The regulation of AMPA-type glutamate receptor (AMPAR) trafficking is a key mechanism by which neurons regulate synaptic strength and plasticity. AMPAR trafficking is modulated through a combination of receptor phosphorylation, ubiquitination, endocytosis, and recycling, yet the in vivo significance of factors involved in these processes are just beginning to be uncovered. Here we identify the ubiquitin-conjugating enzyme variant UEV-1 as a regulator of AMPAR trafficking in vivo, and in a related project find a possible link between AMPAR phosphorylation and ubiquitination-driven internalization. We identified mutations in uev-1 in a genetic screen for mutants with altered trafficking of the AMPAR subunit GLR-1 in C. elegans interneurons. Loss of uev-1 activity results in elongated accretions of GLR-1 in neuron cell bodies and along the ventral cord neurites. Mutants also have a corresponding behavioral defect – a decrease in spontaneous reversals in locomotion – consistent with diminished GLR-1.
function. We provide evidence that GLR-1 accumulates at RAB-10-containing endosomes in *uev-1* mutants. UEV-1 homologs in other species bind to the ubiquitin-conjugating enzyme Ubc13 to create K63-linked polyubiquitin chains on substrate proteins. We find that whereas UEV-1 can interact with *C. elegans* UBC-13, global levels of K63-linked ubiquitination throughout nematodes appear to be unaffected in *uev-1* mutants, even though UEV-1 is broadly expressed in most tissues. Nevertheless, we find that mutations in *ubc-13* result in accumulations of GLR-1 in a similar pattern to that found in *uev-1* mutants. Our results suggest that UEV-1 may regulate a small subset of K63-linked ubiquitination events in nematodes, at least one of which is critical in regulating GLR-1 trafficking. In another project, we used a reverse genetics approach to study the regulation of phosphorylation state on the trafficking of GLR-1. We found that mutation of a single GLR-1 serine (S891) to alanine is sufficient to strongly reduce GLR-1 puncta in the ventral nerve cord. We also determined that this effect is precluded if the C-terminal portion of GLR-1 is mutated such that it can no longer be ubiquitinated and endocytosed. This suggests that S891 phosphorylation may regulate endocytosis and/or ubiquitination and subsequent degradation of GLR-1. This may provide a link between ubiquitination and phosphorylation in the trafficking of GLR-1, with possible implications for similar links in mammalian AMPAR trafficking.
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DEDICATION

To my father, Edward Kramer, who always loved me and encouraged me to persevere and who I wish could have lived to see me finish this thesis. Hope you are seeing from heaven and enjoying this!
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CHAPTER I

INTRODUCTION
AMPA Type Glutamate Receptors

AMPA Receptor Structure and Function

Due to their unique roles in humans and other organisms, neurons are perhaps the most specialized of all cells in both morphology and physiology. With long and often richly branched neurites (axons and dendrites), these cells are able to transmit signals as well as integrate and store sensory or cognitive information. The specialized cell junction that is crucial to neuronal function is the synapse, which has on the sending side a pre-synaptic area where messages are transmitted by the release of neurotransmitters. On the receiving side of the synapse, there is a post-synaptic area where the messages are received largely via trans-membrane ligand-gated receptors that flux sodium, potassium and other ions to control the polarization state of the post-synaptic membrane. Understanding the cell biological machinery underlying synaptic transmission and reception is a key area of study in neurobiology.

The main excitatory neurotransmitter in mammals and many other organisms is glutamate. Glutamatergic synapses show substantial plasticity, even in adult animals, where synaptic strength can be increased or decreased in an activity-dependent manner. Ligand-gated glutamate receptors (GluRs) important in plasticity include N-methyl-D-aspartate (NMDA) type glutamate receptors
(NMDARs) and alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) type glutamate receptors (AMPARs). NMDARs function as coincidence detectors that require both glutamate and depolarization to activate, whereas AMPARs are involved in routine glutamatergic transmission (Dingledine et al., 1999).

AMPARs are tetrameric protein complexes (Rosenmund et al., 1998), that can be composed of a variety of protein subunits. In mammals, the AMPAR subunits are named GluR1, GluR2, GluR3 and GluR4 (Wenthold et al., 1996). Each subunit is a trans-membrane protein with an extracellular N-terminal region, four “trans-membrane” regions (one of which is a pore region that only partly crosses the membrane) and a carboxyl-terminal (cterm) “tail” sequence. The subunits have a high degree of sequence similarity, differing largely in their cterm tails, which are either long (GluR1 and 4) or short (GluR2 and 3) (Dingledine et al., 1999).

AMPAR subunits have various splice forms and are also subject to RNA editing. In the case of GluR2, splice forms produce variations in cterm tail length, and RNA editing results in the change of an ion channel pore region glutamine to an arginine (R607), which among other effects, changes the calcium permeability of the channel (Gomes et al., 2008; Swanson et al., 1997). The subunit composition of each AMPAR can influence both its functioning and trafficking (Chang and Rongo, 2005; Lee et al., 2004; Passafaro et al., 2001; Shi et al.,
Subunit combinations often found in adult mammals are GluR1/2 and GluR2/3, with GluR4 typically having more of a role early in development (Wenthold et al., 1996).

There are many forms of AMPAR–mediated synaptic plasticity, varying in the brain regions in which they have been found, as well as in their actual mechanisms. Two forms of these forms of plasticity are Long Term Potentiation (LTP) and Long Term Depression (LTD). In both of these phenomena, calcium influx via activation of NMDARs leads to changes in the number (AMPAR “trafficking”) and/or single channel conductance of AMPARs (Malenka and Bear, 2004). AMPAR trafficking can result in a weakening (LTD) of synaptic strength via a reduction in the number of receptors in the post-synaptic density (PSD), or strengthening (LTP) via an increase in the number of receptors. In fact there is evidence that “silent synapses” that contain only NMDARs can be converted to active synapses by the addition of AMPARs (Liao et al., 1995; Liao et al., 2001; Song and Huganir, 2002). Both LTP and LTD have been implicated as cellular mechanisms for learning and memory (Rumpel et al., 2005; Takahashi et al., 2003; Whitlock et al., 2006), and could well be important in the course of some neurodegenerative diseases including Alzheimer's (Hsieh et al., 2006; Kessels and Malinow, 2009; Wei et al., 2010).

Many proteins interact with AMPARs to influence subunit folding, localization and function (Bruneau et al., 2009; Jiang et al., 2006). For example, there are
several PDZ domain containing proteins that bind the PDZ binding motif at the cterm tail of each AMPAR subunit, including GRIP/ABP (Dong et al., 1997; Srivastava et al., 1998), PICK1 (Xia et al., 1999), SAP97 (Colledge et al., 2000), PSD-95 (Beique et al., 2006) and MAGI-1 (Emtage et al., 2009). Other proteins that can interact with AMPARs include NSF (Song et al., 1998), protein 4.1N (Shen et al., 2000), AP-4 (Matsuda et al., 2008), Stargazin (Chen et al., 2000) and Cornichon proteins (Tigaret et al., 2009).

Proteins that are synthesized in cells can be reversibly modified by the addition of various other molecules in a process known as post translational modification (PTM). AMPAR PTMs can affect stability, sub-cellular localization, channel properties, protein interactions and other features (Jiang et al., 2006). Examples of PTMs affecting AMPARs directly include the phosphorylation of GluR1 at residues S818, S831 and S845 (Boehm et al., 2006; Lee et al., 2000) and the phosphorylation of GluR2 at residues Y876 and S880 (Chung et al., 2000; Hayashi and Huganir, 2004). In addition, palmitoylation at cysteine residues (Hayashi et al., 2005; Lin et al., 2009) and ubiquitination (Burbea et al., 2002; Schaefer and Rongo, 2006) may be important.

PTMs on proteins that interact with AMPARs can also affect AMPAR function, sometimes by changing binding affinities. Examples of such PTMs include the S-nitrosylation, and phosphorylation of Stargazin (Chetkovich et al., 2002; Kessels et al., 2009; Selvakumar et al., 2009; Tomita et al., 2005b), the palmitoylation of
PSD-95 (El-Husseini Ael et al., 2002), and the phosphorylation of GRIP/ABP (Kulangara et al., 2007).

**AMPA Receptor Synthesis and Trafficking To Dendrites**

AMPA subunits, like other proteins, have their translation coupled with translocation into the Endoplasmic Reticulum (ER), where they become properly folded and assemble with other subunits to form complete receptors (Greger et al., 2007). Mutations in the pore or ligand-binding domains of AMPAR subunits can cause increased retention in the ER, implying some form of quality control to ensure that only correctly folded and functional AMPARs exit the ER to traffic to the synapse (Grunwald and Kaplan, 2003).

Two pathways that regulate protein folding in the ER are the Endoplasmic Reticulum Associated Protein Degradation (ERAD) pathway and the Unfolded Protein Response (UPR). The ERAD pathway targets misfolded proteins for ubiquitination, retro-translocation into the cytoplasm, and subsequent degradation by the 26S proteasome (Hoseki et al., 2010; Vembar and Brodsky, 2008). The UPR is a related pathway that can up-regulate ER resident folding chaperones as well as increase degradation of misfolded proteins via ERAD (Kohno, 2010). The UPR pathway regulates the amount of AMPARs found at synapses (Shim et al., 2004).
Exit from the ER is also controlled by pathways and proteins other than the UPR. In one case, the lack of functional Stargazin, a protein which associates with AMPARs and is important for their localization and function, has been found to cause an up regulation of the UPR pathway and retention of AMPARs in the ER (Vandenberghe et al., 2005a). Also, for GluR2, the residue at position 607, controlled by RNA editing, can control exit (Q607) or retention (R607) of GluR2 containing heteromers from the ER (Greger et al., 2002).

Upon exit from the ER, correctly folded AMPARs are transported through various Golgi compartments and out to the dendrites for insertion into the postsynaptic membrane. Interestingly, it appears that some mRNAs coding for AMPAR subunits are also transported into dendrites, where they may be locally translated and modified in dendritic ER and Golgi compartments. Thus, additional AMPARs may be synthesized locally as needed during LTP (Hirokawa and Takemura, 2005). Indeed, the abundance and translation of these mRNAs in dendrites is regulated by synaptic activity (Grooms et al., 2006; Ju et al., 2004; Kacharmina et al., 2000).

AMPARs might reach the plasma membrane (PM) at synaptic sites by various means, which may include: insertion into the PM in the soma followed by diffusion to the synapses, insertion at extra-synaptic sites in the dendrites followed by diffusion to synapses, and insertion at synaptic sites (Bruneau et al.,
2009; Makino and Malinow, 2009; Shepherd and Huganir, 2007). If all three of these mechanisms exist in neurons then it is possible that regulation of the trafficking of receptors to distal versus proximal synapses in dendrites may differ in the mechanisms involved.

The subunit composition of AMPARs affects how these receptors are trafficked into or out of synapses (Chang and Rongo, 2005; Lee et al., 2004; Passafaro et al., 2001; Shi et al., 2001). It is likely that in GluR1/GluR2 heteromers, the GluR1 subunit controls insertion, with slow insertion in basal conditions up-regulated by synaptic activity and activation of NMDARs during LTP. For GluR2/GluR3 heteromers, GluR2 is the key subunit and insertion is constitutively fast. Perhaps the substitution of GluR1/GluR2 heteromers for GluR2/GluR3 heteromers provides for long term stability of the changes in density of receptors caused by LTP or LTD.

**AMPA Receptor Anchoring, Endocytosis, Recycling and Degradation**

AMPAReceptors that are incorporated into synapses require interactions with other proteins to anchor them in their proper positions post-synaptically. One such anchoring protein is PSD-95, a PDZ domain containing protein that can bind to Stargazin and other members of the trans-membrane AMPAR regulatory proteins (TARP) family, which in turn bind AMPARs (Chen et al., 2000). The interaction
of TARPs with AMPARs stabilizes AMPARs at the synapse and has been shown to be important for the regulation of the abundance, as well as the function, of AMPARs (Bats et al., 2007; Schnell et al., 2002; Tomita et al., 2005a; Tomita et al., 2003; Vandenberghe et al., 2005b; Walker et al., 2006a).

Endocytosis of synaptic AMPARs can be mediated by clathrin-dependent mechanisms (Burbea et al., 2002; Grunwald et al., 2004; Juo and Kaplan, 2004), as well as by clathrin-independent mechanisms (Glodowski et al., 2007). The clathrin-dependent endocytosis of AMPARs can be positively regulated by another protein, Arc, whose expression levels are increased by strong synaptic activation and which is likely involved in synaptic scaling (Chowdhury et al., 2006; Rial Verde et al., 2006; Shepherd et al., 2006). The internalization of one subunit, GluR2, is also known to require AP2, a clathrin adaptor complex (Lee et al., 2002) which is thought to directly bind to the C-terminal portion of GluR2.

After endocytosis, AMPARs located in the early endosome can either be recycled back to the synapse via a recycling endosome or enter a degradation pathway via late endosomes, multivesicular bodies (MVB), and finally lysosomes. The choice of pathways appears to be partially dependent on synaptic activity level and NMDA receptor activation (Ehlers, 2000, 2003; Park et al., 2004). Up-regulation of recycling, as found in LTP, can lead to both re-insertion of AMPARs at the synapse and to insertion of lipids and proteins needed to expand dendritic spines as part of stabilizing the increase in synaptic strength caused by LTP.
Proteins that may be important for AMPAR recycling in some organisms include Rab8 and RME1 in mammals (Brown et al., 2007; Park et al., 2004), as well as LIN-10 and RAB-10 in *C. elegans* (Glodowski et al., 2007; Glodowski et al., 2005; Rongo et al., 1998; Stricker and Huganir, 2003).

Internalization and subsequent degradation of AMPARs is up-regulated during LTD and can be mediated by proteosome-dependent degradation of associated proteins such as PSD-95 or GRIP (Colledge et al., 2003; Guo and Wang, 2007). Alternatively, AMPAR degradation may be more directly regulated by ubiquitination of the receptors themselves (Burbea et al., 2002; Grunwald et al., 2004; Juo and Kaplan, 2004; Schaefer and Rongo, 2006).

The influence of GluR1 vs. GluR2 on AMPAR trafficking was examined in two studies. In one it was found that GluR1/2 receptors are added to synapses in an activity-dependent manner requiring interactions between GluR1 and group I PDZ-containing proteins. By contrast, GluR2/3 receptors replace existing receptors continuously only at non-silent synapses and require interactions by GluR2 with NSF & group II PDZ-containing proteins. A proposed model posited that under basal conditions there is an equilibrium between synaptic and non-synaptic receptors that is maintained by GluR2/3 receptors cycling in and out of synapses. The number of receptors is determined by the available putative “slot proteins”, which are proteins hypothesized to be placeholders at the synapse for later insertion of receptors. LTP induction inserts GluR1/2 receptors, dependent
on the GluR1 cterm. This also inserts more “slots”, which then sets a new equilibration level of receptor number, maintained by a receptor exchanging and recycling mechanism in which GluR2/3 receptors can replace GluR1/2 receptors. The subsequent recycling process mediated by GluR2/3 requires the GluR2 cterm (Shi et al., 2001). Similarly, in the second report, GluR1 insertion occurs extra-synaptically and slowly in an activity driven manner, whereas GluR2 exocytosis is constitutively rapid and occurs directly at synapses. Thus, GluR1 controls the exocytosis and GluR2 controls the recycling and endocytosis of AMPA receptors. Therefore, GluR1/2 insertion may be important in LTP, whereas GluR2/3 removal is essential for LTD (Passafaro et al., 2001).

**Phosphorylation Can Regulate AMPA Receptor Function and Trafficking**

**Phosphorylation Can Reversibly Affect Proteins In Various Ways**

Protein function is often regulated by reversible post translational modifications (PTMs), including the acetylation, methylation or ubiquitination of lysine, the methylation of arginine, the hydroxylation of proline and the phosphorylation of serine, threonine or tyrosine. Phosphorylation was the first PTM to be studied extensively (Cohen, 2002).

The phosphorylation state of a protein can have direct and indirect effects on sub-cellular localization, binding partners, degradation, and enzymatic activity.
For example, the addition of a negatively charged phosphate group can directly alter the three-dimensional structure of a protein, thereby changing its enzymatic activity. Alternatively, phosphorylation of one protein might create a binding site for one of several proteins with phosphate-binding domains (PBDs) (Pawson and Scott, 2005).

Protein phosphorylation is catalyzed by protein kinases, of which there are over 500 in human cells. Phosphate groups are removed by protein phosphatases, 120 of which have been identified in human cells (Manning et al., 2002). Protein kinases generally are either serine/threonine or tyrosine kinases, which often recognize specific amino acid consensus sequences in their substrates (Kennelly and Krebs, 1991). The kinases themselves are often regulated by phosphorylation, which may be auto-phosphorylation or phosphorylation by other kinases in kinase cascades. In addition, it is not uncommon to have a kinase linked to its substrate by a scaffolding protein, which increases the specificity of phosphorylation (Pawson and Scott, 2005). Finally, one or more kinases can target a substrate such that a specific effect on the protein is only achieved after phosphorylation of multiple residues, either simultaneously or in series (Chen et al., 1999).
Phosphorylation of AMPA Receptors Regulates Function and Trafficking

AMPA receptors (AMPARs) are regulated by phosphorylation of their C-terminal (cterm) domains, which extend into the cytoplasm of neurons. It appears likely that the kinases and phosphatases involved in regulating the phosphorylation state of AMPARs, as well as the effect of phosphorylation on synaptic plasticity, varies based on cell type and brain regions (Shepherd and Huganir, 2007). While much work remains, there is increasing evidence that synaptic plasticity, as partially mediated by the phosphorylation state of AMPARs, is important for various forms of learning and memory as well as addiction and neurological diseases (Kessels and Malinow, 2009).

The fact that hippocampal Long Term Potentiation (LTP) is dependent on calcium flux into neurons via NMDAR activation led to early studies which indicated a role for Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) in LTP (Malenka et al., 1989; Malinow et al., 1989) and showed that CaMKII phosphorylates GluR1 \textit{in vitro} (McGlade-McCulloh et al., 1993). Subsequently, Serine 831 (S831) on the cterm of GluR1 was found to be phosphorylated by CaMKII at a site that differs from the typical CaMKII consensus sequence, which possibly allows for more fine regulation by requiring scaffolding proteins to cause the kinase to effectively interact with GluR1 (Barria et al., 1997a; Mammen et al., 1997). This phosphorylation was shown to be important for LTP (Barria et al., 1997b). Interestingly, the accumulation of Abeta1-42, which is linked to cognitive
impairment in Alzheimer's, inhibits dentate gyrus LTP and CaMKII phosphorylation of GluR1 S831 (Zhao et al., 2004), demonstrating a possible link between phosphorylation-mediated AMPAR trafficking and neurological diseases.

Protein Kinase A (PKA) also specifically phosphorylates GluR1 S831 and S845 (Roche et al., 1996). Phosphorylation of GluR1 by PKA at S845 and by CaMKII at S831 is necessary for synaptic localization (Esteban et al., 2003). The phosphorylation of GluR1 by PKA can be mediated by recruitment of both the receptor and kinase through an interaction between PDZ domain containing proteins PSD-95 and SAP97, and AKAP79/150 (Colledge et al., 2000). Importantly, the internalization of AMPARs from the PSD to early endosomes is triggered by NMDAR activation, which is regulated by PKA and accompanied by de-phosphorylation and re-phosphorylation of GluR1 by PKA. By contrast, activation of AMPARs without NMDAR activation targets AMPARs to late endosomes and lysosomes, independent of protein phosphatases or PKA (Ehlers, 2000).

The physiological significance of GluR1 phosphorylation has been further shown in mice with knockin (KI) mutations in the GluR1 phosphorylation sites (S831, S845) which showed deficits in LTD and LTP, and memory defects in spatial learning tasks (Lee et al., 2003). Also, phosphorylation at S845 in GluR1 containing AMPARs correlates with chemically induced LTP, changes in the
surface expression of AMPARs, and selective delivery of AMPARs to extra synaptic sites (Oh et al., 2006). Phosphorylation of GluR1 S845 by PKA is required for increased cell-surface expression by promoting receptor insertion and decreasing receptor endocytosis, while de-phosphorylation at S845 was found to trigger NMDAR activation induced AMPAR internalization (Man et al., 2007).

Interestingly, phosphorylation of GluR1 at S831 and S845 is reversible and depends upon the state of the synapse. LTD induction in naive synapses correlates with de-phosphorylation of the PKA site S845, while in potentiated synapses the CaMKII site S831 is de-phosphorylated. LTP induction in naive and depressed synapses increases phosphorylation of S831 and S845, respectively (Lee et al., 2000). A model advanced to explain this suggests that, in naive synapses, LTP inducing stimuli leads to activated CaMKII, which phosphorylates S831, resulting in LTP, whereas LTD inducing stimuli activates protein phosphatases (including PP1/2A), which de-phosphorylates S845. In previously potentiated synapses, LTD inducing stimuli causes de-phosphorylation of S831 and in previously depressed synapses, LTP inducing stimuli causes phosphorylation of S845 (Lee et al., 2000). Presumably the actual trafficking of AMPARs into or out of the post-synaptic density (PSD) in each situation is mediated by accessory proteins that aid their internalization from or re-insertion into the PSD.
The phosphorylation state of GluR1 affects not only AMPAR trafficking, but also the channel properties of AMPARs. For example, PKA-mediated phosphorylation at S845 can result in a 40% potentiation of the peak whole cell glutamate gated current through GluR1 homomeric channels (Roche et al., 1996) as well as regulating open channel probability (Banke et al., 2000). Likewise, GluR1 phosphorylation by CaMKII at S831 increases single-channel conductance (Derkach et al., 1999; Derkach, 2003).

Recent studies identified additional GluR1 residues that are phosphorylated as well as alternative kinases. Phosphorylation of GluR1 by PKC at the S818 residue is critical for LTP expression, and promotes GluR1 synaptic incorporation (Boehm et al., 2006). Also, cGMP-dependent protein kinase II (cGKII) phosphorylates GluR1 at S845. Activation of cGKII by cGMP increases the surface expression of AMPARs at extra synaptic sites, whereas inhibition of cGKII blocks the surface increase of GluR1 during LTP and reduces LTP in the hippocampal slice (Serulle et al., 2007).

In addition to GluR1, other AMPAR subunits are also phosphorylated. For example, GluR4, which is widely expressed early in development, is known to be phosphorylated (Carvalho et al., 1999; Esteban et al., 2003), whereas relatively little is known about phosphorylation of GluR3. GluR2 is phosphorylated by PKC at S880 (Matsuda et al., 1999) and S863 (McDonald et al., 2001), and PKC
phosphorylation of GluR2 S880 is a critical event in the induction of cerebellar LTD (Chung et al., 2003).

**Proteins Interacting With AMPA Receptors and Their Phosphorylation**

A number of proteins interact with AMPARs. Such proteins often contain PDZ domains, some of which are subunit specific (Bruneau et al., 2009; Jiang et al., 2006). One of these proteins is PDZ domain-containing Protein interacting with C kinase (PICK1), which interacts with and clusters AMPARs (Xia et al., 1999). Similarly, the PDZ binding motif in the cterm of GluR2 also interacts with GRIP (glutamate receptor interacting protein), which has seven PDZ domains (Dong et al., 1997), and a related protein, AMPA receptor-binding protein (ABP) (Srivastava et al., 1998). The association of GluR2 with ABP and/or GRIP but not PICK1 is needed to maintain synaptic surface receptors, in a way that is dependent on S880 (Osten et al., 2000).

Phosphorylation of GluR2 S880 decreases GluR2 binding to GRIP1 but not to PICK1, and thus PKC stimulation in neurons results in rapid internalization of surface GluR2 subunits by exchanging GRIP for PICK1 (Chung et al., 2000; Matsuda et al., 1999). LTD induction increases S880 phosphorylation, which suggests that the phosphorylation state of GluR2 S880 regulates GluR2 containing AMPAR internalization during LTD by differentially regulating GluR2
binding to GRIP/ABP or PICK1 (Kim et al., 2001). This is supported by the fact that mimicking GluR2 S880 phosphorylation excludes these receptors from synapses, depresses transmission, and partially occludes LTD (Seidenman et al., 2003), and that mutation of PICK1 eliminates cerebellar LTD in mice (Steinberg et al., 2006). In addition, phosphorylation of GluR2 tyrosine 876 (Y876) by Src family tyrosine kinases decreases GluR2 association with GRIP1/2 without affecting interaction with PICK1 and is important for internalization of GluR2 containing receptors (Hayashi and Huganir, 2004).

