DISSERTATION

THE PHOSPHATASE PTP-3 REGULATES AMPA RECEPTOR TRANSPORT IN CAENORHABDITIS ELEGANS

Submitted by

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ABSTRACT

THE PHOSPHATASE PTP-3 REGULATES AMPA RECEPTOR TRANSPORT IN CAENORHABDITIS ELEGANS

Glutamate mediates the majority of excitatory neurotransmission by activating the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtype of ionotropic glutamate receptors. AMPAR trafficking, which includes local synaptic trafficking and long-distance transport, can be one of many cellular pathways important for cognition. Since the majority of AMPARs are made at the cell body, this exerts a challenge on neurons to correctly transport receptors to often far-reaching synapses. To ensure correct synapse function, the interplay between long-distance AMPAR transport, delivery, removal, and retention need to be tightly regulated. However, how long-distance transport mechanisms communicate with local synaptic delivery and removal is unknown. Previous work has shown a critical role for the receptor-type protein tyrosine phosphatase (RPTP) leukocyte common antigen-related protein (LAR), in regulating AMPAR numbers at synapses. However, these studies have not identified a mechanism for this process. Therefore, my thesis sets out to test how AMPAR transport impacts local synaptic trafficking and the role that the *C. elegans* homologue of vertebrate LAR, Protein Tyrosine Phosphatase 3A (PTP-3A), has on this regulatory pathway.

Chapter 1 is an introduction and its purpose is to provide background knowledge and scientific context for the main questions of my thesis.

In Chapter 2, we investigate the question of how does glutamate receptor transport impact local synaptic glutamate receptor numbers. Tyrosine phosphorylation is known to play an important role in glutamate receptor trafficking at the synapse, but not much is known about tyrosine phosphorylation on glutamate receptor transport. The vertebrate phosphatase LAR is known to be important in glutamate receptor transmission in cell culture, but its role in glutamate receptor transport is unknown. Here we investigate the role of the sole *C. elegans* LAR-RPTP homologue, PTP-3A, on glutamate receptor transport and how this affects local synaptic delivery. We show that PTP-3A mutants display decreased transport as well as decreased delivery of Glutamate Receptor-1 (GLR-1) to synapses. Since PTP-3A is a large structure, 3 IgG domains, 9 fibronectin domains, and 2 phosphatase domains, we sought out to determine what domains were necessary for GLR-1 transport. Domain analysis of LAR revealed that the phosphatase domain is not required for GLR-1 transport but is required to stabilize GLR-1 at synapses. Surprisingly, the Ig-like external domains are sufficient to cell-specifically rescue GLR-1 transport. Interestingly, LAR mutants exhibit decreased short-term and long-term memory whereas mutants lacking the phosphatase domain only show decreased long-term memory. This could be due to a mechanism where efficient GLR-1 transport is sufficient to sustain the synaptic receptor pool during short-term synaptic activity, but stabilization of GLR-1 at synapses is required long-term consolidation. Taken together, our results show a critical role of LAR in long-distance synaptic AMPAR transport.

Chapter 3 seeks to identify a role of a known regulator of vertebrate LAR, liprin-α, in glutamate receptor transport. Vertebrate liprin-α is known to bind LAR and correctly localize it to synapses. Previously, the *C. elegans* sole liprin-α homologue, SYD-2, has

only been identified as a regulator of active zone maintenance presynaptically. However, vertebrate liprin-α is known to colocalize with postsynaptic proteins such as PSD-95, GRIP-1, LAR, and GluA2. Since LAR and liprin-α colocalize at the synapse, we reasoned that this interaction might happen in *C. elegans* as well and that SYD-2 might be in the same regulatory pathway as PTP-3A. Therefore, we asked if loss of SYD-2 would cause a defect in GLR-1 localization *in vivo* in *C. elegans*. We show that loss of SYD-2 leads to ~1.5-fold increase in GLR-1 transport but has a ~70% reduction in synaptic and surface GLR-1. This leads to an interesting model where synaptic activity might not be directly correlated with GLR-1 transport. Also, our data suggest that SYD-2 might have additional synaptic roles other than correctly localizing PTP-3A.

Finally, Chapter 4 aims to discuss the impact of the data I have generated in my thesis and how experiments from each chapter complement each other and how these have opened new experimental paths.

Overall, the work in my dissertation expands on the limited knowledge of how AMPAR transport and synaptic trafficking interact to control synaptic AMPAR numbers. It furthers our knowledge of the role of the phosphatase PTP-3A/LAR in regulating excitatory synaptic maintenance. It also challenges the idea which recent studies have shown that correlates decreased synaptic activity with decreased AMPAR transport. Altogether, it outlines the general importance of understanding how AMPAR transport relates to synaptic plasticity and behavior.

ACKNOWLEDGEMENTS

If you look up the definition of homebody in the dictionary, you will find a picture of me. Six years ago, I took a leap of faith, leaving my friends and family to chase a dream, which was no easy task. Along this journey, each person that has come into my life has helped me in one way or another. However, I want to take the time to personally thank everyone who has helped me immensely:

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CHAPTER 1: Introduction

1.1 Learning and memory in *C. elegans*

This first chapter is an introduction with background knowledge needed to understand the fundamental premise of my thesis. This is not meant to be an exhaustive review, but it sets the framework for my thesis with key pieces of information in both the vertebrate and *Caenorhabditis elegans* (*C. elegans*) field of study.

In 1965, Eric Kandel and colleagues made a significant step towards showing that studying the nervous systems of invertebrates had significant potential to understand learning and memory, enabling the discovery of key proteins in synaptic plasticity using the mollusk *Aplysia californica*[1]. Around this same time, Sydney Brenner began studying neural development in the nematode *Caenorhabditis elegans* (*C. elegans*) which now has a well-established and well understood nervous system[2]. *C. elegans* is a simple model organism that is small in size (~1mm), has a short life cycle (<3 days egg to adult), and is easy and inexpensive to house. In an adult hermaphrodite, there are 302 neurons. Coupled with its transparent cuticle, this makes it an ideal model to study neuronal processes by using fluorescent tags and microscopy[3]. Although a simple model organism, many of the key proteins involved in vertebrate learning and memory are conserved in *C. elegans* which allows for these molecular mechanisms to be studied in an intact nervous system *in vivo*[4].

C. elegans learning and memory can be divided into associative and nonassociative learning. The first form of learning and memory in worms was discovered in 1990 where Rankin and colleagues studied the tap-withdrawal response (TWR)[5]. This form of nonassociative learning is a behavior where worms crawl backward in response

to a mechanical stimulus generated by tapping the plate containing the worms. Repeated tap stimulus decreases the amplitude and frequency of the response. Thus, the stimulus impacts how far the worms crawl backward and how often they respond, respectively. These studies of habituation found key neurons important in learning and memory. Furthermore, these studies laid the foundation for the discovery of *C. elegans* associative learning which is a higher order form of learning and memory.

Associative memory was first made famous by Ivan Pavlov who showed that an uncontrolled stimulus (US) triggers an uncontrolled response (UR). A neutral conditioned stimulus (CS), can be paired with an US and with repeated attempts, the neutral CS will now elicit a conditioned response (CR)[6]. *C. elegans* has an associative memory mechanism that is similar to what Pavlov observed and exhibits a remarkable capacity to learn and remember environmental features that predict good food, no food, or even aversive stimuli[4]. Worms will move towards volatile organic compounds released by their bacterial food source of which one potent chemoattractant is diacetyl. Morrison and colleagues showed that worms could learn to avoid the diacetyl if previously presented with an aversive solution[7]. A simple experimental design based off that study is showed in **Figure 1.1**: in a naïve state, worms will naturally be drawn to the diacetyl over a control odor. To begin the experimental test for learning, **1)** worms are placed on a plate with no food but a drop of diacetyl on the top of the lid so they can smell the diacetyl, but cannot reach it, for one hour. Within this hour, they begin to associate starvation to the smell of diacetyl. **2)** They are then transferred to an experimental plate with diacetyl and a control. If they have learned the association of starvation to the smell of diacetyl, they will travel away from the diacetyl and towards the control odor. To test for short-term memory, the

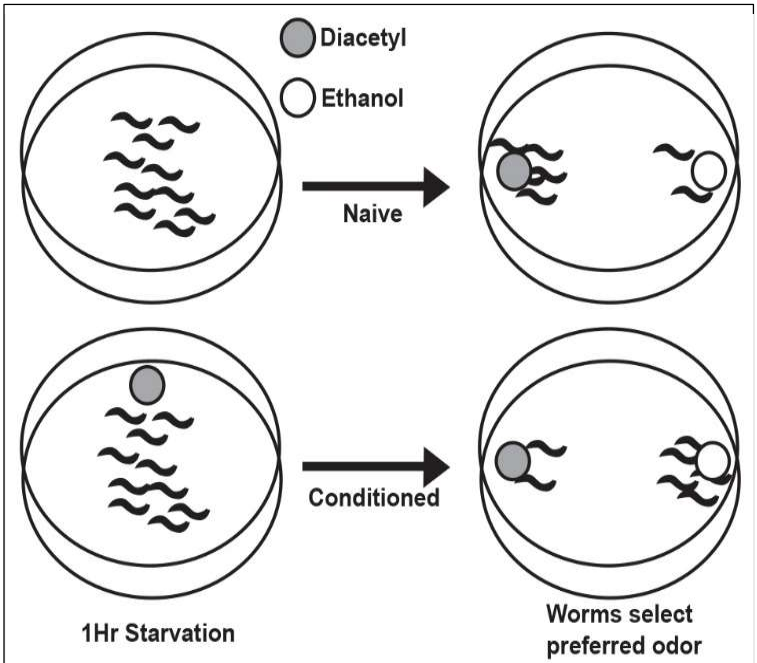

Figure 1.1: Olfactory associative memory in *C. elegans.* Top – naïve worms are placed on an experimental plate with a chemoattract (diacetyl) or control. Bottom – worms are conditioned for one hour with no food and diacetyl on top of the lid. Worms are then placed on an experimental plate to test if they have learned the association between starvation (negative effect) and diacetyl. Adapted from Bargmann *et. al.,* 1993

same procedure is followed except after the one-hour conditioning, worms are placed on a normal plate with food for one hour. These worms are then placed on an experimental plate and if they have remembered the association of starvation to the smell of diacetyl, they will travel towards the control odor.

The establishment of memory experiments in worms led to many candidate-driven approaches to establish a core mechanism of associative memory in *C. elegans*[4]*.* As will be discussed in **chapters 1.3** and **1.4**, many of the critical proteins responsible in vertebrate learning and memory are conserved and important in *C. elegans* learning and memory[8–14].

1.2 Excitatory neurotransmission

Glutamate mediates the majority of fast excitatory transmission in the brain by activating postsynaptic glutamate receptors. The dysregulation of this neurotransmission is known to be a main component of neurodegenerative diseases, as well as impaired learning and memory[15]. Upon presynaptic depolarization reaching the nerve terminal, packets of the neurotransmitter glutamate are dumped into the synaptic cleft which bind to postsynaptic receptors which allow for the influx of positively charged ions, mainly Na^+ and Ca^{2+} [16]**.** Each neuron has thousands of synaptic connections that receive both positive and negative inputs, and it is the culmination of all these inputs that determine whether the neuron will depolarize and thus propagate its signal to postsynaptic targets. Since glutamate receptors are known to be a key factor in this signal propagation which underlies learning and memory, which is an evolutionarily conserved mechanism, it is critical to understand how neurons regulate synaptic glutamate receptor numbers.

1.2.1 *C. elegans* neural network

C. elegans is a very simple model organism which is why its neuronal anatomy is one of the most well understood among all model organisms. Although considerable time could be spent to discussing each neuron's fate and function, I will be focusing on the neurons that are critical in my thesis work.

Most of the glutamatergic signaling in worms is governed by the command interneurons AVA, AVB, AVD, AVE, and PVC, which control forward and backward movement. The AVA interneurons are a central component of the withdrawal response to noxious stimuli (as outlined in **Figure 1.2**)[17] and the activation of this pathway is highly dependent on glutamate receptors (glutamate receptors discussed more in **Chapter**

1.4)[18]. Interestingly, the AVA interneurons are critical for the expression of long-term memory (LTM). Indeed, disrupting presynaptic glutamate input onto AVA or deletion of glutamate receptors in AVA is enough to abolish LTM[19]. The establishment that glutamate receptors are required for LTM as an evolutionarily conserved mechanism make it possible to study the molecular pathways that regulate this behavior in a simpler system.

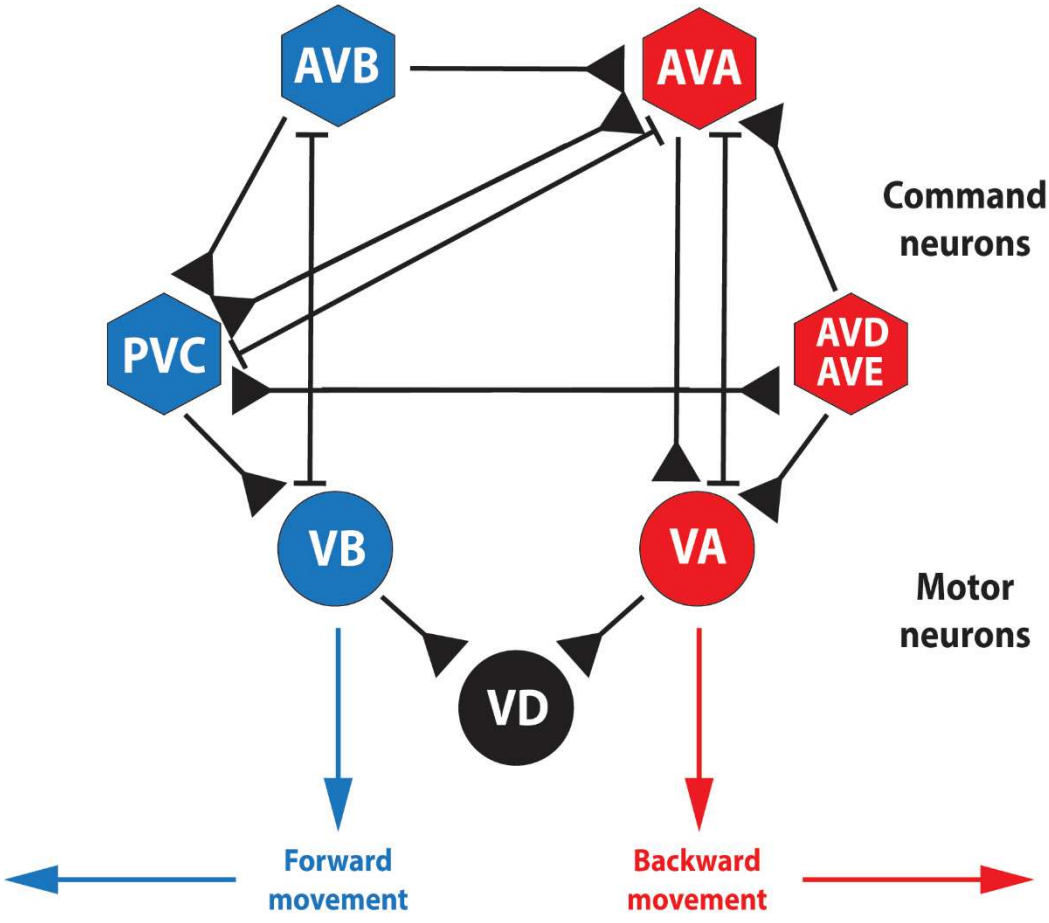

Figure 1.2: *C. elegans* neural control of forward and backward movement. Connectivity between command interneurons (hexagons) and motor neurons (circles). Lines with triangles represent chemical synapses and lines with blunt ends represent gap junctions. Neurons in blue are responsible in the forward movement pathway while neurons in red are responsible in backward movement. The VB, VA, and VD neurons innervate ventral body-wall synapses. Adapted from de Bono *et. al.,* 2005.

1.3 Synaptic plasticity

One of the most fascinating aspects of the brain is its plasticity, which is the capability for a neural activity to modify neural circuit function and thereby modify feelings, behavior, and memory[20]. Synaptic plasticity refers to the activity-dependent modification of the efficacy of synaptic transmission at preexisting synapses and has been observed at virtually all synapses in organisms ranging from worms to mammals[20]. The concept of plasticity was pioneered by Donald Hebb in 1950 when he found, “an often repeated specific stimulation [at synapses] will give rise to the slow development of a diffuse structure[21].” The idea behind this finding was that repeated synaptic stimulation causes the postsynapse to increase in size to house more proteins that are critical in strengthening the transmission between pre and postsynaptic cells. Increasing the postsynaptic spine is not an energy favorable mechanism, however, which is why synaptic plasticity is not an all-or-none process. There is a form of short-term plasticity where the postsynaptic cell determines if the increase in input from the presynaptic cell is coincidence or an increase in activity.

1.3.1 Short-term synaptic plasticity

Long-lasting plasticity is largely a postsynaptic process, but the most common form of short-term plasticity is presynaptic. Most presynaptic terminals have receptors that control the probability of neurotransmitter release and depending on the type of receptor, can either depress or enhance synaptic transmission and thus acts as a feedforward pathway. However, synaptic transmission can be bidirectional. Indeed, upon activation, postsynaptic cells can release neuromodulators, such as serotonin and dopamine, that influence the characteristics of the presynaptic receptors and acts as a feedback pathway.

In many cases, presynaptic activation via neuromodulators act to decrease the release probability of neurotransmitters[20,22]. In a review, Abbot and Regehr stated that, “synaptic plasticity assures that current activity [across a synapse] reflects both the current and previous history of activity within the neural circuit[22].” The neural circuit is defined as an ensemble of neurons that the brain uses to encode external and internal events as spatio-temporal patterns of activity. The mechanism described by Abbot and Regehr roughly translates to a mechanism where the presynaptic cell can “prime” a synapse depending on what type of signal it has received previously so it can respond accordingly to incoming signals. In this model, short-term plasticity can be thought of as a trial period to determine if long-term restructuring of the connection between pre and postsynapse should occur, acting as a check point for long-term plasticity.

1.4 Glutamate receptors

Glutamate receptors are a critical component of my thesis as they are fundamental in understanding the process of learning and memory. Although knowing the biochemical properties of glutamate receptors is important in understanding their function, I will only briefly describe some of these properties since my thesis work did not revolve around the physics of receptor function. Within the glutamate receptor family in vertebrates exists three main classes of ionotropic receptors, the N-methyl-D-aspartate (NMDA) receptor type, the non-NMDA receptor subtypes of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors, with a fourth lesser known δ glutamate receptor[23–25]. All receptors are composed of four large subunits: the extracellular amino-terminal domain (ATD), the extracellular ligand-binding domain (LBD), a transmembrane domain (TMD), and an intracellular carboxyl-terminal domain

(CTD). They assemble as tetrameric subunits and are formed by assembly of subunits within the same functional receptor.

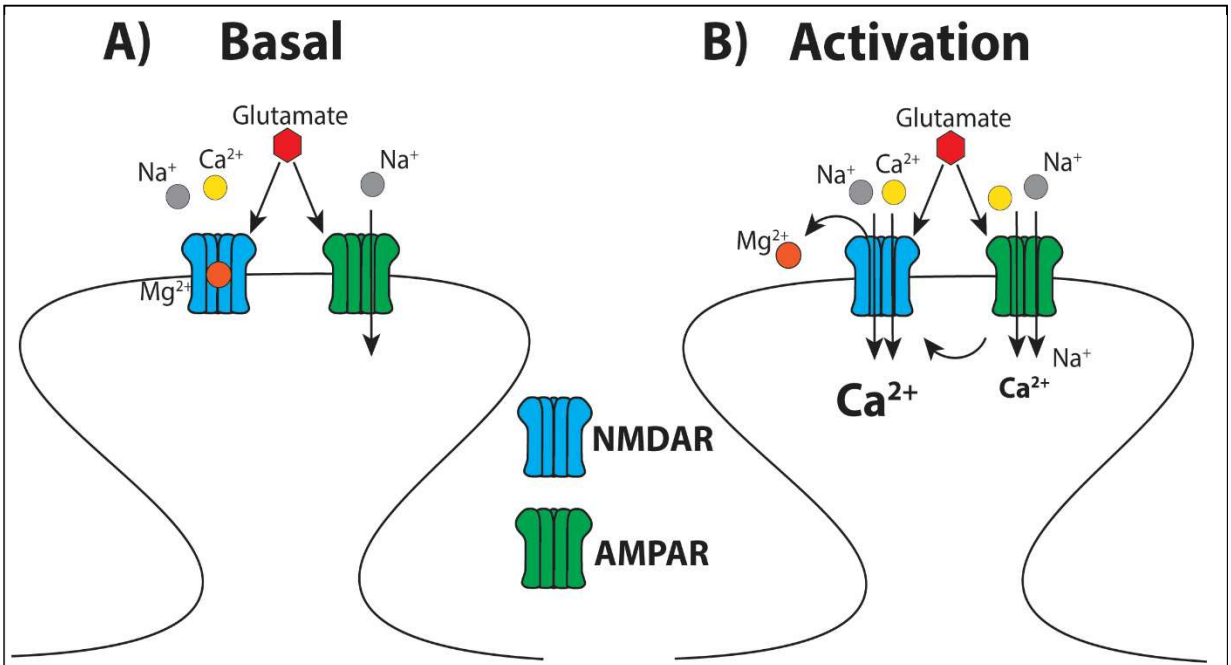

Figure 1.3: AMPAR and NMDAR activation under basal and depolarization conditions. A) Upon glutamate binding to AMPARs and subsequent weak activation, the Mg^{2+} block is not dislodged and thus NMDAR activation does not occur. **B)** When a strong enough AMPAR-mediated activation leads to a large enough depolarization, the Mg^{2+} block is removed and a subsequent large influx of intracellular Ca^{2+} occurs.

NMDARs are glutamate-gated ion channels that are critical in excitatory neurotransmission. There is extensive literature on the critical role of NMDARs in synaptic plasticity which I will not be detailing since my thesis work is primarily on AMPARs. In the next two paragraphs, I will outline the basic properties of NMDARs and metabotropic glutamate receptors (mGluRs) respectively. There are three subunits, NR1-3, that form heteromers with differing physiological properties. Synaptic depolarization has two initial components which include a fast AMPAR activation and a slower NMDAR activation. This slower activation is due to NMDARs containing a pore loop that is blocked by a Mg^{2+} ion during steady state. Opening of receptors requires a strong enough depolarization to

relieve the Mg^{2+} block and allow for an influx of Ca^{2+} **(Figure 1.3)**. Activation of NMDARs is responsible for the large amount of intracellular Ca^{2+} and is essential in long-lasting synaptic plasticity[23]. Upon NMDAR activation, many downstream signaling proteins are activated, which include the metabotropic class of glutamate receptors (mGluRs).

mGluRs are G-protein coupled glutamate receptors that contain an extracellular N-terminal domain (termed the Venus flytrap domain VFD), seven transmembrane-spanning domains, and an intracellular C-terminus[26]. There are three groups of mGluRs with group I often localized postsynaptically and activation leads to cell depolarization. Upon glutamate binding, the G-protein-coupled receptor (GPCR) cascade leads to calcium mobilization and activation of multiple downstream signals, two of importance are protein kinase C (PKC) and components of the mitogen-activated protein kinase (MAPK). These pathways have been heavily implicated in learning and memory and knockout studies of mGluR1 show reduced induction of LTP in mice hippocampal slices[27]. This phenotype is region specific however, as mGluR1 deficiency in the cerebellum leads to deficits in LTD due to regression of cerebellar Purkinje neurons[28,29].

1.4.1 AMPA receptors

The last major class of glutamate receptors are AMPARs which contain four subunits (GluA1-4) and are assembled as dimers-of-dimers and form hetero-tetrameric receptors[30]. The four subunits are highly homologous with 70% amino acid identity with conserved extracellular and transmembrane domains[31]. Receptors are assembled in the endoplasmic reticulum (ER) where correct subunit assembly is tightly regulated[32]. The GluA2 subunit has the most impact on biophysical properties since AMPARs lacking GluA2 are Ca^{2+} permeable. AMPARs that contain GluA2 are largely Ca^{2+} impermeable

(>99%) owing to a unique arginine (R) at amino acid position 607 that is RNA edited from a glutamine (Q) often labeled as Q/R editing[33,34]. This unique glutamine is located in the pore loop of the subunit and editing changes the neutral charged glutamine to a positively charged arginine which opposes the strongly positively charged Ca^{2+}. RNA editing not only controls channel conductance, but efficient exit from the ER as well. Unedited GluA2 rapidly traffics through the ER and edited GluA2 is mostly retained in the ER. However, if edited GluA2 is assembled with GluA1, it traffics rapidly through the ER[35].

AMPARs are largely located in excitatory neurons and enriched at the postsynaptic site. Since AMPARs mediate a large majority of excitatory synaptic transmission, changes in the number of receptors at a given synapse can influence the efficacy of this transmission. Receptors are not static and have a surface half-life of tens of minutes[36], and depending on the pattern of neuronal activity, they will shuttle into or out of the synapse which can lead to long lasting changes in synaptic strength[37]. Upon glutamate binding, the receptor undergoes a conformational shift that opens the pore and allows the influx of Na^{+} ions which in turn can depolarize the postsynaptic compartment[38]. This depolarization leads to multiple cascades that contribute to LTP (discussed later) such as the alleviation of the Mg^{2+} block of the NMDA receptor which allows for a large influx of Ca^{2+} in the postsynaptic compartment[39,40]. Increased intracellular Ca^{2+} caused from high frequency stimulation causes a cascade of downstream protein activation that is dependent on the amount of Ca^{2+}; one such effect is controlling the number of surface synaptic AMPARs[41–43].

