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Synaptic Homeostasis at the Drosophila Neuromuscular Junction: Molecular Mechanisms and Developmental Adaptation

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**Synaptic Homeostasis at the *Drosophila* Neuromuscular Junction: Molecular
Mechanisms and Developmental Adaptation**

By

Lindsay A. Gray

A culminating thesis submitted to the faculty of Dominican University of California and Buck
Institute for Research on Aging in partial fulfillment of the requirements for the degree of Master
of Science in Biology

San Rafael, CA
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CERTIFICATION OF APPROVAL

This thesis, written under the direction of the candidate's thesis advisor and approved by the thesis committee and the MS Biology program director, has been presented and accepted by the Department of Natural Sciences and Mathematics in partial fulfillment of the requirements for the degree Master of Science in Biology at Dominican University of California. The written content presented in this work represent the work of the candidate alone.

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Abbreviations

AD	Alzheimer's Disease
ALS	Amyotrophic Lateral Sclerosis
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP	Action Potential
CNS	Central Nervous System
EJC	Excitatory Junctional Current
EJP	Excitatory Junctional Potential
LTD	Long Term Depression
LTP	Long Term Potentiation
MEJC	Miniature Excitatory Junctional Current
MEJP	Miniature Excitatory Junctional Potential
NMDA	N-methyl-D-aspartate
NMJ	Neuromuscular Junction
OE	Over Expression
PD	Parkinson's Disease
WT	Wild Type

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Abstract

The cognitive and motor decline associated with aging can be said to reflect the failure of synaptic homeostasis, which refers to the coordinated pre- and post-synaptic mechanisms that maintain activity levels at the synapse within a precise range. An important mediator of synaptic homeostasis is a compensatory signal from the post- to pre-synaptic nerve terminal or muscle for diminished postsynaptic responses to neurotransmitter that increases presynaptic transmitter release. The magnitude of the retrograde response and the homeostatic set point have been shown to change over the course of the life span, however the precise mechanisms that promote changes in retrograde signaling and the developmental consequences of impaired homeostatic compensation have yet to be determined.

At the *Drosophila melanogaster* neuromuscular junction (NMJ), a strong retrograde response is observed in mutants lacking the GluRIIA glutamate receptor subunit. Glutamate receptors lacking this subunit also exhibit decreased permeability to calcium, an established physiological mediator of the retrograde response. In mammals, the permeability of AMPA-type glutamate receptors is largely governed by the presence of a pair of glutamine (Q) residues within the transmembrane pore of the channel. Preliminary work suggests that calcium permeability can be restored to *Drosophila* GluRIIA $-/-$ mutants by expressing a mutated GluRIIC subunit construct containing point mutations to glutamine within the channel pore-forming region of the subunit. Our examination of the mechanisms underlying synaptic signaling during the lifespan in *Drosophila* may provide insight into the processes underlying declines in cognitive and motor function associated with human aging and age-related pathologies.

Background

Synaptic strength is maintained by the coordinated activity of excitatory and inhibitory signals. In the mammalian central nervous system (CNS), glutamate is the primary excitatory neurotransmitter. Glutamatergic transmission plays an established role in the maintaining synaptic plasticity and memory formation. The efficiency of glutamatergic transmission depends, in large part, on the conductance properties and composition of postsynaptic glutamate receptors.

In the mammalian nervous system, glutamate receptors are classified by their responses to pharmacological agents, such as N-methyl-D-aspartate (NMDA) or α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). The majority of excitatory transmission in the mammalian CNS is mediated by ionotropic glutamate receptors of the NMDA, AMPA, or Kainate subtypes (Ozawa *et al*, 1998). These receptors are generally tetrameric ligand-gated ion channels that express different subunits which in turn contribute to the conductance properties of the channels (Cull-Candy *et al*, 2006). A critical property of glutamate receptors is that they exhibit varying degrees of permeability to calcium, which is critical not only for strong post-synaptic depolarization but also participates in signaling pathways that promote local protein synthesis.

As discussed briefly, mammalian the conductance properties of AMPA receptors are governed by the representation of subunits which assemble as tetramers. AMPA receptors mediate the majority of excitatory transmission in the brain, and the incorporation of the GluR2 subunit has been associated with varying degrees of permeability to calcium (Kumar *et al*., 2002). Specifically, Kumar *et al*. demonstrated in 2002 that early in development in murine hippocampal cells, AMPA receptors are more permeable to calcium than at later time points. The authors found that, accompanying the age-related shift in calcium permeability of AMPA

receptors, there was a greater abundance of GluR2 subunits in older mice relative to younger mice, suggesting that the calcium permeability of the receptor decreases over time, and that this shift is attributable to GluR2 expression.

Significant RNA editing has been demonstrated in glutamate receptor subunits; within the GluR2 transmembrane region, this process converts a glutamine to an arginine in most of the regions in which it is expressed. It has been shown in several studies that mature brains contain a higher proportion of the edited form of this subunit, and that this modification renders the receptors calcium impermeable (Pachernegg et al., 2015). Similar editing of the Kainate subunit GluR6 was shown by Egebjerg & Heinemann in 1993. The authors found that the permeability of the unedited form of the subunit to calcium was significantly higher than the arginine-containing subunit, again implying a functional role of amino acid sequence within the channel pore in the conductance properties of glutamate receptors.

Excessive glutamate receptor activity is associated with excitotoxicity, which refers to glutamate signaling-induced neuronal damage and death (reviewed in Lewerenz & Maher, 2015). Excitotoxicity is observed in cases of traumatic brain injury and epileptic events, but the severity of tissue damage can be ameliorated by pharmacologically antagonizing NMDA-type glutamate receptors, which are highly permeable to calcium (Lewerenz & Maher, 2015). Glutamate-induced excitotoxicity is associated with the progression of amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (AD). Our study of the calcium permeability and activation states of glutamate receptors could therefore have significant implications for the study of neurodegeneration and neurodegenerative disease.