A likely mechanism to explain the above involves the BAR domain of PICK1, acting as an auto inhibitory domain, which can interact with ABP/GRIP as well as with the PDZ domain of the same PICK1 molecule. Binding of PKC or GluR2 to the PICK1 PDZ domain disrupts the PICK1 internal interaction and facilitates the PICK1 BAR domain association with ABP/GRIP (Lu and Ziff, 2005). Interference with the PICK1-ABP/GRIP interaction impairs S880 phosphorylation of GluR2 by PKC, and decreases the constitutive surface expression of GluR2, the NMDA-induced endocytosis of GluR2, and recycling of internalized GluR2.

The proposed model is that PKC binds PICK1, disrupting the PICK1 PDZ/BAR interaction, followed by the BAR domain targeting the complex to the GRIP-GluR2 complex. Following this, PKC phosphorylates GluR2 S880, which hinders its binding to GRIP, but still allows the binding of PICK1 which in turn might target (via the BAR domain) the PICK1-GluR2 complex to curved (pre-
budding?) membrane areas (Lu and Ziff, 2005). This is likely not the entire story since GluR2 can interact with other molecules, including N-ethylmaleimide-sensitive fusion protein (NSF) which regulates synaptic transmission (Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998) and negatively regulates receptor endocytosis (Braithwaite et al., 2002) at least partly via disrupting PICK1-GluR2 interactions in this complex (Hanley et al., 2002).

Other proteins can also interact with AMPAR subunits and are often themselves subject to PTMs. One such AMPAR-interacting protein is Stargazin, which regulates AMPARs in a way that is dependent on binding to the PDZ domain containing scaffolding protein PSD-95 (Chetkovich et al., 2002). Synaptic activity can induce both Stargazin phosphorylation, via activation of CaMKII and PKC, and Stargazin de-phosphorylation, by activation of a phosphatase in a way that is required for LTP and LTD respectively (Tomita et al., 2005b). Stargazin was the first member found of a family of trans-membrane AMPAR regulatory proteins (TARPs). The TARPs are differentially expressed in various cell types and brain areas and mediate surface expression of AMPARs (Tomita et al., 2003). TARPs are stable at the plasma membrane (PM) even when AMPARs are internalized in a glutamate-regulated manner (Tomita et al., 2004). Thus, they may work together with PSD-95 to serve as placeholders for AMPARs in the PSD. While the cytoplasmic tail of Stargazin regulates receptor trafficking, the ectodomain controls channel properties, affecting synaptic responses by increasing the rate of channel opening (Tomita et al., 2005a).
Recent work shows that Stargazin is phosphorylated on up to 9 serines, which then decreases its interaction with the PM, thereby stimulating binding to PSD-95, which stabilizes AMPARs at the PSD (Sumioka et al., 2010). The physiological role of Stargazin phosphorylation demonstrates the importance for AMPAR regulation of not only AMPAR phosphorylation, but of proteins with which AMPARs interact.

A cytoskeletal protein, 4.1N, also interacts with GluR1 in vivo and colocalizes with AMPARs at excitatory synapses. Disruption of the interaction of GluR1 with 4.1N, or disruption of actin filaments, decreased the surface expression of GluR1, suggesting that protein 4.1N links AMPARs to the actin cytoskeleton (Shen et al., 2000). Additional work suggests that another AMPAR associated PDZ domain containing protein, Sap97, may also be involved (Daw et al., 2000). More recent work shows that 4.1N is required for activity-driven GluR1 insertion, and that PKC phosphorylation of GluR1 S816 and S818 enhanced 4.1N binding and facilitated GluR1 insertion. In addition, disrupting 4.1N dependent GluR1 insertion decreased the expression of LTP (Hayashi et al., 2005; Lin et al., 2009).
Ubiquitination Can Regulate AMPA Receptor Trafficking

Ubiquitination Pathways and Their Biological Role

Ubiquitination is another well studied PTM that can affect AMPAR trafficking. Ubiquitination is the reversible addition of a small 76 amino acid protein, ubiquitin, to the ε-amino group of a substrate lysine (K) residue, via a covalent isopeptide bond to the C-terminal glycine of ubiquitin. Ubiquitination was first found to regulate the proteosomal-dependent degradation of substrates, but is now known to have wide ranging effects on modified substrates (Hershko and Ciechanover, 1998).

Ubiquitination occurs via a three step enzymatic cascade, beginning with ATP-dependent activation of ubiquitin by an E1 ubiquitin activating enzyme, which results in formation of a thioester linkage between ubiquitin and the E1. Next, the ubiquitin is transferred to the active site cysteine of an E2 ubiquitin conjugating enzyme. Finally, an E3 ubiquitin ligase functions either autonomously or in concert with the E2 to recognize a specific substrate and transfer the ubiquitin to the substrate. An isopeptide bond is then formed between the C-terminus of ubiquitin and an acceptor lysine in the substrate. There are various types of E3 ligases including those that contain RING (Really Interesting New Gene), U-box or HECT (homologous with E6-associated protein
C-terminus) domains. E3 ligases can be single polypeptides or multiple subunit proteins (Hershko and Ciechanover, 1998).

The human genome encodes two E1 enzymes, 37 E2 enzymes and over 600 E3 ligases (Komander, 2009). Protein ubiquitination is reversible, and about 85 deubiquitinases, specialized proteases that act on ubiquitin, are found in the human genome. Some deubiquitinases are specific in recognizing and cleaving off individual ubiquitins or various types of poly-ubiquitin chains from substrates (Komander et al., 2009).

As with phosphorylation, ubiquitination can affect substrate proteins by changing how the target protein interacts with other proteins that contain ubiquitin binding domains (UBDs). Ubiquitinated proteins are recognized by at least 20 specialized UBD proteins, which often have specificity for mono-ubiquitin vs. poly-ubiquitin. In addition, there can be specificity as to the type of poly-ubiquitin chain, discriminating between K48 linked and K63 linked chains or other linkages (see below for more information on these linkages) (Dikic et al., 2009; Hurley et al., 2006).

In addition there are a number of ubiquitin like (UBL) molecules in humans and other organisms, including SUMO (small ubiquitin-related modifier) 1/2/3 and Nedd8 (neural-precursor-cell-expressed developmentally down-regulated 8) (Geiss-Friedlander and Melchior, 2007; Kirkin and Dikic, 2007). UBL
modification also occurs on the lysines of substrates, and at times this can act as a switch such as in the case of IκB (inhibitor of NF-κB) in which sumolation can occur at an IκB lysine that is also subject to ubiquitination (Komander, 2009).

Mono-ubiquitination on a single substrate lysine or on multiple lysines in the same protein (multi-mono-ubiquitin) can have varied physiological results. One study found that a single ubiquitin appended to a trans-membrane receptor (an alpha-factor receptor in yeast) was sufficient to mediate internalization of the receptor (Shih et al., 2000). Another type of transmembrane receptor, receptor tyrosine kinases, can undergo internalization and subsequent recycling to the surface or degradation in lysosomes regulated by the multi-mono-ubiquitin modification (Acconcia et al., 2009).

The earliest studies on ubiquitination found that degradation via the proteasome is initiated not by the addition of a single ubiquitin to a substrate, but rather by the addition of a chain of ubiquitins (poly-ubiquitin). A chain of four ubiquitins, linked through the lysine 48 (K48-poly-ubiquitin), is sufficient to target the substrate to the proteasome for degradation (Pickart, 2000). However, the K48 residue is only one of seven lysine residues found in ubiquitin and it turns out that poly-ubiquitin chains can be formed using any of the 7 lysines present in ubiquitin. The first of the other lysine modifications to be characterized was K63 linked poly-ubiquitin chains, early on discovered to enhance the endocytosis of a yeast membrane protein (uracil permease) (Galan and Haguaener-Tsapis,
1997). Since then, K63 linked poly-ubiquitin has been found to be important in a number of non-degradative roles, including endocytosis, the DNA damage response pathway, NF-kB signaling, modification of chromatin via histone poly-ubiquitin, and routing ubiquitinated receptors through the multi-vesicular body (MVB) / lysosomal pathway via “endosomal sorting complex required for transport” (ESCRT) components (Chen and Sun, 2009; Komander, 2009; Mukhopadhyay and Riezman, 2007).

In addition to K48 and K63 linked poly-ubiquitin, other linkages are also found in vivo. In a recent study, mass spectrometry was used to analyze the ubiquitination of yeast proteins and the types of poly-ubiquitin chains attached to them. Results showed that K48 linkages accounted for 29% of all ubiquitin linkages (possibly under-represented since this linkage often leads to quick degradation), followed by K11 (28%), K63 (17%), K6 (11%), K27 (9%), K29 (3%), and K33 (3%) (Xu et al., 2009). Relatively little is known about the in vivo significance of poly-ubiquitin chains linked through ubiquitin residues other than K48 or K63, but this will likely change as further studies are done. Linear poly-ubiquitin chains can also be assembled, linked end to end with a peptide bond, rather than an isopeptide bond by the E3 LUBAC (linear ubiquitin chain assembly complex) (Kirisako et al., 2006). Recently there is also evidence for ubiquitin chains with mixed linkages which alternate linkage types in a poly-ubiquitin chain or have branched ubiquitin chains (Kim et al., 2007b; Kirkpatrick et al., 2006).
**Ubiquitination and Phosphorylation: Similarities and Crosstalk**

Ubiquitination and phosphorylation, which are very different covalent PTMs, share a number of similarities as well as differences in mechanisms and physiological effects. Both modifications are covalent, reversible, have enzymes attaching the modification present in a 5-6 fold excess over those removing it, and can affect protein interactions via specialized binding domains in other proteins that recognize modified proteins. While phosphorylation can occur at a greater diversity of amino acids (most commonly serines, threonines and tyrosines), ubiquitination occurs only on lysine residues and has the additional level of complexity of occurring as polymeric chains, linked through any of seven different lysines within the ubiquitin molecule (Komander, 2009).

Interestingly, there is growing evidence of cross talk between forms of PTMs in regulating protein function and stability. This cross talk can be both positive, in which one PTM becomes a signal for a second PTM, and negative, in which one PTM can inhibit another PTM. For example, phosphorylation of a residue may decrease or increase the likelihood of recognition by an ubiquitin E3 ligase. A phosphorylated sequence that is preferentially recognized by an E3 ligase is referred to as a phosphodegron. Also, phosphorylation can directly affect E3 ligase enzymatic catalytic activity and thereby increase or decrease the ubiquitination of the targets of that ligase (Hunter, 2007).
An example of negative regulation of ubiquitination by phosphorylation is the phosphorylation of beta-catenin by PKA, which can inhibit its ubiquitination and thereby stabilize beta-catenin (Hino et al., 2005). In contrast to this, phosphorylation of Ste2p in yeast has been shown to promote internalization and down regulation of this protein via an increase in Ste2p ubiquitination (Hicke et al., 1998).

Likewise, ubiquitination can influence the phosphorylation state of a protein. This is often negative, where ubiquitination causes protein kinase internalization or degradation. However, ubiquitination of a protein kinase can also activate it, such as in the NF-κB pathway, in which TRAF6 catalyzes addition of K63 linked poly-ubiquitin chains, which then recruits TAK1 kinase, leading to the phosphorylation of TAK1 and IKKβ by TAK1. This phosphorylation then results in the degradation of IκB and the activation of NF-κB (Skaug et al., 2009).

UEV Family Proteins: Biological Roles and Mechanisms of Action

Discovery and Structure of UEV Proteins

As discussed previously, ubiquitination was first thought to occur through K48 linked poly-ubiquitin chains. Such linkages lead to degradation of the
ubiquitinated protein. The subsequent studies that found alternative forms of ubiquitination included the discovery of K63 linked poly-ubiquitin chains. These K63 linked chains were discovered to enhance the endocytosis of a yeast membrane protein (uracil permease) more than mono-ubiquitin of the same protein (Galan and Haguenauer-Tsapis, 1997).

About the same time as the discovery of a physiological role for K63 linked poly-ubiquitin chains, the Ubiquitin E2 Variant (UEV) family proteins were discovered. The UEV proteins are homologous to ubiquitin E2 conjugating enzymes, but lack the cysteine in the active site to which ubiquitin is normally conjugated. Thus, UEV proteins are not by themselves catalytically active (Sancho et al., 1998). Yeast UEV protein MMS2 was found to have two human homologs, hMMS2 and hUEV1A (with similar names such as Uev1 in other systems), and is well conserved, being also found in widely diverse organisms such as plants and protists (Andersen et al., 2005; Villalobo et al., 2002; Wen et al., 2008a; Xiao et al., 1998). Early roles found for Uev1 included control of cell differentiation and DNA damage repair regulation (Broomfield et al., 1998; Sancho et al., 1998; Thomson et al., 1998; Xiao et al., 1998). While there are some physiological roles for UEV domain containing proteins functioning independently, the primary physiological roles of Uev1 relate to its ability to form a heterodimer with an active E2 ligase, Ubc13, and thereby catalyze the formation of K63 linked poly-ubiquitin chains (Hofmann and Pickart, 1999; Pickart, 2000, 2004).
A number of structural studies have revealed how Uev1 and Ubc13 interact with each other, and with ubiquitin molecules. One important study found that the heterodimer has the active site of Ubc13 at the intersection of two channels that are potential binding sites for the two substrate ubiquitins. Mutations, including one changing the 8th Phe in Uev1 to Ala (F8A) disrupted heterodimer binding and function (VanDemark et al., 2001). Other studies confirmed the importance of key residues in the heterodimer interface and showed that Uev1 is important for correctly positioning a second ubiquitin in a chain to ensure use of the K63 ubiquitin residue (McKenna et al., 2001; Moraes et al., 2001; Pastushok et al., 2005).

**Biological Roles of UEV Proteins and K63 Linked Poly-Ubiquitination**

There are a wide variety of functions reported for Uev1, including a role in regulating cell signaling (in the NF-kB Pathway), DNA Damage Tolerance, lysosomal transport, and regulation of autophagy (Broomfield et al., 1998; Deng et al., 2000; Duncan et al., 2006; Hofmann and Pickart, 1999; Sundquist et al., 2004; Tan et al., 2007; Thomson et al., 1998). In addition to these, which are discussed further below, Uev1/Ubc13 dimers are important for modulating the frequency of DNA homologous recombination (Ertongur et al., 2010). Not surprisingly, a number of ubiquitin E3 ligases have been found to work with
Uev1/Ubc13 dimers to catalyze addition of K63 linked poly-ubiquitin chains to various substrates. These E3 ligases include BRCA1, RNF8, CHIP, TRAF2, TRAF6, and Parkin (Christensen et al., 2007; Lim et al., 2005; Plans et al., 2006; Skaug et al., 2009; Zhang et al., 2005)

Stalled DNA replication forks activate post-replication repair pathways that bypass DNA damage, which helps to prevent double strand breaks. In yeast, proliferating cell nuclear antigen (PCNA) is mono-ubiquitinated by the Rad6/Rad18 complex, leading to an error prone repair of the damaged DNA. PCNA can also be further poly-ubiquitinated by the Ubc13/Mms2/Rad5 complex, which adds a K63 linked poly-ubiquitin chain to PCNA and directs a different pathway known as error-free damage avoidance, using the sister chromatid as a template to bypass DNA damage (Lee and Myung, 2008). This pathway was one of the first discovered roles for Uev1 (Hofmann and Pickart, 1999; Ulrich and Jentsch, 2000), and is also found to be conserved in humans and other organisms (Chiu et al., 2006; Hoege et al., 2002; Moldovan et al., 2007; Unk et al., 2008; Unk et al., 2006; Wen et al., 2008a). Rad5 functions as an ubiquitin E3 ligase in this pathway, although another ubiquitin E3 ligase, RNF8, is at times found to work with Uev1/Ubc13 (Kolas et al., 2007; Plans et al., 2006; Wang and Elledge, 2007; Zhang et al., 2008). Interestingly, it has recently been shown that K63 linked poly-ubiquitin of histones may also be important in regulating the response to DNA damage (Pinato et al., 2009).
A key role for UEV proteins is that of regulating transport of proteins, including internalized membrane receptors, to the lysosome for degradation. Often, UEV-mediated trafficking occurs via the endosomal sorting complex required for transport (ESCRT). This complex is important for routing internalized ubiquitinated receptors from the early endosome to the multi-vesicular body (MVB) and onto the endosome, and this complex includes a subunit (Vps23) that contains a UEV domain (Malerod and Stenmark, 2007; Raiborg and Stenmark, 2009).

K63 linked poly-ubiquitin chains, perhaps created via the Uev1/Ubc13 heterodimer, are important for the lysosomal targeting of various proteins including the aquaporin-2 water channel, MHC class I molecules and other membrane surface proteins (Barriere et al., 2007; Duncan et al., 2006; Kamsteeg et al., 2006; Varghese et al., 2008). In particular, K63 linked poly-ubiquitin chains are an important trigger to allow optimal rate endocytosis and lysosomal routing for membrane transporters (permeases) in yeast (Barriere et al., 2006; Galan and Hagueuauer-Tsapis, 1997; Hawryluk et al., 2006; Hicke and Dunn, 2003; Lauwers et al., 2010; Lauwers et al., 2009; Paiva et al., 2009).

Another important known role for Uev1 is in the nuclear factor kappa enhancer binding protein (NF-κB) pathway, which is important for cellular response to microbial infection and exposure to cytokines. NF-κB transcription factor subunits form dimers that are retained in the cytoplasm by IκB proteins
under basal conditions. Stimulation of cells with various agonists leads to a cascade of IκB phosphorylation, poly-ubiquitination, and proteosomal degradation, which frees NF-κB dimers to translocate to the nucleus and regulate transcription of target genes. In the canonical NF-κB pathway, stimulation of the TNF receptor (TNFR), IL-1 receptor (IL-1R), and Toll-like receptors (TLRs) leads to activation of the transforming growth factor-β-activated kinase-1 (TAK1) complex through TNF receptor-associated factors (TRAF) proteins.

TRAF proteins contain an N-terminal RING domain and are ubiquitin ligases. TRAF6 can, in conjunction with Uev1/Ubc13, catalyze synthesis of K63 linked poly-ubiquitin chains onto itself, other substrates in the pathway, and also free chains in the cytoplasm. These K63 linked chains function as a scaffold to recruit the TAK1 and IKK complexes by binding the regulatory subunits TAB2 and NEMO. The kinase complexes phosphorylate TAK1 and IKKβ, leading to phosphorylation of IκB, which in turns causes IκB to be K48 linked poly-ubiquitinated and subsequently degraded by the proteosome. The K63 linked poly-ubiquitin chains formed by TRAF6/Uev1/Ubc13 can be disassembled by K63 specific deubiquitination enzymes including CYLD and A20. In this, as well other NF-κB pathways, the role of Uev1/Ubc13 in the formation of K63 linked poly-ubiquitin chains on various substrates is a key part of the pathway (Conze et al., 2008; Deng et al., 2000; Fan et al., 2010; Skaug et al., 2009; Sun and Chen, 2004; Syed et al., 2006; Wertz et al., 2004; Wu et al., 2006; Xia et al., 2009; Zhou et al., 2004).
Finally, several studies have linked Uev1/Ubc13 produced K63 poly-ubiquitin chains with both the formation of aggregations of misfolded proteins, important in several neurodegenerative diseases, and with their clearance via autophagy (Chin et al., 2010). For example, Parkin (a ubiquitin E3 ligase) works together with Uev1/Ubc13 to form K63 linked poly-ubiquitin chains on several proteins which are found in aggregations of misfolded proteins linked with Parkinson’s disease (Lim et al., 2005; Olzmann and Chin, 2008; Olzmann et al., 2007). Also, in a *C. elegans* study it was found that RNAi of UBC-13 or UEV-1 leads to a reduction of polyglutamine protein aggregations, suggesting that the Uev1/Ubc13 heterodimer may be important in regulating these aggregations, which in humans is linked to Huntington’s disease (Howard et al., 2007).

**Regulation of AMPA Receptor Trafficking By Ubiquitination**

No published studies link UEV family proteins or K63 linked poly-ubiquitin with the regulation of AMPAR trafficking. However, AMPAR trafficking is generally known to be regulated by ubiquitination. It is clear that the ubiquitin proteosome system (UPS) is important for activity-dependent changes affecting synaptic signaling (Ehlers, 2003), but it is not yet known if ubiquitination of the receptors themselves is important for trafficking in mammals. However, internalization and subsequent degradation of AMPARs is up regulated during LTD and can arise by
proteosome-dependent degradation of associated proteins such as PSD-95 or GRIP (Colledge et al., 2003; Guo and Wang, 2007).

**C. elegans as A Model for Studying AMPA Receptor Trafficking**

**GLR-1 Is a C. elegans AMPA Type Glutamate Receptor Subunit**

*Caenorhabditis elegans* has homologs to a number of glutamate receptor subunits (Brockie et al., 2001; Brockie and Maricq, 2003; Sprengel et al., 2001). These include the AMPAR subunits GLR-1 (Hart et al., 1995; Maricq et al., 1995) and GLR-2 (Mellem et al., 2002). Studies of GLR-1 function have employed both genetic tools, readily available for this model organism, as well as the use of a functional GLR-1::GFP fusion (Rongo et al., 1998) that allows visualization of GLR-1 localization in a live animal. In addition, GLR-1 has been shown to function in a neuronal circuit that controls the forward vs. backward movement of the animal. Therefore, measuring the rate of spontaneous reversals is a behavioral measure that correlates with the amount of functional GLR-1 containing receptors found at synapses in *C. elegans* (Zheng et al., 1999).

In addition to homologs for AMPARs, *C. elegans* also has proteins that are similar in sequence to various proteins important for AMPAR function or implicated in neurodegenerative diseases, including Stargazin and Parkin.
(Springer et al., 2005; Walker et al., 2006a; Wang et al., 2008). There are also *C. elegans* homologs to many mammalian enzymes involved in PTMs such as phosphorylation (Juo et al., 2007; Reiner et al., 1999; Rongo and Kaplan, 1999; Sakaguchi et al., 2004; Sieburth et al., 2007; Umemura et al., 2005) and ubiquitination (Gudgen et al., 2004; Jones et al., 2001).

**GLR-1 Is Regulated By Similar Mechanisms as Mammalian Receptors**

As with mammalian AMPARs, GLR-1 trafficking is regulated by multiple factors including the processes of endocytosis, recycling and degradation. The C-terminal “tail” (cterm) of GLR-1 is important for trafficking (Chang and Rongo, 2005), and, as discussed further below, the ubiquitination of this area helps to regulate GLR-1 localization (Burbea et al., 2002; Grunwald et al., 2004; Juo and Kaplan, 2004; Park et al., 2009; Schaefer and Rongo, 2006).

Phosphorylation is also at least somewhat important for GLR-1 trafficking since CaMKII was found to regulate GLR-1 by regulating transport of GLR-1 from cell bodies to neurites and by regulating the addition to or maintenance of GLR-1 at the post synaptic area (Rongo and Kaplan, 1999). In addition, a recent study has shown that a kinase, CDK-5, is indirectly important for the trafficking of GLR-1 (Juo et al., 2007).
Various accessory proteins have been found to be important for GLR-1 localization, including a Stargazin homolog, and SOL-1 (Walker et al., 2006a; Walker et al., 2006b; Wang et al., 2008; Zheng et al., 2006; Zheng et al., 2004). LIN-10, a homolog of mammalian Mint proteins, and RAB-10, have been found to be important in controlling the recycling of GLR-1 from early endosomes back to the synapse by pathways downstream, respectively, of clathrin-dependent and independent endocytosis (Glodowski et al., 2007; Glodowski et al., 2005; Rongo et al., 1998; Stricker and Huganir, 2003). RAB-2 may also be involved in this process (Chun et al., 2008). Recently, an S-SCAM protein, MAGI-1, was found to be important for the regulation of GLR-1 localization in response to mechanical stimulation (Emtage et al., 2009).

