decrease was used. EPSP traces were analyzed in IGOR Pro (Wave-Metrics) and with previously published routines in MATLAB (Mathworks) (Ford and Davis, 2014). mEPSP traces were analyzed using MiniAnalysis 6.0.0.7 (Synaptosoft), averaging at least 100 individual mEPSP events. EPSC amplitudes were analyzed in IGOR Pro (Wave-Metrics) with previously routines (Müller et al., 2015). Quantal content was calculated by dividing mean EPSP by mean mEPSP. The RRP was estimated by cumulative EPSC analysis, as described previously (Müller et al., 2012; Schneggenburger et al., 1999). In brief, muscles were clamped at −65 mV in two-electrode voltage clamp during a stimulus train (60 Hz, 30 stimuli). RRP for each muscle was calculated by dividing cumulative EPSC amplitude in TEVC by mEPSP amplitude in current clamp. $P_{\text{train}}$ was calculated by dividing mean first EPSC amplitude by mean cumulative EPSC. Best-fit curves for mEPSP amplitude versus quantal content were fit in Prism 6 (GraphPad) using a power function for all wild-type data points ± PhTX. 95% data intervals were fit in IGOR Pro (Wave-Metrics) using a power function.

**Immunohistochemistry**

Standard immunohistochemistry was performed as previously described (Pielage et al., 2005). In brief, filleted third instar larvae were fixed in either Bouin’s fixative (Sigma-Aldrich, 5 min) or 4% PFA (Affymetrix, 30 min), as indicated for each antibody below. Preps were washed in PBT (PBS with 0.1% Triton) for 1 hr, then incubated overnight at 4°C in primary antibody in PBT. Larval fillets stained for the following primary antibodies were fixed with Bouin’s: mouse anti-BRP (1:100, Developmental Studies Hybridoma Bank, [Kittel et al., 2006]), rabbit anti-Discs large (Dlg, 1:1,000, [Budnik et al., 1996]), mouse anti-GFP (1:500, Invitrogen clone 3E6), mouse anti-GluRIIA (Developmental Studies Hybridoma Bank [Marrus et al., 2004]), rabbit anti-GluRIIB (1:2500, a gift from Aaron DiAntonio [Marrus et al., 2004]). Larval fillets stained for the following primary antibodies were fixed with PFA: rabbit anti-CLC (1:1000 [Heerssen et al., 2008]), mouse anti-CSP (1:250 [Zinsmaier et al., 1990]), rabbit anti-Syt1 1:1000 (a gift from Troy Littleton), guinea pig anti-Rab5 1:1000 (a gift from Tsubasa Tanaka), rabbit anti-Rab7 1:1000 (a gift from Tsubasa Tanaka), rabbit anti-Rab11 1:1000 (a gift from Tsubasa Tanaka). Preps were washed in PBT for 1 hr and incubated in secondary antibody in PBT for 1 hr at room temperature. Alexa-conjugated secondary antibodies were used at 1:500 and FITC-, Cy3- and Cy5-conjugated HRP was used at 1:100 (Jackson Immuno-research Laboratories). Preps were mounted in Vectashield (Vector). Immunolabeling of surface GFP was performed as described in (Wang et al., 2014) by incubating larval preparations in rabbit anti-GFP antibody (1:500) before permeabilization of cell membranes.

**Image acquisition and analysis**

Deconvolution wide field imaging for synapse morphology was performed using a 100x (1.4 NA) plan Apochromat objective (Carl Zeiss) on an Axiovert 200 inverted microscope (Carl Zeiss) equipped with a cooled CCD camera (CoolSNAP HQ; Roper Scientific). Image acquisition and analyses were performed in SlideBook software (Intelligent Imaging Innovation). Structured illumination fluorescence microscopy was performed using an N-SIM System (Nikon) with an Apo TIRF 100x/1.49 oil objective on a Ti-E microscope (Nikon) and an Andor DU897 camera. Z-stacks of 120 nm were collected for muscle 4 or 6/7. Images were reconstructed in NIS-Elements 4.12. Maximum projection images were made.

**NMJ morphology analysis**

Quantification of BRP and bouton number was performed as previously described (Wang et al., 2014). Boutons were counted manually on a Zeiss axioskop 40 compound microscope (40x, 1.1nA lens). Boutons of type 1b and 1 s were independently quantified for abdominal segments A2 and A3. Active zone number was calculated by counting individual BRP puncta (100x, 1.4nA lens) from
maximum intensity projection deconvolved images, as previously described (Wang et al., 2014). Synaptic Clathrin Light Chain was quantified by masking for the neuronal membrane with HRP, then quantifying average fluorescence intensity using Slidebook Software in maximum projection images (Intelligent Imaging Innovation). Syt1 and CSP were quantified as previously described (Harris et al., 2015).

FYVE-GFP, CLC, Rab7, Rab11, GalT-YFP, Pi3K68D-GFP, and FYVE-GFP puncta number and size were quantified with maximum projections from a fixed number of image planes. A region of interest in muscles 6, segments 2 and 3, was chosen not inclusive of muscle nuclei. Images were thresholded to the same value and puncta were analyzed with Fiji (Schindelin et al., 2012). The number of puncta was counted per ROI, and each puncta area was also measured. Rab5, Rab7, and Rab11 mean intensity per ROI were quantified by taking sum projections of the same number of slices of images (Fiji).

Electron microscopy tomography
Third instar w^{360S} larvae were prepared for electron microscopy as described in (Harris et al., 2015). For EM tomography, 200 nm sections cut with a Diatome diamond knife using a Leica UC-T ultramicrotome were picked up on Piafoform films with 2 nm C on Synaptek slot grids (Ted Pella, Inc). Sections were post-stained with 7.5% uranyl acetate followed by Sato’s lead sa (Sato, 1968). Dual-axis tilt series images (±60 deg) were acquired with an FEI T20 electron microscope at 200 kV equipped with a Tietz F816 digital camera. Tomograms were reconstructed using the eTomo package in IMOD (Mastronarde, 1997).

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Author ORCIDs
Graeme W Davis http://orcid.org/0000-0003-1355-8401

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Additional files
Supplementary files
• Supplementary file 1. Source data for all electrophysiological data for all figures and supplemental figures. Data includes mEPSP amplitudes, EPSP or EPSC amplitudes, quantal contents, and sample size (N).
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