Although vertebrate models have provided a plethora of information in the AMPAR field, the lack of inexpensive genetic techniques coupled with the complexity of the

vertebrate nervous system has led to many holes in our understanding. By using a simpler model such as *C. elegans* which only possess 302 neurons in an adult hermaphrodite, these obstacles can somewhat be overcome. There are at least 10 presumed ionotropic glutamate receptors expressed in *C. elegans.* These include GLR-1 – GLR-8 members of the non-NMDA class which are most similar to AMPA or kainate receptors and the two NMDA subunits, NMR-1 and NMR-2, which belong to the NR1 and NR2A subfamilies[18,44]. *C. elegans* ionotropic glutamate receptor subunits show high amino acid sequence similarities to vertebrate ionotropic glutamate receptors. Sequence alignment shows the highest conservation in areas of importance such as the transmembrane segments, pore region, and ligand-binding domain[44]. Also, C-terminal amino acid sequences that are responsible in receptor gating and receptor localization are highly conserved between *C. elegans* and vertebrates. Therefore, using a simpler model organism such as *C. elegans* confers benefits to studying regulatory properties of AMPARs.

Correctly regulated AMPAR synaptic expression is a tightly regulated mechanism and almost any defect in the control of correct AMPAR localization can have dire consequences on brain function. Most neurodegenerative and neurological disorders, such as Schizophrenia and Alzheimer's, involve synaptic malfunction that can be linked to AMPAR abnormalities[45]. Indeed, neurons that have increased seizure predisposition downregulate GluA2 subunit expression. This loss of GluA2-containing AMPARs leads to the increase in intracellular Ca^{2+} and thus have increased excitability and excitotoxicity[46]. Therefore, it is critical that we understand the regulatory mechanisms that underly correct AMPAR localization as it pertains to neurological and neurodegenerative diseases.

1.4.2 Long-term depression (LTD)

Although a lasting increase in synaptic transmission is often thought of as learning, weakening of synapses is also a form of learning. Long-term depression (LTD) is a process that weakens synapses depending on the molecular state of the synapse. There is evidence that a reduction in the probability of glutamate release can induce LTD which can be triggered by changes in the presynaptic terminal or retrograde messengers sent from postsynapses[47]. LTD is dependent on weak NMDAR activation that leads to low intracellular Ca^{2+} and can be induced by prolonged periods of low-frequency stimulation. This allows for LTD specific proteins that have high affinity to low Ca^{2+} levels to induce removal of surface AMPARs[48]. What is the purpose then of this lasting decrease in synaptic potentiation? Although this answer is still debated, it is known that LTD is required for hippocampus-dependent learning and memory. In a study where the NMDAR subunit GluN2B was selectively ablated and resulted in impaired LTD, also resulted in deficits in learning and memory tasks[49]. Disrupting key proteins that are responsible in LTD such as calcineurin and PP2A also impairs episodic memory which is the ability to recall and mentally reexperience specific episodes from one's personal past[50,51]. Interestingly, when the phosphatase PP2A is inhibited and mice are subjected to a hidden platform Morris water maze regime over 17 days, the mutant mice learn to swim to the new platform location just as efficiently as the wild-type but the time it takes for them to acquire the new location is severely decreased[52]. This suggested to the authors that NMDAR-dependent LTD may have a role in behavioral flexibility which refers to how animals learn to adapt their behavior to their varying environments[53].

There exists a second form of LTD that is mGluR activation-dependent that stimulates a similar cascade as the NMDAR-dependent form but seems to be synapse

type specific[54]. The activation patterns of mGluR-LTD and NMDAR-LTD are generally similar but are synapse specific, for example in the cerebellum, mGluR-LTD causes a decrease in postsynaptic sensitivity to L-glutamate whereas in the hippocampus it is not associated with this reduction[55].

1.4.3 Long-term potentiation (LTP)

The most widely studied form of activity-dependent change of synaptic strength is Hebbian plasticity which includes LTP and LTD. LTP is a long-lasting increase in synaptic transmission elicited by repeated electrical stimulation and is the most widely accepted mechanism of memory storage and one of several different forms of long-term synaptic plasticity[25]. Hebbian plasticity, compared to homeostatic plasticity, is confined to active synapses where synaptic strength change is input specific[56]. The concept of LTP was first introduced by Bliss and Lomo in 1973 where they concluded that, “There exists at least one group of synapses in the hippocampus whose efficiency is influenced by activity which may have occurred several hours previously, a time scale long enough to be potentially useful for information storage[57].” This translates to the conclusion that the long-lasting change in synaptic activity they observed is due to an increase in the strength of synaptic transmission. Although this laid the foundation for the study of synaptic plasticity and memory, it outlined a very critical question: Is the change in synaptic strength due to an increase in pre-synaptic neurotransmitter release, or an increase in the postsynaptic response to the neurotransmitter? This question was difficult to answer at the time since the major excitatory neurotransmitter responsible for LTP, glutamate, would not be discovered until 20 years later. However, there were studies that showed LTP could be prevented by blocking postsynaptic depolarization even with high frequency presynaptic

stimulation[58,59]. Once glutamate was determined to be the major excitatory neurotransmitter, it was found that it acted primarily on NMDARs and AMPARs. The interplay of activation between NMDAR and AMPARs is the basis for LTP induction.

As outlined in **Figure 1.3,** AMPAR activation causes an increase of intracellular Na^+ and Ca^{2+} which depolarizes the postsynapse. This depolarization allows for the Mg^{2+} block within NMDARs to be removed and subsequently activated. LTP induction requires NMDAR activation which causes a large Ca^{2+} influx and activates Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). Rapidly after CaMKII activation, there is a large increase in the number of synaptic AMPARs (**Figure 1.4**)[60–62]. CaMKII is a very promiscuous protein which is why its precise mechanism in LTP is difficult to answer. However, it's known that CaMKII can phosphorylate Ser831 of GluA1 which increases its channel conductance and is required for LTP induction in certain brain regions[63]. Another

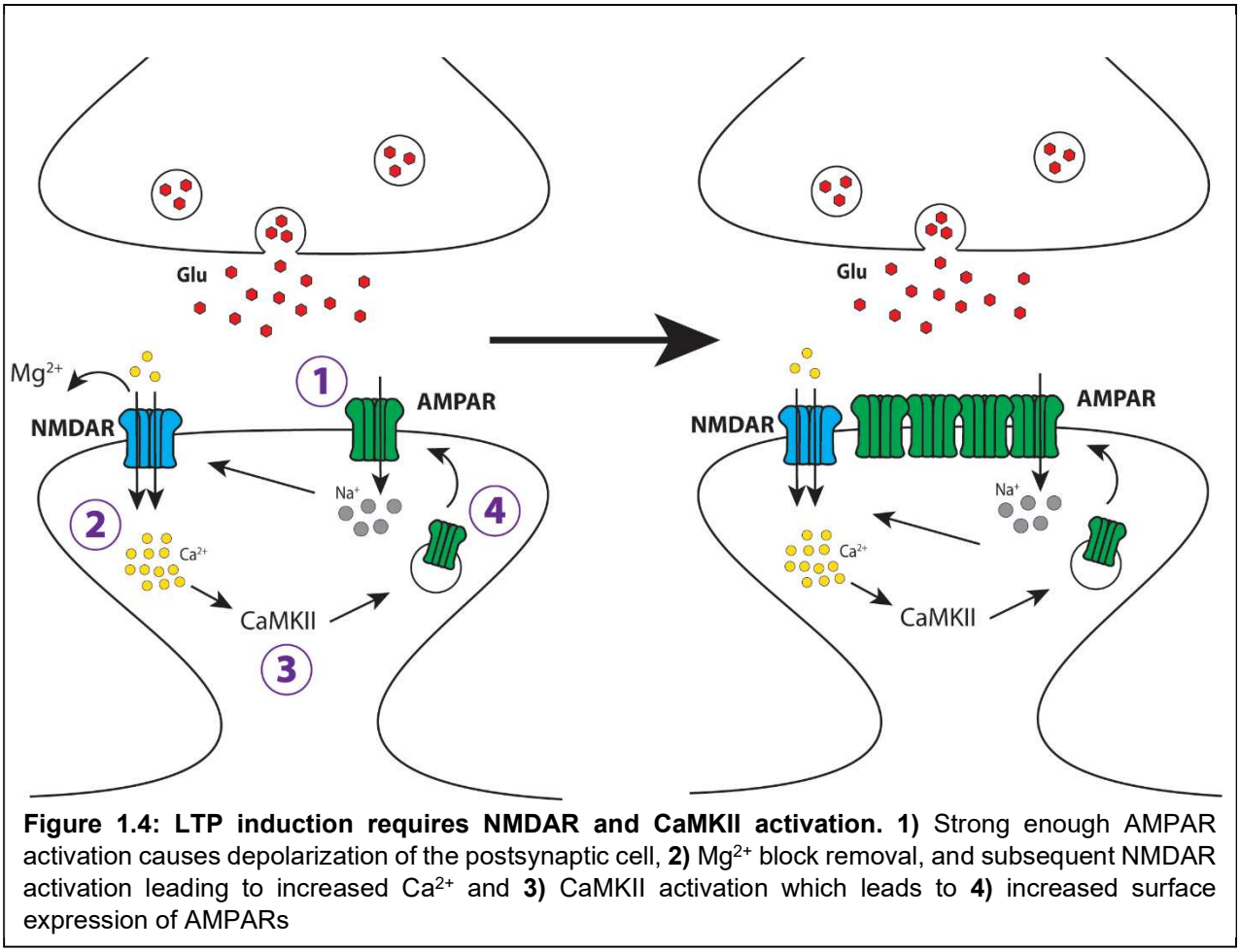

Figure 1.4: LTP induction requires NMDAR and CaMKII activation. 1) Strong enough AMPAR activation causes depolarization of the postsynaptic cell, **2)** Mg^{2+} block removal, and subsequent NMDAR activation leading to increased Ca^{2+} and **3)** CaMKII activation which leads to **4)** increased surface expression of AMPARs

important phosphorylation site on GluA1 is Ser845 which is governed by protein kinase A (PKA)[64]. Ser845 phosphorylation promotes GluA1 surface insertion and synaptic retention whereas Ser845 dephosphorylation causes endocytosis of GluA1-containing AMPARs[63].

Not surprisingly, CaMKII phosphorylation of AMPAR accessory proteins has implications in LTP. AMPARs are largely associated with transmembrane AMPAR-regulatory proteins (TARPs) which have critical roles in AMPAR synaptic trafficking. Indeed, phosphorylation of the TARP stargazin serves as a chaperone for AMPARS in synaptic targeting and surface expression[65]. The trafficking of AMPARs is a critical process in LTP induction and maintenance, which is why many of the proteins involved

are evolutionarily conserved. Although the exact equivalent of LTP has not been determined in worms, the proteins responsible for correct AMPAR localization, that are important for LTP, are similar between vertebrates and worms. Therefore, by using a simpler model like *C. elegans* to understand the basics of AMPAR trafficking, a better understanding of higher order processes such as LTP can be obtained.

1.4.4 AMPAR trafficking and transport

Neurons are complex entities that employ a wide range of subcellular movements to move receptors throughout the cell. This encompasses local synaptic trafficking of receptors through dendritic ER and Golgi, diffusion at the surface of the plasma membrane, and long-distance transport from cell body to distal synapses[66]. From here forward, I will use the term “synaptic trafficking of AMPARs” to indicate the local movement of receptors within and around the synapse. The term “transport” will indicate any movement of AMPARs that is kinesin or dynein dependent. The machinery used in and in-between these two processes are slightly different and need to communicate so that the end result of receptor localization is correct. The precise mechanisms as to how the cell signals for increased AMPAR transport, and how AMPAR-containing-vesicles pivot from long-distance kinesin/dynein-driven transport to local synaptic trafficking to the dendritic or synaptic surface are largely unknown.

AMPARs at the synapse are not static and are constantly recruited and dispersed from the surface to intracellular compartments[62,67–71]. Matthew Kennedy and Michael Ehlers demonstrated that there exists an intracellular pool of AMPARs in endosomes that are readily available for exocytosis and that this receptor-containing endosome is mobilized to the spine during LTP[69,72,73]. This pool can be populated from recycling

AMPARs or newly synthesized receptors[74]. What are the signals that control whether receptors are expressed at the membrane surface or internalized? One key pathway is phosphorylation of the C-terminal tail of AMPARs. Phosphorylation of S845 of GluA1 has shown to promote surface insertion and synaptic retention whereas dephosphorylation of S845 promotes receptor endocytosis[63]. How are receptors then trafficked from intracellular pools to the surface? Although the precise mechanism is unknown, phosphorylation of GluA1 at S831 by calcium calmodulin-dependent kinase II (CaMKII) is thought to promote trafficking of AMPARs from extrasynaptic sites to the synapse[63]. This would then require both S831 and S845 phosphorylation to traffic receptors from extrasynaptic pools to the cell surface.

Phosphorylation of postsynaptic proteins is a known regulatory element that influences synaptic plasticity. Indeed, tyrosine phosphorylation of the NR2B subunit of NMDARs enhances its function and is required for LTP. The GluA2 and GluA3 AMPAR subunits are phosphorylated upon activation and phosphorylation at GluA2 Y876 by Src kinase regulates basal synaptic GluA2 internalization from the surface. GRIP-1 is critical for the correct localization of GluA2-containing AMPARs and GluA2 Y876 phosphorylation increases this binding, establishing a possible mechanism of synaptic plasticity that regulates GluA2 localization within the synapse. Interestingly, phosphorylation at GluA2 Y876 is not required for LTP/LTD but is needed for homeostatic synaptic scaling[75].

Most neurons have long dendritic processes sometimes reaching lengths of tens of millimeters and thus are far removed from the cell body. This creates a challenge to supply synapses with the correct amount of AMPARs. Aside from a larger intracellular

pool, local translation of receptors is the quickest way to supply synapses with new AMPARs. Although there are polyribosomes at synapses, the number is relatively small, estimated one per dendritic shaft. Since it is thought that each polyribosome translates a single mRNA, this places time constraints on timing and diversity for local synthesis[76]. Most AMPAR mRNA is located in the neuronal cell body and thus newly synthesized AMPARs need to be transported to their correct location[77]. During synaptic activation, receptors that are critical in maintaining the activation are readily trafficked to the surface, while receptors that are not required are endocytosed and even degraded. This exhibits a need for a readily replenished intracellular pool of receptors since surface receptors need to be rapidly replaced.

Early studies hypothesized that lateral diffusion of AMPARs from the cell body to synapses was the predominant form of long-distance AMPAR trafficking[78]. This study showed that receptors could diffuse to synapses in tens of seconds. However, LTP induction occurs much more rapidly than this, so although lateral diffusion is a method for supplying receptors to synapses, it cannot be the only form of long-distance trafficking[79]. The main long-distance mode of transport cells utilize are the microtube-dependent motors kinesin and dynein. The idea that long-distance AMPAR transport can populate synapses has been known for 20 years[80,81]. However, insight into the regulatory elements that could possibly control this were not understood until recently[80,82–84]. A key aspect of my thesis was building on the work that the Maricq lab showed in 2013 and 2015. I will now briefly summarize these studies and a recent study by the Choquet lab in vertebrate cell culture supporting evolutionary conservation of regulated AMPAR transport as a mechanism to regulate excitatory synaptic transmission.

In 2013, Hoerndli and colleagues showed the importance of the kinesin motor KIF5 on long-distance AMPAR transport *in vivo* using *C. elegans*[85]. Although there are 21 known kinesin-like motors in the *C. elegans* genome, only few have been studied in detail and only UNC-104/KIF1, KLP-4 (a kinesin-like protein similar to UNC104), and UNC-116/KIF5 are known to be expressed in the pair of AVA glutamatergic interneurons responsible for learning and memory in worms[86]. A candidate driven approach was taken and it was found that only UNC-116 loss-of-function mutants showed decreased overall GLR-1 (GluA-1 homologue) transport. Not only was long-distance transport of AMPARs decreased in these mutants, but they also observed that transport of receptors from synapse to synapse was disrupted. Surprisingly, they observed that removal of GLR-1 from synapses was decreased in *unc-116* mutants. This result demonstrated that motor-driven AMPAR transport not only precedes local synaptic trafficking, but can also influence synaptic AMPAR recycling[87].

In 2015, the same group demonstrated the effects of calcium calmodulin-dependent kinase II (CaMKII) mutants on the long-distance AMPAR transport mechanism. The sole CaMKII homolog in *C. elegans* is encoded by the *unc-43* gene and its role in AMPAR long-distance trafficking was originally posited by Rongo and Kaplan in 1999, where they discovered that loss of function CaMKII/UNC-43 resulted in accumulation of GLR-1 at the cell body[88]. Hoerndli and colleagues found that in *unc-43* mutants, the transport of GLR-1 was severely decreased and almost nonexistent while a gain of function *unc-43* nearly doubles the GLR-1 transport compared to wild-type[9]. To determine if CaMKII was working upstream or downstream of *unc-116/KIF5*, GLR-1 transport was tested in *unc-116* RNAi knockdown with and without the presence of *unc-*

43(gf) mutation. They observed no distinguishable difference between *unc-116(RNAi)* mutants compared to the double mutants with *unc-43(gf)* which places UNC-43 upstream of UNC-116. Not only was UNC-43 acting at the cell body to control AMPAR transport, it also was crucial for the delivery and removal of GLR-1 at synapses[9].

These two studies were critical in finding that altering AMPAR transport can change synaptic properties, namely the number of AMPARs at synapses. However, this left unanswered the effects that synapse activation has on AMPAR transport. It wasn't until 2018 when the Choquet lab answered this question in cell culture. They quantified multiple transport paradigms after selectively activating synapses by uncaging glutamate. They demonstrated that upon chemical LTP induction (defined by chemically inducing LTP by activating enzymes downstream from Ca^{2+} entry) AMPAR-containing vesicles arrested which is due to increased intracellular Ca^{2+}. Interestingly, 15 minutes post LTP induction, the mean speed of AMPAR-containing vesicles increased. Thus, this led to their model that during synaptic activation, AMPAR-containing vesicles are stalled which allows them to survey their environment to determine if they need to be delivered to nearby synapses. The increased transport speed of the receptors is then a positive feedback to supply synapses with receptors to keep the activated synapses strengthened[89,90]. This work not only reinforced what the Maricq lab found in *C. elegans* but also showed that kinesin-1-dependent transport of AMPARs is an evolutionarily conserved molecular mechanism for regulating AMPARs at synapses.

1.5 LAR/PTP-3 family of phosphatases

The leukocyte common antigen-related receptor protein tyrosine phosphatases (LAR-RPTP), also classified as type IIa RPTPs, are cell adhesion molecules that are

important in the developing nervous system and known to be important in excitatory synapse maintenance[91]. Although there are eight subfamilies of RPTPs in vertebrates, I will be focusing on the type IIa RPTPs and before I explain how LAR-RPTPs are involved in the nervous system, I will briefly describe some of their structural properties. In vertebrates, this family consists of three members, LAR, PTP-σ, and PTP-δ and are characterized by an N-terminal extracellular portion containing three immunoglobulin-like (Ig) domains, two to nine fibronectin III domains, and a transmembrane domain with an intracellular dual tyrosine phosphatase domain, D1 and D2, with D1 providing 99% of the catalytic activity of LAR and D2 binds to multiple downstream partners[92].

Interestingly, LAR is known to be proteolytically cleaved which cuts the membrane-proximal part of the extracellular domain into a 150kDa E-subunit and an 85kDa P-subunit[93]. There is a penta-arginine sequence (amino acids 1148-1152) that is just before the cleavage site which is between the arginine at position 1152 and the glutamine at position 1153[93]. This is a posttranslational modification that ultimately leads to the two subunits to reconstitute and be stably bound, but noncovalently associated. The E-subunit is known to be shed from the cell surface following cell activation but is cleaved a second time at a more membrane proximal site at amino acid 1223[93]. Shedding of ectodomains is not an uncommon feature of transmembrane proteins under both normal and activated conditions. In fact, activation of the epidermal growth factor receptor (EGFR) has been shown to cause shedding of the E-subunit and disassociation of the P-subunit from the transmembrane domain[94]. Although other CAMs such as neuroligin and neurexins can be cleaved and are thought to be regulated by shedding, this process is poorly understood.

1.5.1 Presynaptic role of LAR/PTP-3 family of phosphatases

There exist multiple lines of evidence from both invertebrate and vertebrate models that suggest RPTPs are critical in presynaptic synapse formation. General functions of vertebrate RPTPs include: mediating cell-cell adhesion and presynaptic differentiation, recruitment of presynaptic vesicles, and postsynaptic differentiation by binding to dendritic partners[95]. The first reported postsynaptic binding partner of LAR was netrin-G ligand-3 (NGL-3)[96]. In co-cultured cells, NGL-3 induces presynaptic differentiation which is suppressed by addition of the LAR ectodomain. Signaling from presynaptic RPTPs to postsynaptic NGL-3 can induce clustering of NGL-3 which is important in clustering of other postsynaptic proteins such as PSD-95, AMPARs, and NMDARs[96].

One of the distinct features of the RPTPs is that they possess motifs that are present in many cell adhesion molecules (CAMs) yet contain an intracellular phosphatase domain (**Fig 1.5**)[97]. This unusual combination allows RPTPs to directly couple extracellular adhesion mediated events to intracellular signaling pathways[98]. A well-known ligand of LAR is the extracellular matrix (ECM) laminin-nidogen complex which specifically binds to the fifth fibronectin domain[99]. The laminin family of glycoproteins are typically associated in extracellular matrices found in intimate cell-cell contacts[100]. The

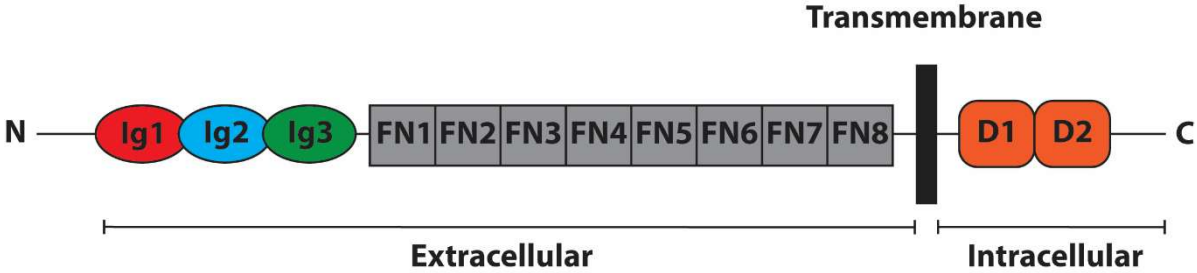

Figure 1.5: Domain structure of LAR-RPTPs. Schematic domain organization containing 3 Ig-like domains, variable FNII domains, a transmembrane domain, and a dual intracellular phosphatase domain. Adapted from Won *et. al.,* 2018

precise mechanism of action of LAR interacting with the laminin-nidogen complex is not well established but is thought to be important in clustering of LAR and correct cellular localization within the synapse[99]. This clustering is a possible mechanism of inhibition of LAR as it is known that the D1 phosphatase domains can homodimerize and block the active catalytic site[101]. Another known ligand of LAR is the heparan sulfate proteoglycan ECM molecule syndecan which is a transmembrane protein but can also be cleaved and shed into the extracellular medium[102]. The binding of syndecan to LAR in *trans* is known to regulate axon guidance in *Drosophila,* however, the authors could not discern if this interaction was when syndecan was still attached to the cell surface or if it was shed[102]*.* A paper that was published at the same time demonstrated that the axon guidance defect seen when disrupting the LAR-syndecan interaction was dependent on the three Ig-like domains and the phosphatase domain, but not the fibronectin domains of LAR[103]. This reiterated what was already known about syndecan binding to the Ig-like domain of LAR but showed that regulation of axon guidance by LAR phosphatase activity is dependent on syndecan binding. The dual property of LAR as a CAM and a receptor give it a variety of ways to stabilize synaptic connections which could explain why it is expressed both pre and postsynaptically and has different mechanisms of action between the two. The complexity and expression patterns of RPTPs could be why their function *in vivo* is not well understood.

The *C. elegans* single RPTP, PTP-3, was originally found to be critical in neuroblast migration during embryogenesis[104]. In 2005 however, the Jin lab demonstrated there were different isoforms of PTP-3 that had different effects on synapse formation aside from neuroblast migration. The *ptp-3* gene encodes two main isoforms

that differ in their extracellular domain, PTP-3A and PTP-3B (with a lesser known C isoform). PTP-3A contains three Ig-like domains, nine fibronectin domains, a transmembrane domain, and a dual phosphatase domain labeled D1 and D2 with D2 being catalytically inactive and is most closely related to vertebrate LAR[91]. PTP-3B has a similar structure except it only contains five fibronectin domains and no Ig-like domains[91]. PTP-3 is highly expressed in the nerve ring and nerve cord of worms and shows high presynaptic expression with overlap of presynaptic proteins such as SYD-2 and synaptotagmin. Interestingly, contrast to *Drosophila,* only the PTP-3B isoform is critical in motor axon guidance and is extrasynaptic. In contrast, PTP-3A, the closest homolog to LAR, is critical in presynaptic development and maintenance. Surprisingly, in the single syndecan homologue in *C. elegans,* there is no interaction with the Ig-like domains of the PTP-3A isoform which is contrary to *Drosophila* and instead only interacts with the PTP-3B isoform[105]. What do we know about postsynaptic roles of LAR-RPTPs? In vertebrates, a few studies have examined LAR's postsynaptic role and its function in synaptic plasticity which will be discussed in the next section.