The sequence homology of many C. elegans neuronal proteins to mammalian proteins, the abundance of genetic and other tools, and the short generation time of C. elegans makes it ideally suited to address some of the questions concerning the effect of ubiquitination and phosphorylation on AMPAR trafficking.

**GLR-1 Is Regulated By Ubiquitination**

As in many other organisms there is only one E1 ubiquitin activating enzyme in C. elegans, over 20 E2 ubiquitin conjugating enzymes and a large number of E3 ubiquitin ligase enzymes (Gudgen et al., 2004; Jones et al., 2001). The
interactions of many of these have been studied using yeast two hybrid assays (Gudgen et al., 2004).

Several studies done in *C. elegans* give considerable information on the role of ubiquitination in the trafficking of AMPARs (Hart et al., 1995; Maricq et al., 1995). One of the earliest of these studies used a version of GLR-1 (GLR-1:4KR) in which all four C-terminal lysines were mutated to arginines, eliminating the possibility of direct ubiquitination of GLR-1. It was found that in animals expressing GLR-1:4KR there is an increase in the number and size of GLR-1 puncta along the ventral cord because of decreased endocytosis (Burbea et al., 2002). In the same study over-expression of ubiquitin under the *glr-1* promoter decreased GLR-1 abundance. Both the effects of over-expressed ubiquitin and of the GLR-1:4KR construct were partially prevented by mutations in the *unc-11* gene, which encodes a clathrin adaptor protein, AP180. These results suggest that direct ubiquitination of AMPARs can regulate their internalization, and that clathrin-dependent endocytosis is at least partially required for this internalization (Burbea et al., 2002). A subsequent study in the same lab found that clathrin mediated endocytosis, but not GLR-1 ubiquitination, is required for compensatory regulation of GLR-1 glutamate receptors after activity blockade (Grunwald et al., 2004).

It was also found that in animals mutant for the APC E3 ubiquitin ligase complex there was an increased abundance of GLR-1 at synapses. Mutations
blocking clathrin mediated endocytosis could block the effects of APC loss of function, indicating APC is important for GLR-1 endocytosis or recycling. However, since APC mutations could not block the decrease in synaptic GLR-1 caused by over-expressed ubiquitin it appears likely that the effect of APC is not via direct ubiquitination of GLR-1 (Juo and Kaplan, 2004).

Interestingly, a *C. elegans* strain lacking the BTB-Kelch protein, KEL-8, also shows increased GLR-1 levels at synapses which was found to be required for ubiquitin mediated GLR-1 turnover. These *kel-8* mutants also had an increased frequency of spontaneous reversals in locomotion, which in *C. elegans* is an indicator of increased levels of synaptic strength for GLR-1 expressing interneurons. An ubiquitin E3 ligase subunit, CUL-3, mediates GLR-1 turnover and binds KEL-8, implying that ubiquitin-dependent internalization and degradation is at least partially dependent on the SCF ubiquitin ligase complex (Schaefer and Rongo, 2006).

In a recent report it was also found that in a *C. elegans* mutant lacking function of another ubiquitin E3 ligase, RPM-1, there is an increased accumulation of GLR-1. However, in this case the accumulations were found to be endosomal, and caused by repression of the protein levels of the MAPKKK DLK-1, which is part of the PMK-3/p38 MAPK pathway in interneurons. The repression of DLK-1 is a consequence of its ubiquitination by RPM-1, which normally leads to its degradation (Park et al., 2009).
Clearly ubiquitination plays a role in directing the trafficking of AMPARs. In addition it is likely that direct ubiquitination of the AMPAR subunits, perhaps via a number of ubiquitin E3 ligases regulating different pathways, is important. To date, much of what we know about the physiological role of ubiquitination in AMPAR trafficking has been provided by studies using *C. elegans*.

The goals of our research were to better understand how phosphorylation and ubiquitination regulate GLR-1 trafficking. We found that phosphorylation of GLR-1 may act as a phosphodegron, effectively a marker for where ubiquitination is to occur, and thereby indirectly regulate GLR-1 localization. This could add to the already known roles for phosphorylation in regulating AMPAR trafficking. Following this we looked into the function of a UEV protein in *C. elegans* which we found regulates GLR-1, in a way that is likely dependent on K63 linked poly-ubiquitination in conjunction with UBC-13. This could indicate a possible role for UEV family proteins in the regulation of mammalian AMPARs.
CHAPTER II

UEV-1 is an Ubiquitin-Conjugating Enzyme Variant That Regulates Glutamate Receptor Trafficking in C. elegans Neurons
PREFACE

The majority of this chapter is contained in a manuscript submitted for publication as Kramer, L. B., et al. (2010). "UEV-1 is an Ubiquitin-Conjugating Enzyme Variant That Regulates Glutamate Receptor Trafficking in *C. elegans* Neurons." PLoS One. The mapping and early work on the project were done by Jaegal Shim, whereas the bulk of the experiments were performed by Lawrence B. Kramer. The yeast-2-hybrid experiment reflected in Figure 8 are the work of Nora Isack, and the results in Figure S3 are the work of Lucy Ming-Chih Lee, both working in the lab of Christopher Rongo. Michelle Previtera, in the lab of Bonnie Firestein, performed the pull downs and western blot experiment reflected in Figure 8. I thank A. Fire, the *C. elegans* Genetics Center, V. Maricq, B. Grant, Y. Jin, Vishva Dixit, and R. Tsien for reagents and strains. I thank Ken Irvine for use of his Odyssey LI-COR and Jo Messing for use of his BioRad Real Time PCR machine.
ABSTRACT

The regulation of AMPA-type glutamate receptor (AMPAR) membrane trafficking is a key mechanism by which neurons regulate synaptic strength and plasticity. AMPAR trafficking is modulated through a combination of receptor phosphorylation, ubiquitination, endocytosis, and recycling, yet the factors that mediate these processes are just beginning to be uncovered. Here we identify the ubiquitin-conjugating enzyme variant UEV-1 as a regulator of AMPAR trafficking in vivo. We identified mutations in uev-1 in a genetic screen for mutants with altered trafficking of the AMPAR subunit GLR-1 in C. elegans interneurons. Loss of uev-1 activity results in the accumulation of GLR-1 in elongated accretions in neuron cell bodies and along the ventral cord neurites. Mutants also have a corresponding behavioral defect – a decrease in spontaneous reversals in locomotion – consistent with diminished GLR-1 function. The localization of other synaptic proteins in uev-1-mutant interneurons appears normal, indicating that the GLR-1 trafficking defects are not due to gross deficiencies in synapse formation or overall protein trafficking. We provide evidence that GLR-1 accumulates at RAB-10-containing endosomes in uev-1 mutants, and that receptors arrive at these endosomes independent of clathrin-mediated endocytosis. UEV-1 homologs in other species bind to the ubiquitin-conjugating enzyme Ubc13 to create K63-linked poly-ubiquitin chains on substrate proteins. We find that whereas UEV-1 can interact with C. elegans
UBC-13, global levels of K63-linked ubiquitination throughout nematodes appear to be unaffected in *uev-1* mutants, even though UEV-1 is broadly expressed in most tissues. Nevertheless, *ubc-13* mutants are similar in phenotype to *uev-1* mutants, suggesting that the two proteins do work together to regulate GLR-1 trafficking. Our results suggest that UEV-1 could regulate a small subset of K63-linked ubiquitination events in nematodes, at least one of which is critical in regulating GLR-1 trafficking.

**INTRODUCTION**

Excitatory synaptic communication in the central nervous system is mediated by the neurotransmitter glutamate and the glutamate receptor ion channels that receive and propagate glutamatergic signaling at the postsynaptic membrane (Dingledine et al., 1999). Glutamatergic synapses show substantial plasticity, becoming dynamically weakened or strengthened in an activity dependent manner (Kerchner and Nicoll, 2008; Kessels and Malinow, 2009; Malenka, 2003; Malenka and Bear, 2004; Shepherd and Huganir, 2007). The trafficking of glutamate receptors, particularly AMPA-type receptors (AMPARs), in and out of the postsynaptic membrane is emerging as a key mechanism underlying synaptic and behavioral plasticity (Rumpel et al., 2005; Shepherd and Huganir, 2007). Thus, robust molecular and cellular models for learning and memory will require
a full understanding of the various mechanisms and molecules that regulate AMPAR trafficking.

AMPAR trafficking involves several steps, including initial delivery to the synaptic membrane, anchoring, endocytosis, and finally sorting either to the lysosomal pathway for degradation or to the synaptic membrane via various recycling pathways (Ehlers, 2000; Park et al., 2004). AMPARs are tetrameric (Rosenmund et al., 1998), and each subunit has cytoplasmic tail sequences that determine its subcellular trafficking (Chang and Rongo, 2005; Dong et al., 1997; Sans et al., 2001; Song et al., 1998; Srivastava et al., 1998; Xia et al., 1999). These tail sequences direct both AMPAR endocytosis and exocytosis in response to neural activity by interacting with kinases and phosphatases, PDZ scaffolding molecules, and the endocytosis machinery (Hanley, 2006; Jiang et al., 2006; Malenka and Bear, 2004).

The Ubiquitin Proteasome System (UPS) also regulates AMPAR trafficking (Ehlers, 2003; Hicke and Dunn, 2003; Jiang et al., 2006). AMPARs, like many membrane proteins, can be post-translationally modified by the addition of single ubiquitin molecules (mono-ubiquitination) (Burbea et al., 2002). Mono-ubiquitination often serves as a signal for their internalization (Haglund et al., 2003; Hicke and Dunn, 2003; Shih et al., 2000). In addition, poly-ubiquitination can occur through the addition of new ubiquitin molecules to the final ubiquitin molecule in a growing poly-ubiquitin chain that is initially attached to a substrate
protein (Hershko and Ciechanover, 1998). Typically this occurs through the formation of a covalent link between the carboxy-terminus of the incoming ubiquitin and the lysine 48 (K48) residue of the final ubiquitin in the chain. Once four or more ubiquitins are added, the poly-ubiquitinated protein becomes a degradation substrate for the 26S proteasome.

Poly-ubiquitin chains can also be formed through linkages other than K48. For example, poly-ubiquitin chains can be assembled through K63 linkages. K63-linked poly-ubiquitination is generally not thought to target substrates to the proteasome; rather, it appears to have a more regulatory role (Xu et al., 2009). For example, K63-ubiquitination has emerged as an additional mechanism by which endocytosed proteins become shunted to Multivesicular Bodies (MVBs) for degradation (Duncan et al., 2006; Kamsteeg et al., 2006; Lauwers et al., 2010; Lauwers et al., 2009; Pickart and Fushman, 2004).

One family of proteins implicated in K63-linked ubiquitination is the UEV protein family. UEV proteins share sequence similarity with ubiquitin E2 conjugating enzymes, but lack the catalytic cysteine present in functional E2 enzymes (Broomfield et al., 1998; Sancho et al., 1998; Thomson et al., 1998; Villalobo et al., 2002; Wen et al., 2008b). One well-studied UEV protein is the ESCRT complex subunit Vps23, which routes ubiquitinated membrane receptors from the endosome to MVBs and eventually to lysosomes for degradation (Malerod and Stenmark, 2007; Teo et al., 2004). Other known UEV proteins
form heterodimers with the E2 enzyme Ubc13 to catalyze the formation of K63-linked poly-ubiquitin chains (Hofmann and Pickart, 1999). UEV/Ubc13 heterodimers are implicated in several biological processes, including the immune response (Deng et al., 2000), lysosomal targeting (Barriere et al., 2007; Kamsteeg et al., 2006; Lauwers et al., 2009; Umebayashi et al., 2008), the formation and clearance by autophagy of protein inclusions (Lim et al., 2006; Tan et al., 2007), DNA repair (Andersen et al., 2005), protein transport (Olzmann et al., 2007), and oncogenic transformation (Syed et al., 2006). While UEV proteins have been examined extensively in yeast and in mammalian cell culture, little is known about their function in vivo in metazoans. Moreover, it is unknown whether K63-linked ubiquitination plays a role in AMPAR trafficking.

The role of ubiquitination in AMPAR trafficking can be studied in vivo in the model organism C. elegans. The C. elegans genome contains two AMPA-type subunits: GLR-1 and GLR-2 (Hart et al., 1995; Maricq et al., 1995; Mellem et al., 2002). Fluorescently tagged versions of GLR-1 are functional and localized to postsynaptic elements when expressed in nematodes (Rongo et al., 1998). GLR-1 is required in the interneuron circuit that regulates backward movement and direction reversal, a key element in the foraging behavior of animals. Mutants with depressed GLR-1 synaptic levels rarely reverse direction, whereas mutants with elevated synaptic levels reverse direction with increased frequency (Hart et al., 1995; Maricq et al., 1995; Mellem et al., 2002; Schaefer and Rongo, 2006; Zheng et al., 1999). The synaptic abundance of GLR-1 is regulated by a
combination of clathrin-dependent endocytosis (Burbea et al., 2002; Grunwald et al., 2004; Juo and Kaplan, 2004) and clathrin-independent endocytosis (Glodowski et al., 2007). Ubiquitination plays several critical roles in regulating GLR-1 trafficking, including the direct ubiquitination of GLR-1 itself on four critical lysines, as well as the ubiquitination of several of the key kinases and signaling molecules that regulate GLR-1 trafficking (Burbea et al., 2002; Dreier et al., 2005; Juo and Kaplan, 2004; Park et al., 2009). Whereas K63-linked ubiquitination specifically has not been implicated in GLR-1 regulation, the C. elegans genome does encode several UEV proteins (Jones et al., 2001), including UEV-1, which can interact in vitro with UBC-13 (Gudgen et al., 2004) and has been implicated in regulating polyglutamine aggregates in C. elegans (Howard et al., 2007).

Here we identify UEV-1 as a regulator of AMPAR trafficking. We show that uev-1 mutants have defects in GLR-1 localization and GLR-1-mediated behavior that are consistent with an intracellular endosomal accumulation of GLR-1. UEV-1 is expressed broadly in most tissues, yet regulates GLR-1 trafficking cell autonomously in the command interneurons. The localization of other synaptic proteins to the central synapses of these neurons appears unaffected in uev-1 mutants. We provide evidence that GLR-1 receptors accumulate in endosomes in uev-1 mutants via clathrin-independent endocytosis, but likely cannot escape such endosomes to either recycle back to the synaptic membrane or be transported for degradation to MVBs and lysosomes. We show that UEV-1 can bind to C. elegans UBC-13, and that ubc-13 mutants have a similar phenotype to
uev-1 mutants. Our results suggest that UEV-1 and UBC-13 function together in C. elegans to regulate GLR-1 trafficking, but that UEV-1 is unlikely to be the sole mediator of global K63-linked poly-ubiquitination in C. elegans.

Results

GLR-1 accumulates in uev-1 mutants

We performed a genetic screen for mutants with defects in the localization of GLR-1 receptors tagged with green fluorescent protein (GLR-1::GFP). GLR-1::GFP receptors are normally localized to postsynaptic clusters at neuron–neuron synapses within C. elegans ventral cord neurites (Burbea et al., 2002; Rongo and Kaplan, 1999; Rongo et al., 1998). In wild-type animals, these receptor clusters are generally seen as small (~0.5-0.7 micron) puncta (Figure 1A). We identified an allele, od10, of a gene that we determined to be uev-1 (please see Materials and Methods for details on mapping and cloning). Mutants for uev-1(od10) accumulate large, elongated accretions (~ 4-6 microns) of GLR-1::GFP, especially in the retrovesicular ganglion region of the ventral nerve cord immediately posterior to the nerve ring (Figure 1B). The average size of puncta and accretions taken together in uev-1 mutants is twice that of wild-type animals (Figure 1I), and there is a slight decrease in the number of GLR-1 puncta (normalized for neurite length; Figure 1J). We also analyzed GLR-1 accretions alone by identifying them based on their size and elongated morphology. Whereas wild-type animals nearly lack these accretions, uev-1 mutants have significant numbers of them (Figure 2G). These results indicate that UEV-1 is
involved in the regulation of GLR-1-containing glutamate receptors in ventral cord neurites.

The change in the pattern of GLR-1 localization could be a consequence of *uev-1* mutants having general defects in protein trafficking or in synapse formation. To test this possibility, we examined the localization of other synaptic proteins: SNB-1 (synaptobrevin), UNC-43 (CaMKII), and LIN-10 (Mint2). We used transgenes that express GFP-tagged versions of these proteins using the *glr-1* promoter (Glodowski et al., 2005; Rongo and Kaplan, 1999; Rongo et al., 1998; Umemura et al., 2005). We introduced these transgenes into wild type and *uev-1* mutants to observe in their ventral cord neurites the subcellular localization of the proteins they encode. SNB-1::GFP is localized to presynaptic terminals (Nonet et al., 1999; Rongo et al., 1998), and we found no significant change in the size or number of SNB-1::GFP-labeled terminals in *uev-1* mutants compared with wild type (Figure 1, C, D, I, and J). UNC-43::GFP and LIN-10::GFP colocalize with GLR-1 at postsynaptic elements in the ventral cord (Glodowski et al., 2005; Rongo and Kaplan, 1999; Umemura et al., 2005), and we found no significant difference in the size or number of either LIN-10::GFP (Figure 1, E, F, I, and J) or UNC-43::GFP (Figure 1, G–J) puncta in *uev-1* mutants compared to wild type. These results lead us to conclude that the aberrant accumulation of GLR-1 in *uev-1* mutants is not because of gross defects in synaptic protein trafficking or in the formation of synapses to GLR-1-expressing neurons. In addition, because the *glr-1* promoter was used to express all of these synaptic
proteins, it is unlikely that the defects observed in \textit{uev-1} mutants are due to an
elevation in \textit{glr-1} transcription.

\textbf{UEV-1 encodes a member of the UEV family of proteins}

The \textit{uev-1} gene contains three exons that encode a 139 amino acid protein in
the UEV family of proteins. UEV proteins are well conserved across species as
diverse as yeast (\textit{S. cerevisiae}), nematodes (\textit{C. elegans}), and humans (Figure
2A) (Broomfield et al., 1998; Sancho et al., 1998; Thomson et al., 1998; Wen et
al., 2008b). We identified \textit{od10} as an allele of \textit{uev-1} by genetic mapping and
transformation rescue using the F39B2 cosmid, which contains \textit{uev-1} under its
own promoter (data not shown).

We confirmed this, as well as the fact that UEV-1 acts cell autonomously, by
transformation with constructs containing \textit{uev-1} coding sequences under the \textit{glr-1}
promoter (\textit{Pglr-1::uev-1}). We found that a cDNA construct did not rescue,
whereas a construct with full length \textit{uev-1} gave partial rescue. A construct with a
“minigene”, consisting of the \textit{uev-1} exon1, intron1, exon2 and exon3 (Figure S1),
rescued robustly (Figure 2B-G).

Following our identification of \textit{od10} as an allele of \textit{uev-1}, a new deletion
allele, \textit{ok2610}, became available from the \textit{C. elegans} Genome Consortium. To
determine the molecular nature of these alleles, we sequenced genomic DNA
from both \textit{uev-1} mutants. The \textit{od10} mutation alters the UEV-1 protein sequence
from an arginine to an opal stop codon at amino acid 70, whereas the ok2610 deletion removes an approximately 500 base pair region that includes the first two exons of uev-1 and the beginning of the third (Figure 2A and Figure S1).

We hypothesized that the phenotype observed in od10 homozygotes was likely due to a complete or severe loss of UEV-1 function, as half of the C-terminus of the protein is predicted to be absent. To confirm that the uev-1(od10) phenotype is due to loss of UEV-1 function, we also examined GLR-1 localization in uev-1(ok2610) and found that it was virtually identical in appearance, number of puncta, and number of accretions observed in uev-1(od10) mutants (Figure 2C, E, F, and G). Since uev-1(ok2610) mutants are deleted for DNA encoding the N-terminus of UEV-1, as well as the promoter and start of transcription, we conclude that both od10 and ok2610 are likely to be null alleles of uev-1.

**UEV-1 is broadly expressed and affects motoneuron development**

In addition to the GLR-1 mislocalization phenotype, we observed that uev-1 mutants have a locomotion defect, flexing their bodies more than wild-type nematodes as they move, suggesting that UEV-1 might function in multiple neuron types. To determine where UEV-1 might function, we created a transgenic reporter containing 2 kilobases of upstream uev-1 promoter sequences and the entire uev-1 transcription unit fused in frame to GFP. We introduced the resulting $P_{uev-1}$::$uev-1$::gfp reporter into the C. elegans germline, and observed that UEV-1 is broadly expressed in the pharynx, neurons, muscle
cells, vulva, embryos, intestine, and in the anus/tail (Figure 3A-F). We found strong expression in the distal tip cell (Figure 3B), which is important in development for gonad morphology (Cram et al., 2006), but upon examination we found that the gonads of uev-1 mutant worms were normal in shape (data not shown). We also found expression in the motoneurons that line the ventral cord (Figure 3B, D, F). We noted that the UEV-1::GFP chimeric protein is localized diffusely throughout the cytosol of most cells, but with some enrichment in the nucleus (Figure 3D-F).

To examine UEV-1 subcellular localization specifically in the command interneurons, we generated a transgene containing the glr-1 promoter sequences driving sequences encoding the fluorescent protein mCherry fused to the complete UEV-1 reading frame sequences. We introduced the $P_{glr-1}$::mCherry::uev-1 transgene into nematodes expressing GLR-1::GFP, and observed that mCherry::UEV-1 protein is enriched in neuronal nuclei (Figure 3G-I). As observed in the cytosol for UEV-1::GFP, we find that mCherry::UEV-1 is diffusely distributed throughout ventral cord neurites (Figure 3J-L).

Given the locomotion defect and the motoneuron expression, we hypothesized that UEV-1 might play a role in regulating motoneuron synapse formation. We examined the subcellular localization of SNB-1 (synaptobrevin) using a transgene, $juls1[P_{unc-25}::SNB-1::GFP]$, that expresses a SNB-1::GFP chimeric protein in the GABAergic motoneurons (Hallam and Jin, 1998). In wild-
type animals, SNB-1::GFP is localized to large neuromuscular junction (NMJ) boutons regularly spaced along both the dorsal and ventral cords (Figure 4A) (Hallam and Jin, 1998). In uev-1(od10) mutants, we observed a decreased number of SNB-1::GFP NMJ boutons and an irregularity in interbouton spacing, similar to the phenotype observed in rpm-1 mutants (Figure 4B, C) (Zhen et al., 2000). We quantified the number of dorsal cord SNB-1::GFP boutons in uev-1(od10) mutants, and found it to be significantly less than that seen in wild-type animals, although not as severely depressed as in rpm-1 nulls (Figure 4E).

RPM-1 is an E3 ubiquitin ligase that directly ubiquitinates the DLK-1 kinase, which is part of the PMK-3/p38 MAPK signaling pathway. RPM-1 is thought to regulate presynaptic bouton formation by repressing PMK-3 activity (Nakata et al., 2005). RPM-1 also acts in the command interneurons to regulate GLR-1 endocytosis by repressing PMK-3 activity (Park et al., 2009). Indeed, both the motoneuron NMJ phenotype and the interneuron GLR-1-trafficking phenotype observed in rpm-1 mutants are suppressed by loss of function mutations in pmk-3 (Nakata et al., 2005; Park et al., 2009). We tested if either the NMJ bouton defect or the GLR-1 accumulation defect is suppressed in animals mutated for both pmk-3 and uev-1, but detected no suppression of either defect (Figure 4D-F). We conclude that UEV-1 can regulate either presynaptic or postsynaptic differentiation depending on the specific neuron type, similar to what has been observed for RPM-1. However, the regulatory targets of UEV-1 are likely to be distinct from those of RPM-1.
**UEV-1 regulates GLR-1 trafficking independent of the ERAD pathway**

One possible explanation for the accumulation of GLR-1 in *uev-1* mutants could be that UEV-1 contributes to the Endoplasmic Reticulum Associated Protein Degradation (ERAD) pathway. This pathway targets misfolded proteins in the ER for ubiquitination, export into the cytoplasm, and subsequent degradation by the 26S proteasome (Feldman and van der Goot, 2009; Hoseki et al.; Vembar and Brodsky, 2008). Consistent with this possibility, we also observed that the cell bodies of *uev-1* mutants have higher levels of GLR-1::GFP than those observed in wild-type animals (Figure 5A, C, K).