AMPA receptors are of particular interest in the progression of ALS, a degenerative condition that primarily affects motorneurons. Carriedo et al (1996) demonstrated that damage to

motorneurons could be attributed to the expression of calcium permeable AMPA receptors in these cells. The authors exposed these neurons in culture to varying concentrations of calcium and found that the extent of tissue damage was directly proportional to calcium concentration, with greater severity resulting from higher concentrations and minimal severity resulting at low concentrations.

Excitotoxicity can result from the sustained activation of NMDA receptors, which exhibit the highest calcium permeability of glutamate receptors in the CNS (Danysz & Parsons, 2012). Excessive calcium conductance through NMDA receptors is linked to the cognitive disturbances characteristic of AD (Danysz & Parsons, 2012). These findings have suggested potential therapeutic use of the NMDA receptor antagonist memantine. (Hu et al., 2010). Increased calcium influx is thought to activate apoptotic pathways via calcineurin or calpain, an apoptotic protease (Dong et al, 2009). The cell death effected by increased calcium influx, as well as the suggested protective effects of the expression of the GluR2 AMPA receptor subunit against excitotoxicity, contribute to the rationale for our present experimental manipulations in *Drosophila* with respect to calcium permeability and glutamate receptor subunit composition.

A concept fundamental to the understanding of memory and how cognition may change across the lifespan is that of long term potentiation (LTP), which refers to the reinforcement of the strength of a given synapse through repeated activation by multiple coordinated inputs (Rosenzweig & Barnes, 2003). LTP has been a reliable experimental measure of memory formation for decades, and the processes underlying its induction and maintenance have important implications for age related memory impairment (Rosenzweig & Barnes, 2003) as well as age related pathologies characterized by cognitive impairment.

Experimental measures of memory retention are usually tests of spatial reasoning, such as the Morris Water Maze or the T-Maze, the objective of which is for a rat to learn the locations of rewards or hazards. The performance of older rats in these tasks is consistently poorer than that of their younger counterparts, and these age-related deficits in spatial reasoning are accompanied by experiments suggesting age-dependent decline in the maintenance of LTP (Rosenzweig & Barnes, 2003). These studies, however, are complicated by differences in experimental protocols used to induce and measure LTP and overall neuronal excitability, and a more consistent set of data would be required to draw meaningful conclusions.

What is more thoroughly established, however, is the role of calcium in LTP induction. The activation of NMDA receptors allows a large postsynaptic influx of calcium, which activates CamKII and initiates signaling cascades required for the maintenance of synaptic potentiation (Luscher & Malenka, 2012). Another property of NMDA-type glutamate receptors, in addition to their high permeability to calcium, is that they are blocked by magnesium. In order to initiate activity-dependent plasticity at a synapse through NMDA receptors, the binding of glutamate must first trigger the opening of a sufficient quantity of postsynaptic AMPA receptors. If the depolarization due to AMPA receptor activation is strong enough, the magnesium block of NMDA receptors at a given synapse will be relieved, allowing for a large postsynaptic influx of calcium. Calcium influx will then activate postsynaptic CamKII, which in turn will phosphorylate AMPA receptor subunits and activate AMPA receptors, as well as facilitate the addition at the synapse of more AMPA receptors. These CamKII-mediated events will further contribute to the strengthening of the synapse by maintaining depolarization at the synapse (Luscher & Malenka, 2012).

While calcium signaling is required for sustained activation and potentiation of synapses, it has been suggested that perturbations to synaptic calcium signaling and the activity of CamKII can promote cell death. Inhibiting CamKII in culture induces exposure-duration dependent apoptosis (Ashpole et al., 2012). The authors also found that acute CamKII inhibition resulted in increased intracellular calcium concentrations. Further, the study examined the relationship between CamKII activation and neuronal excitability; it was shown that when CamKII was inhibited and then NMDA receptors were pharmacologically activated, neuronal death was significantly higher than in cells exposed to NMDA without CamKII inhibition. The authors showed that this effect is not present when extracellular glutamate was buffered, implying that the effects of altered calcium signaling at excitatory synapses is dependent on glutamate.

The excitotoxic effects of CamKII inactivation have been observed in epilepsy and ischemia following traumatic brain injury and stroke (Ashpole et al., 2012). CamKII has also recently been investigated with respect to Alzheimer's disease. Immunoreactivity and expression analyses have been inconclusive and display region-specific variation, however correlations have emerged between altered CamKII expression at dendrites and synapses in hippocampal regions associated with memory formation (Ghosh & Giese, 2015). Studies in cultured murine cells have been recapitulated in cells taken from AD patients (Ghosh & Giese, 2015). Postmortem tissue analyses suggest deficiencies in CamKII autophosphorylation in AD patients; experimental impairment of CamKII autophosphorylation has, indeed, been correlated with impaired cognitive function in mice (Ghosh & Giese, 2015).

A prominent characteristic of AD pathology is amyloid-beta (AB) peptide aggregation. Interestingly, it was shown that when cortical neurons are exposed to AB oligomers in culture, expression of the GluR1 AMPA receptor subunit is decreased, corresponding to decreased

AMPA receptor conductance and decreased levels of synaptically localized CamKII (Gu et al, 2009). The electrophysiological responses observed at synapses where AMPA receptors were deficient in the GluR1 subunit were similar to responses elicited by CamKII knockdown (Gu et al, 2009). These results implicate glutamate receptor-mediated calcium signaling in the maintenance of cognitive function and the induction of pathological cognitive symptoms observed in age-related diseases where cognitive decline is a prominent symptom.

Mammalian AMPA receptors are comprised of four different subunits, designated GluR1-GluR4 (Cull-Candy *et al*, 2006). Typically, a functional AMPA receptor can be comprised of varying combinations of these subunits, and variable abundance of each subunit is associated with different receptor properties and tissue types (Grossman *et al.*, 1999). Each of these subunits contains a genetically-encoded pair of glutamine residues (QQ) within the putative channel pore of the receptor; however the GluR2 subunit undergoes RNA editing at this site, converting one of these glutamine (Q) residues to arginine (R) (Isaac *et al*, 2007). The calcium permeability of AMPA receptors is dictated by the relative abundance of the unedited GluR2 subunit. (Isaac *et al*, 2007). It has therefore been proposed that the calcium permeability of glutamate receptors depends on the presence of the signature QQ pair in the channel pore.