1.5.2 Postsynaptic role of LAR/PTP-3 family of phosphatases

LAR is known to coimmunoprecipitate with postsynaptic proteins such as PSD-95, GRIP and GluA2/3, what role does it have then in postsynaptic maintenance? There is evidence that LAR dephosphorylation of β-catenin recruits it to the synapse and causes anchoring of the pre and postsynaptic cells[106]. The mechanism of action of LAR is not fully understood but is thought to depend on the liprin-α family of proteins and is activity-dependent. Liprin-α is known to bind to the D2 domain of LAR and regulate its subcellular localization. It was originally thought that liprin-α needs to be degraded to allow for LAR

to reach the synapse. However, recent structural analysis shows that liprin-α can bind LAR while it's at the cell surface. The liprin-α/LAR binding causes clustering of LAR which induces dimerization of its D1 domains and inactivates its catalytic activity[107]. Thus, activity-dependent degradation of liprin-α causes declustering of LAR which allows it to be catalytically active. What causes the degradation of the liprin-α then? Morgan Sheng and colleagues demonstrated that activated CaMKII, a general sign of an "active" synapse, degrades liprin-α[108]. What led us to study this phosphatase's mechanism of action as it applies to synaptic plasticity?

Although LAR has not been directly related to a mechanism in synaptic plasticity, LAR attracted us for a few reasons. First, it is known that the liprin-α/LAR complex binds to glutamate receptor interacting protein (GRIP) and thus has implications as being co-transported with AMPARs and possibly regulate AMPAR transport[109]. Secondly, knockout studies of LAR show that it is critical in maintaining excitatory transmission presumably by regulating AMPAR numbers at synapses[108]. Indeed, when LAR is knocked down in hippocampal neurons, there is loss of dendritic protrusions and spines. Lastly, most of the studies of LAR are from *in vitro* cell culture and almost nothing is known about its role in *in vivo* excitatory synapse maintenance.

The mechanism of LAR-RPTP activation in vertebrate excitatory neurons is not well established, but recent studies have started to uncover its possible interaction with NMDA-receptor-mediated responses in hippocampal neurons. The Sudhof lab was able to conjure a triple knockout of all three LAR-RPTPs in hippocampal neurons where they show that synaptic connectivity was unchanged but NMDA-receptor-mediated responses were decreased[110]. In a parallel study, the Kaiser lab also conjured a triple knockout and

found that ablation of the LAR-RPTPs does not alter synapse density and synaptic vesicle release[111]. Although this is contrary to results found previously, the authors denote that this phenotype could be cell specific or that other RPTPs could possibly compensate for this loss. This shows that the mechanism of LAR-RPTPs is not well-understood.

The regulation of PTP-3A activity has not been well studied in worms. However, there has been a study identifying possible direct targets or downstream targets of PTP-3's phosphatase activity[112]. A paper by Christopher Mitchell and colleagues analyzed genome-wide phosphorylation levels of proteins in *ptp-3* deletion mutants to find potential targets of PTP-3. Although there were 264 hyperphosphorylated sites in the mutants, the authors wanted to analyze if any of the sites were evolutionarily conserved between humans. There are currently nine known substrates of the LAR family and they identified orthologs of four of the nine known substrates: NTRK2, MET, INSR, and STAT3, showing a possible evolutionarily conserved role for PTP-3[112].

1.6 Thesis work

My thesis work was largely aimed at better understanding the role of long-distance AMPAR transport on synaptic plasticity *in vivo.* I originally set out to try and understand how dephosphorylation impacts this mechanism. Since there was no knowledge about the role of phosphatases in AMPAR transport but studies showing their importance for plasticity, finding any phosphatase impacting AMPAR transport might be a novel discovery. My work has suggested the importance of the phosphatase PTP-3A in regulating not only synaptic retention of AMPARs, but also in AMPAR transport. Before, PTP-3A was mainly known to be responsible in presynaptic axon guidance. Its role in

excitatory neurons, let alone its role in synaptic plasticity, had not been known in *C. elegans.*

My work was mainly focused on the role of the phosphatase PTP-3A in regulating long-distance AMPAR transport. This led to asking fundamental questions of how AMPAR transport can affect synaptic plasticity *in vivo.* It expands upon previous hypotheses that AMPAR transport is a critical step for regulating synaptic AMPAR numbers while establishing a possible role for AMPAR transport in learning and memory. In **Chapter 2,** I investigate the postsynaptic role that PTP-3A has on AMPAR transport and delivery. **Chapter 3** consists of unpublished data investigating the possible role that SYD-2 has on AMPAR transport and delivery and understanding the genetic pathway it shares with PTP-3A. Although I did not end up unequivocally determining the mechanism of how PTP-3A is controlling AMPAR transport or synaptic AMPAR delivery/removal, my studies have established a basic understanding of its role, laying the foundation for a model that help test many aspects of the role of LAR-RPTP/PTP-3 in AMPAR transport and synaptic plasticity (discussed further in **Chapter 4**).

CHAPTER 2: A Dual Role for LAR/PTP-3 in Regulating Long-distance AMPAR Transport and Synaptic Retention Essential for Long-Term Associative Memory[1]

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[1]This chapter is an article in review as contributed by:
Pierce DM., Doser RL., Lenninger Z., Knight K., Stetak A., and Hoerndli F.J.

2.1 Summary

The AMPA subtype of synaptic glutamate receptors (AMPAR) plays an essential role in excitatory synaptic transmission, learning, and memory. Most of these receptors are synthesized in the soma of neurons and transported to branching, often far-reaching, dendrites. Coordinated transport, delivery, and removal of receptors is required to ensure synapse function and maintain homeostatic balance. This process is a major logistics problem for neurons, but it is essential for circuit function. Although recent studies have shown that long-distance synaptic transport is regulated by neuronal activity, we know very little about the mechanisms of coordinated transport and delivery. Here we show that loss of the PTP-3A isoform of the receptor tyrosine phosphatase PTP-3 (the *C. elegans* homologue of vertebrate LAR-RPTP) leads to a ~60% decrease in the GLR-1 AMPAR transport; this affects synaptic delivery and synaptic functions necessary for long-term associative olfactory memory (LTAM) in *C. elegans*. We reveal that PTP-3A is necessary postsynaptically in adult neurons for the regulation of AMPAR transport, delivery, and removal. While loss of PTP-3A leads to defects in transport and local synaptic trafficking of GLR-1, a mutation affecting all PTP-3 phosphatase domains only influences local synaptic cycling and retention. Finally, we show that the N- and C-terminal of PTP-3A

have differing functions in regulating transport and synaptic retention of GLR-1. Our results suggest a model in which PTP-3/LAR-RPTPs coordinate transport and delivery of AMPARs to synapses via two domains, possibly released by synaptic activity, that are essential for long-term associative learning.

2.2 Introduction

The AMPA subtype of ionotropic glutamate receptors are the workhorse of excitatory synaptic transmission. Regulating the quantity of AMPA receptors (AMPARs) at synapses is essential for both synaptic input-dependent plasticity and network-dependent homeostatic plasticity. However, the majority of AMPARs are produced in the cell body and therefore must be trafficked to the synapses. This complex, multistep process is regulated on many levels, with the ultimate goal of finely tuning the form and function of receptors at the synapses. Although all steps are essential, arguably the most central phase of this process is the transport of AMPARs by molecular motors to be distributed at synapses. However, this is probably the least well studied step in AMPAR trafficking. New studies have started to reveal that this central transport is regulated by neuronal activity, calcium entry, and CaMKII[89,113,114]. Although much of the mechanism of how this pathway exerts control over transport is still unknown, these studies have revealed that phosphorylation of both the cargo and motor-adaptors contribute to transport regulation[89,114]. This implies that many pathways that were previously documented to affect AMPAR synaptic recruitment might also be involved in AMPAR transport in addition to their role in local synaptic mechanisms of stabilization and recruitment.

Recent studies have highlighted the role of tyrosine phosphorylation of AMPARs in synaptic and homeostatic plasticity mechanisms[75,115–117]. Bidirectional phosphorylation

of a single tyrosine residue in the AMPAR GluA2 was observed in up and downscaling homeostatic *in vivo*[75] and has been shown to be critical for AMPAR recruitment during LTP[118]. Interestingly, tyrosine phosphatases such as Receptor-type Protein Tyrosine Phosphatases (RPTPs), in particular the LAR (leukocyte common antigen-related) family, have been documented to recruit and stabilize AMPARs at synapses during development[119]. the exact mechanism of how that is achieved is unknown. LAR-RPTPs and GRIP-1 can form a complex together with the protein scaffold liprin-α[120]. GRIP-1 and liprin-α have been shown to bind to Kinesin-1, and it has been suggested that CaMKII phosphorylation of liprin-α releases LAR-RPTP from the complex to be recruited at synapses[109,120–122]. However, there is no direct recent evidence for the roles of LAR-RPTPs, liprin-α, or GRIP-1 in AMPAR transport. As mentioned above, new approaches to AMPAR transport might shed more light on their role and the importance of Tyrosine phosphorylation in AMPAR transport, delivery, and retention.

The mammalian family of LAR-RPTPs includes LAR, PTP-δ, and PTP-σ which are part of the type IIa class of receptor tyrosine phosphatases. The type IIa RPTPs are single transmembrane domain phosphatases with a large N-terminal extracellular domain containing 3 Ig-like domains at the very tip followed by a variable number of Fibronectin III repeats[97,123]. The intracellular domain contains two tandem phosphatase domains called D1 and D2, with only D1 being catalytically active but D2 being important for substrate specificity. LAR-RPTPs are normally cleaved post-translationally into 2 non-covalently linked subunits: the external E-subunit containing the 3 Ig-likes and Fibronectin repeats and the intracellular P-subunit containing the transmembrane domain and the tandem phosphatases. The D2 domain of LAR-RPTP has been found to bind to liprin-α

and colocalize with AMPARs in cell culture and at synapses. In addition, splicing variations give rise to many different isoforms of LAR-RPTPs that have different functional effects. Altogether, an overwhelming number of studies present a presynaptic role for LAR-RPTPs in synaptogenesis and synaptic function, with single gene-knock outs leading to behavioral defects[124,125]. It is thought that different RPTPs might complement each other, and one important caveat so far has been the lack of a viable triple genetic knock-out. However, two recent studies with triple conditional knock-outs of LAR-RPTPs did not lead to defects in synaptogenesis nor a major effect on glutamatergic or GABAergic transmission in the hippocampus[110,111]. These recent studies further underscore our lack of understanding of the mechanisms by which LAR-RPTPs act on synaptic function.

The *C. elegans* sole type IIa RPTP gene, *ptp-3,* encodes three isoforms that differ in the extracellular domain (**Fig. 2.1A**)[104]. PTP-3A is most similar to vertebrate LAR, with 3 Ig-like domains and 9 fibronectin repeats, followed by a transmembrane domain, and the tandem phosphatase domain (**Fig. 2.1A**). PTP-3 has been shown to have presynaptic roles in synaptogenesis[105,126–128] but has not been studied in relation to glutamate receptor regulation *in vivo*.

Here we report a postsynaptic role for the PTP-3A isoform coordinating GLR-1 transport, synaptic delivery, and synaptic recycling necessary for short and long-term associative memory in *C. elegans*. Using *in vivo* real-time analysis of GLR-1 transport, FRAP, photoconversion, and N and C-terminal cell specific rescues, we show that the extracellular N-terminal domain promotes GLR-1 transport from the cell body, while the intracellular C-terminal phosphatase regulates synaptic GLR-1 recycling. We link these

molecular observations with specific effects on associative olfactory memory, demonstrating that different effects on delivery and synaptic cycling of GLR-1 correspond to different effects on memory retention. We propose a model in which PTP-3A plays a dual role in regulating transport and synaptic retention, which is essential for long-term associative memory in *C. elegans*, that has profound implications for understanding synaptic plasticity mechanisms in vertebrate systems.

2.3 Results

2.3.1 The largest isoform of PTP-3, PTP-3A, modulates GLR-1 transport

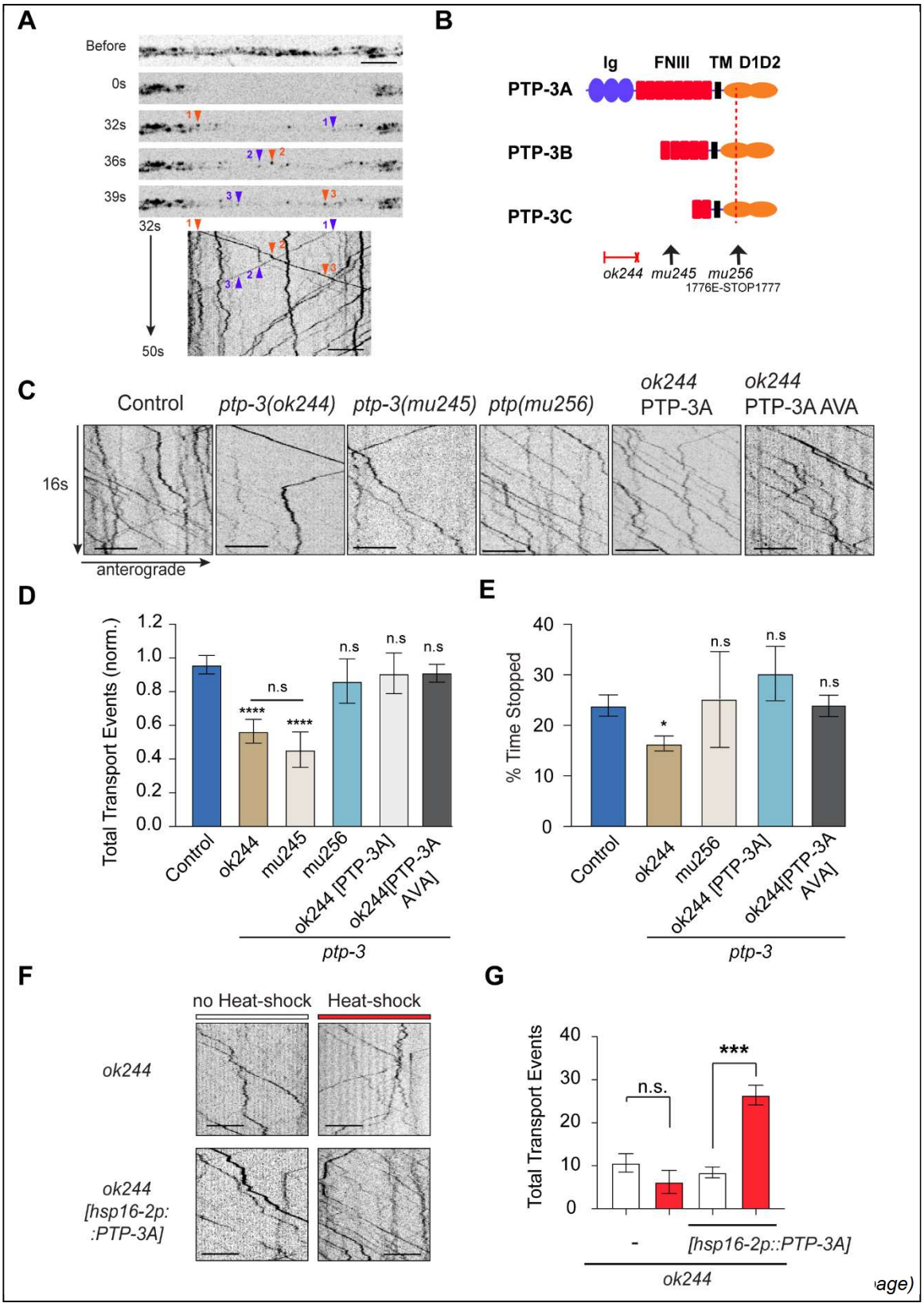
Initial studies in hippocampal cell culture documented a postsynaptic specific role for LAR, PTP-δ, and PTP-σ in recruiting GluA2 to synapses[119,129]. Aside from a requirement for the tandem phosphatase domain to function and interact with other partners, no additional mechanism was proposed[119]. To elucidate how LAR receptors regulate AMPAR synaptic recruitment, we started by quantifying GLR-1 transport *in vivo*, in a single pair of neurons, as previously described[113], using isoform specific loss-of-function mutations in the sole *C. elegans* homologue of LAR: PTP-3. The *ok244* allele leads to a premature stop in translation and loss of expression of the PTP-3A isoform (**Fig. 2.1A and Table 2.1 see Methods**[126]). The *mu245* mutant results in a premature stop codon and thus leads to loss of both PTP-3A and B isoforms (**Fig. 2.1 and Table 2.1 see Methods**[105]). The *mu256* mutation causes a frameshift and premature stop in the first intracellular phosphatase domain affecting all 3 isoforms of PTP-3 (**Fig. 2.1 and Table 2.1 see Methods**[105]). In the *glr-1 (ky176)* null background, using SEP::mCherry::GLR-1 expression in the AVA command interneurons only, we combined photobleaching and continuous imaging of mCherry::GLR-1 to observe single vesicle

GLR-1 transport events as previously reported[113]. The streams were then converted into kymographs displaying lateral displacement on the x axis and time on the y axis, illustrating moving single transport events as diagonal lines (**Fig. 2.1A,**) and immobile vesicles as vertical lines (**Fig. 2.1A**). A first look at the number of transport events (**Fig. 2.1D**), shows that *ok244* and *mu245* lead to a ~50% decrease in GLR-1 transport events. Surprisingly, the *mu256* mutation affecting all 3 isoforms did not significantly affect GLR-1 transport. These results suggest that the loss of the phosphatase domain does not affect GLR-1 transport. In addition, the similarity of effect between *ok244* and *mu245* suggest that PTP-3A is the main isoform modulating GLR-1 transport.

The loss of transport observed in *ok244* mutants, resulting in a PTP-3A null, is rescued by expressing PTP-3A full length under the native promoter and under the pFlp-18 promoter (**Fig. 2.1D**). Flp-18 is specifically expressed in AVA[85] indicating a postsynaptic requirement of PTP-3A for GLR-1 transport regulation. Although transport numbers are an important determinant of GLR-1 delivery, we also recently showed that transport dynamics play a role in GLR-1 delivery and synaptic contingent[113]. Using this same analysis, we illustrate that similar to its effect on transport numbers, the *ok244* allele leads to a significant decrease in stopping (Control= 24%± 2.1; *ok244*= 16%± 1.5). On the contrary the *mu256* allele did not lead to a significant decrease in stopping (*mu256*= 30%± 5.6) nor did a cell specific rescue of the PTP-3A isoform (PTP-3A AVA= 25%± 2.9) (**Fig. 2.1E**). We also quantified anterograde and retrograde velocities in all alleles but saw no major differences in overall instantaneous velocities (**Figure 2.2A-D**). A more detailed analysis plotting the different velocities showed that *ok244* and *mu256* do not differ from controls in terms of velocity distribution, whereas *mu245* seems to have a reduction in

transport events with velocities > 1.5 μm/s (**Figure 2.2B**). In addition, we did not see any defects in total outgrowth of the AVA neurons nor microtubule dynamics in *ok244 mutants* using EBP-2::GFP (**Figure 2.2E-F**) as described previously[85], suggesting that the GLR-1 transport decrease we observed is not due to microtubule defects or neurite outgrowth. Taken together, these experiments suggest that PTP-3A is important for regulating 50% of GLR-1 transport in dendrites by stimulating transport numbers out of the cell body and facilitating stops in dendrites that might favor delivery of receptors.

Previous work in *C. elegans* has shown a role for PTP-3 in neuronal and synaptic development[104,126]. To address the possibility that partial loss of GLR-1 transport may be due to developmental roles of PTP-3 we utilized an inducible heat-shock approach to temporally express PTP-3A in the *ok244* background. Briefly, transgenic *C. elegans* animals containing *hsp16-2P::PTP-3A* were heat-shocked for 1 hour at 32ºC and left to recover for 1 hour at room temperature (as previously published in Hoerndli *et. al.,* 2015). GLR-1 transport was then quantified as described in **Fig. 2.1D**. Heat-shock treatment had no significant effect in control and *ok244* animals without the array (**Fig 2.1F-G**) but showed a strong increase of GLR-1 transport in transgenic animals (**Fig. 2.1E and 2.1F**) that was comparable to levels of GLR-1 transport in control animals (**Figure 2.2G**). Interestingly, a quick analysis of transport stopping for heat shocked treated animals with the transgenic *hsp16-2p::PTP-3A* showed no rescue of the stopping behavior (**Figure 2.2H**), suggesting that the short time scale of this protocol might not be enough for enabling synaptic changes. Overall, our results suggest that PTP-3A has an ongoing role in modulating GLR-1 somatic export as well as dendritic transport dynamics.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36s
39s
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50s
B
Ig
FNIII
TM
D1D2
PTP-3A
PTP-3B
PTP-3C
ok244
mu245
mu256
1776E-STOP1777
C
Control
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ptp-3(mu245)
ptp(mu256)
ok244 PTP-3A
ok244 PTP-3A AVA
16s
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Total Transport Events (norm.)
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ptp-3
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no Heat-shock
Heat-shock
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Total Transport Events
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[hsp16-2p::PTP-3A]
ok244

Figure 2.1: PTP-3A isoform modulates GLR-1 transport. **A)** Sequential images and kymograph of the mCherry signal from SEP::GLR-1::mCherry in the proximal portion of the AVA processes shortly after entering the ventral nerve cord. The images shows GLR-1::mCherry signal prior to 1s photobleaching and 39s after with intermediate pictures showing single GLR-1 mCherry transport vesicles (anterograde orange, retrograde purple arrowheads). The kymograph shows all transport events starting at T = 32 seconds. The numbering of the arrowheads corresponds to the precise position and time of transport events shown in the static images above. **B)** diagram of the different PTP-3 isoforms and approximate localization and nature of different genetic alleles used in this study (and methods). Black arrows indicate point mutations. **C-D)** Representative kymographs and subsequent quantification of total GLR-1 transport in control (n=39) and different *ptp-3* genetic backgrounds including *ok244* (n=26) *, mu245 (n=13), mu256* (n=7) and *ok244* rescued either with PTP-3A expressed under its native promoter (n=7) or under the *flp*-18 promoter (n=16). All kymographs originate from the conversion of 16s streaming imaging of GLR-1::mCherry signal from SEP::GLR-1::mCherry in the *glr-1(ky176); akIs201* background. ****p < 0.0001. **E)** Kymograph quantification of the percentage of time each transport events spends stopped compared to the total time spent traveling (methods). The number of transport events were for controls (n=15), *ok244* (n=17)*, mu256* (n=7) and *ok244* rescued either with PTP-3A expressed under its native promoter (n=5) or under the *flp*-18 promoter (n=8), *p < 0.05. **F-G)** representative kymographs and quantification of the total number of GLR-1 transport events with and without 1hr 32ºC heat shock treatment of adult animals in the *ok244* background alone (no heat-shock n= 12, with heat-shock n=4) or with *hsp16-2p::PTP-3A* (no heat-shock n= 11, with heat-shock n=16). Only heat-shock treated adult animals showed an increase in transport events ***p <0.001. All statistics used an ordinary one-way ANOVA with Dunnett's multiple testing corrections. Error bars represent SEM and all scale bars represent 5µm.