A closely related pathway, the Unfolded Protein Response (UPR), regulates the expression of ER resident chaperones that assist in dealing with ER stress (Kohno; Ma and Hendershot, 2004). GLR-1 requires proper UPR signaling, even in the absence of stress, to exit the ER (Shim et al., 2004). In mutants for the UPR gene *ire-1*, GLR-1::GFP becomes trapped in the ER, visible as a “donut” of fluorescence (Figure 5E) surrounding neuronal nuclei, whereas very little GLR-1 is found in puncta along the ventral nerve cord (Figure 5F) (Shim et al., 2004). In addition, the level of GLR-1 in the cell bodies of *ire-1* mutants is lower than that in wild-type cell bodies, suggesting that much of the ER-trapped receptor in *ire-1* mutants is removed by ERAD (Boelens et al., 2007; Shim et al., 2004). We reasoned that if UEV-1 regulates GLR-1 as part of ERAD, then in the absence of both UEV-1 and UPR function, GLR-1 receptors that would normally have been
degraded would instead accumulate to higher levels in the ER. To address this question, we captured fluorescent images of cell bodies from wild type, *uev-1* single mutants, *ire-1* single mutants, and *uev-1 ire-1* double mutants. While we could not find a single microscope setting with the dynamic range to quantify all four genotypes relative to each other, we were nevertheless able to compare *uev-1* single mutants to wild type, and *uev-1 ire-1* double mutants to *ire-1* single mutants. We found that, unlike *uev-1* single mutants, which have elevated cell body levels of GLR-1 relative to wild type (Figure 5C, K), instead *uev-1 ire-1* double mutants have slightly decreased levels of GLR-1 relative to *ire-1* single mutants (Figure 5G, L). Our results suggest that mutations like *ire-1* that trap GLR-1 receptors in the ER preclude those receptors from regulation by UEV-1, suggesting that UEV-1 regulates GLR-1 trafficking at a step after the ER, making it unlikely that UEV-1 is part of the ERAD pathway.

An alternative possibility for how UEV-1 regulates GLR-1 is that it regulates trafficking at a step further down in the secretion pathway, perhaps by mediating GLR-1 turnover after endocytosis. Indeed, when we analyzed the fluorescence levels of GLR-1::GFP in profile along the ventral cord, we found that the unlocalized baseline in wild type and *uev-1* mutants is similar, but that the peaks of GLR-1::GFP fluorescence are higher and more frequent in *uev-1* mutants compared to wild-type animals (Figure 5B, D, I). We also noticed a spatial asymmetry in the *uev-1* mutant phenotype, with GLR-1 accretions occurring
more anterior along the ventral cord and hence more proximal to the neuron cell bodies (Figure 5I, J; Figure S2 for overview of ventral cord spatial organization).

As described above, mutations in *ire-1* dramatically reduce the amount of GLR-1 that reaches ventral cord neurites (Figure 5F). Interestingly, while double mutants for both *ire-1* and *uev-1* have similar levels of GLR-1 in their cell bodies compared to *ire-1* single mutants, they have elevated levels of GLR-1 in their ventral cord neurites along the same proximal region at which GLR-1 accretions accumulate in *uev-1* single mutants, and decreased GLR-1 at more distal sites (Figure 5H, M). We examined the levels of *glr-1* mRNA and GLR-1::GFP protein in wild type and *uev-1* mutants, and found no significant difference in their levels between the two genotypes (Figure S3). We also examined GLR-1::GFP localization in a strain homozygous for *uev-1* but heterozygous for the *Pglr-1::glr-1::gfp* reporter, hence producing a lower level of GLR-1::GFP than the homozygous reporter strain. We found that the phenotype in the PVC cell bodies is intermediate between wild-type and *uev-1* (Figure S4 A, B, and C), as is the phenotype in the ventral nerve cord (Figure S4 D-K), indicating that the absence of UEV-1 is able to cause a phenotype even in a background of a reduced amount of GLR-1. We also found that the majority of heterozygous animals showed a spatial pattern in their GLR-1::GFP distribution (Figure S4 F, G), confirming that the regulation of spatial location of GLR-1 receptors accumulation is not completely linked to total GLR-1 protein. Taken together, our results indicate that UEV-1 regulates the spatial location at which GLR-1 receptors
accumulate along the proximal-distal axis of the neurites rather than just affecting overall GLR-1 protein levels throughout the neuron.

**GLR-1 accumulates at RAB-10-containing endosomes in uev-1 mutants.**

The GLR-1::GFP accretions that we observed in uev-1 mutants could represent receptor trapped at an intracellular location, perhaps endosomal in nature (Glodowski et al., 2007; Park et al., 2009). To test this possibility, we examined several endosomal markers, each labeled with mRFP and expressed under the glr-1 promoter. We coexpressed these with GLR-1::GFP in our wild-type and uev-1 animals, allowing us to check for colocalization. While these endosomal markers were too dim to observe colocalization in the neurites, we were able to examine colocalization in the neuron cell bodies (Chun et al., 2008; Glodowski et al., 2007). We observed an increase in the amount of GLR-1::GFP that colocalized with mRFP::RAB-10 in neuron cell bodies from uev-1 mutants compared to wild-type animals (Figure 6A-J); RAB-10 is a small Rab GTPase that associates with the early endosomes that are involved in recycling clathrin-independent cargo (Chen et al., 2006; Glodowski et al., 2007). By contrast, we observed no significant difference in the amount of colocalization between GLR-1::GFP and SYN-13::mRFP (Figure 6L, M; Figure S5) and little difference for RME-1::mRFP (Figure S6), which are associated with early and recycling endosomes, respectively, and are involved in recycling clathrin-dependent cargo (Chun et al., 2008; Glodowski et al., 2007; Lee et al., 2005). Given that we see no increase in the levels of GLR-1 colocalization with SYN-13 or RME-1, it is
unlikely that the increased colocalization of GLR-1 with RAB-10 observed in *uev-1* mutants is simply due to elevated levels of GLR-1 in the cell body. We confirmed this by independently analyzing colocalization using randomization through a confined displacement algorithm (Figure S5) (Ramirez et al., 2010).

If the GLR-1::GFP accretions observed in *uev-1* mutants indicate that receptors are becoming trapped at endosomes, then we would expect a decrease in GLR-1 function in *uev-1* mutants (Glodowski et al., 2007; Park et al., 2009). Normally, GLR-1 functions to regulate changes in locomotion direction. As *C. elegans* move, they generally go forward, but will occasionally spontaneously reverse direction; GLR-1 signaling positively induces these spontaneous reversals in locomotion (Mellem et al., 2002; Zheng et al., 1999). Mutants with reduced GLR-1 signaling or reduced levels of GLR-1 at the synapse have a lower frequency of spontaneous reversals, whereas mutants with increased GLR-1 signaling or higher levels of cell surface GLR-1 have a higher frequency of spontaneous reversal (Burbea et al., 2002; Hart et al., 1995; Juo and Kaplan, 2004; Maricq et al., 1995; Schaefer and Rongo, 2006; Zheng et al., 1999). We measured the spontaneous reversal rate of *uev-1* mutants, and found that the rate was significantly lower in *uev-1* mutants than in wild type, similar to *glr-1* mutants, which entirely lack GLR-1 (Figure 6K). Expression of a wild-type *uev-1* minigene via the *glr-1* promoter is sufficient to rescue this behavioral defect. Therefore, little receptor appears to be functioning at the postsynaptic
membrane in \textit{uev-1} mutants. Instead, it is likely that a large amount of the receptor is in an intracellular compartment, possibly endosomal in nature.

\textbf{UEV-1 regulates GLR-1 trafficking independent of clathrin-mediated endocytosis}

GLR-1 is endocytosed by a combination of clathrin-dependent and clathrin-independent mechanisms, with the PDZ protein LIN-10 mediating the recycling of receptors endocytosed by the former mechanism, and RAB-10 mediating the recycling of receptors endocytosed by the latter mechanism (Figure S7) (Chen et al., 2006; Glodowski et al., 2007). In \textit{uev-1} mutants, GLR-1 is colocalized with RAB-10, suggesting that it might regulate GLR-1 recycling along the clathrin-independent pathway, and that the accumulation of GLR-1 that occurs in \textit{uev-1} mutants does not occur through clathrin-mediated endocytosis. To examine this possibility, we used the \textit{itsn-1} mutation, which impairs the function of the ITSN-1 Intersectin protein – an adaptor that helps mediate the clathrin-dependent endocytosis of GLR-1 (Glodowski et al., 2007; Koh et al., 2004; Marie et al., 2004; Sengar et al., 1999; Simpson et al., 1999; Yamabhai et al., 1998). We introduced an \textit{itsn-1} mutation into \textit{uev-1} mutants to determine whether it would suppress the accumulation of GLR-1 in accretions. We found that \textit{itsn-1} did not have any significant effect on either the average size or number of accretions in \textit{uev-1} animals (Figure 7 A-D, I-J). We had previously generated a transgene, \textit{P\textit{glr-1}::rfp::rab-5(GDP)}, which contains a mutated form of the \textit{rab-5} cDNA that can suppress the clathrin-dependent endocytosis of GLR-1 (Park et al., 2009). We
expressed this transgene in both wild-type and *uev-1* animals, but as with the *itsn-1* experiment, this did not result in the suppression of the *uev-1* mutant phenotype (data not shown), suggesting that UEV-1 regulates GLR-1 trafficking at a step independent of clathrin-mediated endocytosis.

As LIN-10 and RAB-10 mediate the recycling of GLR-1 receptors along the clathrin-dependent and clathrin-independent endocytosis pathways, respectively, double mutants for *lin-10* and *rab-10* show an additive effect on GLR-1 accumulation by simultaneously blocking both pathways (Glodowski et al., 2007). Given that UEV-1 does not appear to be downstream of clathrin-dependent endocytosis, and that GLR-1 colocalizes with RAB-10 in *uev-1* mutants, we reasoned that UEV-1 might function in the same pathway as RAB-10. To test this possibility, we made double mutant strains of *uev-1* with either *lin-10* or *rab-10* null mutations. We found that in a *lin-10 uev-1* double mutant, there was a significant increase in GLR-1 accretion size and number compared to either single mutant alone (Figure 7E, F, K, L), making it likely that LIN-10 and UEV-1 do not work in the same pathway. However, we found that in a *rab-10 uev-1* double mutant, there was a significant increase in GLR-1 accretion size and number only compared to the *rab-10* single mutant, but not compared to the *uev-1* single mutant alone (Figure 7G, H, K, L). This suggests that UEV-1 and RAB-10 might function in the same pathway, with the more severe phenotype in *uev-1* mutants indicating the possibility that UEV-1 also works in an additional pathway.
**UEV-1 and ubiquitin-mediated turnover of GLR-1**

The ubiquitination of GLR-1, like many membrane receptors, is necessary for its endocytosis (Burbea et al., 2002; Ehlers, 2003; Hicke and Dunn, 2003; Shih et al., 2000). GLR-1 ubiquitination and hence turnover can be accelerated by the overexpression of ubiquitin monomers from a transgene containing the *glr-1* promotor, resulting in reduced number and size of GLR-1::GFP puncta (Figure 8A, C) (Burbea et al., 2002). We introduced this *P_{glr-1}::ubiquitin* transgene into *uev-1* mutants and found that overexpression of ubiquitin can decrease the number and size of GLR-1 accretions in *uev-1* mutants (Figure 8D, G, H). However, *uev-1* mutations partially block the turnover of GLR-1 caused by increased ubiquitin levels (Figure 8D,G), suggesting that UEV-1 is required for one or more ubiquitin-mediated processes that regulate GLR-1 trafficking.

GLR-1 ubiquitination occurs at four critical lysines on the intracellular tail of the receptor, and expression of GLR-1(4KR)::GFP, a mutant version in which each of the four lysine residues has been changed to arginine to preclude direct receptor ubiquitination, results in an increase in the number and size of GLR-1 puncta along the ventral cord because of decreased endocytosis (Figure 8E) (Burbea et al., 2002). There is also a slight increase in the number of accretions relative to wild type (Figure 8I). We introduced the *P_{glr-1}::glr-1(4kr)::gfp* transgene into *uev-1* mutants to determine whether the phenotype of *uev-1* was directly dependent on receptor ubiquitination. We found that GLR-1(4KR)::GFP, like wild-type GLR-1::GFP, accumulates in accretions in *uev-1* mutants (Figure 8F, I,
J), although with some reduction in number. Furthermore, expression of GLR-1(4KR)::GFP in *uev-1* mutants does not suppress the low reversal rate observed in *uev-1* mutants containing wild-type GLR-1::GFP (data not shown). Taken together, these data indicate that (1) preventing the ubiquitination of GLR-1 tails cannot mimic the *uev-1* mutant phenotype, and (2) the ubiquitination of GLR-1 itself does not seem to alter the internalized accumulation of receptors in *uev-1* mutants dramatically; thus, UEV-1 probably does not participate in the ubiquitination of GLR-1 itself, at least via the four ubiquitinated lysines that have been identified on the receptor so far.

**UEV-1 might function by regulating the K63-linked ubiquitination state of one or more substrates**

UEV-1 is similar to other UEV proteins in a variety of organisms (Figure 2A), including the ESCRT protein Vps23 (Kim et al., 2007a; Thomson et al., 1998). ESCRT proteins are important for routing ubiquitinated receptors from the endosome to multivesicular bodies and lysosomes, and Vps23 is a subunit of ESCRT-1, which binds ubiquitinated receptors in the early endosome (Malerod and Stenmark, 2007). We reasoned that if UEV-1 is working as part of the ESCRT complex, then mutants for homologs of other ESCRT complex genes or related vesicular trafficking proteins might have a GLR-1::GFP localization phenotype similar to that seen in *uev-1*. However, when we introduced *Pglr-1::glr-1::gfp* into several of these mutants, including *vps-34* (Roggo et al., 2002), *vps-54* (Conboy and Cyert, 2000; Walter et al., 2002), *alx-1* (Shi et al., 2007) and
stam-1 (Govindan et al., 2006; Hu et al., 2007), we found that GLR-1::GFP localization was similar to that found in wild-type (data not shown).

UEV proteins in other organisms can function by forming heterodimers with Ubc13 so as to catalyze the formation of K63-linked poly-ubiquitin chains. There are C. elegans homologs for several E3 ligases including Parkin (pdr-1) and TRAF (trf-1), that work in pathways with UEV proteins as heterodimers with Ubc13 to catalyze ubiquitination events (Deng et al., 2000; Olzmann et al., 2007). However, when we introduced P_{glr-1::glr-1::gfp} into each of these strains we found that both had at best a mild phenotype with slightly more or larger GLR-1::GFP puncta in the ventral nerve cord, unlike the extreme phenotype seen in uev-1 mutants. This makes it less likely that uev-1 interacts with either C. elegans pdr-1 or trf-1, while leaving open the possibility that UEV-1 and UBC-13 interact in C. elegans to catalyze ubiquitination working with other E3 ligases.

We performed two different assays to test whether C. elegans UEV-1 and UBC-13 can physically interact. First, we expressed either GST or GST::UBC-13 in bacteria, and then tested the ability of these proteins when bound to glutathione agarose beads to pull down GFP-tagged UEV-1 from lysates of transfected COS7 cells. We found that GST::UBC-13 specifically pulls down GFP::UEV-1 (Figure 9A). The binding between Mms2 (a yeast homolog of UEV-1) and Ubc13 can be disrupted by mutating the eighth residue (Phenylalanine) of Mms2 to Alanine (VanDemark et al., 2001). We introduced this change into the
corresponding residue of GFP::UEV-1, and found that it abolished the interaction with GST::UBC-13 (Figure 9A). Similar results were observed in five different pull down experiments. Second, we introduced UBC-13 and UEV-1 into bait and prey vectors for yeast two-hybrid, using growth on –Leu plates as an indicator of an interaction. We found that wild-type UEV-1, but not UEV-1 with the F8A mutation, interacted with UBC-13 regardless of which protein was bait and which was prey (Figure 9A). Our results indicate that *C. elegans* UEV-1 and UBC-13 can interact in a similar fashion as their homologs in other species do.

Based on the subtle phenotypes and overall viability and fertility of *uev-1* mutants, we reasoned that UEV-1 might have a smaller, more selective set of targets for K63-linked ubiquitination than would UBC-13, and that other proteins might function with UBC-13 to conduct most K63-linked ubiquitination events in nematodes. To test this possibility, we generated lysates of well-fed, mixed stage nematodes from either wild type or *uev-1* mutants, and then separated the proteins from these lysates by SDS-PAGE. We used an antibody specific for K63-linked poly-ubiquitin chains to probe these lysates on Western blots (Newton et al., 2008). As controls for specificity, we used SDS-PAGE to separate pure K63-linked and K48-linked tetraubiquitin proteins on the same gels. The anti-K63-linked ubiquitin antibody detected multiple bands of various sizes and quantities (Figure 9B). Interestingly, we found the same pattern of K63-linked ubiquitinated proteins in both wild-type and *uev-1*-mutant lysates. Importantly, the antibody detected K63-linked but not K48-linked tetraubiquitin control
proteins, supporting its specificity. Similar results were found in five independent immunoblots. Our results indicate that UEV-1 does not affect the overall levels of K63-linked poly-ubiquitination in *C. elegans*.

A viable mutant for *C. elegans ubc-13* has recently been identified (Trujillo et al., 2010a). To directly test whether UEV-1 might regulate GLR-1 trafficking by working with UBC-13, we introduced the $P_{glr-1::glr-1::gfp}$ transgene into *ubc-13* mutants. Similar to *uev-1* mutants, *ubc-13* mutants accumulate GLR-1::GFP in their neuron cell bodies and proximal neurites (Figure 9C-F), suggesting that UEV-1 and UBC-13 might work as a heterodimer to regulate GLR-1 trafficking, most likely by mediating K63-linked poly-ubiquitination. Given that we did not observe changes in global levels of K63-linked poly-ubiquitinated proteins in *uev-1* mutants, we speculate that UEV-1 controls the ubiquitination of a small number of specific substrates (present at low enough levels to escape detection by our anti-K63 Western analysis), and that one or more of these substrates has a key role in regulating GLR-1 trafficking out of RAB-10-containing endosomes.
Discussion

We have identified an *in vivo* role for UEV-1 in a metazoan, *C. elegans*, in regulating the trafficking of an AMPA-type glutamate receptor subunit, GLR-1. We find that while UEV-1 is broadly expressed in *C. elegans* cells and tissues, it causes a specific defect in the localization of GLR-1, which can be rescued cell autonomously in GLR-1-expressing interneurons by expression of a *uev-1* cDNA. Our evidence indicates that UEV-1 likely does not function as part of the ERAD pathway, but in determining this we uncovered a strong spatial preference for where GLR-1 accretions form along the ventral cord of *C. elegans* in *uev-1* mutants. We provide evidence that GLR-1 accumulates at RAB-10-containing endosomes in *uev-1* mutants, and that receptors arrive at these endosomes independent of clathrin-mediated endocytosis. UEV-1 homologs found in other species are thought to function by binding to Ubc13, with the resulting heterodimers forming K63-linked ubiquitin chains. We find that whereas UEV-1 can interact with *C. elegans* UBC-13, global levels of K63-linked ubiquitination seem unaffected in *uev-1* mutants. Nevertheless, UBC-13, like UEV-1, is required to regulate GLR-1 trafficking. Our results suggest that UEV-1 and UBC-13 could regulate a small subset of K63-linked ubiquitination events in nematodes, at least one of which is critical in regulating GLR-1 trafficking.
**UEV-1 might work in conjunction with UBC-13 in C. elegans to regulate the K63-linked ubiquitination state of one or more substrates**

UEV-1 is similar in sequence to other proteins in the highly conserved UEV family, which are similar in sequence and structure to the ubiquitin E2 conjugating enzymes, but lack the catalytic cysteine (Broomfield et al., 1998; Sancho et al., 1998; Thomson et al., 1998; Villalobo et al., 2002; Wen et al., 2008b). UEV family members have been implicated both in generating K63-linked ubiquitin chains, as well as in the recognition and binding of previously ubiquitinated substrate proteins. For example, UEV-1 is similar to TSG101, itself a homolog of the yeast ESCRT protein Vps23 (Kim et al., 2007a; Thomson et al., 1998). ESCRT proteins function to route ubiquitinated receptors from the early endosome to multivesicular bodies and eventually to lysosomes, and Vps23 is a subunit of ESCRT-1, which binds ubiquitinated receptors in the early endosome (Malerod and Stenmark, 2007). Therefore, one possible mechanism of action for UEV-1 in regulating GLR-1 trafficking could be by functioning as part of the ESCRT complex. However, mutations in known ESCRT complex genes do not appear to affect GLR-1 trafficking at a gross level. In addition, UEV-1 does not appear to be localized to endosomes, as would be expected for a Vps23 homolog and ESCRT complex protein. Finally, the overall levels of GLR-1 are not different in *uev-1* mutants compared with wild type, suggesting that the GLR-1 accretions observed in *uev-1* mutants are not simply a result of elevated GLR-1 protein levels, but instead are due to a change in GLR-1 trafficking and subcellular localization.
Another possibility is that UEV-1 forms a heterodimer with UBC-13 (Hofmann and Pickart, 1999) to catalyze the formation of K63-linked poly-ubiquitin chains. In other organisms, K63-linked poly-ubiquitination is important in several processes, including lysosomal targeting (Barriere et al., 2007; Kamsteeg et al., 2006; Lauwers et al., 2009; Umebayashi et al., 2008), regulating protein inclusions (Lim et al., 2006; Tan et al., 2007), and protein transport (Olzmann et al., 2007). Consistent with this possible function, RNAi screens have uncovered a role for UEV-1 and UBC-13 in regulating polyglutamine inclusions in C. elegans (Howard et al., 2007). Importantly, we find that ubc-13 mutants have the same GLR-1 trafficking defects as uev-1 mutants, supporting the idea that a UEV-1/UBC-13 heterodimer complex is regulating GLR-1 trafficking. We find that the levels of K63-linked poly-ubiquitin are not grossly different in uev-1 mutants compared to the levels found in wild type, suggesting that UEV-1 is not needed for most K63-linked ubiquitination events in nematodes. C. elegans does not contain other homologs of Uev1/Mms2 in its genome. However, the ubiquitin conjugating enzyme UBC-1 contains a short N-terminal amino acid stretch that is similar to that of UEV-1, and UBC-1 can interact with UBC-13 in a yeast two-hybrid assay (Gudgen et al., 2004; Li et al., 2004). UBC-13 can also interact with the E3 ubiquitin ligase NHL-1, and together UBC-13, UBC-1, and NHL-1 can form poly-ubiquitin conjugates in vitro (Gudgen et al., 2004). Thus, one possible explanation is that UBC-13 conducts most K63-linked ubiquitination while working with UBC-1 rather than when partnered with UEV-1. We examined
GLR-1 localization in null mutants containing deletions for *ubc-1* and *nhl-1*, but did not observe defects (data not shown). We also generated *uev-1 ubc-1* double mutants and found that they were viable and had similar GLR-1 trafficking defects to *uev-1* single mutant animals (data not shown). We speculate that UEV-1 regulates the K63-linked ubiquitination of a small number of substrates, one or more of which is involved in GLR-1 trafficking, although we acknowledge that we cannot rule out the alternative possibility that UEV-1 and UBC-13 regulate GLR-1 trafficking by binding ubiquitinated proteins rather than catalytically modifying their K63 linkages.