Furthermore, the magnitude of homeostatic regulation of neurotransmitter release changes as a function of age at the *Drosophila* NMJ, abruptly increasing later in the life of the fly (Mahoney, Rawson, & Eaton, 2014). In this study, the authors examined electrophysiological responses at the NMJ at different time points across the lifespan of the organism. They found that while miniature excitatory junctional potential amplitudes did not differ, there was a presynaptic neurotransmitter release did increase between the ages of 35 and 42 days. They further found that this change could not be attributed to age-dependent changes in innervation

patterns or synaptic morphology, based on staining patterns for the synaptic markers VGlut and Dlg.

Homeostatic compensation at the synapse has been studied not only in flies but in the mammalian CNS as well (Macleod & Zinsmaier, 2006). The complexity of the murine nervous system combined with a broader availability of behavioral assays in mice allows for comparisons to be made between age, performance on memory tests, and electrophysiological read-outs for memory function such as LTP. The subunit composition of glutamate receptors has also been extensively characterized in mice; it has been shown through biochemical assays that a developmental shift occurs in the assembly of subunits in mammalian glutamate receptors, with AMPA receptors favoring configurations that lack GluR2 subunits (Deak & Sonntag, 2012). It would therefore be interesting to comprehensively characterize both electrophysiological responses over time and receptor subunit composition in *Drosophila*, since the *Drosophila* NMJ represents a relatively simple analogue for glutamatergic central synapses.

Figure 1. Synaptic homeostasis at the *Drosophila* NMJ.

Responses to single units of neurotransmitter are severely limited in the GluRIIA^{-/-} mutant, as evidenced by decreased mEPSP amplitudes, which are indicators of spontaneous responses to single vesicles of neurotransmitter (bottom right) relative to wildtype (bottom left). Summed evoked release does not differ significantly between the GluRIIA^{-/-} mutant (top right) and wildtype (top left). Adapted from Petersen *et al.*, 1997).

As in mammalian central excitatory synapses, the *Drosophila* neuromuscular junction (NMJ) is glutamatergic. *Drosophila* glutamate receptors bear a high degree of genetic similarity to mammalian AMPA/Kainate receptors, further contributing to the accessibility of this system as a model for the mammalian CNS. Robust homeostatic responses as well as retrograde signaling have been observed in *Drosophila* as a result of impaired postsynaptic glutamate receptor function (Petersen *et al.*, 1997, Fig. 1), deficiencies in presynaptic proteins in pathways associated with neurotransmitter release (Ball *et al.*, 2010; Penny *et al.*, 2012), and inhibition of calcium calmodulin kinase II (CamKII) in muscle (Haghighi *et al.*, 2003).

There are currently five known genetically-encoded *Drosophila* ionotropic glutamate receptor subunits, GluRIIA-GluRIIE (Collins & DiAntonio, 2007). GluRIIC, GluRIID, & GluRIIE are obligatory, however GluRIIA and GluRIIB are redundant and are present in variable proportions. (Marrus *et al.*, 2004). Mutants lacking the GluRIIA subunit exhibit severely limited

responses to spontaneous release of single units of neurotransmitter, as evidenced by diminished amplitude of miniature excitatory junctional currents or potentials (mEJCs or mEJPs, respectively); however, their responses to evoked release, measured in terms of excitatory junctional currents or potentials (EJCs or EJPs) resulting from an action potential, do not differ significantly from wildtype (Fig 2) (Frank, 2014).

Figure 2: Postsynaptic Glutamate Receptor Composition in *Drosophila*

Drosophila glutamate receptors are characterized by the relative abundance of different subunits. Those possessing the GluRIIA subunit (yellow) exhibit robust responses in muscle to single units of neurotransmitter. Those composed solely of the GluRIIB subunit (green) and lacking the IIA subunit exhibit diminished responses to spontaneous release, as evidenced by significantly smaller miniature excitatory junctional current (mEJC) amplitudes (right) relative to wildtype (left).

The decreased response to spontaneous neurotransmitter release, which is reflected in the amplitude of mEJPs or mEJCs and is referred to as quantal size, observed in these mutants is accompanied by a complementary upregulation of presynaptic neurotransmitter release, implying the presence of a muscle-to-motorneuron retrograde signal in these mutants (Frank, 2014; Petersen *et al*, 1997). The presence of the retrograde signal is inferred from the equivalence of

evoked responses between wildtype and GluRIIA^{-/-} mutants despite the significantly decreased spontaneous activity in the mutant. This amounts to an upregulation in what is termed quantal content, which is calculated by dividing the amplitude of the evoked response by that of the spontaneous response at a given neuromuscular junction and yields a quantifiable estimate of neurotransmitter release (DiAntonio *et al.*, 1999; Fig 3). Similar studies examining the loss of the GluRIIB subunit revealed insignificant differences in quantal size compared to wildtype, implying a unique role for GluRIIA in the biophysical properties of the receptor (Fig 2).

Figure 3: Quantifying homeostatic regulation at the *Drosophila* NMJ. Representative EJC and mEJC traces in wildtype (left) and GluRIIA^{-/-} mutant (right) 3rd instar larvae. Quantal content (grey bars) is calculated by dividing the mean amplitude of the EJC by the mean amplitude of the mEJC for each NMJ. In GluRIIA^{-/-} mutants, mEJCs (white bars) are significantly reduced compared to wildtype, however EJCs (black bars) are approximately equivalent between genotypes. This corresponds to a significant upregulation in quantal content at the GluRIIA^{-/-} mutant NMJ.

These studies implicate the GluRIIA subunit in the induction of a retrograde response that maintains synaptic homeostasis. The variable *Drosophila* GluRIIA and GluRIIB subunits share similar amino acid sequences, however the residues present in the channel pore differ by one amino acid; while the GluRIIA subunit contains the QQ pair associated with calcium permeability in mammalian AMPA receptors, the GluRIIB subunit instead expresses an asparagine-glutamine pair (NQ) at this site (Han et al., 2015). We therefore wondered whether

the effect on retrograde signaling observed in the GluRIIA^{-/-} mutant may be an effect of the calcium permeability potentially conferred on the receptor complex by the presence of a glutamine pair in this subunit.