2.3.2 PTP-3A modulates synaptic number of GLR-1 receptors

To determine whether these transport defects would result in reduced synaptic GLR-1, we quantified synaptic SEP::mCherry::GLR-1 in the alleles described above (**Fig. 2.1**). The double N-terminal tag of GLR-1 with SEP (Super Ecliptic Phluorin), which is a pH sensitive GFP whose fluorescence is quenched at pH 6.5 and below, and mCherry allows the quantification of the synaptic surface GLR-1 receptors and the total pool of GLR-1 receptors at synapses[113,130] as described in (**Fig. 2.3A**). Loss of PTP-3A (*ok244)* led to a decrease in synaptic surface GLR-1 (SEP = 0.73± 0.06, **Fig. 2.3C**) as well as a decrease in the total synaptic pool including the endosomal subsynaptic receptors (mCherry**=** 0.66± 0.08, **Fig. 2.3D**) compared to same day controls. We also determined

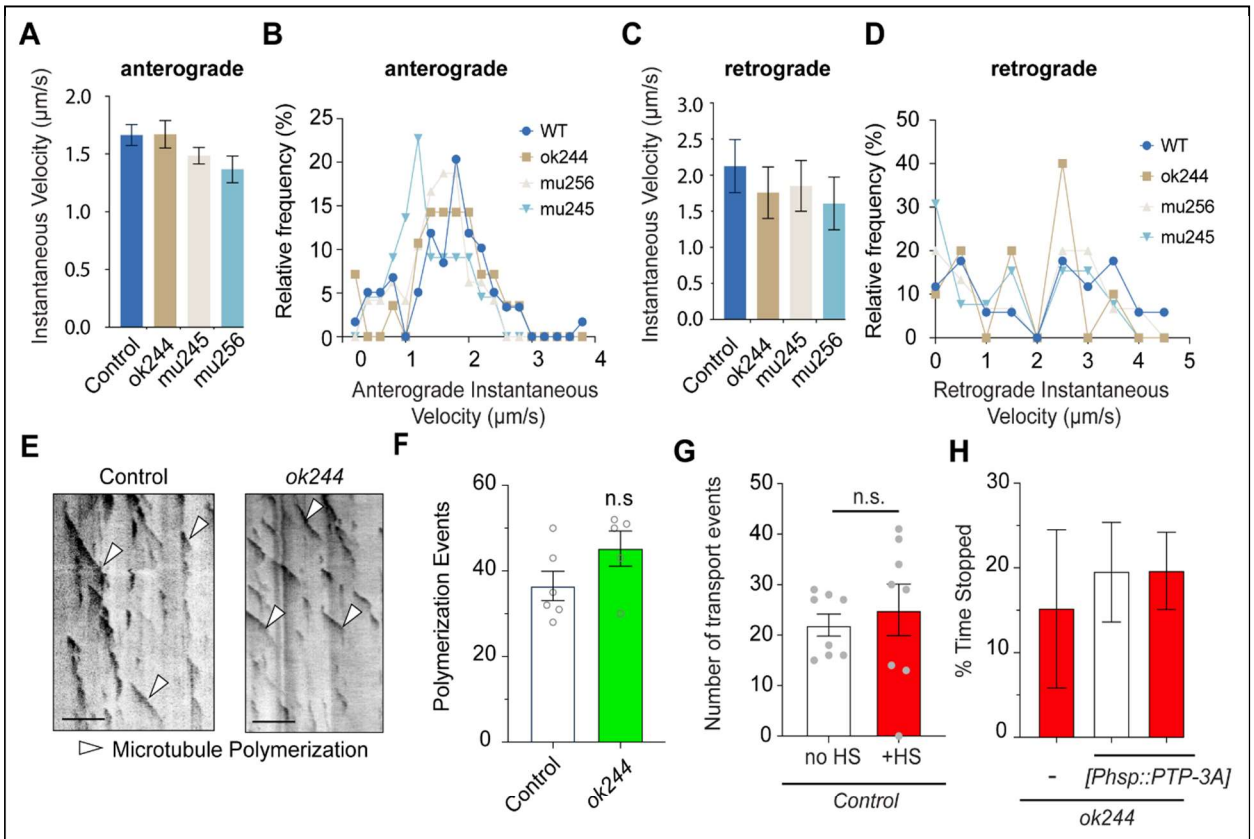

Figure 2.2: Loss of PTP-3A does not affect velocity of GLR-1-containing vesicles A) Instantaneous velocity of GLR-1-containing vesicles traveling anterogradely and **B)** distribution of instantaneous velocities or **C-D)** retrogradely. **E)** Representative kymographs of microtubule polymerization and **F)** quantification of events using EBP-2::GFP. **G)** Quantification of transport events with and without 1-hour 32ºC heat-shock treatment in **G)** control *ky176* and **H)** *ok244+ Phsp::PTP-3A.* Error bars represent SEM and all scale bars represent 5µm.

whether the loss of both PTP-3A and PTP-3B (*mu245)* would lead to a more pronounced loss of synaptic GLR-1 receptors. Our data (**Figure 2.4A** and **2.4B**, SEP= 0.75 ± 0.08, mCherry= 0.75 ± 0.07) show that, similar to what we found with transport, synaptic GLR-1 levels are not more affected by the double loss of PTP-3A and B in the *mu245 allele,* indicating that loss of PTP-3A is the main driver of the synaptic phenotype we observed. The *mu256* allele showed no effect on the synaptic endosomal GLR-1 pool marked by mCherry (**Figure 2.3D**, mCherry = 1.0± 0.15, compared to control= 1.0± 0.07) but did affect the number of GLR-1 surface receptors, as the SEP signal is reduced (**Fig. 2.3C**,

SEP = 0.57± 0.07, compared to control= 1.0± 0.07). Similar to transport, this effect of PTP-3A loss on synaptic GLR-1 could be rescued by expression of PTP-3A under the native promoter as well as the AVA cell specific promoter pFlp-18 (**Fig. 2.3B-D**). This suggests that PTP-3A is required postsynaptically for its effect on synaptic GLR-1 trafficking.

Impaired transport can have a differential effect on proximal and distal distribution of synaptic receptors[85]. To determine if this is the case for the loss of PTP-3A (*ok244)* allele, we quantified both the proximal and distal synaptic GLR-1 numbers (**Fig. 2.3E-H**). Indeed, *ok244* showed a decrease in both surface and total number of synaptic receptors (SEP= 0.73± 0.06, mCherry= 0.66± 0.08, **Fig. 2.3E-H**) compared to controls at proximal synapses, whereas both surface and total pool were increased in distal synapses receptors (SEP= 1.59± 0.22, mCherry= 1.64 ± 0.21, **Fig. 2.3E-H**). This suggests a possible defect in receptor delivery which then results in distal accumulation. We also measured GLR-1 mCherry at the soma of the AVAs in control and *ok244* but found no difference in expression level suggesting that loss of PTP-3A does not affect translation of GLR-1 (**Figure 2.4C-D**).

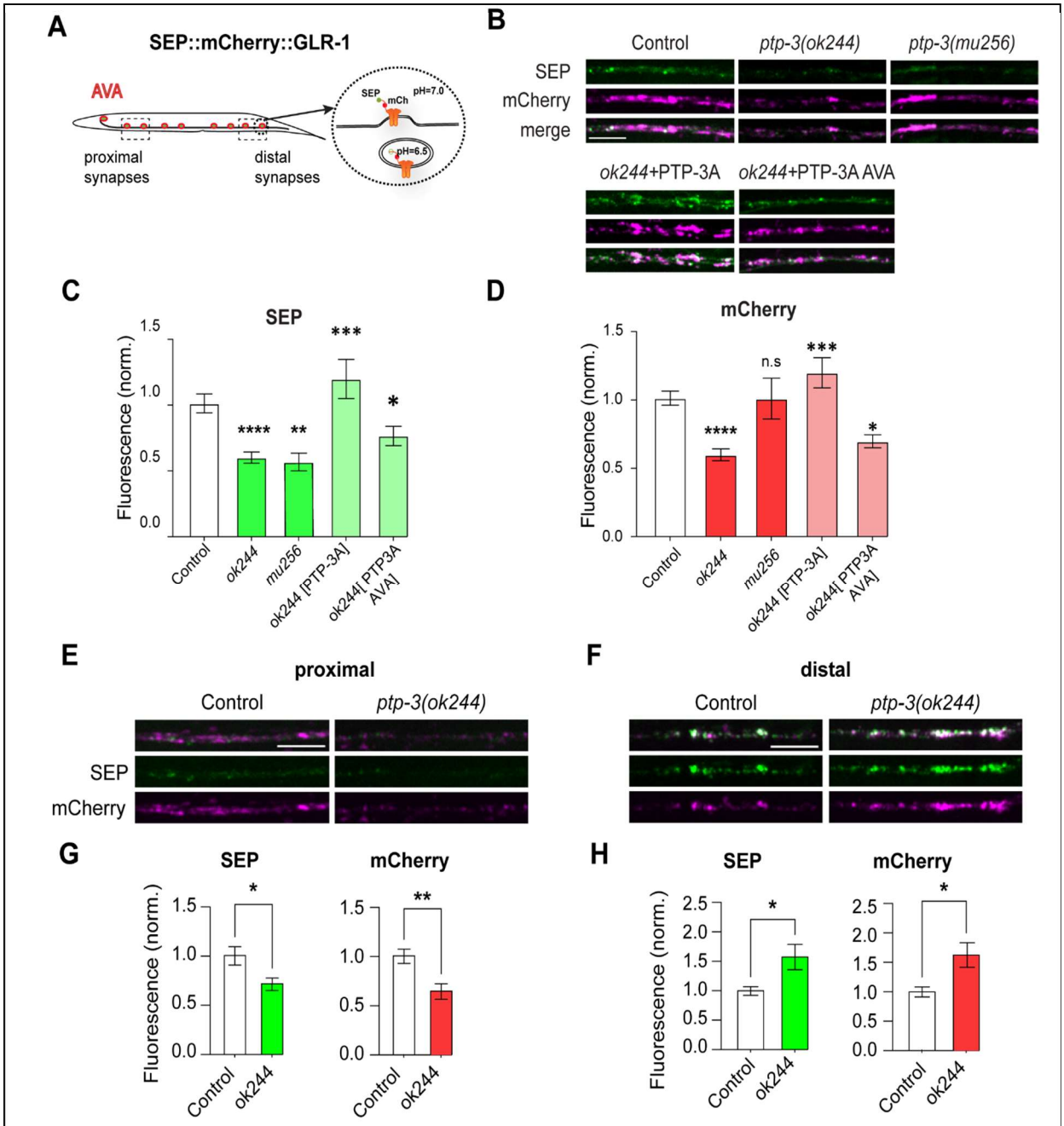

Figure 2.3: PTP-3 modulates synaptic GLR-1 levels. A) All worms express SEP::mCherry::GLR-1 in the *glr-1(ky176)* knockout background. Control worms represent *glr-1(ky176)* expressing SEP::mCherry::GLR-1 with no further mutations. **B)** Confocal images of SEP::GLR-1 and mCherry::GLR-1. **(C and D)** Quantification of **(C)** SEP fluorescence and **(D)** mCherry fluorescence, normalized to control. Control (n=45), *ok244* (n=26), *mu256* (n=19), *ok244*[PTP-3A] (n=15), *ok244*[PTP-3A AVA] (n=45). **(E and F)** Representative confocal images of SEP::GLR-1 and mCherry::GLR-1 of *ok244* in **(E)** proximal portion of the AVA process and **(F)** distal portion that is located at the end of the process as outlined in (A). **(G and H)** Quantification of SEP fluorescence and mCherry fluorescence in the **(G)** proximal process and **(H)** distal process normalized to control.
*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. All statistics used an ordinary one-way ANOVA with Dunnett's multiple testing corrections. Error bars represent SEM and all scale bars represent 5µm.

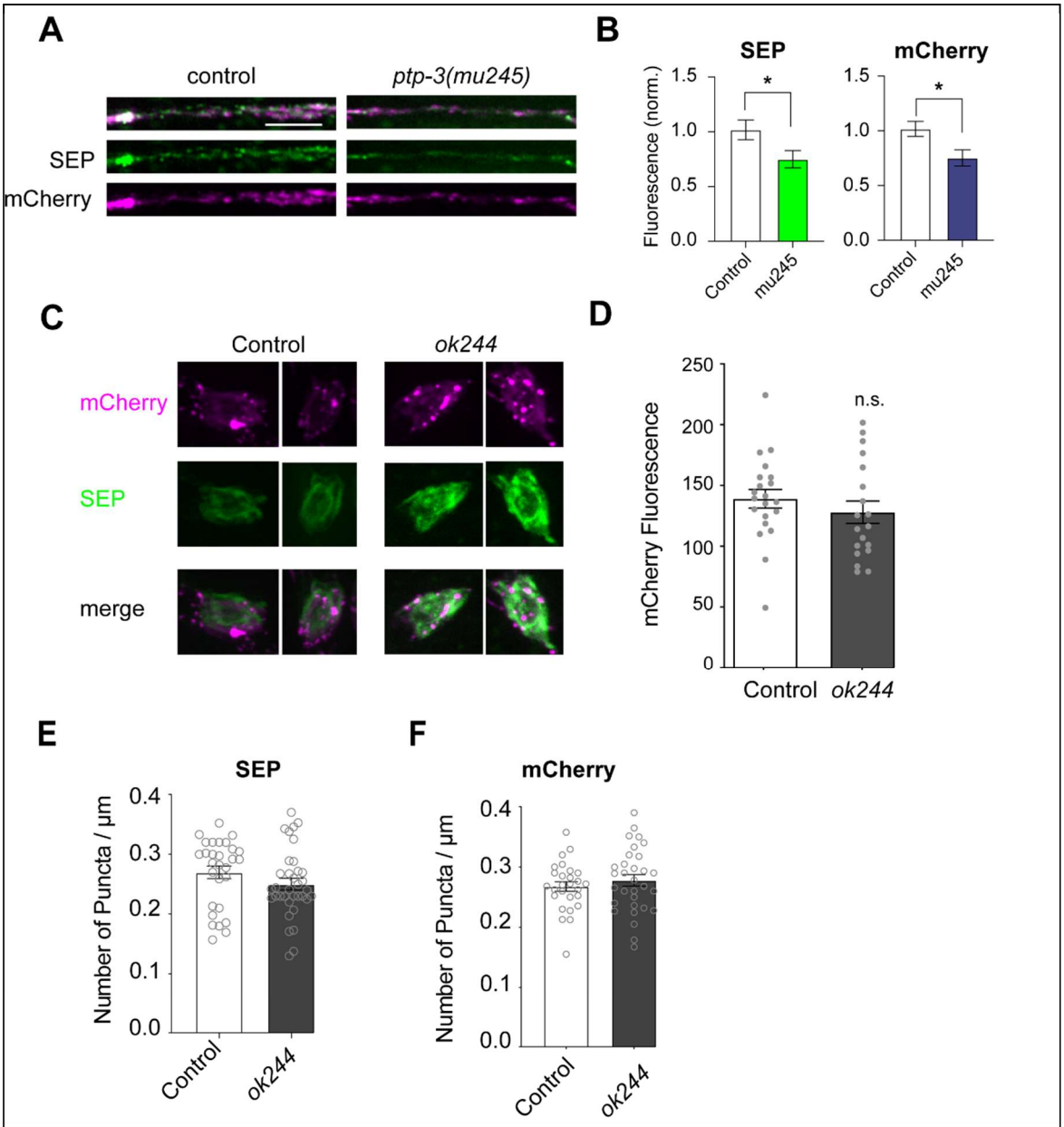

Figure 2.4: Loss of PTP-3A does not affect synapse density of GLR-1 A) Confocal images of SEP::GLR-1 and mCherry::GLR-1. **B**) Quantification of (C) SEP fluorescence and mCherry fluorescence, normalized to control. **C)** Confocal images of SEP::GLR-1 and mCherry::GLR-1 in the cell bodies of the AVA neurons and **D)** mCherry fluorescence quantification of GLR-1. **E)** Quantification of SEP puncta amount per µm and **F)** mCherry puncta amount. Error bars represent SEM and all scale bars represent 5µm.

As mentioned previously, PTP-3 function has been shown to be important for synaptic development of the neuromuscular junction[126,127] but its role in synaptic development of glutamatergic command interneuron development is unknown. We

quantified the density of synapses marked by surface SEP::GLR-1 and synaptic endosomal pools marked by mCherry::GLR-1 (**Figure 2.4 E-F**) to test for evidence of a developmental role of PTP-3A in excitatory synapse development. However, we did not observe significant differences between *ok244* and controls suggesting that PTP-3A is not of major importance for the development of glutamatergic synapses in the AVA command interneurons. Based on these results, we predicted that loss of PTP-3A would modify excitatory synaptic function and therefore behaviors associated with excitatory synapses in *C. elegans.*

2.3.3 PTP-3A is necessary for olfactory associative memory but not learning

A proper contingent of GLR-1 receptors in AVA has been tied to normal olfactory associative memory[11,131]. To determine if the synaptic GLR-1 defects due to loss of PTP-3A are associated with learning and memory, we used population based olfactory attraction assays[11,132–134]. We quantified olfactory attraction to 0.1% diacetyl in naïve animals without training, immediately after diacetyl coupled starvation training, and after 1-hour recovery on normal food plates (**Fig. 2.5A**). Our results, show that loss of PTP-3A (*ok244)* or all phosphatase domains (*mu256),* did not affect naïve attraction nor did it prevent learning the negative association of diacetyl with starving. However, loss of PTP-3A did significantly decrease retention of this memory, whereas loss of the phosphatase domains was more mitigated in its effects (**Fig. 2.5B**). One hour of conditioning and 1-hour of recovery reveal effects on short term associative memory (STAM,[11]). Long-term associative memory (LTAM) can be assayed by repeating negative association with an hour of recovery in between and then quantifying attraction 24 hours after training (**Fig. 2.5C**). Here, loss of PTP-3A and PTP-3 phosphatase function led to a strong decrease in

memory retention again without any effects on naïve attraction or immediate learned avoidance after conditioning. To determine if this effect on STAM and LTAM requires pre- or postsynaptic expression of PTP-3A, we used transgenic animals expressing PTP-3A under the Pflp-18 promoter, which drives PTP-3A expression in AVA [85]. By Repeating the previous approach with a new set of controls (wild-type animals and *ok244*), we quantified learning, STAM, and LTAM for animals with PTP-3A expression only in AVA (**Fig. 2.5D and E**). Expression of PTP-3A in AVA alone had no effect on learning in either STAM (**Fig. 2.5D**) or LTAM (**Fig. 2.5E**). However, it rescued both STAM and LTAM defects observed in animals lacking PTP-3A completely (1h memory and 24hrs memory in **Fig. 2.5D and E**). These data confirm the defects we observed with the loss of PTP-3A and show that postsynaptic expression of PTP-3A is essential for both short and long-term olfactory associative memory.

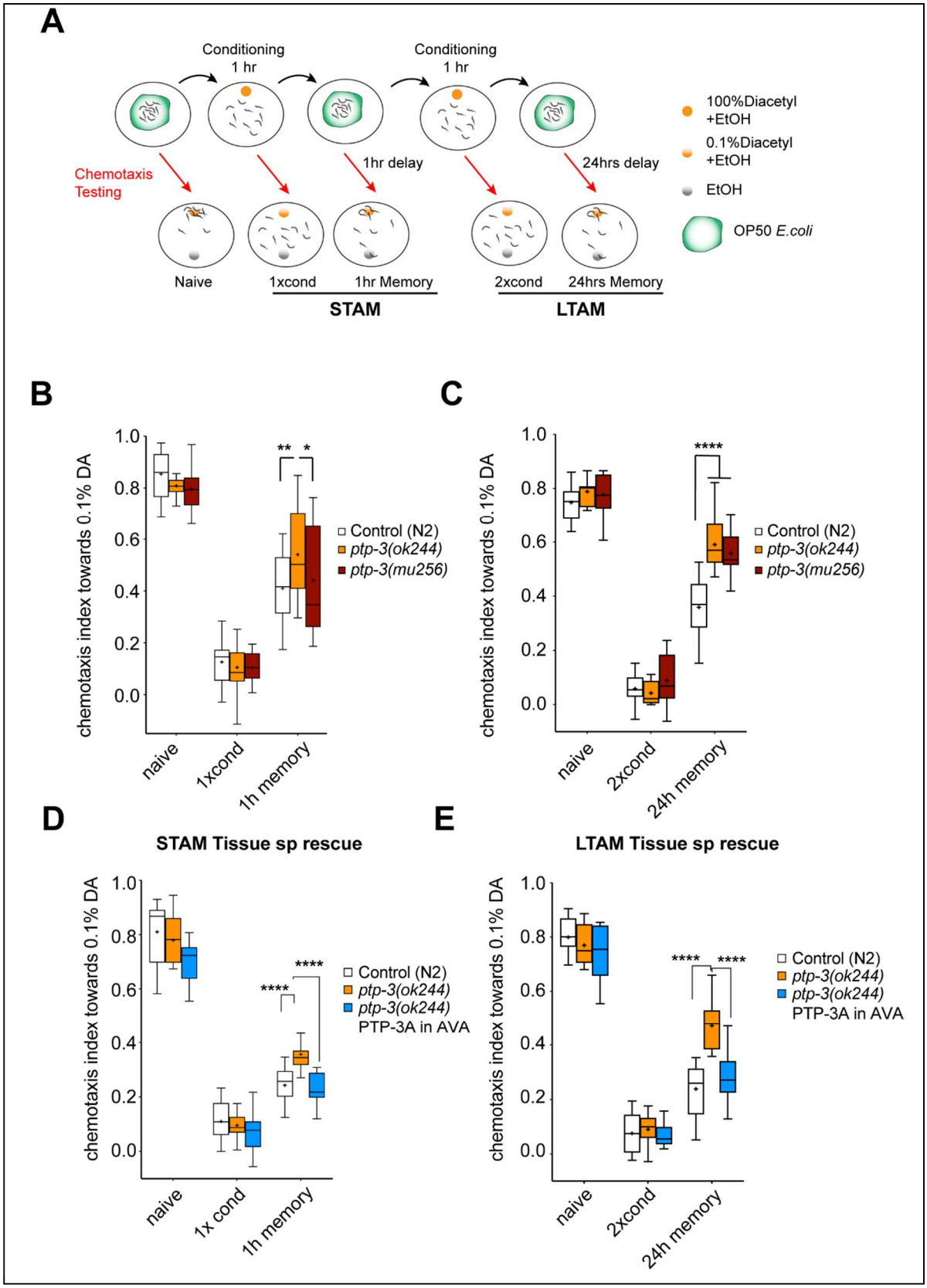

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Figure 2.5: Loss of PTP-3 function affects short and long-term associative memory.
A) Illustration of the chemotaxis associative memory protocol. **B)** Starvation conditioning of worms was assayed towards 0.1% diacetyl (DA) after preincubation with no DA (naïve) or 1-hour preincubation (1xcond). For 1h memory, worms were starvation conditioned with 0.1% DA for 1 hour then were presented with food and no DA for 1 hour. These worms were then subjected to the chemotaxis trial to test short-term memory. **C)** two 1-hour preincubation with 0.1% DA trials with a 30-minute rest period between trials. Worms were allowed food and no DA for 24hr then subjected to the chemotaxis trial (24h memory). **(D and E)** Tissue specific rescue of the **(D)** short and **(E)** long-term associative learning defect in *ok244* with [PTP-3A AVA]. Chemotaxis index = (worms at DA – worms at EtOh)/Total number of worms. All stats were done using a 2-way ANOVA with Bonferroni correction for multiple testing.

2.3.4 PTP-3A is necessary for both delivery and retention of GLR-1 at synapses

Our results so far show that transport and synaptic levels of GLR-1 are affected by the loss of the PTP-3A isoform. More precise analysis of the proximal and distal contingent of receptors as well as stops during transport in the proximal AVA sections indicate that loss of PTP-3A could lead to decreased GLR-1 delivery. To better determine what is happening at synapses and how this relates to different mutations in PTP-3, we used FRAP of SEP::mCherry::GLR-1 in the same proximal region of AVA in which GLR-1 synaptic fluorescence was quantified (**Fig. 2.3**). As described in the Methods and previously, we quantified SEP and mCherry FRAP before, immediately after, and at several time points over a total of 20 minutes[113]. Our results indicate that, compared to controls, loss of PTP-3A(*ok244)* showed a distinct decrease in mCherry GLR-1 recovery which was matched by a similar decrease in SEP recovery (**Fig. 2.6B-E**). On the other hand, the *mu256* allele showed no effect on mCherry recovery but a strong decrease in SEP recovery (**Fig. 2.6B-E**). The mCherry FRAP is consistent with the phenotypes observed for these alleles in which *ok244* but not *mu256* (**Fig. 2.1B**) showed decreased transport. The SEP analysis shows that in addition to delivery, exocytosis and maybe retention play an important role partially explaining why both *ok244* and *mu256* decrease olfactory associative memory retention (**Fig. 2.5**).

To determine whether retention of GLR-1 might be affected by loss of PTP-3A or PTP-3 phosphatase domains, we utilized GLR-1::Dendra2, which allows single synapse photoconversion of GLR-1 receptors[85,114]. Two to three single synapses with similar GLR-1::Dendra2 expression were photoconverted from green to red and image stacks were acquired after 1, 2, 4, 8, 12 and 16 minutes as described previously[9] and in the online methods. *ok244* mutants displayed a quick loss of GLR-1 receptors resulting in 90% of receptors lost in 8 minutes (15.7% $\pm$ 3.7 fluorescence remaining, compared to 63 % $\pm$ 4.6 in control animals **Fig. 2.6H**). *mu256* mutants also showed a loss of GLR-1 receptors, much slower than *ok244* (54.5 % $\pm$ 3.5 after 8 minutes **Fig. 2.6H**), but nevertheless faster than controls. Indeed, when we quantified the number of remaining photoconverted receptors still present after 2 hours, both *ok244* and *mu256* animals had significantly lower number of receptors remaining compared to controls but not significantly different from each other (**Figure 2.7B**). In this assay, images were captured before and immediately after photoconversion, and animals were then left to recover on food plates for 2 hours before imaging photoconverted synapses again as previously described[9]. In addition, since *ok244* did lead to decreased GLR-1 delivery, we tested whether the area of photoconverted GLR-1::Dendra2 differed between controls, *ok244,* and *mu256* but found no significant differences (**Figure 2.7A**).