Does UEV-1 regulate the poly-ubiquitination of GLR-1 subunits directly? We found that *uev-1* mutants contain GLR-1 in accretions and possess the same defects in spontaneous reversal frequency even when the four lysines that are normally ubiquitinated on the tail of GLR-1 are mutated to arginine to prevent their ubiquitination. Preventing direct ubiquitination of GLR-1 does not substantially suppress the *uev-1* mutant phenotype. Thus, it seems unlikely that UEV-1 regulates GLR-1 trafficking via direct poly-ubiquitination of the receptor itself. Several E3 ligases regulate GLR-1 trafficking indirectly by ubiquitinating key regulators of trafficking (Dreier et al., 2005; Park et al., 2009). One of these regulators is the PMK-3/p38 MAPK pathway, which is a downstream target of the E3 ligase RPM-1 in both the command interneurons and the motoneurons (Nakata et al., 2005; Park et al., 2009). Mutants for *uev-1* have similar defects to *rpm-1* mutants with regard to these different neuron types, and mutations in *pmk-*
3 suppress the defects in *rpm-1* mutants in both neuron types. However, mutations in *pmk-3* do not suppress the GLR-1 trafficking defects or the motoneuron synaptic differentiation defects in *uev-1* mutants, suggesting that p38 MAPK signaling is not the target of UEV-1 activity.

Interestingly, we detected UEV-1 enrichment in nuclei. K63-linked ubiquitination of histones has been observed in response to DNA damage (Panier and Durocher, 2009; Pinato et al., 2009; Yan and Jetten, 2008). While we have not found any evidence to suggest that UEV-1 functions in a DNA damage response pathway, it remains possible that UEV-1 regulates GLR-1 by affecting changes in transcription in the nucleus. We do not see an effect of *uev-1* on other reporters expressed by the *glr-1* promoter or on *glr-1* mRNA levels, so it is unlikely that UEV-1 regulates *glr-1* transcription; however, we cannot exclude the possibility that UEV-1 regulates the transcription of yet unknown factors that in turn regulate GLR-1 trafficking.

**UEV-1 controls the exit of GLR-1 from early endosomes.**

A lower frequency of spontaneous reversals in *C. elegans* has previously been shown to correlate with less GLR-1 in the postsynaptic membrane of GLR-1 expressing neurons (Hart et al., 1995; Maricq et al., 1995; Mellem et al., 2002; Zheng et al., 1999). We found that the rate of reversals was significantly lower in *uev-1* mutants than in wild type, suggesting that a large amount of the receptor is in an intracellular compartment. In addition, we found that in *uev-1* mutants there
is a significant increase in the colocalization of GLR-1::GFP with mRFP::RAB-10, which is associated with early endosomes, suggesting that UEV-1 regulates the flow of GLR-1 receptors into or out of the early endosome. The increased colocalization of GLR-1 with RME-1 in *uev-1* mutants may indicate UEV-1 also functions to regulate the flow of GLR-1 into or out of the recycling endosome, but the effect is less than in RAB-10 and may in this case be due to the large amount of GLR-1::GFP accumulating in the *uev-1* mutant.

We also found that mutations or transgenes designed to suppress clathrin-dependent endocytosis were not able to suppress the internal accumulation of GLR-1::GFP seen in *uev-1* mutants. In addition, we found that the effect of *uev-1* mutations on GLR-1 trafficking is additive when combined with *lin-10* mutations (in which the recycling of clathrin-dependent cargo is impaired), suggesting that these two genes regulate GLR-1 trafficking by different genetic pathways. By contrast, GLR-1 trafficking defects in *rab-10 uev-1* double mutants are not significantly greater than in *uev-1* single mutants, suggesting that RAB-10 and UEV-1 might function in the same genetic pathway. RAB-10 mediates the recycling of clathrin-independent cargo (Glodowski et al., 2007); thus, if UEV-1 and RAB-10 work in the same pathway, this would position UEV-1 as a mediator of GLR-1 recycling in the clathrin-independent endocytosis pathway from endosomes back to synaptic membranes. Consistent with this hypothesis, *uev-1* mutants, like *rab-10* mutants, have a reversal phenotype indicative of reduced GLR-1 synaptic function. It should be noted that *uev-1* null mutants have a
stronger GLR-1 accumulation defect than *rab-10* null mutants, suggesting either that UEV-1 is a more critical component than RAB-10 in this recycling pathway, or UEV-1 regulates GLR-1 trafficking by one or more additional mechanisms than just the clathrin-independent recycling pathway.

This leads us to entertain a model in which UEV-1 functions in GLR-1-expressing interneurons to facilitate the exit of GLR-1 from early endosomes (Figure 10A). A fraction of GLR-1 receptors is normally endocytosed by a clathrin-independent pathway and sent to RAB-10-containing early endosomes (Glodowski et al., 2007). Subsequently, GLR-1 receptors exit these endosomes and are either recycled to the synapse or sent to lysosomes for turnover. In the absence of UEV-1 function, a fraction of GLR-1 receptors become endocytosed into these endosomes, but cannot exit them, resulting in a net build up of receptor in the neurons while simultaneously depleting the synaptic surface of receptor and resulting in decreased GLR-1 signaling (Figure 10B). Interestingly, we observed a spatial bias in the localization defects in *uev-1* mutants, suggesting that these endosomes might lie more proximal along neurites. This finding raises the possibility that neurons can control the spatial localization of glutamate receptors along neurites by regulating the balance of the different endocytosis pathways that regulate the receptors. Our model is consistent with work showing a role for K63-linked poly-ubiquitination in the trafficking of renal aquaporin-2 water channels (Kamsteeg et al., 2006), the epidermal growth factor receptor (Umebayashi et al., 2008), Gap1 permease (Lauwers et al., 2009), and
other proteins (Barriere et al., 2007), with the novel feature that the GLR-1 receptor itself is not the likely K63-linked ubiquitination target. Finally, this model raises the possibility that UEV proteins are important for regulating AMPAR function, which could have implications for synaptic plasticity and neurodegeneration in other organisms, including mammals. Most studies of UEV protein function to date have been in single celled organisms or have been in tissue culture. Our study indicates that the in vivo roles of UEV proteins in metazoans are likely to be even broader than originally expected.
Materials and Methods

Strains

Animals were grown at 20°C on standard NGM plates seeded with OP50 *E. coli*. Some strains were provided by the Caenorhabditis Genetics Center. Most strains were backcrossed multiple times to our laboratory N2 strain to minimize other genetic variation. The following strains were used: *alx-1(gk338), ire-1(ok799), itsn-1(ok268), juls1[P*unc-25::SNB-1::GFP], lin-10(e1439), nhl-1(gk15), nuls108[GLR-1(4KR)::GFP], nuls24[P*glr-1::GLR-1::GFP], nuls25[P*glr-1::GLR-1::GFP], nuls68[P*glr-1::UNC-43::GFP], nuls89[P*glr-1::MUb], odEx[F39B2 cosmid, rol-6dm], odEx[P*glr-1::mCherry::UEV-1, rol-6dm], odEx[P*glr-1::SYN-13::RFP], odEx[P*glr-1::UEV-1(cDNA), rol-6dm], odEx[P*glr-1::UEV-1(F8A cDNA), rol-6dm], odEx[P*glr-1::UEV-1(F8A mini-gene)], odEx[P*glr-1::UEV-1(mini-gene)], odEx[P*glr-1::UEV-1(mini-gene)], odEx[P*glr-1::UEV-1(genomic)], odEx[P*glr-1::UEV-1::GFP], odEx[Rab-5(S23N)], odEx[rfp::rme-1], odIs1[P*glr-1::SNB-1::GFP], odIs22[P*glr-1::LIN-10::GFP], odIs42[P*glr-1::RAB-10::RFP], pdr-1(tm395), pdr-1(tm598), pmk-3(ok169), stam-1(ok406), rab-10(q373), rpm-1(js317), trf-1(nr2014), ubc-1(gk14), uev-1(od10), uev-1(ok2610), ubc-13(tm3546), and the CB4856 Hawaiian strain.

Transgenes and Germline Transformation

Transgenic strains generated in this study were isolated after microinjecting various plasmids (5–50 ng/ml) using *rol-6dm* (a gift from C. Mello, UMass), *ttx-3::rfp* (a gift from O. Hobert, Columbia Univ.), or *lin-15(+)* (a gift from J. Mendel, CalTech) as a marker. Plasmids containing the *glr-1* or *uev-1* promoters,
followed either by a *uev-1* minigene (wild type or F8A mutant), *uev-1* cDNA (wild type or F8A mutant), *uev-1* genomic, or *mCherry::uev-1*, were generated using standard techniques. All resulting transgenes were introduced into the germline and followed as extrachromosomal arrays.

**Isolation and mapping of *uev-1*(od10)**

P0 *nuls25* nematodes were EMS mutagenized using standard procedures. F2 animals from individual plates were sampled (n = 30-50) by mounting on 2% agarose pads containing levamisole. Animals were scored by fluorescence microscopy for defects in GLR-1::GFP localization. Mutants were recovered either directly from the slide or by isolating siblings from the parental F1 plate. Mutants were further characterized after 4 rounds of backcrossing.

The *od10* mutation was closely linked to *unc-54* (map position +27.4), and was three-factor mapped between SNP *pkP1071* (map position +23.4) and SNP *pkP1072* (map position +28.48) on the right arm of LGI by crossing marked *uev-1* strains to the polymorphic strain CB4856. We detected 20 recombination events between *pkP1071* and *od10*, and 2 recombination events between *od10* and *pkP1072*, suggesting that *od10* mapped to around +27.0. We injected genomic cosmids covering ~200 kb around the *unc-54* locus, and found that cosmid F39B2 rescued the *uev-1* mutant phenotype. This cosmid contains 12 genes, including *uev-1*. We sequenced several candidate genes from *od10* mutant genomic DNA, and identified a nonsense mutation in the *uev-1* gene.
Expression of a *uev-1* minigene containing *uev-1* coding sequences and 2 kilobases of promoter was also sufficient to rescue the *uev-1* mutant phenotype.

**Fluorescence Microscopy**

GFP- and RFP-tagged fluorescent proteins were visualized in nematodes by mounting larvae on 2% agarose pads with levamisole. Fluorescent images were observed using a Zeiss Axioplan II. A 100X (N.A. = 1.4) PlanApo objective was used to detect GFP and RFP signals. Imaging was done with an ORCA charge-coupled device (CCD) camera (Hamamatsu, Bridgewater, NJ) using IPLab software (Scanalytics, Inc, Fairfax, VA) or iVision v4.0.11 (Biovision Technologies, Exton, PA) software. Exposure times were chosen to fill the 12-bit dynamic range without saturation. In most cases, maximum intensity projections of z-series stacks were obtained and out-of-focus light was removed with a constrained iterative deconvolution algorithm (Vaytek). For most images, we captured the ventral cord neurites in the retrovesicular ganglion region surrounding the RIG and AVG cell bodies.

The quantification of ventral nerve cord fluorescent objects (i.e., puncta and accretions) was done using ImageJ (Collins, 2007) to automatically threshold the images and then determine the outlines of fluorescent objects in ventral cord neurites. ImageJ was used to quantify both the shape and the size of all individual fluorescent objects along the ventral cord. This allowed us to distinguish between the small GLR-1::GFP puncta in wild-type animals and the large, aberrant accretions (which have an elongated shape not observed in wild
type) in *uvev-1* and other mutants. Object size was measured as the maximum diameter for each outlined cluster. Object number was calculated by counting the average number of clusters per 100 microns of dendrite length.

To quantify the fluorescent intensities of individual GLR-1::GFP puncta and accretions along the ventral cord neurites, a 20X objective was used to capture images of animals, and a median filter was used to subtract away the background nematode and coverslip autofluorescence. Line profiles were drawn along the length of the ventral cord starting from the anterior end of the retrovesicular ganglion and moving posterior for approximately 250 microns to show the fluorescent intensity peaks for individual puncta and accretions. For quantification of ventral cord fluorescence, ImageJ was used to measure the integrated fluorescent density (the sum of all detectable pixel intensities along a given ventral cord process) normalized to the length of that process.

The quantification of PVC cell body fluorescence was done using ImageJ to measure the integrated fluorescent density (the sum of all detectable pixel intensities per cell body) for each neuron. For the quantification of GLR-1::GFP and mRFP::RAB-10 colocalization, we fixed animals with ice cold 1% paraformaldehyde in PBS for 10 minutes and imaged them using a previously published protocol (Chun et al., 2008; Park et al., 2009). Images for neuronal cell bodies were taken using a Carl Zeiss confocal microscope equipped with the BD CARV II TM Confocal Imager and a Carl Zeiss 100X Plan-Apochromat objective (N.A. = 1.4).
For quantitative colocalization analysis, all image manipulations were performed with iVision v4.0.11 (Biovision Technologies, Exton, PA) software using the FCV colocalization function. We applied an empirically derived threshold to all images for both the GLR-1::GFP channel and the mRFP::RAB-10 channel to eliminate background coverslip fluorescence and other noise (typically, 5% of pixels for each channel). The fluorescent intensity values for both the GLR-1::GFP and mRFP::RAB-10 channels were then scatter plotted for each pixel. Pixels with similar intensity values for both channels (within an empirically established tolerance factor) were counted as colocalized. To acquire the fraction of GLR-1::GFP colocalized with mRFP::RAB-10, the number of colocalized pixels was normalized to the number of GLR-1::GFP pixels under threshold. To maximize our resolving power while observing the relatively small C. elegans neuron cell bodies, we restricted our analysis to a single focal plane taken through the middle of each cell body. As an alternative approach, we also analyzed the same images using a confined displacement algorithm run in ImageJ as previously described (Ramirez et al., 2010). Manders colocalization coefficients were determined for the real data and for the randomized image, and then compared statistically to obtain $p$ values.

**Behavioral Assays**

The reversal frequency of individual animals was assayed as previously described, but with some modifications (Zheng et al., 1999). Single young adult hermaphrodites were placed on NGM plates in the absence of food. The animals
were allowed to adjust to the plates for 5 minutes, and the number of spontaneous reversals for each animal was counted over a 5-minute period. Twenty animals were tested for each genotype, and the reported scores reflect the mean number of reversals per minute.

**GST Pull Downs**

Complete sequences for *ubc-13* were introduced into the GST expression vector pGEX-GW (a gift from Barth Grant), and either GST alone or GST::UBC-13 were expressed in *E. coli* strain BL21 and purified using glutathione-Sepharose as described previously (Firestein et al., 1999). Complete sequences for *uev-1* or *uev-1* mutated F8A were introduced into the pcDNA3.1-GFP expression vector (a gift from Barth Grant), and transfected in COS7 cells for 48 hours. Cell were harvested and lysed in TEEN (20 mM Tris, 1 mM EDTA, 1 mM EGTA, 100 mM NaCl) plus 1 mM PMSF, and Triton X-100 was added to 1% for 1 hour at 4°C after needle aspiration. For each experiment, 12 micrograms of GST protein was added to 400 microliters of solubilized supernatant, incubated for 45-60 minutes at 4°C, and washed several times in TEEN plus 0.2% Triton X-100. Proteins were eluted into loading buffer and separated by SDS-PAGE for Western blot analysis using anti-GFP antibodies.

**Yeast Two-hybrid Interactions**

Yeast two-hybrid experiments were performed by placing *ubc-13* and *uev-1* cDNA sequences into the pEG202 bait vector and pJG4-5 prey vector. The
resulting plasmids were cotransformed, along with the reporter plasmid pSH18-34, into yeast strain EGY48, and transformed yeast were recovered on –His –Trp –Ura dropout plates. Resulting colonies were diluted in series on –Leu –His –Trp dropout plates to test for interactions based on growth.

**K63-linked Anti-ubiquitin Western Blotting**

Western blots with Apu3.A8 were performed essentially as described previously with modifications (Newton et al., 2008). Nematodes were dounce homogenized, needle aspirated, and sonicated in 20 mM Tris (pH 7.4), 1 mM EGTA, 10% glycerol, 135 mM NaCl, 1.5 mM MgCl2, 1% Triton X-100, 6M urea, 2 mM N-ethylmaleimide, 25 μM MG132, and a protease inhibitor cocktail (Roche). Total protein was quantified by Bradford assay, and then SDS was added to a final concentration of 1%. The lysates was centrifuged at 13,000xg to remove insoluble material, and equal quantities of extracted proteins were separated on SDS-PAGE gels. After electrophoresis, resolved proteins were transferred to PVDF membranes (Immobilon-P) and blocked with 2% BSA in PBS with Triton X-100. Membranes were incubated with Apu3.A8 anti-K63-linked ubiquitin antibodies (Millipore) at 1:300 for 1 hour, or C4 monoclonal anti-actin antibodies (MPBiomedic) at 1:1000 overnight at 4°C. After washing, HRP-conjugated secondary antibody was applied at 1:2000 for 1 hour at room temperature. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) system (GE Healthcare).
GLR-1::GFP Western Blotting

Lysates were prepared from adult worms using a Wheaton DuraGrind stainless steel dounce homogenizer and buffer A (50 mM Hepes pH 7.7, 50 mM potassium acetate, 2 mM magnesium, 1mM EDTA, 250 mM sucrose), a protease inhibitor cocktail (Roche), and 10 mM N-ethylmaleimide. Membranes were isolated from clarified lysates by ultracentrifugation, and then suspended in buffer A plus β-mercaptoethanol, SDS, and DTT. Proteins were separated from membrane lysates by SDS-PAGE, and GLR-1::GFP or actin were simultaneously detected by Western blotting using a combination of anti-GFP antibodies (GeneTex Inc.) and anti-actin antibodies (MP Biomedicals). Secondary antibodies conjugated to infrared-emitting tags were used to detect both primaries on the blot using an Odyssey LI-COR. Quantitation was performed by comparing samples to a standard curve generated by diluting samples from wild-type lysates.

Real-time PCR

Total RNA from adult worms was prepared using Trizol (Invitrogen). First strand reverse transcription was performed using iScript (Biorad) as per the manufacturer's instructions. Real-time PCR reactions were performed using iQ SYBR Green (Biorad) and a BioRad real-time PCR cycler as previously described (Umemura et al., 2005). Primer sets detected either glr-1 mRNA or mRNA for dlg-1, a control transcript of an adherens junction protein (Firestein and Rongo, 2001). Primers spanned intron junctions so as not to detect genomic
DNA, and no product was detected in the absence of reverse transcription.

Standard curves were generated and analyzed in triplicate by dilution. Concentrations were derived by comparing Ct values of samples to standard curves. For each genotype, mean glr-1 expression was normalized to that of dlg-1.
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Figure 1. UEV-1 regulates GLR-1 Trafficking.

The fluorescence of (A, B) GLR-1::GFP (GluR), (C, D) SNB-1::GFP (synaptobrevin), (E, F) LIN-10::GFP (Mint2), and (G, H) UNC-43::GFP (CaMKII) was observed along ventral cord neurites of (A, C, E, G) wild-type or (B, D, F, H) *uev-1*(od10)-mutant animals. The mean size (I) of fluorescent puncta and accretions (arrows) combined are plotted for adult nematodes of the given genotype (stippled bars for wild type, gray bars for *uev-1* mutants) and for the given fluorescent reporter (indicated below the graph). (J) The mean density (number per 10 microns of ventral cord length) of fluorescent puncta is plotted for adults of the given genotype. For each genotype, the values have been normalized to the mean value of the wild-type control. Whereas wild-type animals have small GLR-1::GFP puncta, *uev-1* mutants accumulate GLR-1::GFP in large accretions (arrows); other synaptic proteins appear not to be affected in *uev-1* mutants. Bar, 5 μm. Error bars are SEM. N=20-30 animals for each genotype. *P<0.01, ***P<0.001 by ANOVA with Bonferroni multiple comparison tests (only shown for wild type versus mutant for each reporter).
Figure 2. UEV-1 is a member of the E2 ubiquitin-conjugating enzyme variant family.

(A) The intron/exon structure of uev-1 based on sequenced cDNAs is shown in the top panel. Gray boxes indicate exonic coding sequences. The arrow indicates the start of transcription. The site of the od10 molecular lesion is indicated. Predicted alpha helices and beta sheets, based on alignment with human MMS2, are indicated (Moraes et al., 2001). The purple line indicates the sequences that are removed by the ok2610 deletion. The bottom panel shows the amino acid alignment of C. elegans (C.e.) uev-1 with its two putative homologs in humans (H.s.), UBE2V1/UEV1 and UBE2V2/MMS2, along with Mms2 from yeast (S.c.). Black highlighting indicates identity, and gray indicates similarity. The locations of the F8 residue (mutated in our binding constructs) and the residue altered in od10 to nonsense are indicated. Predicted alpha helices and beta sheets are indicated below the aligned sequence. (B-E) GLR-1::GFP fluorescence in the ventral cord from (B) wild type, (C) uev-1(od10) homozygotes, (D) uev-1(od10) containing the $P_{glr-1}::uev-1(\pm )$ transgene, and (E) uev-1(ok2610) homozygotes. The mean number of (F) GLR-1::GFP puncta and (G) GLR-1::GFP accretions per 100 micron of ventral cord length is indicated for the given genotypes. Cell autonomous expression of wild-type uev-1 via the glr-1 promoter is sufficient to rescue uev-1 mutants. Bar, 5 μm. Error bars are SEM. N=20-30 animals for each genotype. **P<0.01 by ANOVA with Dunnett’s multiple comparison to wild type.
Figure 3. UEV-1 is broadly expressed.

(A-F) Fluorescence from animals transgenic for $P_{uev-1}::uev-1::gfp$. Expression is detected in (A) pharynx and multiple head neurons, (B) distal tip cell, vulval epithelia, and ventral cord motoneurons (arrowheads), (C) most cells of embryos (gastrulating embryo shown from ventral view), (D) intestinal epithelia and motoneurons (arrowheads), (E) body wall muscle, and (F) ventral cord motoneurons. (D-F) Arrows indicate nuclear enrichment of the UEV-1::GFP protein. (G-L) Fluorescence from $uev-1$-mutant animals co-expressing (G, J) GLR-1::GFP and (H, K) mCherry::UEV-1 via the $glr-1$ promoter. (I, L) Merged images. (G-I) UEV-1 is enriched in nuclei (arrow), but can be found at punctate structures in the cell body cytoplasm. (J-L) UEV-1 is uniformly distributed along ventral cord neurites. Bar, 5 μm.
Figure 4. UEV-1 is required for motoneuron synaptic bouton differentiation.

(A-D) Fluorescence from animals transgenic for $P_{unc-25}::snb-1::gfp$ (synaptobrevin). (A) Presynaptic boutons labeled with SNB-1::GFP are distinct along the dorsal and ventral cords of wild-type animals. Such boutons are missing, irregular in shape and size, and/or abnormally spaced in (C) $uev-1(od10)$ mutants, a phenotype reminiscent of that observed in (B) $rpm-1$ mutants. (D) Mutations in $pmk-3$ do not alter the $uev-1$ phenotype. (E) The total number of
dorsal cord SNB-1::GFP puncta is plotted for the indicated genotypes. (F) GLR-1::GFP accumulation in accretions in uev-1 pmk-3 double mutants is similar to that observed in uev-1(od10) single mutants. Bar, 5 μm. Error bars are SEM. N=20-30 animals for each genotype. ***P<0.001 by ANOVA with Bonferroni multiple comparison tests.
GLR-1::GFP in PVC Cell Body

**A** Wild Type

**C** uev-1

**E** ire-1

**G** ire-1 uev-1

**B** GLR-1::GFP in Neurites

**Proximal**

**Distal**

**I** GLR-1::GFP Levels Along the Ventral Cord

**J** GLR-1::GFP Levels Along the Ventral Cord

**K** Integrated Fluorescence Density (AU)

**L** Integrated Fluorescence Density (AU)

**M** Number of GLR-1::GFP Puncta along the Ventral Cord

- **Wild Type**
- **uev-1**
Figure 5. UEV-1 regulates both GLR-1 levels and spatial distribution.