A 2003 study by Haghighi *et al* investigated the relationship between electrophysiological response properties and postsynaptic calcium activity. The authors expressed either an inhibitor of CamKII or constitutively active CamKII in muscle and observed electrophysiological response properties under each condition. They demonstrated that when CamKII is inhibited in muscle, it causes an increase in excitatory junctional potential (EJP) amplitude without affecting mEJP amplitude, correlating to an upregulation of quantal content. Conversely, constitutively active CamKII decreased the amplitude of the EJs, corresponding to a downregulation of quantal content. These data imply a role for calcium activity in the maintenance of the retrograde signal.

In addition to electrophysiological aberrations, previous work has shown that GluRIIA^{-/-} mutants exhibit limited permeability to calcium (personal communication). Of the *Drosophila* glutamate receptor subunits, GluRIIB and GluRIIC are the only subunits lacking the signature QQ pair in the channel pore region that is associated with calcium permeability in mammalian AMPA receptors (Marrus *et al*, 2004). Following the rationale that the QQ pair is necessary for calcium permeability, we have expressed a mutated GluRIIC containing point mutations to glutamine at critical positions within the channel pore region in a GluRIIA^{-/-} mutant background. We chose to express our construct in a GluRIIA^{-/-} mutant background because the GluRIIC, GluRIID, and GluRIIE have been demonstrated to be essential subunits for synaptic transmission at the larval NMJ (Marrus *et al*, 2004), and due to the unique synaptic phenotype observed specifically as a result of GluRIIA loss of function. (Fig. 4)

DGluRIII	(635)	SIM	TA	GCDILPR	SP
DGluRIIA	(612)	SIM	QQ	GCDILPR	GP

Figure 4. Generation of UAS GluRIIC construct.

Sequence alignment is shown for the pore loop of the GluRIIC (DGluRIII) subunit and the GluRIIA subunit. At positions 638 and 639, a point mutation was made from threonine to glutamine (T638Q), alanine to glutamine (A639Q) or both residues were mutated to glutamine. (Adapted from Marrus *et al.*, 2004)

SynapGCamp3 is a synaptically targeted, genetically encoded calcium indicator that reports postsynaptic calcium influx through glutamate receptors in response to action potentials. Its construction is similar to a previously developed reporter, SynapCam, which is a Cameleon FRET-based calcium reporter targeted to the muscle with a CD8 N-terminus region and to the postsynapse with a C-terminus PDZ domain of the *Shaker* potassium channel (Guerrero *et al.*, 2005). The reporter used instead of Cameleon is GCamp3, a non-ratiometric fluorescent calcium indicator with higher signal-to-noise than previous reporters (Peled & Isacoff, 2011). Using confocal microscopy coupled with electrophysiology, it is possible to generate *in vivo* activity maps of postsynaptic calcium activity in response to nerve stimulation. Preliminary calcium imaging data suggest that the GluRIIC mutant construct restores calcium permeability to the GluRIIA^{-/-} mutant. We are investigating the effect of restored calcium permeability on the magnitude of the retrograde response in the GluRIIA^{-/-} mutant. If calcium is a trigger of the retrograde response, we expect to see abolition of the homeostatic response observed in the GluRIIA^{-/-} mutant. Preliminary electrophysiological data suggests that this is the case; in GluRIIA^{-/-} mutants expressing the modified GluRIIC^{QQ} construct, we observe decreased EJC amplitudes relative to wildtype larvae and GluRIIA^{-/-} mutants. The amplitude of the mEJCs

remain equivalent to those observed in the GluRIIA^{-/-} mutant alone, implying that retrograde compensation for this decreased spontaneous activity has been interrupted by the addition of our construct.

Materials & Methods

Methods

Fly stocks. SynapGCamp3 (MHCGC3, gift of Ehud Isacoff) recombined with the 24b gal4 muscle driver in a *w1118* or *df^{(2L)Clh4}* background were used to generate the following lines: *Df(2L)Clh4/cyogfp;MHCGC3/+*, *Df(2L)Clh4/sp16; MHCGC3/+*, *Df(2L)Clh4/sp16;UAS-GluRIICQQ/MHCGC3*, *Df(2L)Clh4/sp16;UAS-GluRIICQQ/MHCGC3, +/-cyogfp; MHCGC3/+; +/-cyogfp; UAS-GluRIICQQ/MHCGC3, +/-cyogfp; +/-UAS-M/R*. Crosses were performed using virgin females of the genotype *Df(2L)Clh4/cyogfp; 24b,MHCGC3/TM6B* or *+/-cyogfp; 24b,MHCGC3/TM6B* were crossed to males of the following genotypes: *w1118*, *sp16/cyogfp; +/-TM3GFP*, *sp16/cyogfp;UAS-GluRIICQQ/TM3GFP*, *+/-cyogfp;UAS-GluRIICQQ/TM3GFP*, or *sp16/cyogfp;UAS-GluRIICQ/TM3GFP*. Crosses were maintained at 25°C.

Electrophysiology. Two electrode voltage clamp recordings were acquired with an Axon Instruments Axon900A amplifier (Molecular Devices, Sunnyvale CA). Wandering 3rd instar larvae were dissected in cold Stewart's HL3 saline (Stewart et al., 1994) containing 70mM NaCl, 5mM KCl, 20mM MgCl₂, 10mM NaHCO₃, 5mM Trehalose, 115mM Sucrose, and 5mM HEPES. Recording solution was buffered to pH 7.4 using KOH. Recordings were performed at room temperature in 0.5mM extracellular calcium (or 1.5mM extracellular calcium for imaging recordings) on muscle 6, segment A2 or A3. Muscles were held at a potential of -80mV and larvae with a resting membrane potential below -60mV or with input resistances below 4 megaohms were not selected for further analysis. Electrophysiology data were analyzed in Clampfit (Molecular Devices). EJC amplitudes were measured using peak-detection functions of the Clampfit software (Molecular Devices), and mEJC amplitudes were analyzed using the peak

detection functions of the MiniAnalysis software program (SynapseSoft, Inc., Fort Lee NJ).