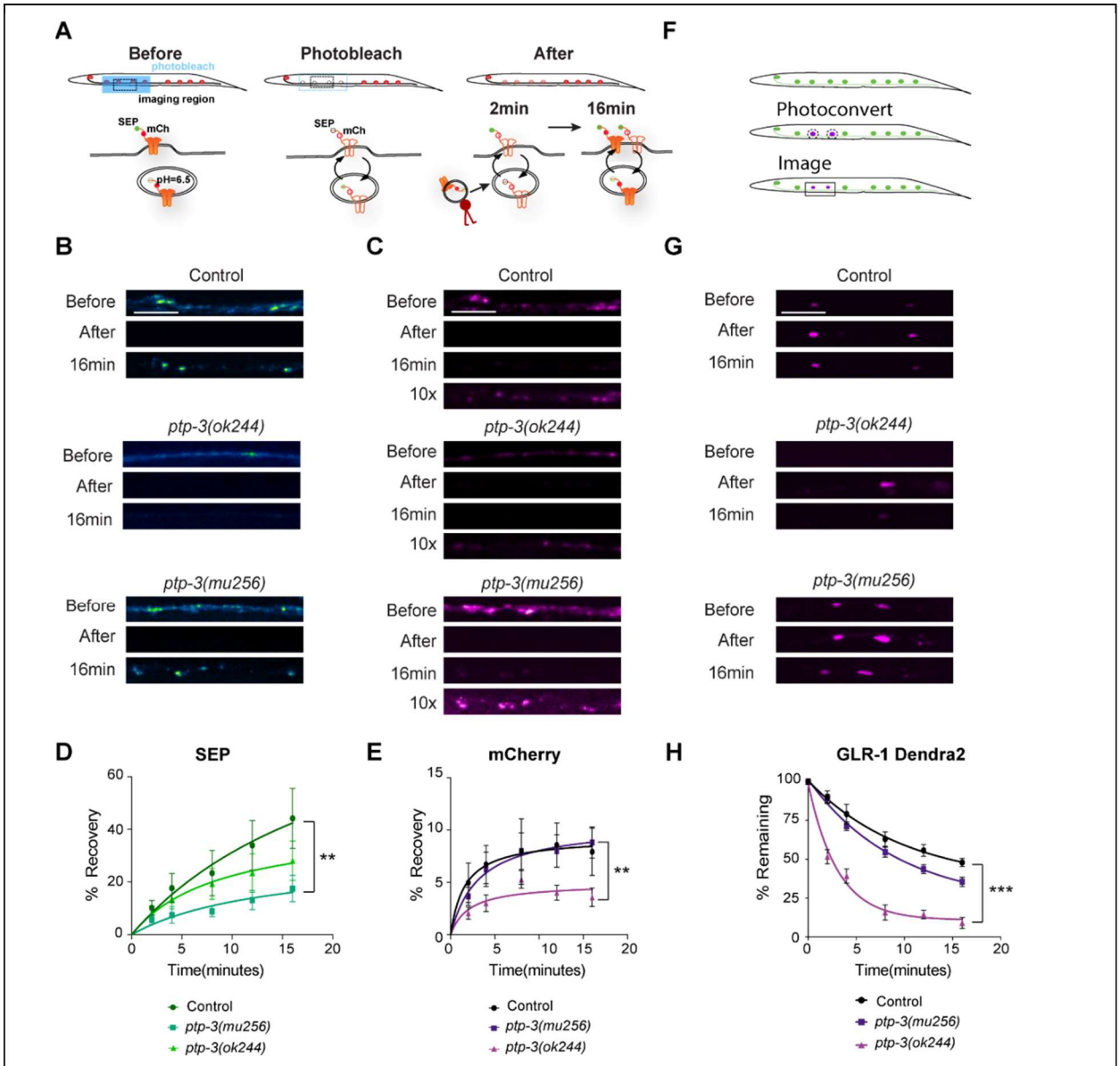

Figure 2.6: Differential regulation of delivery and removal of synaptic GLR-1 by PTP-3.
A) Cartoon illustration of the FRAP imaging location within AVA. **(B and C)** Confocal images taken before photobleach, directly after photobleach, and 16min post photobleach of (B) SEP::GLR-1 and **(C)** mCherry::GLR-1. Post processing of a 10x exposure increase of some pictures is required to see fluorescence. **(D and E)** Quantification of the %Recovery at each time point of **(D)** SEP fluorescence and **(E)** mCherry fluorescence. Time points are 0min, 2min, 4min, 8min, 12min, 16min. Control (n=7), *ok244* (n=9), *mu256* (n=9). %Recovery is calculated by fluorescence of (Before – After photobleach) – (Fluorescence at time point) / (Before – After photobleach). **F)** Cartoon illustration of the dendra photoconversion protocol. **G)** Confocal images of GLR-1::Dendra2 in the proximal region of AVA before UV photoconversion, directly after UV photoconversion, and 16 minutes post photoconversion. **H)** Quantification of the red fluorescence at time points 0min, 2min, 4min, 8min, 12min, 16min. %Remaining is calculated by fluorescence of (Fluorescence at time point)/(Before – After photoconversion). Control (n=10 puncta), *ok244* (n=10 puncta), *mu256* (n=16puncta). **p < 0.01, ***p < 0.001. Error bars represent SEM and all scale bars represent 5µm.

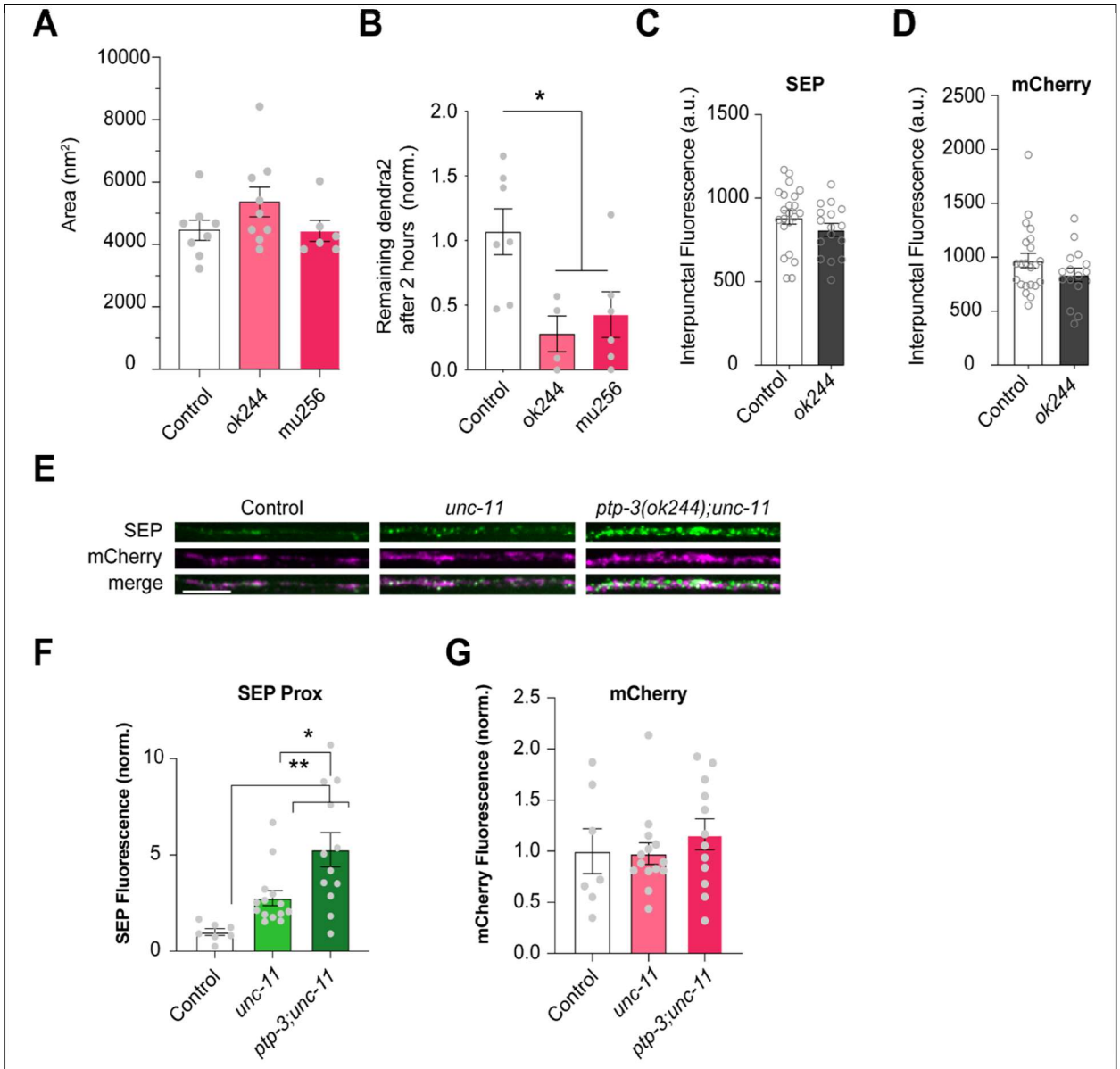

Figure 2.7: PTP-3A affects removal and not exocytosis of GLR-1 A) Quantification of the photoconverted area of puncta. **B)** Quantification of photoconverted GLR-1::Dendra2 fluorescence remaining after 2 hours post photoconversion. **C)** Quantification of SEP and **D)** mCherry fluorescence between puncta, termed interpunctal. **E)** Confocal images of SEP::GLR-1 and mCherry::GLR-1 in *unc-11* and *ptp-3(ok244);unc-11* double mutants and subsequent quantification of **F)** SEP and **G)** mCherry fluorescence. *p < 0.05, **p < 0.005. Error bars represent SEM and all scale bars represent 5µm.

Taken together, FRAP and quantification of synaptic GLR-1 content in animals lacking PTP-3A show decreased synaptic GLR-1 in endosomes and at the synaptic surface due to a defect in synaptic retention. To try to distinguish between a role of PTP-

3A in regulating recycling or GLR-1 synaptic anchoring, we measured interpunctal SEP::GLR-1 levels in PTP-3A(*ok244*) animals. We reasoned that loss of GLR-1 synaptic anchoring in animals lacking PTP-3A would lead to increased diffusion out of the synapse into extra synaptic space. Our results (**Figure 2.7C**) did not support this model, nor did it show increased levels of GLR-1 between synaptic endosomes (**Figure 2.7D**). These results suggest that PTP-3A affects synaptic recycling of GLR-1.

Since there is both enhanced removal and decreased delivery, it is not clear which of these two mechanisms contributes more to the decreased synaptic GLR-1 numbers. To better understand how synaptic cycling is affected by loss of PTP-3A, we used a genetic loss-of-function of UNC-11, the *C. elegans* homologue of a clathrin adaptor protein, AP180, necessary for the endocytosis of GLR-1[135]. We made double mutants of *unc-11* and *ptp-3*, as well as single *unc-11(lf),* all containing SEP::GLR-1::mCherry (**Figure 2.7E-G**). Quantification of SEP and mCherry signals in *unc-11(lf)* showed that a lack of endocytosis led to higher levels of SEP::GLR-1 compared to controls (**Figure 2.7E-G**) but did not affect GLR-1::mCherry signal. This indicates that loss of *unc-11* clearly affected only synaptic surface levels of GLR-1 without affecting endosomal retention. Here we can make a few predictions. First, if loss of PTP-3A mostly affects synaptic GLR-1 by acting on delivery and exocytosis, then double mutants with *unc-11(lf)* would have less synaptic SEP::GLR-1 signal than *unc-11(lf)* alone. Second, if delivery and exocytosis defects are secondary to increased removal, then double mutants with *unc-11(lf)* would appear to have normal or increased SEP::GLR-1. Our results indicate that *ptp-3A(ok244);unc-11(lf)* show increased SEP::GLR-1 compared to controls and *unc-11(lf)* (**Figure 2.7E**). In addition, total levels of GLR-1::mCherry are indistinguishable

between controls, *unc-11(lf)* and *unc-11(lf);ptp-3(ok244)* doubles. Altogether, these experiments suggest that PTP-3A may regulate synaptic endocytosis of GLR-1.

2.3.5 Differential roles of PTP-3A N-terminal and C-terminal domains in regulating GLR-1 transport and synaptic retention

Our results show that the *ok244* allele and not the *mu256* allele leads to decreased GLR-1 transport, synaptic stability, and behavioral memory dysfunction. In addition, the loss of both PTP-3A and B isoforms is no different than the loss of PTP-3A alone for GLR-1 transport or synaptic numbers. PTP-3A differs from PTP-3B by its N-terminal ectodomain, containing three Ig-like and five fibronectin domains. This suggests that these domains could play an important role in GLR-1 transport. Indeed, a previous study has reported that a short N-terminal fragment of LAR-RPTP plays a functional signaling role *in vivo* in vertebrates[136]. To test this hypothesis, we engineered a construct that would lead to extracellular secretion of the two most N-terminal Ig-like domains of PTP-3A by AVA neurons (**Fig. 2.8B**) and expressed it in animals lacking PTP-3A, *ptp-3(ok244)*. We first quantified GLR-1 transport in controls, *ok244,* and *ok244* with the N-terminal constructs (**Fig. 2.8A-C**). Interestingly, postsynaptic expression of this limited N-terminal domain alone rescued GLR-1 transport almost to control levels (**Fig. 2.8C**, *ok244*+[flp-18p::Nterm PTP-3] (n=16) 0.863 ± 0.066 compared to control (n=39) 1.00 ± 0.058). Although rescue of the number of transport events carrying GLR-1 is of central importance for GLR-1 delivery, other signaling mechanisms at synapses can also control delivery (Doser *et. al.*, 2020 and **Fig. 2.1**). Therefore, we proceeded to quantify synaptic GLR-1 levels in controls, *ok244,* and *ok244* expressing our N-terminal construct (**Fig. 2.8D-F**). Analysis of SEP and mCherry GLR-1 signals show that AVA specific expression

of only the two most N-terminal Ig-like domains of PTP-3A could partially rescue total GLR-1 levels at synapses (**Fig. 2.8F**) as well as the number of GLR-1 receptors at the synaptic surface (**Fig. 2.8E**). Our previous analyses of GLR-1 synaptic contingents and transport showed that the *mu256* allele, with a premature stop before the D1 D2 phosphatase domain, did not affect global transport of GLR-1 but did affect synaptic surface retention of receptors. Therefore, we were curious if we could rescue this phenotype by sole expression of the C-terminal phosphatase domain with its membrane tether in AVA (**Fig. 2.8B**). We first quantified GLR-1 transport (**Fig. 2.8A and 2.8D**) and saw no effect on transport due to *mu256* nor in *mu256* animals expressing phosphatase domains. However, expression of the C-terminal domain did partially rescue the numbers of synaptic GLR-1 surface receptors (**Fig. 2.8E**). Taken together, these results suggest a cell specific role for the N-terminal domain of PTP-3A in regulating transport and synaptic recruitment of GLR-1, whereas the phosphatase domain of PTP-3A has a more local, synapse specific, role in retention of GLR-1 receptors at the synaptic surface.

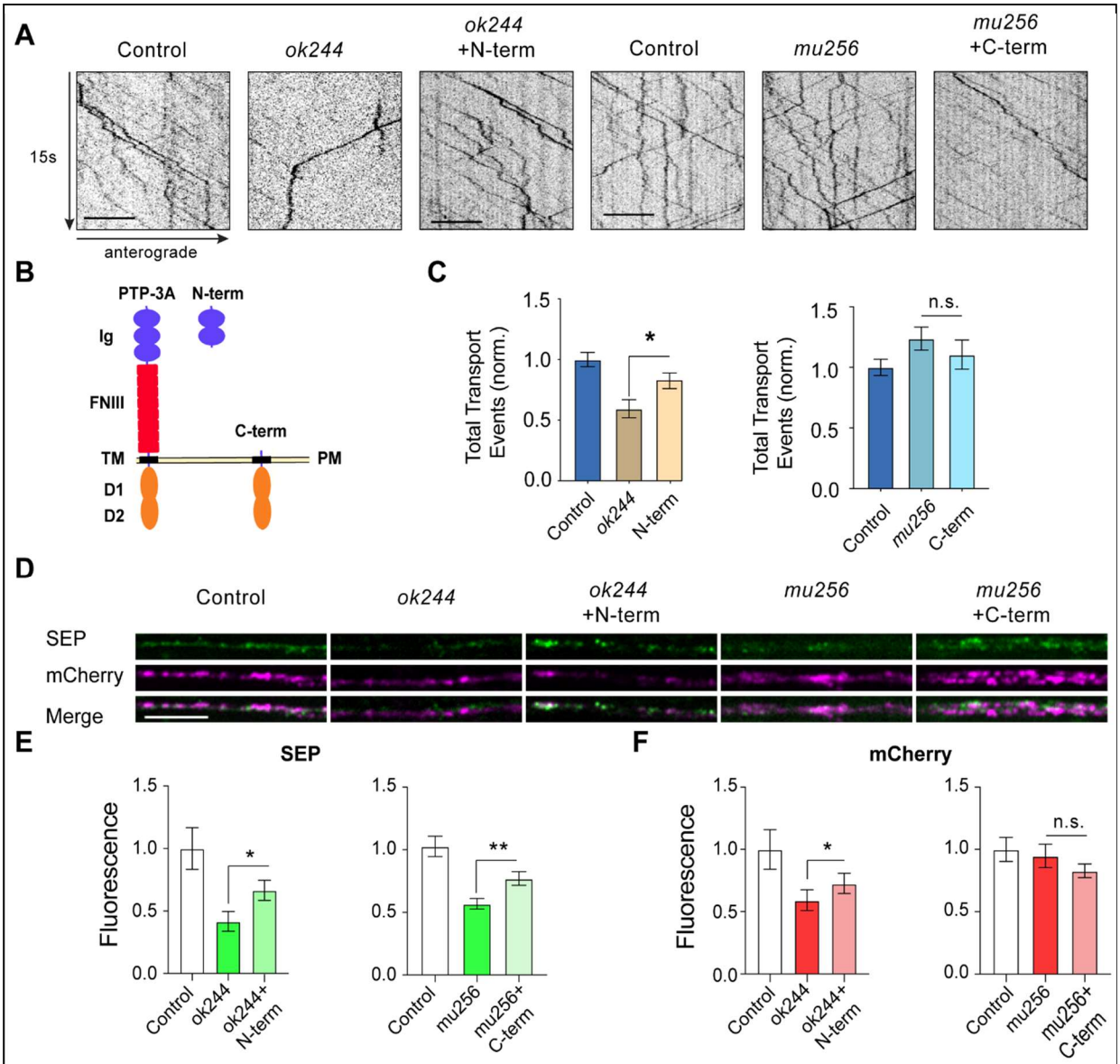

Figure 2.8: The N-terminal and C-terminal domain of PTP-3A regulate different aspects of GLR-1 transport to synapses. A) 15s of representative kymographs from each group. *ok244* N-term represents the three Ig-like domains of PTP-3A expressed under the AVA specific *flp-18* promoter in the *ok244* background. *mu256* + C-term represents the dual-phosphatase domain of PTP-3A expressed under the AVA specific *flp-18* promoter in the *mu256* background. Cartoon schematic of the expression is outlined in **(B)** Control (n=25), *ok244* (n=26), *ok244* + N-term (n=16), *mu256* (n=10), *mu256* + C-term (n=10). **C)** Different day transport event quantification of *ok244* and *ok244* + N-term (N-term) and *mu256* and *mu256* + C-term (C-term) normalized to control. **D)** Confocal images of SEP::GLR-1 and mCherry::GLR-1 of the different groups. **(E and F)** Quantification of (E) SEP fluorescence and (F) mCherry fluorescence, normalized to control. Control (n=20), *ok244* (n=10), *ok244* + N term (n=24) *mu256* (n=24), *mu256* + C term (n=24) *p < 0.05, **p < 0.01. Error bars represent SEM and all scale bars represent 5µm.

Our previous analyses of synaptic GLR-1 numbers and transport dynamics in animals lacking PTP-3A correlated with functional consequences in memory retention (**Fig. 2.5**). To test whether this holds true for the N-terminal AVA expression of PTP-3A, we tested both short and long-term associative olfactory memory in controls, *ok244,* and *ok244* with AVA expression of the PTP-3A Ig-like domains (**Fig. 2.9A-B**). Consistent with our previous analyses, animals lacking PTP-3A(*ok244*) exhibited defects in short- and long-term memory, without any defects in learning compared to controls. AVA expression of the Ig-like domains completely rescued these defects back to control levels. Altogether, our data have uncovered a new role for PTP-3A in regulating long-distance GLR-1 transport and synaptic retention that is important for synaptic GLR-1 function. More importantly, our analyses with genetic alleles and cell specific rescue of PTP-3A domains revealed a completely new role for the N-terminal domain of PTP-3A in regulating GLR-1 transport and synaptic delivery that is important for excitatory synaptic function and associative memory. Our results suggest a model (**Figure 2.9C**) in which the N-terminal domain of PTP-3A regulates GLR-1 transport numbers and receptor delivery whereas the C-terminal phosphatase domains regulate local synaptic retention.

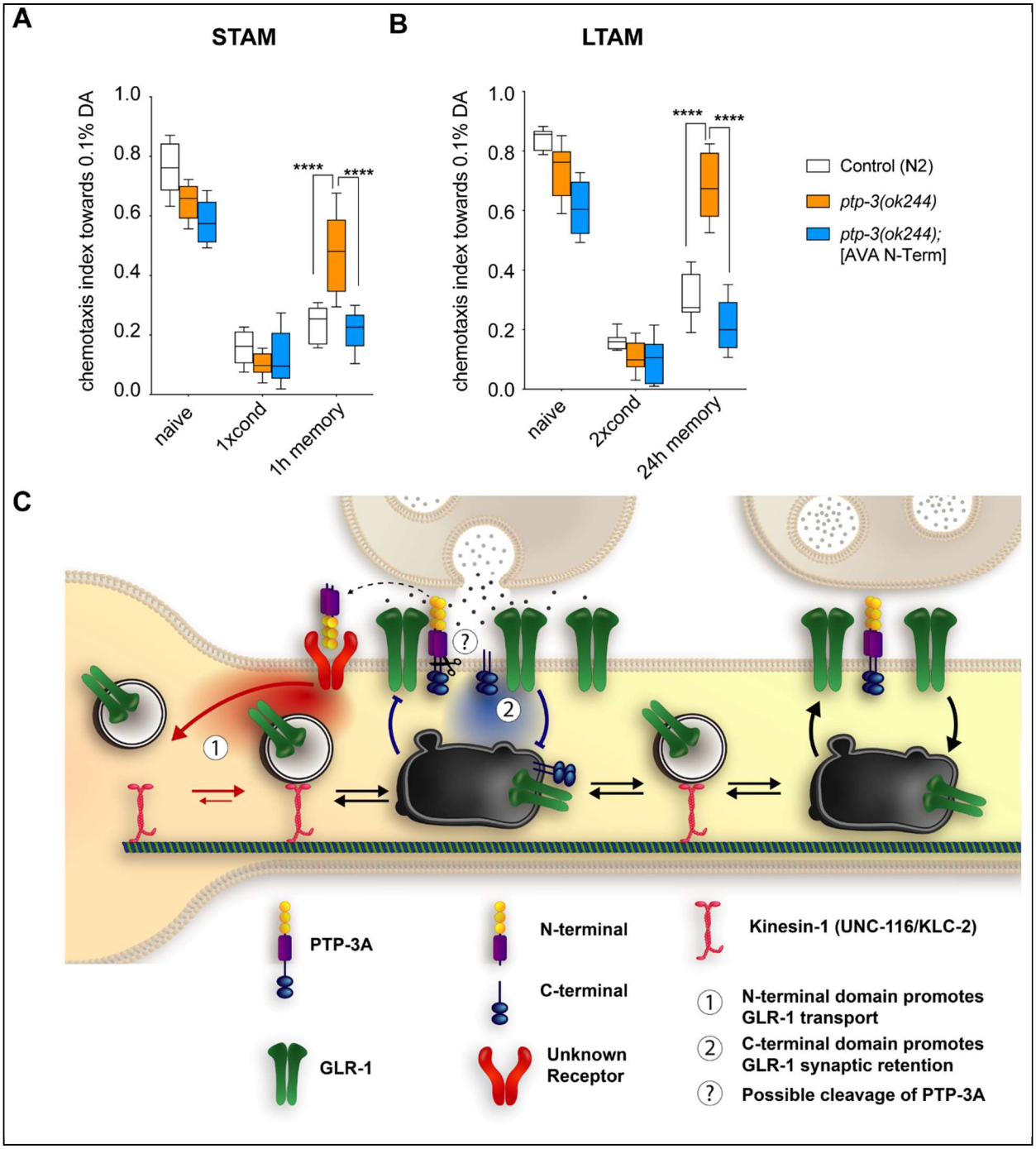

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Figure 2.9: Postsynaptic cell specific expression of PTP-3A N-terminal domain rescues short and long-term associative memory. A) Quantification of the chemotaxis towards 0.1% DA of unconditioned worms (naïve), worms conditioned for 1 hour with 1% DA without food (1xcond) and animals conditioned but transferred to food for 1 hour (1h memory, STAM). The following genotypes were tested: control (N2), *ptp-3(ok244)* and *ptp-3(ok244)* with AVA expression of the N-term 2Ig-like domains (N=12 trials, for all genotypes). **B)** Quantification of the chemotaxis towards 0.1% DA of unconditioned worms (naïve), worms conditioned twice for 1 hour with 1% DA without food with 30 minutes rest between conditioning (2xcond) and animals conditioned but transferred to food for 24 hours (24h memory, LTAM). The following genotypes were tested: control (N2) trials), *ptp-3(ok244)* and *ptp-3(ok244)* with AVA expression of the N-term 2Ig-like domains (N=9 trials, for all genotypes). Chemotaxis index = (worms at DA – worms at EtOH)/Total number of worms. * $p < 0.05$, .** $p < 0.01$, .*** $p < 0.001$, .**** $p < 0.0001$, using a 2-way ANOVA with Bonferroni correction for multiple testing. **C)** Hypothetical model of the dual function of PTP-3A in regulating GLR-1 transport and synaptic retention. Synaptic activity and glutamate release may lead to cleavage of the extracellular and intracellular domains of PTP-3A (?), the N-terminal domains could bind to an unknown receptor which then stimulates GLR-1 transport out of the neuronal soma (1), the cleaved C-terminal D1D2 phosphatase domains either remain at the synaptic surface or get internalized (2), where they dephosphorylate unidentified substrates leading to retention of GLR-1 at synapses.

2.4 Discussion

Excitatory synaptic transmission is essential for all animal behavior including cognition, learning, and memory. Reliable synaptic transmission and plasticity depend on the continuous function of hundreds of synaptic proteins, which all have a limited lifetime, and the majority of which are produced in the soma of neurons. Since some neurons have upwards of thousands of synapses, sometimes far away from the cell body, this presents a particularly formidable logistical challenge. In some ways, this is reminiscent of the famous optimization problem called the Traveling Salesman Problem (TSP) in which a traveling salesman must find the shortest path to visit a certain number of cities. This problem was first formulated in the 1930s and then applied in computer sciences[137]. Although this supply chain issue applies to all synaptic proteins, neurotransmitter receptors, particularly the ionotropic AMPA receptors at synapses, are especially important since they are critical components of excitatory neurotransmission and synaptic plasticity. Although neurons usually use local pools of receptors (either at the membrane or in endosomes) to rapidly respond to local synaptic demands[66], these pools still need

continuous targeted supply to be functional[81,84]. Several key studies have now shown that activity regulated molecular motor-dependent transport of AMPARs is an evolutionarily conserved mechanism essential for synaptic function and plasticity[9,89,90]. However, although these studies present good evidence that this transport system is necessary and global transport levels are regulated by neuronal activity, they have not identified the regulatory mechanisms of AMPAR delivery to synapses nor how neurons integrate synaptic demands to achieve proper homeostatic balance.