(A-H) GLR-1::GFP fluorescence in (A, C, E, G) neuron cell bodies and (B, D, F, H) along the ventral cord for (A, B) wild type, (C, D) uev-1(od10) mutants, (E, F) ire-1 mutants, and (G, H) ire-1 uev-1 double mutants. To quantify the unusual spatial distribution of GLR-1 in uev-1 mutants, the region 65 microns anterior (left in the figure) of the RIG/AVG cell bodies (these neurons contribute little GLR-1::GFP to the ventral cord, used here solely as a landmark) was considered as “proximal” to the neuron cell bodies of the command neurons in the head (AVA, AVB, AVD, AVE, and PVC, which contribute almost all of the GLR-1::GFP along the ventral and are outside of the images in this figure), whereas the region 65 microns posterior (right in the figure) of the RIG/AVG cell bodies were considered as “distal” to the command neuron cell bodies. The images in E-H were collected at several times the exposure as the images in A-D as no single exposure time possessed the dynamic range to precisely capture all four genotypes. (I) Line profiles for GLR-1::GFP fluorescent intensity along the ventral cord for a wild-type animal (blue) and a uev-1(od10) mutant (red) after removal of background autofluorescence. Baseline is a measure of unlocalized GLR-1::GFP levels. Brighter GLR-1::GFP puncta are observed more proximal along the neurites to the cell bodies. This proximal-distal bias is increased in uev-1 mutants compared to wild type. (J) Mean GLR-1::GFP fluorescence (sum of pixel values) along the ventral cord for either the total cord, the proximal region, or the distal region for wild type (stippled) or uev-1 mutants (gray). (K, L) GLR-1::GFP fluorescence
(sum of pixel values) observed in the PVC neuron cell bodies for the indicated genotypes. (M) Mean number of GLR-1::GFP puncta along the ventral cord (per 100 micron length) for either the total cord, the proximal region, or the distal region for wild type (stippled) or uev-1(od10) mutants (gray). Bar, 5 μm. Error bars are SEM. N=20-30 animals for each genotype. (J, M) **P<0.01, ***P<0.001 by ANOVA with Bonferroni multiple comparison tests (only shown for wild type versus mutant for each reporter). (K, L) *P<0.01, ***P<0.0001 by t-test.
Figure 6. UEV-1 regulates GLR-1 colocalization with endosomal protein RAB-10.

(A, E) GLR-1::GFP and (B, F) mRFP::RAB-10 fluorescence was observed in single-plane confocal images of PVC neuron cell bodies from (A-D) wild-type animals and (E-H) uev-1(od10) mutants. (C, G) Merged images. (D, H) Binary masks (yellow) were created to highlight pixels with matching intensity values for both GLR-1::GFP and mRFP::RAB-10, indicating colocalization. The mean percent of (I) GLR-1 colocalized with RAB-10, and (J) RAB-10 colocalized with GLR-1 is plotted for the indicated genotypes. More GLR-1 is found colocalized with RAB-10 in uev-1(od10) mutants. (K) The mean spontaneous reversal frequency as an indication of GLR-1 function is plotted for the indicated genotypes. The mean percent of (L) GLR-1::GFP colocalized with Syntaxin-13::mRFP, and (M) Syntaxin-13::mRFP colocalized with GLR-1::GFP is plotted for the indicated genotypes. Bar, 1 μm. Error bars are SEM. N=20-30 animals for each genotype. (I, J) ***P<0.0001, *P<0.05 by t-test. (K) **P<0.01, *P<0.05 by ANOVA with Dunnett’s multiple comparison test to wild type.
Figure 7. UEV-1-dependent trafficking of GLR-1 is independent of clathrin-mediated endocytosis.

(A-H) GLR-1::GFP ventral cord fluorescence is shown for the indicated genotypes: (A) wild type, (B) uev-1, (C) itsn-1, (D) itsn-1 uev-1, (E) lin-10, (F) lin-10 uev-1, (G) rab-10, and (H) rab-10 uev-1. The uev-1(ok2610) allele was used in these experiments. Mean (I, K) size and (J, L) number of GLR-1::GFP accretions observed along the ventral cord of the indicated genotypes. Bar, 5 μm. Error bars are SEM. N=20-30 animals for each genotype. ***P<0.001, **P<0.01, *P<0.05 by ANOVA with Bonferroni multiple comparison tests. For (K) and (L), only comparisons between double mutants and their corresponding single mutants are shown for clarity.
Figure 8. UEV-1 and ubiquitin-mediated turnover of GLR-1.

(A-D) GLR-1::GFP ventral cord fluorescence in (A) wild type, (B) uev-1(od10) mutants, (C) wild type that overexpress free ubiquitin, and (D) uev-1(od10) mutants that overexpress free ubiquitin. (E, F) GLR-1(4KR)::GFP ventral cord fluorescence in (E) wild type and (F) uev-1(od10) mutants. The mean (G, I) number and (H, J) size of GLR-1::GFP accretions is plotted for the indicated genotypes. In (I-J), “GLR-1(+)” indicates the presence of the wild-type GLR-1::GFP-expressing transgene, whereas “GLR-1(4KR)” indicates the presence of the GLR-1(4KR)::GFP-expressing transgene; GLR-1(4KR)::GFP cannot be ubiquitinated. Bar, 5 μm. Error bars are SEM. N=20-30 animals for each genotype. ***P<0.001 by ANOVA with Bonferroni multiple comparison tests.
Figure 9. **UEV-1 interacts with UBC-13 to regulate GLR-1.**

(A) Top left panel shows a Coomasie-stained SDS-PAGE for GST and GST::UBC-13 used in the pull down assay. Top right panel shows a Western blot using anti-GFP antibodies to detect GFP::UEV-1 or GFP::UEV-1(F8A) pulled down from COS7 lysates using GST or GST::UBC-13 bound to beads. “Input” indicates 10% of the lysate used in the binding reaction. Arrow indicates the specific GFP::UEV-1 protein. Arrowheads indicate non-specific bands in the lysate that are pulled down by GST::UBC-13 and detected on the Western blot. Bottom panel shows growth on media selecting for interaction (–Leu –His –Trp) or without selection (–His –Trp) for 10 fold serial dilutions of yeast cultures co-expressing the indicated bait and prey plasmids. Similar results were found in 3 independent experiments. (B) Western blots for K63-linked poly-ubiquitinated proteins or actin as a loading control. Purified tetra-ubiquitin (Ub4) is present on the SDS-PAGE in either the K63-linked form (~25 kDa) or the K48-linked form (~30 kDa). Coomasie-stained SDS-PAGE for each Ub4 protein is also shown. Similar results were found in 5 independent experiments. (C) GLR-1::GFP fluorescence from PVC cell bodies (left side) or neurites around the retrovesicular region (right side) are shown for the indicated genotypes. Like *uev-1* mutants, *ubc-13(tm3546)* mutants accumulate GLR-1::GFP in their cell bodies and at proximal regions along their ventral cord neurites. Bar, 5 μm. The (D) number of GLR-1::GFP puncta, (E) number of GLR-1::GFP accretions, and (F) size of GLR-1::GFP accretions are indicated for the given genotypes.
*P<0.01 by Student-t test. ***P<0.001 by ANOVA with Dunnett's comparison to wild type. Error bars are SEM. N=20-30 animals for each genotype.
Figure 10. A model for UEV-1 function.

Previous results indicate that GLR-1 is endocytosed and recycled by two pathways: a clathrin-dependent pathway (which involves ITSN-1 and RAB-5) that requires LIN-10 for recycling, and a clathrin-independent pathway that requires RAB-10 for recycling. Based on genetic and cell biological data, we suggest that UEV-1 functions in the clathrin independent pathway to move receptors out of endosomes for recycling and for turnover. (A) Cartoon illustrating both pathways
at different synapses along the ventral cord of wild-type animals. GLR-1 receptors (red) are being endocytosed and recycled by either the clathrin-independent endocytosis pathway (CIE) and RAB-10 (the synapse on the left) or the clathrin-dependent endocytosis pathway (CDE) and LIN-10 (the synapse on the right). Our results suggest that the more proximal synapses favor the clathrin-independent, UEV-1 dependent pathway. (B) Cartoon illustrating that the clathrin-independent pathway is disrupted in *uev-1* mutants, resulting in the internalization of GLR-1 receptors into accretions due to their failure to exit endosomal compartments.
**Figure S1. The *uev-1* transcription unit.**

Genomic sequences in and around the *uev-1* transcription unit, from the start of transcription until the final nucleotide present in the mRNA, are shown. Capital letters highlighted in gray indicate coding sequences within exons. Numbers are based on nucleotides starting from the ATG and containing only exonic sequences. The horizontal bar indicates sequences missing in the *ok2610* deletion. The nonsense mutation in *od10* is also indicated.
Figure S2. Overview of GLR-1-Expressing Neurons.

Top panel diagram indicates the position of the pharynx (green), command interneuron cell bodies (red circles, only left side shown for clarity), RIG cell bodies (blue circles), AVG cell body (purple circle), and the various neurite projections into the nerve ring and along the ventral cord in the head region. The PVC command neuron cell body, which is located in the tail, is not shown; however, the PVC neurite would belong to the bundle of fibers along the ventral cord and projecting into the nerve ring as indicated in red. Note that the RIG neurites do not enter the ventral cord until the most anterior portion of the lateral ganglion; thus, no RIG synapses are included in our analysis (White, 1986). Also note that AVG makes only a single synapse in the retrovesicular ganglion region.
of the ventral cord; thus, the contribution of AVG to our analysis is minimal (White, 1986). Bottom panel diagram indicates the position of cell bodies and neurites from a dorsal view. The circular nerve ring has been flattened out to lie in the same plane as the ventral cord so that the posterior face of the nerve ring and the dorsal face of the ventral cord are directed out of the page. The lateral ganglion and retrovesicular ganglion regions are indicated by brackets.
Figure S3. GLR-1 mRNA and protein levels do not vary significantly in uev-1 mutants compared to wild type.

(A) The levels of glr-1 mRNA relative to dlg-1 (a control adherens junction protein) mRNA as detected by qRT-PCR are shown for the indicated genotypes. N=5 trials. (B) The levels of GLR-1::GFP protein relative to actin protein as detected by quantitative Western blotting are shown. (C) A sample Western blot for GLR-1::GFP (top) and actin (bottom) is shown. N=3 trials.
Figure S4. UEV-1 regulates GLR-1 levels in the background of reduced GLR-1 expression.

(A-G) Fluorescence from GLR-1::GFP, either (A, C, D, and E) two copies or (B, F, and G) one copy of the transgene in the (A-C) PVC neuron or (D-G) ventral cord bundle of (A and D) wild-type animals or (B, C, E, F, and G) uev-1 mutants. (H, I) The mean size of (H) puncta or (I) accretions and (J, K) the mean number of (J) puncta or (K) accretions in the ventral cord is plotted for adult nematodes of the given genotype and expressing the transgenes indicated beneath the graph. Under each graph “double” refers to nematodes homozygous for the \textit{Pglr-1::glr-1::gfp} transgene (two copies), whereas “single” refers to nematodes heterozygous for the \textit{Pglr-1::glr-1::gfp} transgene (one copy). (E) This image is of the indicated genotype, but was acquired on a different day than the other images. Error bars are SEM for all graphs. Statistical analysis was done using (H, J, and K) ANOVA, followed by a Bonferroni Multiple Comparison test or (I) t-test. * = p<0.01, ** = p<0.01, and *** = p<.001.
Figure S5. GLR-1 colocalization with RAB-10 and SYN-13 as analyzed by a confinement displacement algorithm.

(A,D) GLR-1::GFP fluorescence and (B,E) SYN-13::RFP fluorescence from (A-C) wild type or (D-F) uev-1 mutants. (C,F) Merged images. The mean Manders colocalization coefficient is shown for (G) GLR-1 that colocalizes with RAB-10, (H) RAB-10 that colocalizes with GLR-1, (I) GLR-1 that colocalizes with SYN-13, and (J) SYN-13 that colocalizes with GLR-1 for the indicated genotypes. Gray bars indicate the coefficients determined from the original fluorescent images. Red bars indicate the coefficients determined from the same images randomized by a confinement displacement algorithm, thus measuring the probability that the correlation coefficients for colocalization are occurring by random chance within the small confined space of these cells (Ramirez et al., 2010). *P<0.05, **P<0.01, ***P<0.001 by ANOVA with the indicated Bonferroni comparisons. N=15-22 animals for each genotype.
Figure S6. UEV-1 regulates GLR-1 colocalization with endosomal proteins.

The mean percent of (A) GLR-1 colocalized with RME-1, and (B) RME-1 colocalized with GLR-1 in PVC cell bodies, is plotted for the indicated genotypes. Error bars are SEM. N=20-30 animals for each genotype. *P<0.05 by t-test.
Figure S7. Clathrin-dependent and clathrin-independent pathways regulate GLR-1 trafficking.

For each genotype, a cartoon is shown of two synapses along the ventral nerve cord bundle, with predictions based on our hypothesis for UEV-1 function. GLR-1 receptors (red) are being endocytosed and recycled by either the clathrin-independent endocytosis pathway (CIE) and RAB-10 (the synapse on the left) or the clathrin-dependent endocytosis pathway (CDE) and LIN-10 (the synapse on the right). (A) Trafficking in wild-type animals, based on our model and previously published results (Glodowski et al., 2007; Park et al., 2009). (B) In *lin-10* mutants, GLR-1 is endocytosed by CDE, including RAB-5 and ITSN-1; however, receptors are not recycled and accumulate in internal endosomes. (C) In *uev-1* mutants, *rab-10* mutants, or *uev-1 rab-10* double mutants, our findings suggest that GLR-1 is endocytosed by CIE; however, receptors are not recycled and accumulate in internal endosomes. (D) Since UEV-1 and LIN-10 are
expected to regulate GLR-1 by these two separate pathways, we would expect an increase in the amount of internalized GLR-1 in uev-1 lin-10 double mutants. (E) Mutations that reduce CDE (e.g., itsn-1) should not block the accumulation of GLR-1 in intracellular compartments in uev-1 mutants, but (F) they do block the internalization of GLR-1 in lin-10 mutants (Glodowski et al., 2007).
CHAPTER III

The Role of Phosphorylation in C. elegans AMPA-type Glutamate Receptor Localization
ABSTRACT

The dependence of glutamate receptor trafficking upon the phosphorylation state of individual amino acids within the receptor sequence has been widely studied, but relatively little is known about the in vivo significance of this in a live and behaving animal. We used a reverse genetics approach to test the potential of phosphorylation sites for their effect on the trafficking of the glutamate receptor subunit GLR-1 in the *C. elegans* ventral nerve cord. We found that mutation of a single GLR-1 serine (S891) to alanine is sufficient to strongly reduce GLR-1 puncta in the ventral nerve cord, whereas mutation of this residue to aspartate does not have this effect. We also determined that this effect is prevented if the C-terminal portion of GLR-1 is mutated such that it can no longer be ubiquitinated and endocytosed. This suggests that S891 phosphorylation may regulate endocytosis and/or ubiquitination and subsequent degradation of GLR-1. This in turn likely serves as a mechanism to control the amount of this receptor available to function at the synaptic membrane, possibly thereby allowing a tuning of synaptic strength.
INTRODUCTION

Synaptic plasticity, the ability of mammalian neuronal synapses to be dynamically weakened or strengthened in an activity-dependent manner (Malenka and Bear, 2004), has been implicated as a cellular mechanism for learning and memory, and is likely affected in various neurodegenerative diseases (Rumpel et al., 2005; Shepherd and Huganir, 2007; Zhao et al., 2004). The plasticity of glutamatergic synapses is at least partially mediated by the “trafficking” or movement of AMPA type glutamate receptors (AMPARs) in and out of the post-synaptic membrane (Kessels and Malinow, 2009). AMPAR trafficking involves several components including initial delivery to the synaptic membrane, anchoring at the synapse, endocytosis, trafficking to early endosomes, and finally sorting to the lysosomal pathway for degradation or via recycling pathways back to the synaptic membrane (Ehlers, 2000; Park et al., 2004). AMPARs are tetrameric (Rosenmund et al., 1998) and each subunit has critical determinants for trafficking found in their cytoplasmic C-terminal (cterm) “tails” (Chang and Rongo, 2005).

AMPARs as well as other proteins often undergo post-translation modification (PTM) by phosphorylation, which can influence localization, binding partners, and protein activity (Blom et al., 2004). The phosphorylation state of certain serine, threonine, or tyrosine residues on AMPAR subunit cterm tails is crucial for regulating AMPAR function (Carvalho et al., 1999; Mammen et al., 1997;
Soderling and Derkach, 2000). This has been intensely studied in mammalian cells and there is evidence that this can influence both the trafficking of AMPARs (Esteban et al., 2003; Hayashi et al., 2000), as well as their single channel conductance (Derkach et al., 1999; Oh and Derkach, 2005). There is a direct relationship between AMPAR tail phosphorylation state and varied processes involved in forms of synaptic plasticity (Barria et al., 1997b; Chung et al., 2003; Hayashi et al., 2000; Lee et al., 2000; Seidenman et al., 2003).

Phosphorylation and regulation of trafficking of AMPARs can be via several kinases, including CaMKII, PKC, and PKA (Esteban et al., 2003; Lisman et al., 2002; Mammen et al., 1997; Matsuda et al., 1999; McDonald et al., 2001; McGlade-McCulloh et al., 1993; Roche et al., 1996; Rongo and Kaplan, 1999). Src family tyrosine kinases have also been implicated in AMPAR tyrosine phosphorylation (Ahmadian et al., 2004; Hayashi and Huganir, 2004). Naturally, regulation of dephosphorylation via phosphatases (Bradshaw et al., 2003; Colbran, 2004; Lee et al., 2000) is also important in these processes. The effect that phosphorylation or dephosphorylation has can be relatively direct, or can be via activating other signal transduction pathways such as the MAP kinase cascade (Thomas and Huganir, 2004; Zhu et al., 2002).

AMPARs can also be post-translationally modified by addition of a single ubiquitin molecule (mono-ubiquitin) via a process involving an E1 ubiquitin activating enzyme, transfer to a cysteine residue on an E2 ubiquitin conjugating
enzyme (Ubc) and finally the action of an E3 ubiquitin ligase to add the ubiquitin to a substrate protein. Mono-ubiquitin of membrane receptors can by itself serve as a signal for internalization (Shih et al., 2000). Poly-ubiquitinated chains can also be formed, which, when utilizing the lysine 48 (K48) residue or possibly other residues of ubiquitin, is a common signal for proteosome mediated degradation (Xu et al., 2009). Interestingly, phosphorylation of a residue may increase or decrease the likelihood of recognition by an ubiquitin E3 ligase. If it increases the likelihood, then the phosphorylated sequence that is preferentially recognized by an E3 ligase is referred to as a phosphodegron (Hunter, 2007).

Much of the work to date on GluR trafficking and phosphorylation has been done in mammalian cell culture, but relatively little has been done in a live behaving organism. The involvement of phosphorylation and ubiquitination in AMPAR trafficking can be studied in vivo in the model organism C. elegans. The C. elegans genome contains two AMPAR-type subunits: GLR-1 and GLR-2 (Hart et al., 1995; Maricq et al., 1995; Mellem et al., 2002), as well as putative homologs to several kinases involved in synaptic plasticity. We previously showed that fluorescently tagged versions of GLR-1 are functional and localized to postsynaptic regions when expressed in nematodes (Rongo et al., 1998). As with mammalian AMPARs, GLR-1 synaptic abundance is regulated by endocytosis and ubiquitination (Burbea et al., 2002; Grunwald et al., 2004; Juo and Kaplan, 2004). In addition, the C-terminal cytosolic tail sequences of both GLR-1 and GLR-2 have been found to be essential for synaptic localization, and
contain PDZ binding motifs as well as other sequences conserved with mammalian AMPAR subunits (Chang and Rongo, 2005).

Here, we take a reverse genetics approach to determining if the phosphorylation state of residues within the GLR-1 and GLR-2 carboxyl-terminal tail sequences regulates trafficking of these C. elegans glutamate receptors to and from post-synaptic areas in C. elegans neurons. Various versions of GLR-1 or GLR-2 were made in which serines that are possible phosphorylation sites are mutated, singly or in groups, to alanine, which can not be phosphorylated, or to aspartate, which can often mimic phosphorylation. Transgenic strains of animals were produced using these GFP tagged constructs and we found that one construct resulted in a marked decrease in the number of GLR-1::GFP puncta found in the neurites of these animals. This phenotype was blocked when using versions of GLR-1 that could not be ubiquitinated. We also examined GLR-1 localization in the background of mutations for several candidate kinases and a phosphastase, but found no effect in any of these. Our findings point to the possibility that phosphorylation at one particular serine of GLR-1 may normally regulate GLR-1 levels by regulating the ubiquitination of GLR-1 itself.
RESULTS

Mutating GLR-1 to prevent phosphorylation reduces the number and size of GLR-1 clusters.

We took a reverse genetics approach to investigate in vivo the potential effect of the phosphorylation of residues in the carboxyl terminal cytoplasmic domain (CTD) of *C. elegans* glutamate receptor subunits (Figure 1A) GLR-1 and GLR-2. We analyzed the CTD sequence of GLR-1 and GLR-2 (Figure 1B) to find potential phosphorylation sites by both manual examination and using the bioinformatics sites Scansite, NetPhos 2.0 and NetPhosK 1.0 (Blom et al., 1999; Blom et al., 2004; Obenauer et al., 2003). We created various cDNA constructs each consisting of the *glr-1* or *glr-2* promoter, followed by DNA coding for amino acids of the wild-type protein through to the end of the TM IV domain (Figure 1A), followed by GFP, and ending with the CTD (Figure 1C).

The first of the GLR-1 constructs, referred to as “GLR-1 WT” had coding for the wild-type GLR-1 CTD. The second and third constructs, “GLR-1-1” and “GLR-1-2” each had three serines mutated to alanines, each of which was predicted to be a possible phosphorylation site. Finally, the fourth GLR-1 construct, “GLR-1-3” had a threonine mutated to alanine and a tyrosine mutated to phenylalanine (Figure 1D).
Likewise, the first of the GLR-2 constructs, referred to as “GLR-2 WT” had coding for the wild-type GLR-2 CTD. The second and third constructs, “GLR-2-1” and “GLR-2-2” each had several serines or threonines mutated to alanines, each of which was predicted to be a possible phosphorylation site (Figure 1E). We introduced both the GLR-1 and GLR-2 cDNA constructs, as free extrachromosomal arrays, into glr-1; glr-2 animals, which completely lack both receptors.

In the case of the GLR-2, it has previously been very difficult to visualize puncta of GLR-2::YFP in the ventral nerve cord (VNC), although the GLR-2::YFP signal can be seen in the cell bodies of animals (Figure 2A). Observing animals expressing GLR-2 WT we saw similar results with the cell bodies visible (Figure 2B), but with the VNC virtually invisible (Figure 2E). We reasoned that this could be because in wild-type animals GLR-2 may not form functional homodimers, but rather may need to dimerize with GLR-1 to form functional heteodimers (Chang and Rongo, 2005). Therefore, we introduced the wild-type construct into GLR-1::RFP animals, reasoning that the GLR-1::RFP might dimerize with our GLR-2::GFP WT protein, and that the RFP signal might make confirming the location of the VNC easier. We found that in this background the GLR-2::GFP was visible in the cell bodies (Figure 2C), very faintly visible in the VNC (Figure 2G), with rare animals in which puncta were more visible and co-localized with GLR-1::RFP puncta (Figure 2F, H, and J). We then introduced our glr-2-1 construct into GLR-1::RFP animals and found that both the signal in the cell bodies and
VNC were similar to that seen for GLR-2 WT (Figure 2C, D, E, G, and I). Based on these results we decided it was unlikely that the phosphorylation of residues mutated in the glr-2-1 construct have a significant effect on the localization of GLR-2. However, given the faint signal of all GLR-2 constructs we decided to discontinue studies of GLR-2 localization and instead focus the balance of this study on GLR-1 localization.

It has previously been demonstrated that a fluorescently tagged version (Figure 3A, D) of GLR-1 can be functional and localized to postsynaptic clusters at neuron–neuron synapses within C. elegans neurites (Rongo and Kaplan, 1999; Rongo et al., 1998). We introduced glr-1 wt and other glr-1 constructs (Figure 1C) into glr-1;glr-2 animals, reasoning that the lack of endogenous receptors would highlight the effect, if any, of the mutated versions of the constructs. We found that the pattern of GLR-1::GFP localization in the cell bodies for GLR-1 WT animals was similar to that for the GLR-1::GFP strain (nuIs25) we use as a control (Figure 3A, B) as well as being similar in GLR-1-2 and GLR-1-3 animals (data not shown). However, the cell bodies in GLR1-1 animals were somewhat dimmer than in GLR-1 WT animals (Figure 3B, C). In the VNC we found that GLR 1-1 WT animals had a punctate expression pattern that was similar to, although dimmer than nuIs25, with GLR-1-2 and GLR-1-3 animals also appearing similar to this (Figure 3D-E and G-H). However, the VNC in GLR1-1 animals was markedly dimmer with few, if any, puncta visible and visible puncta appearing smaller than those seen in GLR-1 WT animals. It is
possible but unlikely that the difference between constructs is due to the amount of DNA injected to make the original transgenic strains since we were careful to use the same concentration for each construct. Therefore, this suggests that the presence of one or more of the serines (S889, S891, S893) mutated in construct *glr-1-1* could be essential for proper trafficking of GLR-1. Our tentative hypothesis at this point was that phosphorylation of one or more of these residues is essential to allow GLR-1 to be maintained stably in the ventral nerve cord.