Quantal content was calculated by dividing the mean EJC amplitude of 40 trials per NMJ by the mean mEJC amplitude for three minutes of continuous recording at the same NMJ.

Statistical analysis was performed in Excel (Microsoft Inc., Redmond WA) or Origin (OriginLab, Northampton MA). For comparison of EJCs and Quantal Content, pairwise Student's T-Tests were conducted. Values reported are means $\pm$ SEM.

Immunohistochemistry. Following electrophysiology, larval fillets were fixed in methanol for 10 minutes and incubated with primary antibody against dGluRIIA or dGluRIIC (gifts of Aaron DiAntonio) for 24 hours at 4°C overnight. Mouse anti dGluRIIA was diluted at 1:250 and Mouse anti dGluRIIC was diluted at 1:1000. Following incubation with primary antibody, Goat anti-Cy3-HRP (Jackson ImmunoResearch Laboratories, West Grove PA) conjugated secondary antibody was added and incubated for 2 hours at room temperature. Neuromuscular junctions were imaged using a Zeiss LSM 780 NLO AxioExaminer (Carl Zeiss, Oberkochen Germany) at 40x or 63x magnification.

Calcium Imaging. Two electrode voltage clamp was performed on muscle 6/7 in extracellular solution containing 1.5mM calcium. 0.2-0.4 micromolar Thapsigargin (Sigma Aldrich, St. Louis MO) was added to the recording solution to prevent muscle contraction. Type 1b boutons were imaged continuously using a Zeiss 5-Live Axioskop line scanning confocal microscope (Carl Zeiss) or an Andor IQ CCD camera coupled to an Olympus BX50WI microscope (Olympus Corporation, Shinjuku, Tokyo Japan) . Boutons were imaged at 63x magnification. Each NMJ was subjected to at least 20 cycles of stimulation at 0.1 Hz. Image series were taken such that one image was taken immediately prior to stimulation and one taken after. Calcium imaging data were analyzed in Matlab (Mathworks, Natick MA) using custom routines written by Einat Peled

(Peled & Isacoff, 2011). Statistical comparison of calcium imaging quantification was done in Excel (Microsoft) using pairwise Student's T-tests. Values reported are means +/- SEM.

qPCR/RT-PCR. RNA extraction was performed on 8 3rd instar larval muscle fillets and 8 adult whole bodies using a Qiagen RNA Easy Plus kit (Qiagen Sciences, Germantown MD). cDNA was synthesized using a BioRad iScript cDNA synthesis kit (BioRad, Hercules CA). qPCR was performed with BioRad Sso Advanced Universal SybrGreen Supermix (BioRad) and a BioRad CFX96 RealTime system (BioRad). qPCR primers used were as follows:

GluRIIA forward: TTCAATCCCTCGGCCTTCAC

GluRIIA reverse: GTCCGGTAATCAGAGCCCAG

RPL32 Control forward: AAGCGGCGACGCACTCTGTT

RPL32 Control reverse: GCCCAGCATACAGGCCCAAG

Results

SynapGCamp3 Expression Does Not Affect Presynaptic Release

SynapGCamp3 is a synaptically targeted calcium indicator that reports calcium influx through glutamate receptors at single active zone resolution (Peled & Isacoff, 2011). It was previously reported that modulation of calcium-dependent processes at the *Drosophila* NMJ via the activation or suppression of CamKII in muscle directly affects synaptic physiology (Haghighi et al., 2003). We therefore sought to assess whether the introduction of a calcium-binding compound at the neuromuscular junction could have a similar effect on baseline electrophysiological responses. We compared mean EJC amplitude, mEJC amplitude, and quantal content between wildtype controls and larvae expressing SynapGCamp3. We found a marginal but statistically insignificant differences in mEJC amplitude, with no difference in quantal content (Figure 5).

Figure 5. SynapGCAMP3 does not affect EJC amplitude or quantal content. Mean mEJC and EJC amplitudes in larvae expressing synaptically targeted GCamp3 (MHCGC3) and wildtype larvae, expressed as a percentage of control. Mean EJC amplitude differed insignificantly between MHCGC3 larvae (n=8) and controls (n=8) (p=0.923). Mean mEJC amplitude also differed insignificantly between larvae expressing GCamp and control larvae (p=0.1456).

GluRIIA^{-/-} Mutants Exhibit Decreased Calcium Permeability

Drosophila larvae lacking functional GluRIIA glutamate receptor subunits exhibit reduced mEJC amplitude and increased presynaptic neurotransmitter release that maintains EJC amplitude at wildtype levels (Petersen et al., 1997). Since inhibition of the activity of CamKII in muscle results in an increase in presynaptic transmitter release (Haghighi et al, 2003), we speculated that this compensatory mechanism may depend on calcium influx at the NMJ. To assess calcium influx through glutamate receptors, we performed calcium imaging experiments on wildtype larvae and GluRIIA^{-/-} mutants expressing SynapGCamp3 and compared changes in fluorescence at each bouton ($\Delta F/F$). Comparison of fluorescence changes in response to neurotransmitter release indicate that GluRIIA-lacking receptor assemblies are significantly less permeable to calcium than those that contain the GluRIIA subunit (Figure 6).

Figure 6. GluRIIA mutants exhibit decreased calcium activity at the NMJ. Representative fluorescence imaging data for wildtype controls (n=10) and GluRIIA^{-/-} mutants (n=10) expressing GCamp3 at the synapse. Control larvae exhibited significantly higher fluorescence changes than GluRIIA^{-/-} mutants (p=.00981). Values are expressed as mean $\Delta F/F$. Means were normalized to baseline fluorescence values for all groups.