Here, we present evidence that PTP-3A, the *C. elegans* homologue of vertebrate LAR, is a key signaling component that enables the coordinated distribution of synaptic AMPARs. By comparing long-distance transport and local synaptic dynamics of GLR-1, we can distinguish PTP-3/LAR-RPTP's role in global trafficking or local, synapse specific, retention. Our data show that two separate domains of the transmembrane PTP-3A/LAR achieve this coordination by regulating different yet overlapping aspects of GLR-1/AMPARs transport. The N-terminal extracellular portion regulates the number of transport vesicles containing GLR-1 that originate from the soma. This process is dependent on the presence of Ig-like domains of PTP-3A/LAR and necessary for the delivery of receptors to synapses. The C-terminal intracellular portion of PTP-3A/LAR containing 2 phosphatase domains regulates synaptic retention of GLR-1/AMPARs by acting locally on receptor delivery, surface delivery, and endocytosis. In addition, we show that postsynaptic PTP-3A functions are necessary for proper associative olfactory memory, which is critically dependent on the glutamatergic function of command interneurons in *C. elegans*. We propose a new theoretical model in which synaptic activity could potentially lead to the cleavage of LAR-RPTP/PTP-3A and subsequent release of

the N- and C-terminal domains, enabling both cellular stimulation of global GLR-1/AMPAR transport and local retention of GLR-1/AMPARs at activated synapses (**Fig. 2.9C).**

2.4.1 Cell specific regulation of GLR-1/AMPAR transport by PTP-3A/LAR-RPTP

AMPA receptor transport from the neuronal soma and intracellular pools is dependent on microtubule-dependent motors[81,84,89,114]. Recent studies in *C. elegans* have shown that this is mostly dependent on Kinesin-1, with some anterograde transport by KLP-4(KIF13), and is regulated by neuronal activity[85,113,114]. Vertebrate studies showing that AMPAR transport is regulated by synaptic activity and important for synaptic plasticity[89,90] further show that activity-dependent regulation of AMPAR transport is evolutionarily conserved. In this study we show that the loss of PTP-3A leads to a 60% decrease in GLR-1 transport (**Fig. 2.1**). We also establish that this decrease can be rescued by cell specific and temporally-limited adult expression of PTP-3A, indicating that PTP-3A modulation of GLR-1 transport is required constitutively and cell specifically.

PTP-3A is the longest isoform of PTP-3 in *C. elegans* and the closest homologue to LAR in vertebrates[104,126]. Previous studies in *C. elegans* have shown a presynaptic role for PTP-3A working with *syd-2*/liprin--α and Nidogen-1 in neuromuscular junction development[105,126]. These previous studies illustrated a presynaptic localization of PTP-3A based on its colocalization with UNC-10 and SNT-1 and only a partial overlap with the $GABA_A$ *C. elegans* homologue, UNC-49. Our data reveal that cell specific expression of PTP-3A in AVA is sufficient to rescue transport and synaptic content of GLR-1, suggesting a postsynaptic function of PTP-3A in GLR-1 regulation. In addition, these previous studies indicated a neuro-developmental role for PTP-3 similar to their homologues, LAR-RPTPs,

in vertebrates[97,123]. More specifically, it was observed that the PTP-3B isoform was necessary for axon guidance whereas the PTP-3A was not. However, PTP-3A was important for regulating synapse formation using UNC-10 and SNT-1 presynaptic markers. Our data using SEP::GLR-1::mCherry expressed in AVA shows that indeed some animals with the *mu245* allele, leading to the loss of both PTP-3A and 3B, showed defasciculated AVA processes in ~20-30% of all animals (data not shown). However, loss of PTP-3A did not induce any defasciculation, nor did it affect synaptic density of GLR-1 (**Figure 2.1 and Figure 2.4**). Furthermore, synaptic GLR-1 levels could be rescued by adult expression of PTP-3A indicating that PTP-3A has more of a constitutive role in GLR-1 transport, recruitment, and retention at the synapse. These results add to other *in vivo* vertebrate studies in which a triple conditional mouse knock-out for all class IIa RPTPs did not affect development of either excitatory nor inhibitory synapses in the hippocampus of mice[110,111].

In contrast to a large body of literature documenting a presynaptic role for LAR-RPTPs[123,138,139], which include LAR, PTP-σ and PTP-δ, a few key studies in dissociated neuronal culture show a postsynaptic role for LAR-RPTPs in AMPA receptor retention, particularly GluA2 retention[109,119,125]. These studies suggested that the internal phosphatase domains D1 D2 and their ability to interact with liprin-α were important for synaptic AMPAR recruitment. The mechanism by which LARs were proposed to increase retention of AMPARs was based solely on a local synaptic role for LARs in dephosphorylating proximal proteins recruited by liprin-α, without mentioning a possible role in AMPAR transport. Our data indicates that PTP-3A function is required postsynaptically for normal GLR-1 transport and synaptic retention in *C. elegans*. In

addition, two sets of experiments suggest that the extracellular domain of PTP-3A is specifically important for normal GLR-1 transport. First, the *mu256* allele with a premature STOP of all isoforms before the D1 D2 phosphatase domains did not alter GLR-1 transport (**Fig. 2.1**). Second, cell specific expression of the N-terminal Ig-like domains of PTP-3A rescued GLR-1-transport (**Fig. 2.8**) and associative memory (**Fig. 2.9**) when expressed in animals with a complete loss of PTP-3A. These results suggest that the N-terminal extracellular domain of PTP-3A interacts with a binding partner that promotes AMPAR transport and synaptic delivery.

One possibility is that the extracellular domain of PTP-3A is cleaved by proteases. Shedding of the extracellular domain of LARs has been documented in non-neuronal cell culture experiments[140,141]. Additional experiments show that PKC activation by TPA phorbol ester and calcium ionophore leads to shedding of the extracellular domain and receptor redistribution in cell [142]. A short form of the ectodomain of LAR corresponding to the first 2 Ig-Like domains of LAR has been documented in rat brains, and it has an active role as a homophilic ligand of LAR-RPTP[136]. Alternatively, it is possible that the N-terminal of PTP-3A could interact with other yet to be determined receptors similar to how PTP-σ interacts with the receptor tyrosine kinase C (TrkC)[143]. Other cell adhesion molecules (CAMs) of the Immunoglobulin superfamily, such as NCAM1, NCAM2, neuroligins and neurexins have been found to be cleaved[144,145]. However, a role for signaling of the shed ectodomains for these molecules *in vivo* is unclear. Altogether, our studies of *in vivo* transport of GLR-1 in *C. elegans* reveal the exciting possibility that LARs could be modulating levels of AMPAR transport by a mechanism akin to autocrine signaling.

2.4.2 Regulation of local synaptic GLR-1 levels by PTP-3A/LAR-RPTP

As mentioned above, a few studies have documented a postsynaptic localization and role for LAR-RPTPs in regulating AMPA receptors[119,129]. These studies suggested that LAR-RPTP function and interaction with local synaptic proteins is the key to recruitment and retention of AMPARs at synapses. Our results show that PTP-3A is acting at two levels of the AMPA receptor trafficking process. The first one pertaining to promoting anterograde transport of AMPARs is discussed above. The second pertains to the regulation of AMPAR synaptic retention by PTP-3A. Our experiments show that loss of PTP-3A not only leads to a 60% decrease in transport events but also changes dendritic transport dynamics, leading to diminished dendritic stops of GLR-1, which we have previously associated with decreased delivery of GLR-1[113]. Indeed, analysis of GLR-1 levels in distal synapses (**Fig. 2.3**) reveals accumulation of GLR-1 in the distal synapses, indicative of a change in GLR-1 distribution in PTP-3A loss of function mutants. Our heat-shock experiments further suggest that rapid translation of PTP-3A is sufficient to rescue transport but not synaptic stopping (**Figure 2.2**). Altogether, these results corroborate vertebrate findings of a local synaptic role of PTP-3A in AMPAR recruitment. In addition, we show that the *mu256* allele of PTP-3 leads to a specific decrease in surface synaptic SEP::GLR-1 signal, indicating lowered exocytosis of PTP-3. This allele of PTP-3 leads to a premature stop at nucleotide position 1776[126]. One possibility is that this leads to a complete loss of all PTP-3 proteins as suggested previously[104]. Alternatively, it is possible that truncated PTP-3 proteins are made and transported to synapses. These could be indistinguishable in the previous studies because antibodies against native PTP-3 were directed to the C-terminal region of PTP-3[104]. Our data show that in *mu256* alleles, transport of GLR-1 is normal, as are dendritic stops (**Figure 2.2**) and total GLR-1 numbers

at synapses (**Fig. 2.3A and 2.3B**). This differs significantly from transport and synaptic levels of GLR-1 in the *ok244* and *mu245* alleles, which only led to loss of PTP-3A or PTP-3A&B. Furthermore, cell specific expression of the C-terminal phosphatase domains of PTP-3 in the *mu256* allele (**Fig. 2.8**) was sufficient to rescue synaptic surface expression of GLR-1. These data are more consistent with *mu256* leading to C-terminally truncated form of PTP-3 which is how we have interpreted our data in this study.

FRAP of SEP::GLR-1::mCherry and Photoconversion of GLR-1::Dendra2 reveal a defect for synaptic surface delivery and synaptic retention of GLR-1 in mutants affecting PTP-3A or deleting the phosphatase domains of all PTP-3 isoforms. These data are consistent with a model in which PTP-3 phosphatases are important at synapses to maintain a contingent of GLR-1 receptors. Acute rescue of PTP-3A loss of function using an inducible heat-shock approach further supports a constitutive role for PTP-3A in maintenance of a contingent of synaptic GLR-1. These effects could be mediated by direct dephosphorylation of downstream targets of PTP-3A or interactors which bind to the D2 domain of PTP-3A, similar to what has been observed for vertebrate LAR-RPTPs[109,119,146]. Genetic evidence has been presented previously for regulation of synaptic SYD-2/liprin-α localization by PTP-3[126]. Since SYD-2 is the *C. elegans* homologue of liprin-α, this speaks to the conservation of the LAR and liprin-α interaction. Thus, it could be that in mutants lacking PTP-3A, a decrease in synaptic GLR-1 levels is due to postsynaptic SYD-2 mislocalization. In vertebrates, LARs, liprin-α, and GRIP-1 form a complex[109] but a close homologue of GRIP-1 in *C. elegans* has not been reported at this point, although *mpz-1* and *magi-1* could potentially be PDZ containing scaffolds found postsynaptically in *C. elegans* that may play that role[11,147–149]. In addition to liprin-

α, a rho/rac guanine nucleotide exchange factor named Trio can interact with the D2 domain of LAR[150]. The *C. elegans* homologue of Trio is UNC-73[151] but has not been reported to interact with PTP-3 or modify GLR-1 transport or synaptic abundance.

Potential substrates of PTP-3 tyrosine dephosphorylation in *C. elegans* neurons have been identified. These include: MAPK15, FER, MAPK7, NTRK2 (TrkB),HIPK1, GSK3A, CDK5, MET and PDGFRB [112]. Interestingly, previous studies have shown that synaptic levels of GLR-1 are decreased in a *cdk-5* loss of function mutant[152]. In addition, this study showed that CDK-5 interacted with the clathrin adaptin AP180, regulating GLR-1 levels by acting on GLR-1 endocytosis. Although classical cyclin-dependent kinases (CDKs) are usually inactivated by phosphorylation at Y15[153], the role of this phosphorylation of Y15 and activation of CDK-5 is unclear in vertebrate neurons[154] and unknown in *C. elegans*. CDK-5's action on the actin cytoskeleton and associated scaffold proteins has been implicated in dendritic spine remodeling[153]. The D1D2 domains of PTP-3A are highly conserved with the D1D2 domains of LAR-RPTP. The D1 domain in vertebrate LAR has many downstream targets including β-catenin which has been shown to co-immunoprecipitate with GLuA2, liprin-α, and GRIP-1[109,119,155]. GluA2 tyrosine phosphorylation is associated with the internalization of GluA2[75,115] but it remains to be shown whether GluA2 and GLR-1 can be dephosphorylated by LAR/PTP-3A. Taken together, PTP-3A could be affecting local GLR-1 retention through interactions with scaffolds or via phosphorylation of key downstream targets, including GLR-1.

Although intensely studied in the last 25 years, the regulation of LAR-RPTP activation is not well understood and has been majorly focused on the role of the extracellular domains acting as ligands for trans-synaptic partners. Overall, it is unclear

whether LAR-RPTPs mediate their effect through binding at their D1D2 domains or through activity of the phosphatase D1 domain. In addition, how activity of the phosphatase domains is regulated is not well understood at all. Early models predicted that de-clustering of LAR-RPTPs led to loss of phosphatase activity[93], but more recent structural studies suggest that tight clustering of LAR-RPTP could inhibit phosphatase activity since D1 domains can form tight homophilic interactions[107]. In this latest model, it is possible that cleavage of LAR could lead to decreased clustering and increased active D1 domains. In this context, it is interesting to note that PKC activation and calcium ionophores lead to internalization of LAR[142]. However, PKC activation leads to additional cleavage and shedding of the ectodomain whereas calcium ionophores only lead to internalization. In this configuration, the D1D2 domains would be facing the cytoplasmic side and thus be able to interact with substrates for dephosphorylation, as it appears in **Fig. 2.9C**. Since it is known that synaptic activity leads to a local increase in PKC activity[156,157] and lysosomal fusion[158], with release of metalloproteases, it is tempting to speculate that synaptic activity might lead to cleavage of the extracellular and intracellular domains of PTP-3A as shown **Fig. 2.9C**. At the very least, this provides an interesting model to test for future studies.

2.4.3 A model for coordination of AMPAR transport and synaptic maintenance

Before we had the ability to track AMPA receptor transport and local synaptic trafficking, several signaling mechanisms and synaptic protein functions were classified as affecting AMPAR “trafficking” because the methods at that time could not distinguish between global effects on transport and local synaptic trafficking effects. By combining transport, FRAP, and photoconversion *in vivo* we are now able to better define the spatio-

temporal effects of signaling pathways and synaptic protein functions. Previous studies put the postsynaptic effect of LARs broadly in terms of affecting AMPAR trafficking to synapses[119]. Here we show that 2 domains of the closest homologue for LAR in *C. elegans*: PTP-3A, have differential effects on transport and local synaptic retention. Our ability to distinguish between transport and local recycling effects enables us to more precisely specify how signaling pathways work to control AMPAR distribution. This powerful approach is now being used in vertebrates with two-photon imaging and shows that AMPARs exist in tunable immobile and mobile fractions[159] consistent with our models in *C. elegans.*

Nevertheless, our approach in *C. elegans* has enabled us to identify 2 domains of PTP-3A with differential effects on GLR-1 trafficking. This dual function could provide an elegant model for the distribution of AMPARs to synapses, in which the cleavage of PTP-3A promotes transport, through the N-terminal autocrine effect, and retention through local liberation of the C-terminal phosphatase domains. This model posits that one signal has global cellular effects on GLR-1 transport, whereas the other only exhibits local effects- seeming to speak to both a homeostatic mechanism and a Hebbian mechanism. The fact that PKC activation has been shown to lead to shedding of the extracellular domain of LAR and internalization of the intracellular phosphatase, whereas calcium chelation by ionophores leads to internalization of the LAR-RPTP without shedding[142], shows that additional synaptic regulatory mechanisms may control the balance between shedding with internalization or only internalization of LAR-RPTPs. Overall, this model is interesting because it bridges homeostatic global modulation and synapse specific

regulation, providing a unified mechanism to maintain synaptic plasticity and a cellular excitatory set point.

2.4.4 How AMPAR transport and synaptic retention affects learning and memory

Although it is now clear that the number and function of AMPARs at synapses can be directly associated with synaptic plasticity and behavioral learning and memory[66,118,160,161], the role of long-distance transport in behavioral learning and memory has not been very well studied. The most recent studies to date show that activity-dependent regulation of AMPAR transport is necessary for synaptic plasticity[89,114]. However, we do not as yet understand how this relates to behavioral learning and memory. Obviously many studies, have validated how synaptic plasticity relates to behavioral learning and memory[161] but what is unclear is how much transport, delivery, and removal are necessary or sufficient for learning, memory or both. Here we show that both short and long-term associative memory in *C. elegans* depend on modulation of GLR-1 transport and synaptic retention by the PTP-3A isoform (**Fig. 2.5**). In addition, we show that PTP-3A is necessary postsynaptically for short and long-term associative memory but not learning. It is interesting to note that loss of PTP-3A does not lead to a complete loss of GLR-1 receptors at synapses but only to 40% decrease. In addition, the *mu256* allele, which does not lead to GLR-1 transport defects but only to a defect in synaptic retention, also shows defects in STAM and LTAM. Taken together these data suggest that synaptic retention of GLR-1 is essential for STAM and LTAM. Surprisingly although restoring expression of PTP-3A N-terminal 2 Ig-Like domains by AVA, only rescues transport to ~80% of Control (**Fig. 2.8**), and 70% of synaptic surface GLR1, it completely rescues STAM and LTAM (**Fig. 2.9A and 2.9B**). Taken together, these results

strongly suggest that transport and mobility provided by GLR-1 transport is essential for this type of behavioral memory with perhaps an additional role for the N-terminal extracellular domain of PTP-3A. Although, seemingly specific to *C. elegans,* this behavioral paradigm has been used to reveal molecular conservation of genes important for memory in both *C. elegans* and humans[133,162,163]. Overall, our study reveals that the PTP-3A isoform of PTP-3, a *C. elegans* homologue of vertebrate LAR, has essential functions in regulating the coordinated transport and retention of AMPARs critical for behavioral associative short and long-term memory. Our data reveal a new function for PTP-3/LAR in regulating AMPAR transport and that AMPAR transport is crucial for short and long-term associative memory.

2.5 Materials and Methods

2.5.1 *C. elegans* culture and strains

C. elegans strains were kept on NGM and fed the *E. coli* strain OP50 at 20ºC. Double and triple mutants were generated by standard genetic methods. For a list of PCR genotyping primers, enzymes and phenotypes see Table 2.1. Transgenic strains were created by microinjection of *lin-15 (n765ts)* worms with plasmids containing the LIN-15 rescue or microinjection of pCT61 encoding *egl-20p::nls::DsRed* to expressing DsRed in the nucleus of four epithelial cells in the tail to visualize rescued worms[85].

2.5.2 *C. elegans* transgenes

akIs201, *rig-3p::SEP::GLR-1::mCherry; akIs154; rig-3p::HA::glr-1::Dendra2*; *CsfEx 14, PTP-3p::PTP-3A::HA; csfEx75, flp-18p::PTP-3A::HA; CsfEx131, flp-18p::PTP-3A::HA; CsfEx132, flp-18p::PTP-3A-Nterm::HA; csfEx133, flp-18p::PTP-3A-Cterm::HA; csfEx135, Hsp16-2p::PTP-3A::HA.*

Table 2.1			
Gene	**Allele**	**Mutation**	**Functional Change**
glr-1	*ky176*	Premature stop	Truncated, unfunctional receptor
ptp-3	*ok244*	Premature stop	Loss of PTP-3A
ptp-3	*mu245*	Premature stop	Loss of Isoforms A and B
ptp-3	*mu256*	Premature stop	Loss of phosphatase domain of gene
lin-15	*n765ts*	Frameshift mutation	Protein null

2.5.3 Plasmids and cloning

All generated plasmids were made by the Takara In-Fusion cloning method. Plasmids were created by PCR linearization and subsequent inserting of desired sequence by complimentary overhangs created by the PCR linearization. Primers were designed using Takara's In-Fusion primer design tool.

The *ptp-3p::PTP-3A* plasmid was generously donated by Dr. Brian Ackley. Using the Taraka Bio's In-Fusion PCR primer tool, the PTP-3 promoter was swapped for the AVA specific FLP-18 promoter. This plasmid was then used to generate the *flp-18p::PTP-3A_Nterm* keeping amino acids 1-232 and deleting the rest using In-Fusion primers- leading to a secretion sequence followed by 2 Ig-Like domains and then a STOP. A similar strategy was used for *flp-18p::PTP-3A_Cterm*, in which In-Fusion primers resulted inresults led to the retention of amino acids 5986-6543 or a stretch of 556 aa. The same replacement strategy using In-Fusion as the one described for pflp-18 was used for swapping in the *hsp-16p* promoter for inducible heat-shock.

2.5.4 Confocal Imaging

Imaging was conducted on a spinning disk confocal microscope (Olympus IX83) equipped with 488 and 561 nm excitation lasers (Andor ILE Laser Combiner). Images were captured using an Andor iXon Ultra EMCCD camera through either a 10x/0.40 or a

100x/1.40 oil objective (Olympus). Devices were controlled remotely for image acquisition using MetaMorph 7.10.1 (Molecular Devices).

Transport imaging: All imaging was performed on worms containing the akIs201 array in glr-1 null (*ky176*) background. Worms were mounted on a 10% agarose pad with 1.6µl of a mixture of polystyrene beads (Polybead, catalog #00876-15, Polysciences) and 30µM Muscimol (catalog #195336, MP Biomedicals). Using a coverslip, worms were positioned such that the AVA neuron was near the coverslip to allow for clear visualization. Through the 100X objective, the neurons were located using the 561 nm excitation laser, then, a cross section in the proximal portion of AVA neurites was photobleached using a 3 W 488 nm Coherent solid-state laser Coherent solid-state laser (Genesis MX MTM) set to 0.5 W output and a pulse time of 1 s targeted using a Mosaic II digital mirror device (Andor Mosaic 3). After photobleaching, a 500-frame stream was collected at 100 ms exposure per frame with the 561 nm excitation laser in a single z plane. Kymographs were generated by the Kymograph tool in MetaMorph with a 20-pixel line width as described in[85,113].

FRAP: Worms containing akIs201 were mounted as described in the **Transport imaging** section. A proximal region of AVA just distal of the bifurcation of AVAL and AVAR was used as the imaging region and was saved using MetaMorph's stage position memory function. An image stack was acquired using the 488nm and 561nm excitation lasers at 500ms exposure (20 total images every 0.25um starting 2.5µm below to 2.5µm above the neuron). Around 60um to the left and right of the process was photobleached using the same parameters described earlier. Finally, using the stage memory position, the initial process location was photobleached and then immediately imaged with both excitation

lasers. Imaging was repeated at 2-, 4-, 8-, 12-, and 16-minutes time points post photobleach.

Photoconversion: All photoconversion was performed on worms containing the akIs154 (GLR-1::Dendra2) array in GLR-1 null (Ky176) backgrounds. 2-3 synaptic puncta were converted using an ROI selection tool a 500ms pulse of 35mW/mm^2 from a 405nm laser (475mW, LDC8, Power Technologies Inc.). Immediately following photoconversion, 20 images were taken using both 561nm and 488 excitation lasers starting 2.5µm below and finishing 2.5µm above the process at 500ms intervals. This was repeated at times 2, 4-, 8-, 12-, and 16-minutes post-conversion. For 2-hour imaging, the same process as above was performed but after pictures were taken immediately after photoconversion, worms are taken off the microscope. Worms were then taken off the pad and let go on a normal plate with food for 2 hours. Once the time had passed, worms were placed back on a new coverslip and the photoconverted synapses were imaged.

2.5.5 Short-term and long-term associative olfactory memory

Assays were conducted as described previously[132]. Briefly, animals were synchronized using the egg preparation method[164]. For all conditions, the chemotaxis of the animals after treatment was tested the following way: 80-150 worms were placed in the center of a 10cm chemotaxis plates (CTX 5 mM KH_2PO_4/K_2HPO_4 [pH 6.0], 1 mM $CaCl_2$, 1 mM $MgSO_4$, 2% agar) with 1 µl of Sodium Azide 1M, and either 1% diacetyl(DIA) in EtOH or EtOH alone on each side of the plate. After 45 min at room temperature with closed lids, animals immobilized at the 0.1% Diacetyl, at the EtOH and outside of the spots were counted. The Chemotaxis index was calculated as follows: CI= (Nb Animals 0.1%DIA- Nb Animals EtOH)/ Total number of animals as described in Bargmann *et. al.,*1993[165].

Counting of animals on the plates was done blind to the genotype. The chemotaxis assay was conducted in triplicate for each genotype and for each condition and repeated a minimum of 3 times. Data from all chemotaxis assays for each condition and genotype were combined to generate the average CI per genotype and condition. For STAM, 80-150 worms were conditioned for 1-hour without food in the presence of 2µl diacetyl on the top of the lid of a 10cm CTX plate. Animals were then either tested immediately (1x cond) or left for 1 hour on NGM with OP50 and tested for STAM (1h memory). For LTAM, the 1hour conditioning was repeated with a 30-minute interval in between conditioning sessions where worms were able to roam on plates with food. After the second conditioning, worms were either immediately test for chemotaxis or put on NGM plates with OP50 for 24 hours before LTAM was tested (24h memory).

2.5.6 Image analysis

Transport: Transport event quantification was performed blinded to the genotype and manually counted. Transport velocities were blindly quantified by manually tracing transport events and subsequently analyzed using the KymoAnalyzer ImageJ plugin (Neumann et al., 2017).

FRAP: Image stacks from all time points were converted to maximum projections using MetaMorph's stack arithmetic function. Average fluorescence from all timepoints was analyzed using the region measurement tool of ImageJ. To account for initial background fluorescence of time 0 post photobleach, the average fluorescence of timepoint 0 was subtracted from each timepoint's average fluorescence.

Dendra: Image stacks from all timepoints were converted to max projections using MetaMorph's stack arithmetic tool. Photoconverted synaptic puncta fluorescence was

quantified using ImageJ's region measurement tool by outlining the puncta. The same area measurement was used for all timepoints of the same image.