**Phosphorylation of GLR-1 may mediate trafficking via regulation of the ubiquitination state of GLR-1.**

It has previously been shown that phosphorylation of serine or threonine residues can regulate the ubiquitination state of nearby lysines in proteins (Hino et al., 2005). In addition, the ubiquitination of membrane receptors can regulate their endocytosis (Ehlers, 2003; Hicke and Dunn, 2003; Shih et al., 2000). Therefore, we reasoned that a possible explanation for the reduction in VNC puncta seen in GLR-1-1 expressing animals could be that in the absence of normal phosphorylation the mutated GLR-1 protein is ubiquitinated to a greater than normal degree and therefore endocytosed and subsequently degraded.

One approach to studying the effect of ubiquitination on GLR-1 trafficking in *C. elegans* has been to over-express a version of GLR-1 (GLR-1:4KR) in which
each of the four lysine residues in the intracellular “tail” of the receptor have been changed to arginines, eliminating the possibility of them being ubiquitinated. In otherwise wild-type animals, this results in an increase in the number, size and brightness of GLR-1 accretions in the ventral cord (Figure 4A and B) (Burbea et al., 2002). We made 4KR versions of both the glr-1 wt and glr-1-1 constructs and introduced these into glr-1;glr-2 animals.

The appearance of the cell bodies in GLR-1 4KR strains were generally similar to that found in nuls25 animals (data not shown). However, we found that, as in nuls25 compared to a glr-1::4kr strain (nuls108), our GLR-1 WT 4KR animals had larger, brighter and more numerous VNC puncta than GLR-1 WT animals (Figure 4A-C, and E). We also found that our GLR-1-1 4KR animals had a very similar phenotype, with a relatively great change from the GLR-1-1 VNC phenotype, to the point that many animals had large patches of GLR-1::GFP signal, similar to those found in nuls108 (Figure 4B, D, and F). This suggests that the GLR-1 serines mutated in the glr-1-1 construct may be essential for proper ubiquitination of GLR-1 since when GLR-1 cannot be ubiquitinated the effect of the glr-1-1 construct on GLR-1 trafficking is lost.
Mimicking or blocking phosphorylation of GLR-1 at a single serine is sufficient to affect trafficking.

Post-translational phosphorylation of proteins can affect protein function, at least partially due to the negative charge that the phosphate groups bestow upon the phosphorylated residue. Therefore, mutating normally phosphorylated residues to aspartate, which has a negative charge, can mimic constitutive phosphorylation at that residue. To test if the affect of the mutated serines in glr-1-1 is truly due to their inability to be phosphorylated, we created constructs with these residues mutated to aspartate, both as a group and individually, and introduced these individually into glr-1;glr-2 animals. We found that animals expressing the 1-1 Dall (all three 1-1 residues mutated – see Figure 1D), or D1, or D2, or D3 (first, second, or third residues mutated respectively from left to right in sequence) each had cell bodies similar to those found in GLR-1 WT animals (data not shown) indicating GLR-1 was able to normally traffic from the cell bodies to the neurites. While there was some variability between constructs (GLR-1-1 D3 animal VNC puncta being perhaps slightly dimmer), in general we found that the VNC for all aspartate mutants were punctate, sometimes with brighter puncta than seen in GLR-1 WT animals (Figure 5F, and K-N). If the mere fact of mutating these residues was what caused a phenotype than the constructs with mutations to alanines and the constructs with mutations to aspartates might be expected to similarly disturb GLR-1 trafficking. Therefore, these results support the hypothesis that it is the phosphorylation state of the
affected residues, rather than the mere fact that they are no longer serines, that causes the phenotype seen in GLR-1-1 animals.

To help identify exactly which residues are important for the GLR-1-1 phenotype we also made constructs in which only one of the three serines were mutated to alanine. These were labeled as GLR-1-1 A1, A2, or A3 (first, second, or third residues mutated respectively from left to right in sequence). We found that the cell body (data not shown) and VNC puncta phenotypes of the A1 and A3 animals were similar to GLR-1 WT (Figure 5F, H, and J). However, the cell body phenotype and VNC puncta phenotype of A2 animals was very similar to that seen in GLR-1-1 animals (Figure 5B-D, F, G, and I). This suggests that it is the “A2” (S891) GLR-1 residue whose phosphorylation state regulates the trafficking of GLR-1.

The effect of various kinases and phosphatases on GLR-1 trafficking.

If the phosphorylation state of GLR-1 S891 or other GLR-1 residues is important in vivo for regulating GLR-1 localization, then we reasoned that strains mutated for the kinase(s) and/or phosphatase(s) that regulate GLR-1 phosphorylation might have a GLR-1 mis-localization phenotype. It is already known that animals mutant for CAMKII have GLR-1 trafficking defects in which GLR-1 accumulates in the cell bodies and there is a lower density of puncta in the VNC (Rongo and Kaplan, 1999). However, this differs from what we saw with our GLR-1-1 construct. We took a candidate gene approach here and
examined a number of kinase or phosphatase gene mutants, each crossed to a GLR-2::YFP strain, *odls18* or a GLR-1::GFP strain, *nuls25*. As previously seen, the GLR-2 strains proved very difficult to evaluate, but we saw no obvious effect for animals mutant for *dgk-1* (which have increased PKC kinase activity) when these mutants were crossed to *odls18* (two *dgk-1* alleles tested – data not shown).

We found that *pkc-1* animals (two alleles tested, data not shown for allele *rt144*) crossed to *nuls25* appeared similar to wild-type (Figure 6A-B). On the other hand, while *dgk-1* animals were also similar to wild-type (Figure 6A, and C), there were in some animals some more large accretions of GLR-1::GFP in the VNC compared to wild-type. Animals mutant for *kin-2*, the gene encoding the regulatory subunit of PKA, also were similar to wild-type (Figure 6A, and D), though occasional animals may have brighter cell bodies or slightly greater size or density of puncta in the VNC. Animals mutant for gene *pptr-2* (PP2A phosphatase) were also similar to wild-type (Figure 6A, and E), with occasional animals that had slightly less puncta in the VNC, and perhaps brighter cell bodies. Finally, animals in which we over-expressed a PP2A regulatory subunit dominant negative construct (dB56) were also similar to wild-type (Figure 6A, and F). Overall, these results do not strongly support PKC, PKA or PP2A being important for GLR-1 trafficking, but given the preliminary nature of these experiments these proteins are not entirely ruled out as GLR-1 localization regulators and could have subtle effects.
DISCUSSION

We took a reverse genetics approach to determine if the phosphorylation state of residues within the GLR-1 and GLR-2 carboxyl-terminal tail sequences regulates trafficking of these *C. elegans* glutamate receptors to and from post-synaptic areas in *C. elegans* neurons. We used versions of GLR-1 or GLR-2 in which serines that are likely phosphorylation sites were mutated to alanine or aspartate. We found that one construct with several serines mutated to alanines caused a reduction in the GLR-1 puncta seen in the ventral nerve cord (VNC), and this phenotype was prevented when using versions of GLR-1 that could not be ubiquitinated. The key residue was found to be GLR-1 S891. In contrast, mutations to aspartate resulted in punctate GLR-1 distribution similar to or brighter than in wild-type. We also examined GLR-1 localization in the background of mutations for several candidate kinases and a phosphatase, but found no effect in any of these.

The effect of various kinases and phosphatases on GLR-1 trafficking in *C. elegans*

Regulation of trafficking of AMPARs can be by means of various kinases and phosphatases regulating the phosphorylation state of individual AMPAR residues (Bradshaw et al., 2003; Colbran, 2004; Esteban et al., 2003; Lee et al., 2000;...
Lisman et al., 2002; Mammen et al., 1997; Matsuda et al., 1999; McDonald et al., 2001; McGlade-McCulloh et al., 1993; Roche et al., 1996; Rongo and Kaplan, 1999). We examined the localization of GLR-1 in the background of mutations that reduced or increased the activity of PKC kinase, increased the activity of PKA kinase or reduced the activity of PP2A phosphatase, but found no significant change in GLR-1 localization. This implies that these kinases and this phosphatase are not important in the regulation of the basal localization of GLR-1. However, the affects of the phosphorylation state of AMPAR’s on synaptic trafficking are often activity dependent (Esteban et al., 2003; Oh et al., 2006) in mammalian cells. Therefore, it is still formally possible that the effects of these particular kinases and phosphatase would only be seen in a situation in which GLR-1 containing synapses are differentially stimulated for mutants and wild-type worms, as has been done in other studies into *C. elegans* synaptic function (Emtage et al., 2009).

**Possible phosphorylation of GLR-1 and its role in regulating GLR-1 localization**

We found that in one of our mutant GLR-1 constructs (GLR-1-1) with three serines in the GLR-1 C-terminal domain (CTD) mutated to alanines there is a marked decrease in GLR-1 puncta in the ventral nerve cord (VNC). We were able to further map the phenotype to mutation of a single serine (S891). The fact that the phenotype maps to a single residue and is not present in a serine to
aspartate mutant suggests that the phenotype is produced due to the inability of S891 to be phosphorylated. The use of immuno-histochemical reagents, not readily available when this research was originally done, might help confirm the phosphorylation of S891. This could be done using antibodies specific for the CTD sequence containing either non-phosphorylated or phosphorylated S891.

Unfortunately, S891 is not in a strong consensus sequence for any of the kinases we have considered. However, lack of a consensus sequence could allow for finer regulation of phosphorylation, perhaps requiring some type of scaffolding protein process to allow this section of GLR-1 to be exposed to a sufficiently high concentration of a kinase at the appropriate time and in the appropriate sub-cellular location as is the case for the mammalian GluR1 subunit that is phosphorylated at S831 by CaMKII in a non-consensus site (Mammen et al., 1997).

**Possible interactions between GLR-1 phosphorylation and ubiquitination state**

Past experiments done to over-express in *C. elegans* a version of GLR-1 (GLR-1:4KR) in which the C-terminal domain (CTD) cannot be ubiquitinated resulted in an increase in the number, size and brightness of GLR-1 accretions in the VNC. We made a version of our expression constructs with the 4KR changes in it and were able to reproduce these results. Interestingly, these 4KR
changes were able to preclude the reduction in GLR-1 puncta we had found using our GLR-1-1 construct. Since the 4KR changes appear to act by reducing endocytosis of GLR-1 (Burbea et al., 2002), the blocking of the GLR-1-1 phenotype suggests that the residues mutated in GLR-1-1 are important for the degradation of GLR-1 downstream of endocytosis, possibly via the phosphorylation of GLR-1 S891. Phosphorylation of proteins can have many affects as mediated by changes to the interactions of the substrate proteins with other proteins, one of these effects being to increase or decrease the likelihood of ubiquitination of these proteins (Hino et al., 2005). Therefore, it is possible that the phosphorylation of GLR1-1 S891 is normally important in regulating the ubiquitination and thereby the degradation of GLR-1, which in turn is important in proper synaptic localization and trafficking of GLR-1.

The studies so far are preliminary in nature, and significant work remains to be done, as detailed further in chapter four of this thesis. However, one model that would explain our results would envision GLR-1 as being constitutively phosphorylated at S891 under basal condition. This phosphorylation may act to inhibit ubiquitination and subsequent internalization. In this admittedly speculative model, it would be the de-phosphorylation of GLR-1 at this residue, perhaps in response to a change in synaptic activity, that would trigger GLR-1 ubiquitination and internalization (Figure 7).
An alternative to the above model is that S891 phosphorylation directly regulates the endocytosis of GLR-1. In this second scenario, the inability of S891 to be phosphorylated may cause an abnormally high level of endocytosis and degradation, similar to that seen when mono-ubiquitin is over-expressed in *C. elegans* (Burbea et al., 2002). Follow-up experiments, some of which are suggested in chapter four, could distinguish between the possibilities suggested here and perhaps lead to a better understanding of how phosphorylation and ubiquitination can work together to regulate GluR trafficking and stability.

Over-expression of ubiquitin under the *glr-1* promoter decreases GLR-1 abundance (Burbea et al., 2002), so one initial experiment could be to create an integrated GLR-1-1 strain then see if the effect of over-expressed ubiquitin is additive with the GLR-1-1 phenotype. If it is then it would be more likely that phosphorylation has a direct effect on endocytosis and/or degradation of GLR-1, not completely mediated through an effect on the ubiquitination of GLR-1. Another experiment that could be done with such an integrated strain is to see the effect of mutations that inhibit endocytosis, such as that found in the *unc-11* mutant (Burbea et al., 2002).

The present study suggests that the phosphorylation state of a single residue of the GLR-1 CTD is able to strongly effect GLR-1 localization. This is reminiscent of how mammalian glutamate receptor (GluR) single residue phosphorylation states have been shown to be important for the trafficking of
GluR to or away from the post-synaptic densities (Carvalho et al., 1999; Mammen et al., 1997; Soderling and Derkach, 2000). This implies that regulation of GluR trafficking in *C. elegans* shares more in common with that found in mammalian systems than had been previously known. Further investigation of this phosphorylation state dependent GluR trafficking in the genetically tractable organism *C. elegans* could give both in vivo confirmation to mammalian cell culture studies and allow for genetic screens leading to further information on accessory proteins involved in this trafficking.

**MATERIALS AND METHODS**

**Strains**

Animals were grown at 20°C on standard NGM plates seeded with OP50 E. coli. Some strains were provided by the Caenorhabditis Genetics Center. Most strains were backcrossed multiple times to our laboratory N2 strain to minimize other genetic variation. The following strains were used: dgk-1(nu62), dgk-1(sy428), glr-1(ky176), glr-2(ak10), kin-2(ce179), n2, nuls108[GLR-1(4KR)::GFP], nuls25[Pglr-1::GLR-1::GFP], odEx[Pglr-1::GLR-1(4KR) (wild-type or 1-1 versions), lin-15(+)], odEx[Pglr-1::GLR-1(wild-type or 1-1, or 1-2, or 1-3 version), rol-6dm], odEx[Pglr-1::GLR-2(wild-type or 2-1 version), rol-6dm],
Transgenes and Germline Transformation

A number of existing transgenic strains were used. New transgenic strains used in this study were isolated after microinjecting various plasmids (5–50 ng/ml) using *rol-6dm* (a gift from C. Mello, UMass) or RFP (monomeric RFP; a gift from R. Tsien, Stanford Univ.), or *lin-15(+)* as a marker. Plasmids to test GLR-2 function contained the *glr-1* promoter, and were followed by the wild-type DNA through to the end of trans-membrane domain four, followed by *gfp*, then by the *glr-2* C-terminal “tail” region and were generated using standard techniques. Plasmids to test GLR-1 function contained the *glr-1* promoter, and were followed by the wild-type DNA through to the end of trans-membrane domain four, followed by *gfp*, then by the *glr-1* C-terminal “tail” region and were generated using standard techniques. The “tail” DNA used in making constructs was either wild-type, or contained one or more point mutations produced using site directed mutagenesis. All resulting transgenes were introduced into the germline and followed as extra-chromosomal arrays.
**Fluorescence Microscopy**

GFP- and RFP-tagged fluorescent proteins were visualized in nematodes by mounting larvae on 2% agarose pads with levamisole. Fluorescent images were observed using a Zeiss Axioplan II. A 1006 (N.A. = 1.4) PlanApo objective was used to detect GFP and RFP signal. Imaging was done with an ORCA charge-coupled device (CCD) camera (Hamamatsu, Bridgewater, NJ) using IPLab software (Scanalytics, Inc, Fairfax, VA). Exposure times were generally chosen to fill the 12-bit dynamic range without saturation.
FIGURES

A  Schematic showing structure of an AMPA type glutamate receptor subunit

B  Sequence of GLR-1 and GLR-2 ctds and alignment to similar sequences in other organisms.

\[
\begin{align*}
glu\text{r}1 &: IEFCYKS\text{ES}KX 837 \\
glu\text{r}2 &: IEFCYKS\text{R}AEK 844 \\
gl\text{r}1 &: GEFLYRS\text{RI}EAR 887 \\
gl\text{r}2 &: VEFLPRKN\text{EN}R 930 \\
\text{glur}1 &: RM\text{KGFC}L\text{IPQ}QS\text{S}--\text{INEA}\text{IRTS\text{T}LPRNSGAGAGSGS\text{GEN}RV\text{VSHDFPKX} 885 \\
\text{glur}2 &: RM\text{K}\text{---}V\text{AKN}\text{----}AQ\text{NI\text{NFS}SSQ\text{S}Q\text{NFAT\text{YKEGYNVG}---\text{-------} 877 \\
gl\text{r}1 &: K\text{SSNSSMV\text{ANFAKNLK}\text{SALSQ}L\text{RLSVEGGAVAQPG\text{OSHAINR}RQVAA} 937 \\
gl\text{r}2 &: E\text{KERNM}R\text{S}-----\text{SRPLKPGLISCE}R---\text{---------AKQK}--- 958 \\
\text{glur}1 &: SM\text{QI}PC\text{MSHSSGMPLG}\text{ATGL}\text{---} 906 \\
\text{glur}2 &: -\text{IESV}KI\text{-------} ------- 883 \\
gl\text{r}1 &: FL\text{PANEKEAFNNVDRPAN\text{TLYNTAV} 962 \\
gl\text{r}2 &: --\text{LQR}T\text{KSLEE}VT\text{PRSDFL}\text{F}---- 977 \\
\end{align*}
\]

C  \text{g}l\text{r}-1/2 \text{promoter} | \text{WT} \text{glr}-1/2 - \text{TM IV} | \text{gfp} | \text{glr}-1/2 \text{WT or mutant tail}

D  GLR-1 constructs

\[
\begin{align*}
\text{Residue} & \quad \text{Mutated} & \text{Construct} & \text{Possible Kinases} \\
S882 & N & 1-1, A1 & \text{** PKG} \\
S889 & Y & 1-1, A2 & \text{** PKA} \\
\underline{S891} & Y & 1-1, A3 & \text{Weak CaMKII} \\
S893 & Y & 1-2a & \text{* PKC} \\
S904 & N & 1-2a & \text{* PKC} \\
S907 & Y & 1-2a & \text{** PKC} \\
S908 & Y & 1-2a & \text{** PKC} \\
S913 & Y & 1-2b & \text{(homologous to GluR1 S845 PKA phos site)} \\
T956 & Y & 1-3 & \text{** PKA (homologous to GluR1 S845 PKA phos site)} \\
Y958 & Y & 1-3 & \text{Mutating since close to c-term PDZ site.} \\
\end{align*}
\]

E  GLR-2 constructs

\[
\begin{align*}
\text{Residue} & \quad \text{Mutated} & \text{Construct} & \text{Possible Kinases} \\
S870 & Y & 2-1 & \text{** CaMKII, PKC} \\
S871 & Y & 2-1 & \text{PKA, PKC} \\
S881 & N & 2-1 & \text{PKC} \\
T895 & Y & 2-2c & \text{PKA} \\
S897 & Y & 2-2c & \text{CaMKII, PKA} \\
S901 & Y & 2-2a & \text{**} \\
T902 & Y & 2-2a & \text{**} \\
S905 & Y & 2-2b & \text{CKI (homologous to GluR2 S880 PKC phos site)} \\
T906 & N & 2-2b & \text{PKA (part of c-term PDZ site, so do not mutate)} \\
\end{align*}
\]
Figure 1. GLR-1 encodes an AMPA type glutamate receptor subunit. (A) Schematic showing the tetrameric structure of AMPA type glutamate receptors. (B) An amino acid alignment of the *C. elegans* GLR-1 and GLR-2 carboxy terminal cytoplasmic domain (CTD) sequences with that of related proteins in other species. ("**" = residues or nucleotides in that column are identical in all sequences in the alignment. ":" = conserved substitutions have been observed, "." = semi-conserved substitutions are observed, bold/italic/underlined residues have been shown to be phosphorylated in GluR1 and GluR2). (C) Text schematic and diagram of the type of constructs used to make transgenic lines to test GLR-1 phosphorylation mutations. (D,E) GLR-1 (D) and GLR-2 (E) C-terminal sequences and residues mutated. **Bolded** numbers are above or below the first mutated residue of the construct the numbers correspond to. **Underlined** = possible phosphorylation site, *italic* = residue choose not to mutate, **bold** = mutated residues. Tables are of selected residues and the kinases that may phosphorylate them. * = residue that is likely phosphorylated per the Net Phos website. Note that the first GLR-1 residue shown is number 878, whereas the first GLR-2 residue shown is number 852.
Figure 2. A mutant version of GLR-2 does not affect receptor localization.

GLR-2::YFP (A) and GLR-2::GFP (B, C, D) interneuron cell body fluorescence in wild-type (A), glr-1;glr-2 (B), or GLF-1:RFP (C, D) worms all visualize only faintly. GLR-2::GFP (E, G, H, I) fluorescence or GLR-1::RFP (F) observed along the ventral cord neurites of glr-1;glr-2 (E) or GLF-1:RFP (F, G, H, I, J) worms. GLR-2 puncta are generally few or absent (E, G, H, I), whereas GLR-1 puncta are readily visible (F), and co-localize with the fewer GLR-2 puncta when merged (J). YFP/GFP constructs are either integrated (A and B) or free arrays (C-E and H-J).
A GLR-1::GFP (nults25)  
B GLR-1::GFP wild-type  
C GLR-1::GFP 1-1  
D GLR-1::GFP (nults25)  
E GLR-1::GFP wild-type  
F GLR-1::GFP 1-1  
G GLR-1::GFP 1-2  
H GLR-1::GFP 1-3
Figure 3. Mutant versions of GLR-1 regulates abundance of GLR-1 puncta in neurites. GLR-1::GFP interneuron cell body (A-C) and ventral cord neurite (D-H) fluorescence in wild-type (A) or glr-1;glr-2 (B-H) worms. Constructs are either integrated (A and D) or free arrays (B-C and E-H).

Figure 4. Phosphorylation of GLR-1 may mediate trafficking via inhibiting the ubiquitination of GLR-1. GLR-1::GFP ventral cord neurite fluorescence in wild-type (A) or glr-1;glr-2 (B-F) worms. GFP constructs are either integrated (A) or free arrays (B-F).
Figure 5. GLR-1 phosphorylation at a single residue is sufficient to regulate GLR-1 puncta abundance.  GLR-1::GFP interneuron cell body (A-D) and ventral cord neurite (E-N) fluorescence in wild-type (A and E) or glr-1;glr-2 (B-D and F-N) worms. Constructs are either integrated (A and E) or free arrays (B-D and E-N).
Figure 6. Mutations affecting kinase and phosphatase activity have little affect on GLR-1 puncta abundance. GLR-1::GFP ventral cord neurite fluorescence. GFP constructs are integrated.
Figure 7. A model for regulation of GLR-1 ubiquitination by S891 phosphorylation.