Expression of UAS GluRIIC in *Sp16* Background Suppresses Quantal Content

In mammalian AMPA receptors, the presence of a pair of glutamine (Q) residues within the channel pore confer calcium permeability on the receptor complex as a whole (Liu & Cull Candy, 2000). The GluRIIA subunit also bears a pair of glutamine residues at a corresponding position within the channel pore; however, the obligatory subunit GluRIIC instead bears a threonine and alanine at these positions. We therefore sought to assess the effect of expressing a UAS-GluRIIC construct wherein either one or both of these residues was mutated to Q. We found that when expressed in an *sp16* mutant background, UAS-GluRIIC^{QQ} results in a suppression of the increased quantal content exhibited by the GluRIIA^{-/-} mutant. Moreover, expression of a GluRIIC construct in which only a single residue has been mutated to Q fails to suppress quantal content upregulation (Figure 7).

Figure 7. Expression of UAS-GluRIIC^{QQ} suppresses quantal content upregulation in GluRIIA ^{-/-} mutant. (a) Sequence alignment of mammalian GluR6 and *Drosophila* GluRIIA-GluRIID subunits within pore loop. (b) Representative traces for EJCs (averaged over 40 trials) and mEJCs in controls (n=12), GluRIIA^{-/-} mutants (n=14), and GluRIIA^{-/-} mutants expressing either UAS GluRIIC^{QQ} (n=21) or UAS GluRIIC^Q (n=11). (c) Bar graph expressing mean mEJC, EJC, and quantal content values as a percentage of control. Expression of UAS GluRIIC^{QQ} in the *Sp16* mutant background result in significant (p=.0014) suppression of quantal content. Quantal content differed insignificantly between *Sp16* mutants and *Sp16* mutants expressing UAS-GluRIIC^Q.

UAS GluRIIC^{QQ} Fails To Suppress Quantal Content in Dominant Negative GluRIIA Mutant

We observed a significant impairment of the quantal content upregulation normally observed in the GluRIIA mutant when we expressed a modified GluRIIC construct at the GluRIIA^{-/-} mutant NMJ. The GluRIIA mutant we used for these experiments represents a genetic deletion of the functional subunit. We also wished to examine the effects of the IIC^{QQ} transgene when expressed with a dominant negative GluRIIA construct. DGluRIIA^{M/R} is a UAS construct wherein an arginine within the channel pore region is substituted with a methionine. Postsynaptic expression of this transgene results in miniature EJC amplitude reduction accompanied by increased EJC amplitudes, corresponding to quantal content upregulation (Haghighi et al., 2003). When we co-expressed DGluRIIA^{M/R} with GluRIIC^{QQ}, we failed to find any significant differences in EJC amplitude or quantal content between flies expressing both UAS-GluRIIC^{QQ} and UAS GluRIIA^{M/R}, and flies expressing only the UAS M/R dominant negative construct. (Fig 8).

Figure 8: UAS GluRIIC^{QQ} Fails to Suppress QC in GluRIIA Dominant Negative Background

Mean amplitudes of mEJC and EJC, and quantal content in larvae expressing UAS GluRIIC^{QQ} and UAS GluRIIA M/R in muscle (n=6 NMJs). Values are expressed as percent of UAS GluRIIA M/R expressed in w1118 background (n=6 NMJs). Mean mEJC amplitude (p=.083115), EJC amplitude (p=.511635), and quantal content (p=.964191) did not differ significantly between genotypes.

UAS GluRIIC^{QQ} Restores Calcium Permeability to GluRIIA^{-/-} Mutants

Having observed a synaptic phenotype in GluRIIA^{-/-} mutants expressing a modified GluRIIC subunit, we next sought to observe the effects of this construct on calcium permeability at the NMJ. To do so, we again performed calcium imaging experiments on wildtype larvae, GluRIIA^{-/-} mutants, and GluRIIA^{-/-} mutants expressing GluRIIC^{QQ}. When expressed in a GluRIIA^{-/-} mutant background, the GluRIIC^{QQ} construct resulted in increased calcium permeability at the NMJ, as inferred from comparison of fluorescence changes in response to stimulation (Figure 9).

Figure 9. GluRIIC Mutant Construct Affects Calcium Permeability in GluRIIA^{-/-} Mutants. Calcium imaging data comparing fluorescence changes in GluRIIA^{-/-} mutants and GluRIIA^{-/-} mutants expressing UAS GluRIIC^{QQ}. GluRIIC^{QQ} larvae exhibit significantly higher mean fluorescence (n=10, p=.0006) than GluRIIA^{-/-} mutants.

Expression of GluRIIC^{QQ} Is Sufficient to Reduce Quantal Content in Wildtype

Background

If regulation of presynaptic neurotransmitter release is dependent on calcium, we conjectured that perhaps the apparent increase in calcium permeability conferred by the expression of the UAS-GluRIIC^{QQ} construct in the *Sp16* background could have an effect on baseline physiological responses in wildtype larvae as well. When we overexpressed UAS-GluRIIC^{QQ} in

a wildtype background, we observed reduced EJC amplitudes without a significant effect on the amplitude of mEJCs (Figure 10).

Figure 10. Effects of GluRIIC^{QQ} overexpression on synaptic physiology in wildtype background. (a) Representative traces for GCamp controls and flies expressing UAS GluRIIC^{QQ} in the same background. (b) Quantification of average values for mEJC, EJC, and QC, expressed as percentage of control. Preliminary data suggests a slight increase in mEJC amplitude and a slight reduction in EJC amplitude in IIC^{QQ} overexpressing flies (n=6) relative to controls (n=12). This equates to an approximately 40% reduction in quantal content when UAS GluRIIC^{QQ} is expressed in a wildtype background.

Glutamate Receptor Subunit Composition Shifts With Age

It has been proposed that the homeostatic mechanism whereby presynaptic neurotransmitter release is maintained changes as a function of age in *Drosophila* (Mahoney et al., 2014).