2.5.7 Statistical Analyses

For all analyses with less or equal to three groups a non-parametric Mann-Whitney *U*-test was used. For all microscopy data analyses with more than 3 groups except FRAP and Dendra2 photoconversion, and data with equal variations an ordinary one-way ANOVA with Dunnett's multiple testing correction was used to determine significance. To determine if the FRAP recovery curve and Dendra2 fluorescence removal were significantly different between genotypes a sum of square F-test applied to the exponential fits of the data were used. For analyses of short term and long-term associative memory (STAM and LTAM), a 2-way ANOVA with Bonferroni correction for multiple testing was used. All statistics were done use Prism Version 9.1.2.

2.6 Author Contributions

Pierce DR and Hoerndli FJ, conceived and designed all experiments except behavioral associative learning experiments for the manuscript. DP did all experiments and recorded data for the manuscript except for the associative learning behavior. Heat-shock synaptic rescue of PTP-3A(*ok244*), was performed and analyzed by ZL and KK. Molecular cloning and creation of transgenic strains was conducted by DP with aid from Doser RL. Stettak A, designed, recorded, and analyzed the data for all associative learning behavior experiments. Hoerndli FJ wrote the manuscript. Stettak A, Pierce DP, Doser RL and Lenninger Z, gave feedback and input on the manuscript. Stacher Hoerndli CN created the Figure model in Figure 2.9C.

CHAPTER 3: The role of SYD-2 in postsynaptic AMPAR transport and synaptic plasticity

3. Summary

Coordinated efforts from synthesis of AMPARs at the cell body to delivery of these receptors at synapses are critical processes in maintaining excitatory neurotransmission. However, this poses a challenge to neurons since these all of these processes need to be working correctly or else it can lead to decreased neurotransmission which can cause cognitive defects and impairments in learning and memory. In **Chapter 2,** we show that loss of the phosphatase PTP-3A causes defects in glutamate receptor-1 (GLR-1) transport, delivery, and removal in *C. elegans.* The liprin-α family of proteins are known to interact with LAR-RPTPs in vertebrates where they have shown to be critical in correct localization of LAR as well as critical in AMPAR trafficking. However, these studies have not determined if the effects seen on AMPAR trafficking are directly due to liprin-α or because of mislocalization of LAR. The *C. elegans* sole homologue of liprin-α, SYD-2, is known to modulate presynaptic active zone maintenance. The interactions between SYD-2 and PTP-3A are not well understood in *C. elegans* and SYD-2's possible role in AMPAR transport has not been identified. The data that I am presenting in this chapter are unpublished and is work that I have performed alone. My data suggest that SYD-2 and PTP-3A are in the same genetic pathway that modulate GLR-1 transport and that SYD-2 may have additional effects on synaptic GLR-1.

3.1 Introduction

When we first discovered that PTP-3A could be regulating synaptic GLR-1 from a retention and transport point of view, I knew an important protein like this had to have tight regulation. Vertebrate literature based on cell culture experiments, suggested that

activity-dependent degradation of liprin-α by CaMKII is responsible for cellular localization of LAR[166]. Indeed, when a non-degradable form of liprin-α is expressed in neurons, there is little LAR present at spines, indicating that the degradation of liprin-α is what is driving LAR to spines[129]. This idea is somewhat confounded by another finding in the same paper[129] where overexpression of liprin-α in heterologous cells causes increased surface expression and clustering of LAR. This could be that the interaction of exogenous expression in non-neuronal cells causes liprin-α to work differently. However, another role of liprin-α suggests it can bind to LAR intracellularly while LAR is at the synaptic surface and inhibit its phosphatase function by clustering LAR[107]. In fact, liprin-α is known to colocalize with PSD-95 and GluA2/3 indicating it is expressed in dendritic spines. Liprin-α could have additional roles apart from just being an LAR regulator, however. A study on the interaction between liprin-α and glutamate receptor-interacting protein (GRIP) revealed that when this binding was inhibited, synaptic AMPAR targeting was disrupted and this could be induced by just mutating the GRIP binding domain on liprin-α[109]. The difficulty in assessing this result is that they did not address if LAR function/localization was changed in these mutants. Since GRIP is known to traffic GluA2/3 to the membrane surface, it is likely that GRIP can bind the liprin-α/LAR complex and traffic it to the surface as well. Therefore, it is suggested that liprin-α may have more than one function on AMPAR localization which may be dependent, or independent of LAR. LAR activity is difficult to study *in vivo* in vertebrates since it is not highly expressed[93,129]. Thus, using the relatively simple *C. elegans* model, which has one sole homologue of liprin-α, we can expand on this knowledge to better understand how the liprin-α/LAR interaction is involved in AMPAR trafficking *in vivo*.

The *C. elegans* sole liprin-α homologue, SYD-2, is mainly known for its presynaptic role in active zone formation and correct localization of neurotransmitter-containing vesicles[128]. The postsynaptic interaction between PTP-3A and SYD-2 is unknown in *C. elegans.* Not only that, but a role for SYD-2 in GLR-1 transport of synaptic localization is also currently unknown in *C. elegans.* Given the evolutionary conservation between these two proteins and results from **Chapter 2**, it is plausible that SYD-2 could be regulating PTP-3A activity and be important in excitatory synapse maintenance.

3.2 Main findings

3.2.1 Loss of SYD-2 causes decreased synaptic GLR-1 numbers

We showed in **Chapter 2** the importance that PTP-3A has on surface synaptic expression of GLR-1 which is largely due to the phosphatase domain. We hypothesize that this phenotype relies on PTP-3A being at active synapses. Since SYD-2 is a possible regulator of PTP-3A localization, this naturally led us to test if SYD-2 is an upstream regulator of synaptic GLR-1.

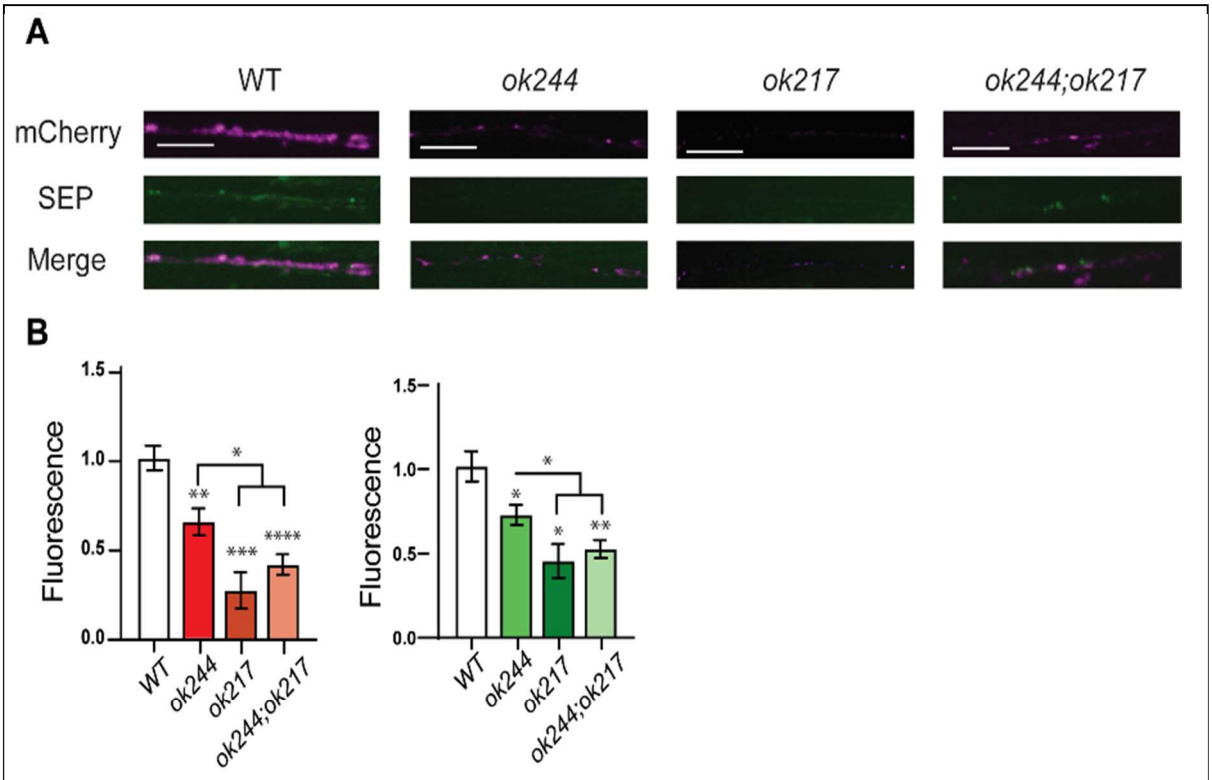

Figure 3.1: Loss of SYD-2 leads to decreased synaptic GLR-1 numbers. All worms express SEP::mCherry::GLR-1 in the *glr-1(ky176)* knockout background. Wild-type (WT) worms represent *glr-1(ky176)* expressing SEP::mCherry::GLR-1 with no further mutations. **A)** Confocal images of SEP::GLR-1 and mCherry::GLR-1. (**B and C**) Quantification of (B) mCherry fluorescence and (C) SEP fluorescence, normalized to control. WT (n=15), *ok244* (n=20), *ok217* (n=10), *ok244;ok217* (n=16). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. All statistics used an ordinary one-way ANOVA with Dunnett's multiple testing corrections. Errors bars represent SEM and all scale bars represent 5µm.

To test this, we used the *ok217* allele of SYD-2 which causes a premature stop and is considered function null[167]. Loss of SYD-2 causes reduced amount of synaptic GLR-1 (mCherry channel) and reduced surface expression (SEP channel) in the proximal portion of AVA **Fig 3.1A.** Double mutants of *ok244* and *ok217* were generated and have significantly reduced mCherry and SEP fluorescence that is similar to the single *ok217* mutants. The *ok244* single mutant has significantly higher mCherry and SEP fluorescence than both the single *ok217* and double *ok244;ok217* mutants. These results suggest that

PTP-3A and SYD-2 may act in the same genetic signaling pathway to regulate synaptic GLR-1 but that SYD-2 may also have additional synaptic roles.

3.2.2 Loss of SYD-2 increases AMPAR transport

The finding that loss of SYD-2 decreases synaptic GLR-1 led us to test trafficking steps that precede synaptic localization of GLR-1, such as long-distance AMPAR transport. If the decreased synaptic GLR-1 phenotype we are observing is similar to that in PTP-3A mutants, then this could be caused in part, due to reduced transport.

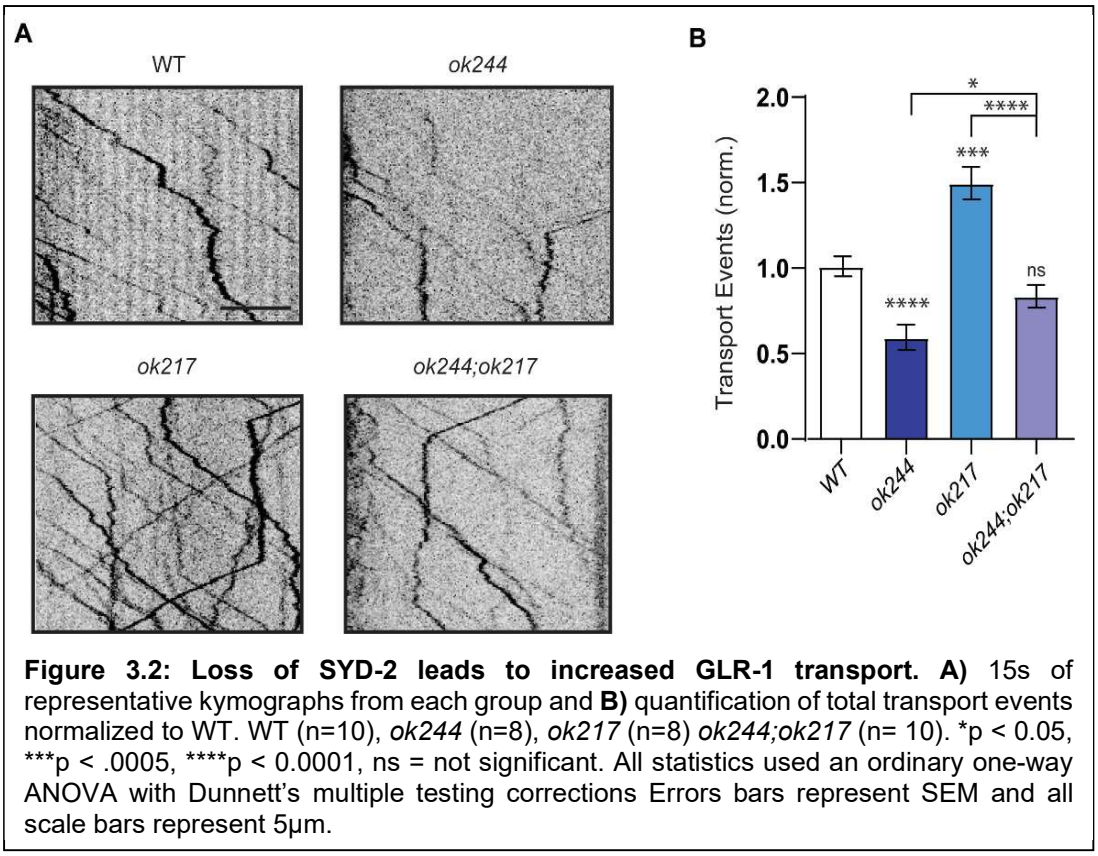

Figure 3.2: Loss of SYD-2 leads to increased GLR-1 transport. A) 15s of representative kymographs from each group and **B)** quantification of total transport events normalized to WT. WT (n=10), *ok244* (n=8), *ok217* (n=8) *ok244;ok217* (n= 10). *p < 0.05, ***p < .0005, ****p < 0.0001, ns = not significant. All statistics used an ordinary one-way ANOVA with Dunnett's multiple testing corrections Errors bars represent SEM and all scale bars represent 5µm.

Surprisingly, loss of SYD-2 causes an almost 50% increase in AMPAR transport compared to wild-type (**Fig 3.2B**). Interestingly, there is no statistical difference between *ok244;ok217* and wild-type. This intermediate phenotype, although significantly different from *ok244*, is trending towards a decrease as seen in the *ok244* single mutants, which

could be due to the limited number of worms analyzed. Differing from **Fig 3.1,** these results suggest that PTP-3A and SYD-2 may not be in the same genetic pathway that regulates GLR-1 transport.

3.2.3 SYD-2 is required for synaptic stabilization of AMPARs

The difficulty of imaging synapses at a single point in time is that it only gives information for that particular moment in time. It could be that there is less GLR-1 at a synapse due to less being delivered, or more being removed, which a snapshot does not tell you. To address this, both delivery and removal of GLR-1 needs to be analyzed to acquire a comprehensive picture. Since we observed less total and surface synaptic GLR-1 in *syd-2(lf)* mutants (**Fig 3.1**), but increased GLR-1 transport in these mutants (**Fig 3.2**), we hypothesized that the decreased synaptic GLR-1 could be due to an increased removal of GLR-1. Unlike in **Chapter 2** where the GLR-1::Dendra2 removal experiment is over a 16-minute trial, the original protocol called for a 2-hour trial. The 2-hour protocol requires animals to be removed from the microscope where they roam and feed freely before imaging again. **Fig 3.3A** briefly outlines the Dendra2 protocol: individual puncta are photoconverted by UV light, the worms are then removed from the confocal microscope and left on a normal feeding plate for 2 hours. After this time, the same puncta are imaged for retention of the photoconverted receptors. As seen in **Fig 3.3B** and quantified in **3.3C**, the amount of photoconverted receptors still present at the synapse after 2 hours in wild-type is 65% whereas both the single *ok217* and double *ok244;ok217* mutants exhibit almost 0% retention after 2 hours.

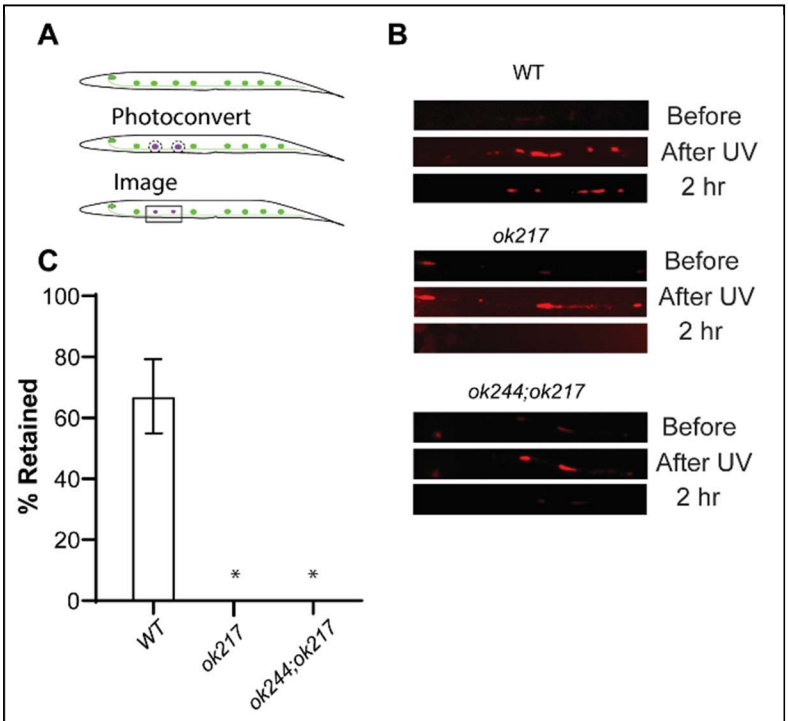

Figure 3.3: Increased removal of synaptic GLR-1 in *syd-2* mutants. A) Cartoon illustration of the dendra2 photoconversion protocol. **B)** Confocal images of GLR-1::Dendra2 in the proximal region of AVA before UV photoconversion, directly after UV photoconversion, and 2hrs post photoconversion. **C)** Quantification of the red fluorescence 2hrs post photoconversion. %Retained is calculated by fluorescence of (Fluorescence at 2hrs) / (Before – After photoconversion). WT (n= 4 puncta), *ok217* (n= 3 puncta), *ok244;ok217* (n= 3puncta). *p < 0.05. All statistics used an ordinary one-way ANOVA with Dunnett's multiple testing corrections. Errors bars represent SEM.

Taken together, these results lead to three key conclusions. First, that PTP-3A and SYD-2 most likely act in the same genetic signaling pathway to regulate GLR-1 synaptic levels. Second, that although GLR-1 synaptic levels are decreased in both SYD-2 and PTP-3A loss of function mutants, loss of SYD-2 does not decrease transport of GLR-1 as does loss of PTP-3A. Third, loss of SYD-2 leads to lowered synaptic GLR-1 not because of impaired transport, but because of impaired synaptic retention. Altogether, these results suggest a possible model where SYD-2 localizes PTP-3A to the synapse. If PTP-3A is unable to be trafficked to active synapses, its function in regulating delivery and

removal of GLR-1 would not occur, which is what is seen in *syd-2(lf)* mutants. However, we know the N-terminal domain of PTP-3A can regulate transport, possibly independent of it being at synapses. Therefore, if PTP-3A is retained at the cell body, it could possibly be activating GLR-1 transport independent of SYD-2.

3.3 Discussion and future directions

In conclusion, these preliminary results suggest that SYD-2 is an important regulator of synaptic GLR-1. The data I have collected establish a foundation on how SYD-2 may interact with PTP-3A to regulate GLR-1 transport and may be an important step towards understanding the role of SYD-2 in synaptic plasticity.

***syd-2(lf)* mutants have reduced synaptic GLR-1 which is likely due to increased GLR-1 removal**. If one of the key functions of SYD-2 is to correctly localize PTP-3A, then in *syd-2(lf)* mutants, PTP-3A would likely not be at synapses unless it can diffuse out from the cell body. This would result in synapses that phenotypically look like *ptp-3(ok244)* mutants which is what we observed **Fig 3.1**. However, the significant decrease of synaptic GLR-1 in *syd-2(lf)* mutants compared to *ptp-3(ok244)* mutants suggests that SYD-2 might possess additional roles for regulating synaptic GLR-1 aside from solely being a PTP-3A chaperone.

We have identified that the phosphatase domain of PTP-3A is the main domain controlling retention of synaptic GLR-1. If SYD-2 is controlling PTP-3A localization, then *syd-2(lf)* mutants should have decreased retention of synaptic GLR-1 since PTP-3A is absent from the synapse, which is what we observed in **Fig 3.3**. This result is consistent to what is observed in vertebrates as Wyszynski *et. al.* suggests liprin-α regulates the synaptic targeting of AMPARs[109]. However, the researchers do not distinguish if liprin-α

regulates the delivery or removal of receptors to the synapse. Therefore, the decreased synaptic AMPAR expression they observe in liprin-α mutants could be due to an increased removal of AMPARs. It is also possible that there is a fundamental difference in *C. elegans* compared to vertebrates since.

Caveats, alternative interpretations, and future directions. A large assumption of the interpretation of these results is that SYD-2 binds to PTP-3A similar to how liprin-α binds to LAR. However, it could be that SYD-2 does not interact with PTP-3A and is acting in an independent pathway of PTP-3A to regulate AMPAR transport and trafficking. To test if SYD-2 has an interaction with PTP-3A, co-immunoprecipitation assays should be performed. However, since proteins can co-immunoprecipitate without directly binding to each other, predicted binding sites of SYD-2 on PTP-3A should be mutated to test if this interferes with co-immunoprecipitation. Another way to test whether SYD-2 and PTP-3A associate *in vivo* is to test their co-localization using fluorescent tags on SYD-2 or PTP-3A or both. Mislocalization of PTP-3A in *syd-2(lf)* mutants would indicate that similar to liprin-α and LAR-RPTPs in vertebrates, SYD-2 is necessary for synaptic localization of PTP-3A. What if SYD-2 is mislocalized in *ptp-3(ok244)* mutants? Then perhaps PTP-3A and SYD-2 localization is interdependent.

There are a few alternative explanations to our results which could explain the observed decreased synaptic GLR-1 phenotype. For instance, we do not know if synaptic delivery of GLR-1 is impaired. Given our strong GLR-1 removal phenotype, it is unlikely that delivery of GLR-1 would be decreased or else the synapse would have significantly less GLR-1 then what is observed in the *syd-2(lf)* mutants. To test this, FRAP experiments should be performed to better understand the effect that SYD-2 has on GLR-1 synaptic

targeting. Further, it is possible that loss of SYD-2 causes decrease transcription/translation of GLR-1. Although there is no known evidence that SYD-2/liprin-α causes decreased overall expression of AMPARs, we cannot rule it out. Thus, cell body and whole neuron fluorescent imaging in *syd-2(lf)* mutants would reveal if there is a global decrease of GLR-1 in the AVA neurons.

SYD-2 is known to be important in presynaptic development and it could be that the phenotype we are observing is largely due to a presynaptic role[128]. SYD-2 is involved in presynaptic active-zone development and helps to recruit neurotransmitter-containing vesicles. Since *C. elegans* exhibit a form homeostatic plasticity, it is possible that loss of SYD-2 disrupts glutamate release onto postsynaptic receptors. The only known inducible form of homeostatic plasticity in worms is when presynaptic glutamate release is inhibited which causes an increase in AMPAR expression on postsynaptic neurons[168]. Given that *syd-2(lf)* mutants have decreased postsynaptic GLR-1, it could be that there is an increase of glutamate release which would presumably cause a homeostatic downscaling of GLR-1. It is difficult in worms to increase presynaptic glutamate release, either by inducible factors or genetic pathways. Therefore, the best way to test this would be for AVA specific expression of SYD-2 to determine if this can rescue the decreased synaptic GLR-1 phenotype.

There is evidence in vertebrates that binding of liprin-α to LAR dimerizes the D1 phosphatase domains and inactivates LAR[107]. If these functions are evolutionarily conserved, this would suggest that SYD-2 can regulate PTP-3A activity. To explore this possibility, a conditional knockout of SYD-2 in the AVA neuron can be engineered with the auxin-induced degradation (AID) system[169]. Proteins can be tagged with an AID and

when exogenously expressed, the F-Box protein TIR1 can recruit the ubiquitin machinery to the AID and causes degradation, but only in the presence of auxin. This would allow for PTP-3A to be present at synapses and if presence of SYD-2 is responsible in inhibiting PTP-3A activity, conditional knockout of SYD-2 would not have the same defect of synaptic GLR-1 seen in *syd-2(lf)* mutants. By conditionally knocking out SYD-2 then testing for GLR-1 retention, this would help to uncover if SYD-2 is controlling PTP-3A activity/localization. If conditional knockout of SYD-2 has wild-type levels of GLR-1 retention, it is hypothesized that the decrease seen in *syd-2(lf)* mutants is likely from mislocalization of PTP-3A.

Loss of SYD-2 increases GLR-1 transport. Our results demonstrate that in *syd-2(lf)* mutants, there is a ~1.5x increase in GLR-1 transport. Vertebrate literature has identified that liprin-α can directly bind to the kinesin-3 motor, KIF1A in the coiled-coiled 2 (CC2) and CC3 regions[170]. Interestingly, the CC2 domain is known to regulate KIF1A activity by blocking interactions with microtubules and contributes to autoinhibition of the motor[171]. Different mutations in the autoinhibition regions of KIF1A cause overactive motors that have increased processivity[172]. Thus, it is possible that liprin-α can bind to the CC3 region (which has no autoinhibition effect), then by some signaling mechanism, liprin-α binds the CC2 region to halt transport, possibly at activated synapses. When liprin-α is not present then, the CC2 domain cannot be inhibited and thus the transport machinery would increase transport of cargo. Although the kinesin-1 motor KIF5 is largely responsible for long-distance transport of GLR-1 in AVA in *C. elegans,* it is possible SYD-2 could interact with the KIF5 autoinhibition domains. Therefore, a possible hypothesis would be that the SYD-2/KIF5 interaction could behave like the mechanism of liprin-α/KIF1A in long-

distance transport. Therefore, the increased transport of GLR-1 in *syd-2(lf)* mutants could be due to autoinhibition being disrupted. Further, if the processivity of receptors is increased, this would cause mislocalization of receptors at synapses which could be why we observe decreased synaptic GLR-1 in *syd-2(lf)* mutants.