Previous results indicate that GLR-1 is endocytosed and recycled by two pathways: a clathrin-independent pathway that requires RAB-10 for recycling and a clathrin-dependent pathway that requires LIN-10 for recycling. In both panels GLR-1 receptors (red) are being endocytosed and recycled by either the clathrin-independent endocytosis pathway (CIE) and RAB-10 (Top Panel) or the clathrin-dependent endocytosis pathway (CDE) and LIN-10 (Bottom Panel). Our preliminary results suggest the possibility that basal phosphorylation of GLR-1 at S891 (shown at left of each panel by green hexagons) may normally function to inhibit ubiquitination of GLR-1 (shown throughout by blue hexagons) and thereby reduce its internalization in one or both of these pathways. De-phosphorylation of this residue would then allow GLR-1 to be ubiquitinated and endocytosed.
CHAPTER IV

Conclusions and future directions
We have identified an *in vivo* role for UEV-1 in a metazoan, *C. elegans*, in regulating the trafficking of an AMPA-type glutamate receptor subunit, GLR-1, by means of a genetic screen. We found that UEV-1 is broadly expressed in *C. elegans* cells and tissues, and that the lack of functional UEV-1 causes a specific defect in the localization of GLR-1, as well as a defect in the formation of motor neuron neuromuscular junctions. The GLR-1 localization defect can be rescued cell autonomously in GLR-1-expressing interneurons by expression of a *uev-1* cDNA. Our evidence indicates that UEV-1 likely does not function as part of the ERAD pathway, but in determining this we uncovered a strong spatial preference for where GLR-1 accretions form along the ventral cord of *C. elegans* in *uev-1* mutants. We provide evidence that GLR-1 accumulates at RAB-10-containing endosomes in *uev-1* mutants, and that receptors arrive at these endosomes independent of clathrin-mediated endocytosis. UEV-1 homologs found in other species are thought to function by binding to Ubc13, with the resulting heterodimers forming K63-linked ubiquitin chains. We find that whereas UEV-1 can interact with *C. elegans* UBC-13, global levels of K63-linked ubiquitination seem unaffected in *uev-1* mutants. Nevertheless, UBC-13, like UEV-1, is required to regulate GLR-1 trafficking. Our results suggest that UEV-1 and UBC-13 could regulate a small subset of K63-linked ubiquitination events in nematodes, at least one of which is critical in regulating GLR-1 trafficking.

Based on our findings, expressed in more detail in chapter two, it is clear that UEV-1 has a role in regulating GLR-1 localization. A model for what processes
are being regulated will be discussed further below. However, a key question is by what physical mechanism UEV-1 is working in regulating GLR-1 in *C. elegans*. UEV family proteins in other organisms have been found to function at times in the recognition and binding of previously ubiquitinated substrate proteins. *C. elegans* UEV-1 is similar to TSG101, itself a homolog of the yeast ESCRT protein Vps23 (Kim et al., 2007a; Thomson et al., 1998). ESCRT proteins function to route ubiquitinated receptors from the early endosome to multivesicular bodies and eventually to lysosomes, and Vps23 is a subunit of ESCRT-1, which binds ubiquitinated receptors in the early endosome (Malerod and Stenmark, 2007). We explored the possibility that UEV-1 is working as part of the ESCRT complex in *C. elegans* or in a similar role by introducing mutations in known ESCRT complex (or related) genes into our GLR-1::GFP reporter strain. In general there were no marked changes in the GLR-1 localization observed in these mutants vs. wild-type. However, there were some subtle differences, and possibly a bit more robust change in the case of *pqn-19/stam-1* mutants, not followed up on at the time due to technical challenges and more promising directions elsewhere. UEV-1 does not appear to be localized to endosomes, as would be expected for a Vps23 homolog and ESCRT complex protein, which argues against investigating this further. Still, in continuing this research it might be worthwhile to re-examine GLR-1 localization more closely in some of these mutant backgrounds, as well as perhaps some additional mutants that may have become available.
UEV family proteins have also been found to form a heterodimer with UBC-13 (Hofmann and Pickart, 1999; Pickart, 2000, 2004) to catalyze the formation of K63-linked poly-ubiquitin chains. In other organisms, K63-linked poly-ubiquitination is important in several processes, including lysosomal targeting (Barriere et al., 2007; Kamsteeg et al., 2006; Lauwers et al., 2009; Umebayashi et al., 2008), as well as the formation and autophagic clearance of protein inclusions (Lim et al., 2006; Tan et al., 2007), and protein transport (Olzmann et al., 2007). As pointed out previously, consistent with this possible function, RNAi screens have uncovered a role for UEV-1 and UBC-13 in regulating polyglutamine inclusions in C. elegans (Howard et al., 2007). However, RNAi of ubc-13 results in embryonic lethality (Rual et al., 2004; Sonnichsen et al., 2005), a far more dramatic phenotype than that observed in uev-1 null mutants, suggesting that UBC-13 has additional functions that are independent of UEV-1. We found the levels of K63-linked poly-ubiquitin are not grossly different in uev-1 mutants compared to the levels found in wild-type, suggesting that UEV-1 is not needed for most K63-linked ubiquitination events in nematodes. As previously discussed in chapter two, UBC-1 can interact with UBC-13 in a yeast two-hybrid assay (Gudgen et al., 2004; Li et al., 2004) and UBC-13, UBC-1, and NHL-1 (an E3 ubiquitin ligase) can form poly-ubiquitin conjugates in vitro (Gudgen et al., 2004). Thus, it is possible that in C. elegans UBC-13 conducts most K63-linked ubiquitination while working with UBC-1 rather than when partnered with UEV-1 and that UEV-1/UBC-13 heterodimers may regulate the K63-linked ubiquitination
of a relatively small number of substrates, one or more of which is involved in GLR-1 trafficking.

While most available mutations in UBC-13 are intronic or lethal, a recent paper reported that a mutant strain (\textit{ubc-13(tm3546)}) previously thought to be lethal may be viable. Crossing this to our GLR-1 strain, we find that \textit{ubc-13} mutants have the same GLR-1 trafficking defects as \textit{uev-1} mutants, supporting the idea that a UEV-1/UBC-13 heterodimer complex is regulating GLR-1 trafficking. Our finding that mutations known to de-stabilize UEV-1/UBC-13 heterodimers also inhibit in vitro binding of \textit{C. elegans} UEV-1 and UBC-13 shows these two proteins can associate closely in \textit{C. elegans}. To look further at this we could do a co-ip assay and see if each is able to pull down the other, and perhaps other interacting proteins. To help determine with which proteins UEV-1/UBC-13 interacts and potentially ubiquitinates, we could do a yeast two hybrid screen using UEV-1 as prey.

An additional experiment would be to obtain a large amount of wild-type and \textit{uev-1} worm lysates and attempt to determine if there is a decrease in GLR-1 K63 linked poly-ubiquitination in \textit{uev-1} mutants. However, if GLR-1 K63 linked poly-ubiquitin is not altered in \textit{uev-1} mutants this would not rule out UEV-1/UBC-13 catalyzed K63 poly-ubiquitin chain formation as a mechanism for UEV-1 function since the effect on GLR-1 localization in \textit{uev-1} mutants could easily be due to K63 linked poly-ubiquitin of accessory proteins rather than that of GLR-1 itself.
It is known that *C. elegans* AMPARs can be heteromers containing both GLR-1 and GLR-2 (Chang and Rongo, 2005). Therefore it might be useful to look at the possibility that it is the K63 linked poly-ubiquitination of GLR-2, rather than that of GLR-1, that is regulated by UEV-1. GLR-2::GFP has not been readily visualized in the ventral nerve cord of *C. elegans*, but as a start toward looking at this we could see if the GLR-1 accumulations seen in *uev-1* animals are altered in the background of a *glr-2* mutant.

Leaving the question of mechanism for the moment, it is clear from our findings that UEV-1 does have a role in regulating GLR-1 localization, and determining the processes that are regulated would be helpful in understanding this better. In *uev-1* mutants there is a decreased spontaneous reversal frequency, indicating that either there is less GLR-1 at synapses or that much of the GLR-1 containing receptors at the synapses is not functional for some reason. The former possibility is more likely given the significant increase in the colocalization of GLR-1::GFP with mRFP::RAB-10, which is associated with early endosomes. The colocalization finding also raises the possibility that UEV-1 regulates the flow of GLR-1 receptors out of the early endosome, regardless of whether the flow leads back to the plasma membrane or to the lysosome. In this model UEV-1 functions in GLR-1-expressing interneurons to facilitate the exit of GLR-1 from early endosomes. A fraction of GLR-1 receptors is normally endocytosed by a clathrin-independent pathway and sent to RAB-10-containing
early endosomes (Glodowski et al., 2007). Subsequently, GLR-1 receptors exit these endosomes and are either recycled to the synapse or sent to lysosomes for turnover. In the absence of UEV-1 function, a fraction of GLR-1 receptors become endocytosed into these endosomes, but cannot exit them, resulting in a net build up of receptor in the neurons while simultaneously depleting the synaptic surface of receptor and resulting in decreased GLR-1 signaling.

The above model is consistent with work showing a role for K63-linked poly-ubiquitination in the trafficking of renal aquaporin-2 water channels (Kamsteeg et al., 2006), the epidermal growth factor receptor (Umebayashi et al., 2008), Gap1 permease (Lauwers et al., 2009), and other proteins (Barriere et al., 2007), with the novel feature that the GLR-1 receptor itself is not the likely K63-linked ubiquitination target. This model raises the possibility that UEV proteins are important for AMPAR turnover, which could have implications for synaptic plasticity and neurodegeneration in other organisms, including mammals. A broad way of looking at the role of UEV-1 might be to do a genetic screen for suppressors of the large GLR-1::GFP accumulations seen in \textit{uev-1}. Some of the mutants isolated from such a screen help us determine what proteins UEV-1 interacts with to regulate GLR-1. For example, such a screen could uncover proteins which use the poly-ubiquitination state of GLR-1 to determine if GLR-1 is to be recycled or routed to the lysosome.
While much of our research on UEV-1 has to do with its role in regulating GLR-1, it would be interesting to also look further at its role in regulating neuromuscular junction formation. Using a Punc-25::snb-1::gfp reporter strain (juIs1) we had found this process to be disrupted somewhat in uev-1 mutants in a way similar to but less than the disruption previously seen in rpm-1 mutants (Nakata et al., 2005). While a recent study found that another C. elegans UEV protein, UEV-3, suppresses the neuromuscular phenotype of rpm-1, this is not surprising given the significant differences in sequence between the two proteins (Trujillo et al., 2010b). An experiment to start looking into this further could include crossing our juIs1; uev-1 strain to rpm-1 to see if the phenotypes are additive, which would indicate RPM-1 and UEV-1 work in different pathways in this process. In addition we would want to examine if the action of UEV-1 here is cell autonomous and look at co-localization with SNB-1 by making and expressing a Punc-25::uev-1::rfp construct in both juIs1 and juIs1; uev-1 backgrounds. Also, we would want to cross a neuromuscular junction reporter strain to ubc-13 strain to help determine if UBC-13 is also important for UEV-1 function in proper formation of neuromuscular junctions.

In the other related project, discussed in chapter three of this thesis, we took a reverse genetics approach to determining if the phosphorylation state of residues within the GLR-1 and GLR-2 carboxyl-terminal tail sequences regulates trafficking of these C. elegans glutamate receptors to and from post-synaptic areas in C. elegans neurons. We used versions of GLR-1 or GLR-2 in which
serines that are likely phosphorylation sites were mutated to alanine or aspartate. We found that one construct with several serines mutated to alanines caused a reduction in the GLR-1 puncta seen in the ventral nerve cord (VNC), and this phenotype was precluded when using versions of GLR-1 that could not be ubiquitinated. The key residue was found to be GLR-1 S891. Parallel mutations to aspartate resulted in punctate GLR-1 distribution similar to or brighter than in wild-type. We also examined GLR-1 localization in the background of mutations for several candidate kinases and a phosphastase, but found no effect in any of these.

Due to the preliminary nature of these investigations we expressed the constructs used as extra-chromosomal arrays, which can lead to variations in protein expression levels between various independent “lines” for a given construct. To help control for this we always looked at several lines for each construct, only reporting a result if it was consistent across multiple lines. However, the use of this expression method precluded quantification of the effects of various constructs on GLR-1 localization. Therefore, before proceeding any further on this project an important step would be to generate strains in which these key constructs (GLR-1-WT, GLR-1-1 and GLR-1 A2 at a minimum) had been integrated into the chromosomes of *C. elegans*. This would be followed by quantifying the differences in GLR-1 localization and protein levels between strains to verify the previous results and better see the exact effects of preventing the phosphorylation of key GLR-1 serines. In addition to this it would
be desirable to check the GLR-1 expression levels in each strain to ensure that the decrease in GLR-1::GFP seen in GLR-1-1 and GLR-1 A2 vs. GLR-1_WT is not simply due to a change in GLR-1 expression levels between strains.

A next step after the above experiments could be to create antibodies which recognize GLR-1 phosphorylated or not phosphorylated at S891. These reagents could be used to biochemically examine the phosphorylation of wild-type vs. GLR-1-1 and GLR-1 A2 to confirm that it is the phosphorylation state of S891 that is causing the GLR-1-1 phenotype. Also, behavioral (spontaneous reversals) assays would help to confirm that the evident virtually absent GLR-1::GFP seen for the GLR-1-1 construct corresponds to little or no functional GLR-1-1 at the synapses.

A key question to resolve would be the relationship between the phosphorylation of GLR-1 and its internalization, which is known to be at least partially dependent on the ubiquitination of GLR-1, and likely other proteins (Burbea et al., 2002; Grunwald et al., 2004; Hart et al., 1995; Juo and Kaplan, 2004; Park et al., 2009; Schaefer and Rongo, 2006). Over-expressing a version of GLR-1-1 (GLR-1-1:4KR) in which the C-terminal domain (CTD) can not be ubiquitinated resulted in prevention of the reduction in GLR-1 puncta we had found with the GLR-1-1 construct. Since the 4KR changes appear to act by reducing endocytosis of GLR-1 (Burbea et al., 2002), the suppression of the GLR-1-1 phenotype suggests that the residues mutated in GLR-1-1 are important
for the degradation of GLR-1 downstream of endocytosis, possibly via the phosphorylation of GLR-1 S891, which can act to increase or decrease the likelihood of ubiquitination of proteins (Hino et al., 2005). Using Western Blots we could check lysates of wild-type and GLR-1 A2 and GLR-1 D2 worms to see if there is a change in the proportion of GLR-1 that is ubiquitinated. We would expect that D2 GLR-1 would be least ubiquitinated and A2 GLR-1 would be most highly ubiquitinated with wild-type being intermediate, but due to the often rapid degradation of ubiquitinated substrates it is possible that this would be inconclusive.

Other experiments that might address the possible relationship between the phosphorylation of GLR-1 and its ubiquitination driven internalization would be to cross the various integrated strains to a Pglr-1::mUb strain or to uev-1. It has been shown by others that GLR-1 ubiquitination and hence turnover can be accelerated by over-expressing ubiquitin monomers from a Pglr-1::mUb transgene, resulting in reduced number and size of GLR-1::GFP puncta (Burbea et al., 2002), and presumably this would preclude the GLR-1-1 phenotype, whereas the GLR-1 D2 strain might be somewhat resistant to this if constitutive phosphorylation of S891 inhibits GLR-1 ubiquitination. Likewise, the cross to uev-1 might yield a GLR-1 phenotype like that seen in uev-1 mutants alone whereas the GLR-1 A2 yields a lesser phenotype if the phosphorylation of S891 is important for poly-ubiquitin of GLR-1.
The lack of changes to GLR-1 localization in the background of mutations for several candidate kinases and a phosphatase might be due to checking this only under basal conditions. Therefore, it might be worthwhile to look at the GLR-1::GFP pattern in each of these mutants when the animals are stimulated as has been done in other studies into *C. elegans* synaptic function (Emtage et al., 2009). However, this would still leave the identity of which kinase phosphorylates S891 unknown. *In vitro* kinase assays using GLR-1 and various candidate kinases could narrow the field to a few possible kinases, which could then be tested *in vivo* by expressing dominant negative forms of each kinase (for example, the regulatory subunit of PKA alone) under the *glr-1* promoter.

In addition to following up on the S891 residue, we might find other residues that are phosphorylated in the GLR-1 C-terminal domain (CTD) by the use of mass spectrometry. Tagging the CTD and adding trypsin cleavage sites within the CTD and between it and the last GLR-1 transmembrane domain would allow us to affinity purify the CTD and then produce fragments that could be analyzed to determine which residues are phosphorylated *in vivo*.

Understanding how phosphorylation regulates synaptic plasticity is a field well studied in mammalian cell culture experiments, but with significant holes in understanding of mechanism (Kessels and Malinow, 2009; Shepherd and Huganir, 2007). Identifying S891 as a GLR-1 residue phosphorylated *in vivo* by a specific kinase to control GLR-1 localization, and perhaps determining a role for
this in modulating ubiquitination of GLR-1, could point the way to a phosphodegron being part of the regulation of AMPAR plasticity in mammals.

Our findings so far have exploited some of the advantages of *C. elegans* as a model system. These advantages include the simple anatomy, cell biological tools and the ability to do forward genetic screens in this organism to look at *in vivo* physiology. Further possible studies as detailed above could use the advantages to significantly extend our knowledge of the role of phosphorylation and ubiquitination in regulating AMPAR plasticity.
Appendix I

Screen for suppressors and enhancers of *ire-1; nuls25*
INTRODUCTION

AMPA receptor subunits, like other proteins, have their translation coupled with translocation into the Endoplasmic Reticulum (ER), where they become properly folded and assemble with other subunits to form complete receptors (Greger et al., 2007). Mutations in the pore or ligand-binding domains of AMPAR subunits cause increased retention in the ER, implying some form of quality control to ensure that only correctly folded and functional AMPA receptors exit the ER to traffic to the synapse (Grunwald and Kaplan, 2003).

Two pathways that regulate protein folding in the ER are the Endoplasmic Reticulum Associated Protein Degradation (ERAD) pathway and the Unfolded Protein Response (UPR). The ERAD pathway targets misfolded proteins for ubiquitination, retro-translocation into the cytoplasm, and subsequent degradation by the 26S proteasome (Hoseki et al., 2010; Vembar and Brodsky, 2008). The UPR is a related pathway that can up-regulate the ER resident folding chaperones as well as increase degradation of misfolded proteins via ERAD (Kohno, 2010). Our lab previously showed that the UPR pathway regulates the amount of AMPARs found at synapses (Shim et al., 2004). Also, the lack of functional Stargazin, a protein which associates with AMPARs and is important for their localization and function, causes an up regulation of the UPR pathway and retention of AMPARs in the ER (Vandenberghhe et al., 2005a).
The UPR pathway involves several different mechanisms for dealing with accumulations of unfolded proteins in the ER of eukaryotic cells when these cells are stressed in various ways. In *C. elegans* and in mammals it has been shown that one of the pathways involves IRE-1, which can promote splicing of introns from Xbp1 mRNA to form an activated form of this transcription factor (Calfon et al., 2002). Xbp1 in turn is involved in activating downstream genes including those for ER chaperone genes, which is important as part of many cellular processes, including inflammatory response (Hetz and Glimcher, 2009).

Our lab previously found that IRE-1 is important to allow the proper exit of GLR-1, an AMPA type glutamate receptor subunit in *C. elegans*), to exit the ER and ultimately be properly localized in the worm (Shim et al., 2004). To better understand what other proteins are important to this process, we chose to do a genetic screen to look for mutants that enhance or suppress the GLR-1 mis-localization phenotype found in *ire-1(ok799)* mutants. This was a preliminary pilot screen, but did yield four mutants in which the effect of *ire-1* on GLR-1 localization was suppressed.

**RESULTS AND DISCUSSION**

We previously showed that fluorescently tagged versions of GLR-1 are functional and localized to postsynaptic clusters at neuron–neuron synapses within *C. elegans* neurites (Rongo and Kaplan, 1999; Rongo et al., 1998). The
normal expression pattern of GLR-1::GFP in this strain (*nuls25*) has moderately bright cell bodies with occasional puncta (Figure 1A) and a ventral nerve cord in which there is a punctate expression of GLR-1::GFP (Figure 1C). In *ire-1(ok799); nuls25* mutants GLR-1::GFP is seen to accumulate in the ER as a ring of fluorescence (Figure 1B), surrounding neuronal cell bodies, whereas very little GLR-1 is found as puncta in the ventral nerve cord (Figure 1D) (Shim et al., 2004).

We performed an EMS clonal screen and initially found potential enhancers or suppressors of the *ire-1; nuls25* phenotype by using a dissecting fluorescence microscope to look at plates of animals to identify those plates containing worms with increased or decreased cell body GLR-1::GFP signal. We confirmed if these were indeed enhancers or suppressors by examining animals on a compound fluorescence microscope. We found in this process that animals with a suppressed *ire-1* phenotype tended to grow faster, even at higher (25°C) temperatures and took advantage of this to help more quickly screen for suppressors.

We found a number of strains that had at least mild suppression of the GLR-1::GFP localization defect, and a few with possibly mild enhancement of the defect. No enhancers seemed significant enough to study further, but we identified four strains in particular that showed moderate to strong suppression (Table 1). It is possible that the four suppressors represent mutations in genes
that code for proteins that interact with IRE-1 or other UPR pathway proteins in a way that bypasses the requirement for functional IRE-1 to allow GLR-1 to exit the ER properly.

Additional screens could be done to identify more suppressors. These screens might best be done by growing the F1 and worms at 25°C, only singling out F1 worms that grew well. The F2 plates could be grown at 20°C and only those plates which grow well examined under the dissection fluorescence microscope which might help to quickly find suppressors. Our inability to find enhancers in our screen to date could be because in *ire-1; nuls25* the defect is so severe that seeing enhancement may not be possible.

The four strains we have identified, along with any additional suppressors found could be back crossed then complementation tested and mapped to identify the genes in which the mutations have taken place. The identity of the genes mutated in our suppressor strains could indicate new proteins interacting with UPR pathway proteins and perhaps give clues to how these bypass the requirement for functional IRE-1 to allow GLR-1 to exit the ER properly.
MATERIALS AND METHODS

Strains

Animals were grown using standard techniques on NGM plates seeded with OP50 E. coli. The following strains were used: nul525 [Pglr-1::GLR-1::GFP], ire-1(ok799).

Isolation of ire-1 suppressors

P0 nul525; ire-1(ok799) L4 stage worms were EMS mutagenized in three rounds of clonal screens. Each screen involved EMS exposure for four hours in a 47mM EMS solution. P0 worms were allowed to recover overnight and allowed the next day to lay eggs for the F1 generation which was then grown at 15°C, 20 °C or 25°C for several days. F1 worms were then singled out and grown on separate plates at either 15°C or 20°C. The F2 plates were initially screened using a dissecting fluorescence microscope to find plates with possible suppressor or enhancer mutants. Possible suppressors were those that had more diffuse or weaker fluorescence in the cell bodies near the pharynx. These possible suppressors were then confirmed by mounting multiple worms from each plate on 2% agarose pads containing levamisole. Animals were scored by fluorescence microscopy for GLR-1::GFP localization to look for reduced cell body fluorescence and enhanced number of GLR-1::GFP puncta in the ventral nerve cord. Mutants were recovered either directly from the slide or by isolating siblings from the plate.
**Fluorescence Microscopy**

GFP-tagged fluorescent proteins were visualized in nematodes by mounting larvae on 2% agarose pads with levamisole. Fluorescent images were observed using a Zeiss Axioplan II. A 1006 (N.A. = 1.4) PlanApo objective was used to detect GFP and RFP signal. Imaging was done with an ORCA charge-coupled device (CCD) camera (Hamamatsu, Bridgewater, NJ) using IPLab software (Scanalytics, Inc, Fairfax, VA). Exposure times were generally chosen to fill the 12-bit dynamic range without saturation.
Figure 1. GLR-1::GFP expression pattern in wild-type vs. ire-1. GLR-1::GFP fluorescence was observed in the neuron cell bodies of (A) wild-type nematodes (A), and (B) ire-1(ok799) mutants as well as along the ventral cord dendrites of (C) wild-type nematodes, and (D) ire-1(ok799) mutants. The above images are representative images of the PVC neuron and ventral nerve and are not the same animals examined during the ire-1(ok799); nuls25 genetic screen since no images of these are available. The cell bodies examined during the screen were at times those in the head of the animals, rather than the PVC neurons which are in the tail, but both sets of cell bodies showed the same phenotype in ire-1 vs. wild-type nematodes.
# Tables

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<td>Homozygous.</td>
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<td>Mild to moderate suppressor.</td>
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Table 1. Strains in which the *ire-1(ok799)* GLR-1 mis-localization phenotype was suppressed.
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Curriculum Vita

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Education

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Publications and Selected Abstracts

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