Specifically, Mahoney et al found that quantal content increases significantly in 42-day old flies relative to 7 day old flies. Developmental shifts in the subunit composition of AMPA and

NMDA-type glutamate receptors have been observed throughout synapse development in mice, with different subunits conferring different kinetic properties and permeability to divalent cations

upon the receptor assembly (Elias et al, 2008). Given the well-established sensitivity of

homeostatic compensation and retrograde signaling to the presence of the GluRIIA subunit at the *Drosophila* NMJ, we speculated that perhaps the upregulation in quantal content observed at

later points in the life of the fly may be attributable to a shift in the expression of GluRIIA. In larvae, impairment of the GluRIIA subunit results in depressed mEPSC amplitude with a compensatory upregulation in EJC amplitude and quantal content. We therefore quantified the relative abundance of the GluRIIA subunit in 3rd instar larval muscle and 40 day adult muscle using qPCR. We found that, in 40 day aged flies (n=8), there is a roughly 2.5-fold decrease in GluRIIA transcript abundance relative to the 3rd larval instar (n=8). (Fig 11).

Figure 11: Decreased Abundance of GluRIIA mRNA at the Adult *Drosophila* NMJ

Relative abundance of GluRIIA transcript quantified using RT-qPCR. Expression of GluRIIA in 3rd instar larval muscle (n=8) is increased 2.43 fold relative to adult muscle (age 40 days, n=8).

Conclusions & Discussion

Sustained synaptic activity and the regulation of neurotransmission is critical throughout development to the maintenance of learning and memory, motor function, and the regulation of complex behaviors (Frank, 2014). Retrograde compensation has been shown to act at the *Drosophila melanogaster* NMJ to maintain synaptic activity in response to a loss of postsynaptic glutamate receptor function (Petersen et al., 1997). CamKII plays a regulatory role in this homeostatic compensation, abrogating the retrograde response when constitutively activated (Haghighi et al., 2003). It is therefore proposed that calcium, the postsynaptic admittance of which relies heavily on glutamate receptors, mediates retrograde signaling. By attempting to restore calcium permeability to glutamate receptors lacking the GluRIIA subunit, known to confer calcium permeability to *Drosophila* glutamate receptors (personal communication), we observe abolition of the retrograde response in glutamate receptor mutants that mimics that observed when CamKII is constitutively active, implying that calcium is indeed a trigger for homeostatic compensation.

Calcium functions in the nervous system not only as a necessity for the propagation of action potentials due to its capacity to carry depolarizing charge, but also as a second messenger that initiates a variety of developmental programs. In glutamate receptor mutants that lack functional GluRIIA subunits, we observe a dramatic decrease in the magnitude of the electrical response to single units of neurotransmitter vesicle fusion. Acutely, we see that this reduction is compensated for by an upregulation in quantal content at GluRIIA^{-/-} mutant NMJs. Based on the amplitude of EJCs in GluRIIA^{-/-} mutants expressing a modified GluRIIC UAS, we no longer see this compensatory upregulation of quantal content, and we also observe concomitant restoration of calcium permeability in these animals based on our imaging data.

Taken together with previous findings that CamKII has similar effects on retrograde compensation, these results strongly implicate the calcium permeability of glutamate receptors as a defining factor in the maintenance of homeostatic compensation. Recent findings also indicate that the homeostatic set-point changes with age at the *Drosophila* NMJ (Mahoney et al., 2014); in the context of these findings, it would be of interest in future studies to determine the relative overall abundance of glutamate receptors, as well as the expression levels of the subunits themselves, at the adult NMJ. Previous work implies that the magnitude of retrograde compensation observed during aging could be attributable to a shift in the relative expression of the GluRIIA subunit at later time points, and that these shifts may be accompanied by changes in the calcium permeability of the receptors.

Fluctuations in homeostatic compensation during normal aging imply that this system may be susceptible to dysregulation during pathological aging as well, and may account for disturbances in motor function and cognition characteristic of neurodegenerative diseases. Excessive calcium entry via glutamate receptors may be cytotoxic in established neural networks, whereas the proliferative neurogenesis observed very early in development requires excessive glutamatergic activity. Indeed, developmental shifts in glutamate receptor composition, with configurations favoring higher calcium permeability early in development and lower calcium permeability later in life, have been observed in the mammalian glutamatergic system.

While it has been electrophysiologically established that homeostatic compensation at the synapse exists, its developmental function has remained unclear. Homeostatic compensation serves to maintain overall network activity during developmentally sensitive periods of muscle and synapse growth, but the potential effects of this regulation as an animal ages may be more

subtle. In GluRIIA^{-/-} mutants, we observe diminished calcium permeability corresponding to an upregulation in quantal content. A key subject of future work would therefore be to determine if this persistent retrograde compensation has any significant effect on lifespan or muscle health. We could then proceed to examine any differences in locomotion or behavior in aging GluRIIA^{-/-} mutants expressing the GluRIIC^{QQ} construct. In expressing this construct, we are effectively restoring wildtype calcium permeability to these receptors and eliminating the need for retrograde compensation. In the absence of appropriate presynaptic neurotransmitter release, however, it is possible that we would observe defects in synaptic strength and muscle health if the dampening of the retrograde response persists throughout the lifespan. We may also observe inappropriate developmental shifts in retrograde compensation, as this process is induced during the course of aging at the wildtype NMJ. Our preliminary observation that, at the level of transcription, GluRIIA expression is diminished in adulthood relative to expression during larval development could correlate to a commensurate change in electrophysiological response properties at the adult NMJ. Mahoney et al.'s 2014 study at the CM9 NMJ produced results consistent with those observed at the larval NMJ in response to impaired GluRIIA function. Further characterization of electrophysiological responses and the biophysical properties of glutamate receptors in wildtype and GluRIIA^{-/-} mutant adults could yield valuable insight into the role of the homeostatic response in the maintenance of nervous and motor function. In future, we seek to establish correlations between glutamate receptor function, calcium permeability, and behavioral and motor function assays that serve as reliable markers of aging and neurodegeneration phenotypes across the lifespan of the adult fly.