Caveats, alternative interpretations, and future directions. In vertebrate cell culture, liprin-α has also been shown to regulate LAR activity, it is possible that in *syd-2(lf)* mutants, PTP-3A is fully active, but is still retained in the cell body **Fig 3.4**. This could possibly explain why GLR-1 transport is increased in *syd-2(lf)* mutants. What if PTP-3A accumulation in the cell body causes off target binding that would normally not happen if it were chaperoned correctly? To test this, different domains of either SYD-2 or PTP-3A could be mutated such that there would be no association between them. This would keep the relative function of both proteins unchanged, except for their binding. If SYD-2 cannot bind PTP-3A, and thus accumulates in the cell body, it is hypothesized that GLR-1 transport would look similar to *syd-2(lf)* mutants.

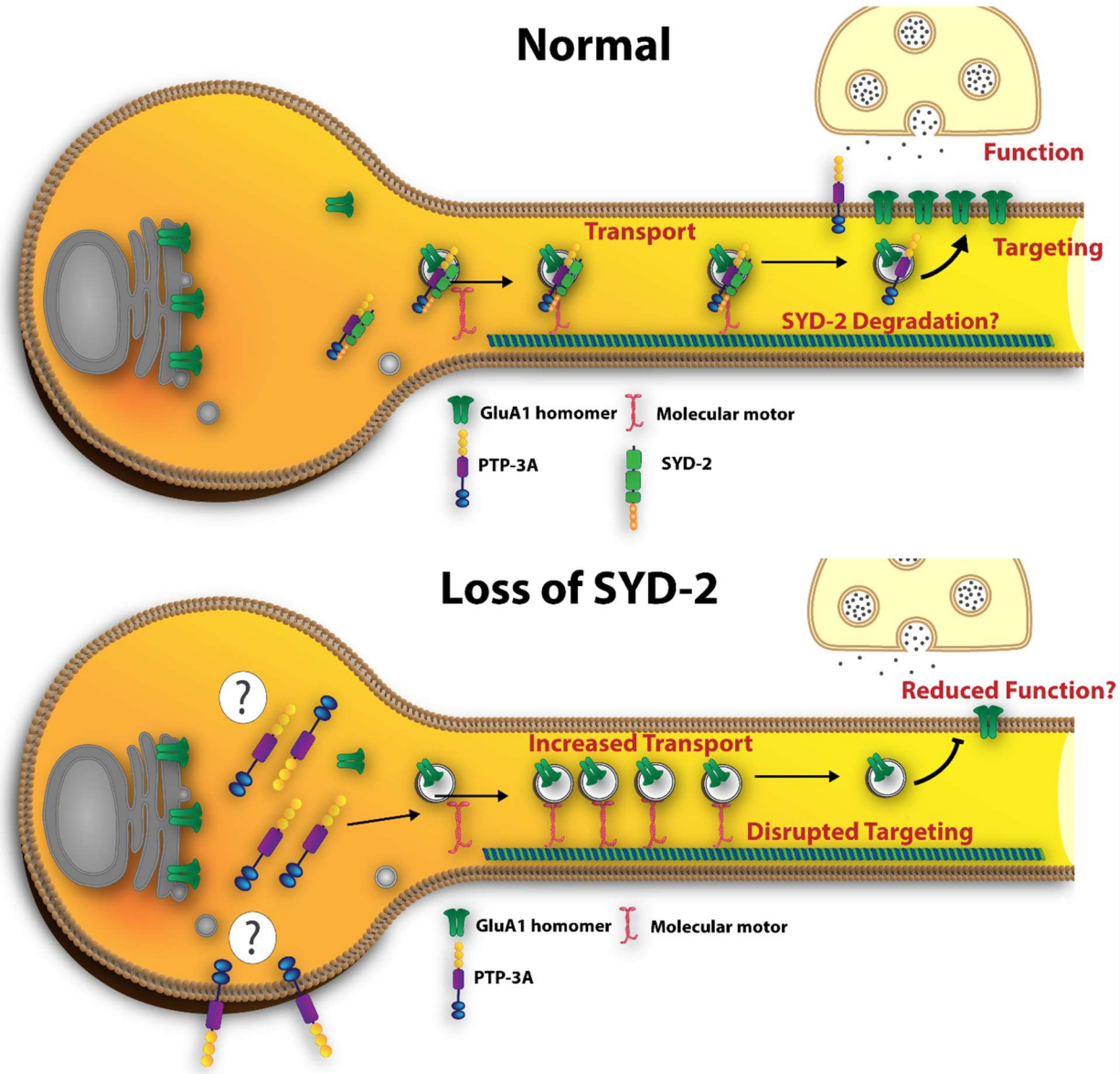

Figure 3.4: Working model of how loss of SYD-2 affects PTP-3A localization. Top: Proposed GLR-1 transport under normal circumstances. SYD-2 transports PTP-3A to synapses where it is potentially degraded so PTP-3A can be inserted into the membrane. **Bottom:** In the absence of SYD-2, PTP-3A may accumulate at the cell body and possibly interact with regulators of GLR-1 transport. It may also be inserted on the surface of the cell body where activation leads to increased GLR-1 transport. Without PTP-3A at the synapse, there is disrupted synaptic targeting and retention of GLR-1. The accumulation of these effects may reduce synaptic function.

3.4 Materials and Methods

3.4.1 *C. elegans* culture and strains

All *C. elegans* strains were kept on NGM and fed the *E. coli* strain OP50 at 20ºC as outlined in **Chapter 2.5.1.**

3.4.2 *C. elegans* transgenes

akIs201, *rig-3p::SEP::GLR-1::mCherry; akIs154; rig-3p::HA::glr-1::Dendra2.*

Table 3.1

Gene	Allele	Mutation	Functional Change
glr-1	*ky176*	Premature stop	Truncated, unfunctional receptor
ptp-3	*ok244*	Premature stop	Loss of PTP-3A
syd-2	*ok217*	Frameshift mutation[173]	Protein null

3.5 Confocal Imaging

Imaging was conducted on a spinning disk confocal microscope (Olympus IX83) and all transport and photoconversion imaging was conducted as described in **Chapter 2.5.4.**

CHAPTER 4: Overall conclusion and future directions

I was once told that a good PhD would open the door to more questions rather than only give answers. Although my work gave a novel outlook on the regulation of long-distance transport and delivery of AMPARs by the phosphatase PTP-3A, it opened the door to many important questions. My thesis began by trying to identify regulators of long-distance AMPAR transport and how this pathway could regulate synaptic plasticity. I was fortunate enough that the regulator I identified and have dedicated my PhD to, the phosphatase PTP-3A, not only has a role in AMPAR transport, but in synaptic stabilization of AMPARs as well. The idea that PTP-3A may be involved in regulating postsynaptic AMPAR expression is not a novel idea, since LAR is shown to regulate synaptic AMPAR numbers in vertebrates. However, its role in AMPAR transport has not been explored in vertebrates nor *C. elegans.* In this conclusion chapter, I review my data presented in **Chapters 2** and **3** and how these data fit in the larger picture of AMPAR transport. I also expand on possible future directions of the project and how these questions can help us better understand the mechanism behind PTP-3A's modulatory effects on AMPAR transport.

Loss of PTP-3 causes reduced synaptic GLR-1. Our results demonstrate a need for the phosphatase PTP-3A in modulating the number of synaptic GLR-1 in *C. elegans in vivo.* RPTP involvement in excitatory postsynaptic transmission was previously documented by Dunah and colleagues, showing reduced expression of surface AMPARs in dissociated neuronal cell cultures using LAR dominant negative constructs and RNAi[174]. They argued that this could be due to an overall reduced synapse density which would cause less AMPARs to be expressed. However, our results *in vivo* indicate that

synapse density is unchanged in PTP-3A mutants and the decreased synaptic GLR-1 we observed is not due to an overall decrease of the GLR-1 protein (**Figure 2.4**). In vertebrates, it is proposed that the decreased synapse density observed in LAR mutants is due to loss of its interaction with extracellular matrix proteins that are responsible in stabilization and growth of the spine. A substrate of LAR is β-catenin which when dephosphorylated, accumulates in the dendritic spine[175]. Therefore, it is hypothesized that upon a strong stimulus, activation of postsynaptic LAR results in β-catenin accumulation which stabilizes and allows the spine to grow. Since there are no known neuronal spines in *C. elegans,* we cannot rule out the possibility of PTP-3A being involved in synapse structure remodeling, however, our results suggest that PTP-3A might be involved in more than just structural organization.

The amount of synaptic AMPARs at a given time is loosely dependent on two main factors: how many are delivered and how many are removed, which then equate to the amount present at a given time. Our results suggest that the decreased synaptic GLR-1 in *ptp-3(ok244)* mutants is from decreased delivery and increased removal of receptors. The interplay between phosphorylation and dephosphorylation is critical for regulating both AMPAR surface expression and endocytosis. Although direct Ser/Thr phosphorylation of AMPARs are the main forms of phosphorylation that control their trafficking, there are tyrosine phosphorylation sites on GluA2 that are responsible for receptor endocytosis[176]. Indeed, phosphorylation of Y876 on GluA2 is required for activity-dependent internalization[177]. Although tyrosines on AMPARs have not been reported to be substrates of RPTPs, the tyrosine phosphorylation mutants of GluA2 outlined by Hayashi and Huganir exhibit behavior similar to GLR-1 that we observe in *ptp-*

3(ok244) mutants[177]. In *C. elegans,* the amino acid conservation between GLR-1 and GluA2 is roughly the same as GLR-1 to GluA1[178]. In Y876F GluA2 mutants, there is less overall GluA2 at synapses and at the surface. However, they did not discern if this was from decreased insertion or increased internalization. Our results suggest that the dual phosphatase domain of PTP-3A is involved in synaptic cell surface expression and also removal of synaptic AMPARs. Support for PTP-3A mainly being involved in removal of GLR-1 comes from the GLR-1::Dendra2 photoconversion and removal experiments showing decreased removal in mutants with loss of PTP-3A. Also, from genetic experiments using loss of function mutants for the clathrin adaptin AP180 (**Figure 2.7**) or *C. elegans* UNC-11 necessary for clathrin-mediated endocytosis of GLR-1. This second set of experiments with *unc-11(lf)* mutants suggest that when clathrin-mediated endocytosis is blocked, there is roughly a two-fold increase in surface expressed GLR-1 in *ptp-3(ok244)* mutants (**Figure 2.7**). In these mutants, if PTP-3A was mainly required for exocytosis of receptors onto the cell surface, then blocking clathrin-mediated endocytosis would not show the large increase in retained surface expression. Interestingly, when the phosphatase domain was overexpressed in the PTP-3 phosphatase deleted *ptp-3(mu256)* mutants, there was rescue of surface expression of GLR-1 but did not affect the total endosomal pool of receptors. Not only does this suggest that the phosphatase domain is important for retaining receptors at the synapse, it also suggests that the N-terminal portion may be regulating delivery of receptors to synapses. This expands on knowledge in both the *C. elegans* and vertebrate literature which previously did not demonstrate how PTP-3A/LAR may be affecting synaptic AMPAR numbers.

Caveats, alternative interpretations, and future directions. Since PTP-3A has components of a cell adhesion molecule (CAM) and a phosphatase domain, it is possible these two domains can act independently of each other. Our result showing that the N-terminal section of PTP-3A can partially rescue synaptic GLR-1 in *ptp-3(ok244)* mutants is difficult to interpret since we do not know how it is affecting the delivery or removal rate of GLR-1. It is possible that the N-terminal section is activating a retrograde signal that induces the delivery of GLR-1 at synapses. This is a more plausible hypothesis than the N-terminal portion controlling removal of GLR-1 since we know from the *ptp-3(mu256)* experiments that it is the phosphatase domain and not the N-terminal portion controlling removal. These results suggest that activation of PTP-3A might be more complicated than what was previously identified. In vertebrates, a leading hypothesis is that when LAR's N-terminal domain is proteolytically cleaved, this leads to disassociation of the phosphatase domain from the surface and subsequent degradation. Although we cannot rule out this possibility, overexpression of a diffuse form of the phosphatase domain can partially rescue surface expression of GLR-1. This suggests that disassociation from the surface membrane and degradation are not directly correlated in our system. If this is true, then overexpression of the phosphatase domain in *ptp-3(ok244)* mutants should show similar rescue of surface GLR-1 which still needs to be tested.

There are a plethora of experiments and questions that can be asked based on our synaptic GLR-1 trafficking results. However, one of the fundamental questions that arise from these data is how is PTP-3A regulating delivery of receptors to synapses? One of the biggest unknowns in the AMPAR trafficking field is the signal that causes receptors to be unloaded from microtubule-driven motors to synaptic endosomal pools. It seems

that the N-terminal section is controlling this process, however, it is difficult to assess if PTP-3A is controlling this from the synapse or the vesicle. A possible way to test this would be to acutely inactivate PTP-3A in a region then assess delivery rates of GLR-1 using FRAP. If PTP-3A is controlling delivery from the synapse, then inactivation would lead to decreased delivery and if it is regulating delivery from the vesicle complex, delivery of GLR-1 would be unchanged from wild type.

How does GLR-1 transport affect synaptic function? Our fundamental knowledge of synaptic plasticity largely comes from the idea that synapses change and adapt depending on the information they are receiving. A large assumption that is made in vertebrate models of AMPAR synaptic trafficking is that upstream pathways, such as long-distance AMPAR transport, are normal. Thus, it is difficult to accurately interpret synaptic results that involve AMPAR trafficking without considering how long-distance AMPAR transport is changed. This is why using a model such as *C. elegans* is such an attractive organism to study this pathway since we can manipulate this mechanism *in vivo*. Although the concept that synaptic defects in AMPAR numbers could be due to impaired long-distance transport has been posited in literature, our understanding of how long-distance AMPAR transport can affect synaptic trafficking is minimal. Our results suggest that PTP-3A is required for normal long-distance AMPAR transport. Thus, I want to use this section to instead explain how our results help to further our knowledge of how AMPAR transport can affect synaptic function.

One of the fundamental papers of my thesis has suggested that the amount of AMPAR transport is correlated with synaptic activity, i.e. hyperpolarization of the postsynaptic neuron decreases AMPAR transport[9]. Further, results published by our lab

in Doser *et. al.* 2020*,* suggest that decreased intracellular Ca^{2+} levels decreases AMPAR transport[179]. Although we cannot definitively say if intracellular Ca^{2+} levels are changed in any of the PTP-3A and SYD-2 mutants, we can assume that there is decreased levels since there is decreased surface expression of GLR-1. If this is true, our *ptp-3(mu256)* and *syd-2(lf)* results suggest that GLR-1 transport is not directly related to the amount of intracellular Ca^{2+}. What are the implications of these results on vertebrate studies? Recently, the evolutionary conservation of CaMKII's activity and the relation between intracellular Ca^{2+} levels and AMPAR transport numbers were shown to be conserved between *C. elegans* and vertebrates[89]. In this study, even 15-minutes after induction of LTP in disassociated neurons, intracellular Ca^{2+} levels are similar to non-LTP induced neurons although there is increased AMPAR transport[89]. Therefore, the mechanism that regulates AMPAR transport may rely on increased intracellular Ca^{2+} to ignite increased AMPAR transport but is not entirely dependent on Ca^{2+} to sustain this increase. Thus, it is possible to speculate that there exists signaling cascades which control AMPAR transport that are specifically activated by extreme intracellular Ca^{2+} levels that remain activated in the absence of Ca^{2+}. Moreover, SYD-2 might be involved in limiting the activation of this pathway and when not expressed, there is increased AMPAR transport.

Caveats, alternative interpretations, and future directions. PTP-3A is known to be important in presynaptic axon guidance and synapse formation in *C. elegans* via connections to the postsynapse*,* therefore it is possible that its activation through retrograde signaling could be a mechanism to regulate AMPAR transport. Since the ectodomain that contains the Ig-like domains, which are a common ligand motif, can be shed, it is logical that it could disassociate from the presynapse and activate a

postsynaptic signaling cascade that regulates AMPAR transport. Although this is a possibility, we hypothesize that this is not the main mechanism of action. First, postsynaptic expression of PTP-3A alone is sufficient to rescue the defect seen in *ptp-3(ok244)* mutants. If presynaptic PTP-3A alone was controlling postsynaptic AMPAR transport, or if it was an additive of both pre and postsynaptic PTP-3A, we would have observed partial rescue of AMPAR transport in our PTP-3A AVA expressed worms. Surprisingly, expression of only the Ig-like domains of PTP-3A in AVA is sufficient to rescue the decreased AMPAR transport phenotype in *ptp-3(ok244)* mutants. Ig-like domains are known to be involved in signal transduction pathways that can communicate between neurons[180]. However, there's no indication that this signaling has any interaction with AMPAR transport. This does lead to a testable model though where a forward genetic screen of the *ptp-3(ok244)* + PTP-3A N-terminal worms can be analyzed for mutations that decrease GLR-1 transport. This could possibly be the substrate that the N-terminal segment is binding and thus regulating transport.

The effect of AMPAR transport on learning and memory. *C. elegans* as a model organism is a powerful tool to study how molecular pathways can influence behavior such as learning and memory. Our results suggest that loss of PTP-3A decreases short and long-term olfactory associative memory *in vivo* in *C. elegans.* This result is what we would expect in mutants with defects in surface GLR-1. However, loss of PTP-3A does not cause a defect in associative learning. A similar phenotype has been observed in worms with a loss-of-function membrane-associated guanylate kinase (MAGI)[11]. In *magi-1(lf)* mutants, the clustering of GLR-1 is disrupted, suggesting the efficiency of GLR-1 in regulating transmission is impaired. Since we know that GLR-1 exocytosis to the surface

is relatively independent of PTP-3A, it is possible that upon increased presynaptic input induced from associative learning, GLR-1 surface expression is normal in *ptp-3(ok244)* mutants, which could explain why learning is normal. However, it is the retention of receptors that is required to maintain memory which is why short-term memory is impaired in *ptp-3(ok244)* mutants. Since our SYD-2 results suggest that impaired localization of synaptic GLR-1 does not entirely correlate with impaired long-distance transport of GLR-1, how then does GLR-1 transport affect learning and memory? Our *ptp-3(mu256)* results suggest that efficient transport is sufficient for normal learning and short-term memory. Although in *ptp-3(mu256)* mutants there is decreased surface GLR-1 due to increased removal, the endosomal pool of receptors is normal compared to wild type. This would suggest that during consolidation into short-term memory, having a readily available supply of receptors to express on the surface can overcome the increased surface removal of GLR-1. However, this process does not seem to be sufficient for maintaining long-term associative memory and is dependent on surface retention of GLR-1 which is controlled by the phosphatase domain. This could be a direct effect of PTP-3A, i.e. retention of surface GLR-1, or an indirect effect of the phosphatase domain of PTP-3A which might be needed to regulate key proteins in the long-term memory cascade.

To my knowledge, the role that LAR has in vertebrate learning and memory is not known. However, the role of PTPδ, which is highly homologous to LAR, has been studied in learning and memory. In fact, mice lacking PTPδ have impaired spatial learning and memory. Interestingly, these mice have normal learning paradigms depending on the spatial task which suggests that PTPδ may not be involved with overall learning, but for certain spatial tasks[181]. Since RPTPs seem to have a region bias on their mechanism of

action in different neurons, it is difficult to interpret how our learning and memory results may be paralleled in vertebrates. Recently, the three main type IIa RPTPs have been deleted in mice to study their effects on synaptic function. It appears that a triple conditional knockout of the type IIa RPTPs in hippocampal neurons have no effect on AMPAR-mediated responses, but decrease NMDAR-mediated responses[110]. Although this is somewhat differing from what we observe in *C. elegans,* the authors cautioned that they were restricting their studies to AMPAR and NMDAR-mediated synaptic responses and not other forms of plasticity. Nevertheless, these RPTPs still have altered NMDAR-mediated responses, which would likely cause learning and memory defects[182]. Therefore, an interesting avenue of study would be to observe NMDAR properties in different *ptp-3* mutants *in vivo*.

Final model. Altogether, my work has led to a working model where PTP-3A needs to be localized at synapses, likely due to SYD-2, in order to stabilize excitatory connections. Upon postsynaptic stimulation, the extracellular matrix (ECM) undergoes structural reorganization to increase the spine size. PTP-3A's connection with ECM may be disrupted which could cause the cleavage of the ectodomain. This ectodomain could possibly act as a ligand for a signaling cascade that retrogradely signals for increased AMPAR transport. This could be a mechanism to supply activated synapses with AMPARs. The phosphatase domain, either still attached to the membrane or now disassociated, invokes a signaling mechanism that keeps receptors at the surface. This retention of receptors at the surface makes it so the synaptic connection is strengthened. Together, PTP-3A is responsible for supplying receptors to the synapse while also retaining them at the surface to maintain excitatory connections **Fig 4.1**.

My work has expanded our knowledge of the role of PTP-3A in glutamate receptor transport while also demonstrating a general importance of glutamate receptor transport on local synaptic receptor numbers. The effects of disrupted LAR-RPTP function have been identified in diseases such as autism and bipolar disorder[138,183–185]. Mechanistically, it could be that loss of LAR-RPTPs causes a disrupted neuronal circuit that then leads to disrupted behavioral outcomes. This disruption of communication could be due to decreased glutamatergic synaptic transmission which arises from inadequate distribution of glutamate receptors and not necessarily disrupting the physical synaptic connections themselves. Thus, it is optimistic to postulate that if distribution of glutamate receptors can be rescued, then so could some of the behavioral outcomes of the diseases.

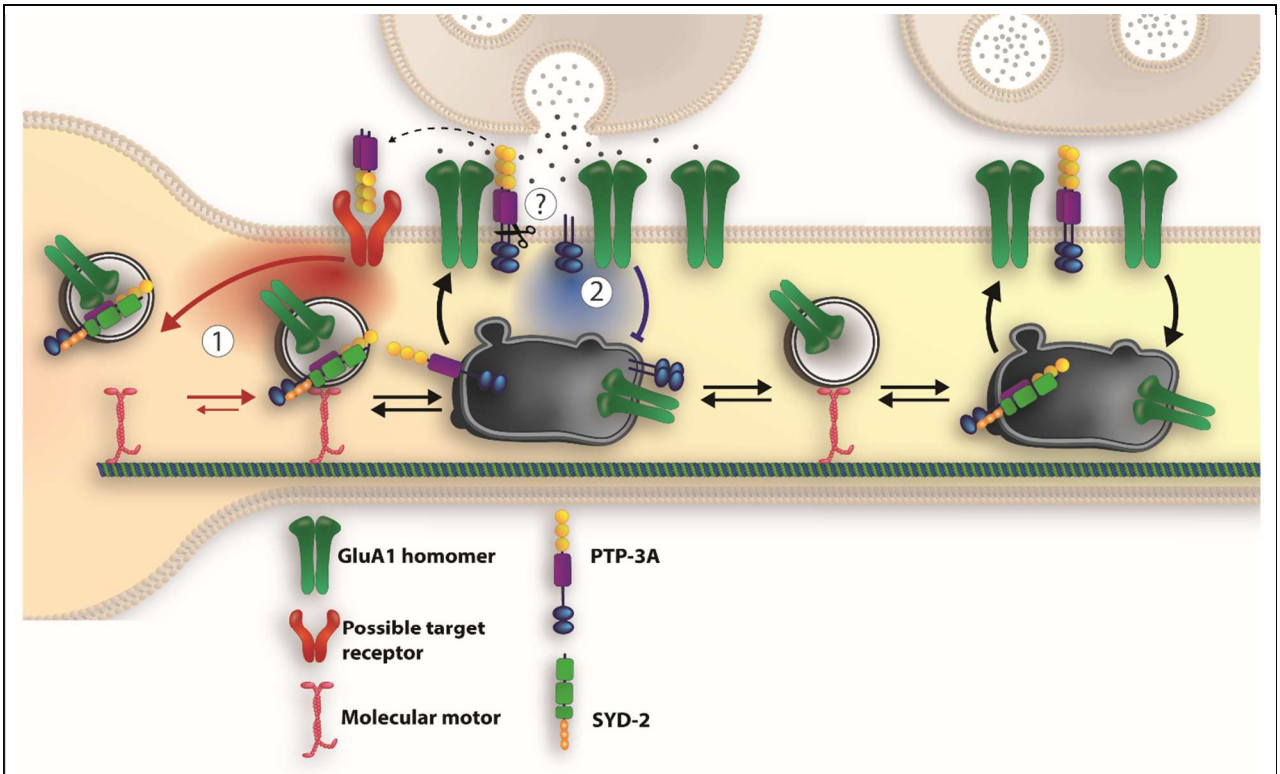

Figure 4.1: Working model of PTP-3A's regulation on GLR-1 transport and GLR-1 retention. 1) PTP-3A is transported to synapses likely by SYD-2. **2)** Upon synaptic stimulation, PTP-3A's N-terminal domain is shed and possibly acts as a ligand to a possible target receptor. The phosphatase domain inhibits endocytosis of receptors to keep them at the surface.

If I had to summarize my PhD with a phrase, it would be the famous quote from Thomas Edison, "I have not failed, I've just found 10,000 ways that won't work." What this dissertation does not include is the hours spent in hardship and failure but outlines the successes that could not be possible without the failures. My graduate work, much like a single synapse, may only be important for a specific connection, but it is the combination of all the synapses that contribute to a working network.

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