An extensive body of literature exists on the physical hallmarks of neurodegenerative disease. Models have been developed on the basis of lifespan and brain lesions in response to

genetic manipulations associated with AD or PD. Specifically, studies of AD related neuropathology have been conducted in mice using human tau protein, a microtubule associated protein thought to cause the neurofibrillary tangles observed in AD. In mice and in flies, studies have also targeted a family of proteins called presenilins, which are believed to facilitate the formation of amyloid beta plaques, another major component of AD pathology (Lu & Vogel, 2009). Tau phosphorylation has been repeatedly shown to be significantly increased in postmortem analyses of AD brain tissue, and the ease of genetic manipulation in *Drosophila* has yielded a variety of promising investigations into the biochemical basis of phosphorylated tau toxicity. The focus of a preponderance of these studies has been on morphological changes and neuronal tissue loss in response to tau phosphorylation or amyloid beta plaque formation, with a comparative dearth of electrophysiological interrogation of the synapses affected by these processes. In the past several decades, it has been shown that amyloid precursor protein is capable of effecting cognitive deficits in mice without accompanying neuronal tissue damage (Lu & Vogel, 2009), suggesting that synaptic dysfunction may be a core feature of AD pathology.

It was also shown that PAR-1, a kinase that phosphorylates tau, can affect synaptic recruitment of the protein discs-large (Dlg). Dlg was shown to be critical for synaptic incorporation of the GluRIIB subunit in *Drosophila*; this protein is also sensitive to phosphorylation by CamKII and is therefore believed to regulate receptor subunit composition in an activity dependent manner (Chen & Featherstone, 2005). Disruption of synaptic protein localization has been observed as a result of beta amyloid accumulation as well; Almeida et al showed in 2009 that cultured neurons expressing mutant APP secrete excessive AB peptide, resulting in reduced PSD-95 expression at mutant synapses. PSD-95 is required for the post-

synaptic anchoring of glutamate receptors. Almeida et al observed reduced GluR1 subunit density in APP mutant synapses, consistent with more recent evidence that PSD-95 is required for the synaptic anchoring of NMDA and AMPA receptors (Chen et al, 2015).

In examining the connections between calcium permeability, the maintenance of network activity at the synapse, and the functional outcomes of these interactions, we must also consider the regulation of synaptic localization of scaffolding proteins and glutamate receptor subunits. These processes are activity dependent, and it will be important to establish the relationship between retrograde signaling and the relative representation of GluRIIA at the NMJ.

In the literature on neurodegenerative disease, synaptic dysfunction is discussed in greater detail with respect to Parkinson's disease. Several genetic markers for PD have been identified recently, the best characterized being leucine-rich repeat kinase 2 (LRRK2). LRRK2 mutations are found in approximately 13% of cases of inherited late-onset PD (Belluzi, Greggio, & Piccoli, 2012). A recent study demonstrated that overexpression of the disease-associated LRRK2 mutation G2019S in mice increased glutamatergic transmission in cultured cortical neurons (Beccano-Kelly et al., 2014), and our lab has observed upregulation of retrograde synaptic compensation as a result of overexpression of human or drosophila LRRK2 transgenes (Penny et al, 2015, in review). These findings indicate a potential role for synaptic dysfunction and retrograde signaling in the development of neurodegenerative disease.

In *Drosophila*, genetic manipulations of LRRK2 represent the most well characterized model system for PD, both mechanistically and electrophysiologically. Expression of mutant LRRK2 shortens the lifespan of the fly and causes a loss of dopaminergic neurons (Liu et al, 2007). LRRK2 also interacts directly with the mTOR pathway via its phosphorylation of 4E-BP (Imai et al., 2008). 4E-BPs are regulatory proteins that ordinarily inhibit the activity of the

translational activator eIF4E. When 4E-BPs are phosphorylated, this suppression of eIF4E is relieved and translation can occur via the helicase activity of eIF4E, which helps to unwind the 5' cap of mRNAs and facilitate translation initiation (Penney et al., 2012). mTOR regulates synaptic strength and retrograde signaling by phosphorylating 4E-BP and facilitating translation initiation; knocking down mTOR in *Drosophila* abolishes retrograde compensation at the NMJ in GluRIIA^{-/-} mutants, while also reducing levels of the GluRIIA subunit in wildtype organisms (Penney et al., 2012). The effect of mTOR loss of function on the retrograde response also depended upon the time course of mTOR inactivation, based on experiments in which GluRIIA^{-/-} mutants were fed rapamycin and their physiological responses were measured at regular intervals (Penney et al., 2012).

A clear role has been established for translational control of synaptic homeostasis and retrograde signaling. Given the observed effects on lifespan in *Drosophila* models wherein this compensation is disrupted, as well as the sensitivity of these pathways to environmental factors, it is also clear that synaptic homeostasis has broad physiological consequences and is subject to activity dependent fluctuation. The experimental evidence we have obtained so far adds a greater dimension of specificity to the current literature on the translational control of synaptic activity. Specifically, if calcium activity and the immediate targets of calcium at the synapse can be placed within the context of pathways that regulate the translation of critical synaptic proteins, new insights could be generated into the interactions between translational control pathways and potential targets within these pathways that depend upon the activity of calcium. Calcium plays distinct roles not only in learning and memory, but in neurodegenerative process via cellular excitotoxicity. Therefore, establishing a causal link between synaptic activity, calcium, and local protein synthesis during development and aging will not only benefit research into

neurodegenerative processes, but also diseases of dysregulated synaptic transmission such as epilepsy and schizophrenia.

Retrograde signaling and the maintenance of synaptic homeostasis are sensitive to environmental factors, and recent experimental evidence supports the idea of an age-dependent change in synaptic function and the maintenance of an ideal level of synaptic activity. In the context of these data, the relationship between calcium permeability and glutamate receptor function and composition becomes relevant not only to the cognitive and motor function deficits seen in otherwise healthy aging, but to disease states as well.

Our preliminary work with our GluRIIC mutant construct strongly suggests a role for calcium activity in the induction and maintenance of the retrograde response. We have not only observed decreases in quantal content on the introduction of the GluRIIC mutant construct into a GluRIIA^{-/-} mutant background, but have also observed a corresponding increase in fluorescence in calcium imaging experiments performed on these same flies. It remains to be seen whether altered calcium permeability of glutamate receptors at the *Drosophila* NMJ will have an effect on behavior or muscle health throughout the course of development and lifespan. We also aim to verify our calcium imaging data with more robust electrophysiological assays of calcium current during voltage clamp. However, results up to this point offer a potentially novel insight into the basic mechanisms underlying the retrograde signal in *Drosophila*.

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