Molecular Mechanisms of Subcellular Development: Down Syndrome-Related Genetic Interactions and Axon-Dendrite Coordination During Neurodevelopment

by

Sarah A Pizzano

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Doctoral Committee:

Assistant Professor Paul Jenkins, Co-Chair
Professor Bing Ye, Co-Chair
Associate Professor Sami Barmada
Assistant Professor Josephine Clowney
Associate Professor Catherine Collins

Sarah A. Pizzano

spizzano@umich.edu

ORCID iD: 0000-0002-8959-6458

Dedication

To Judy Tsao who knew graduate school would be the right call for me before I ever considered it. And to the family and friends who have been the most rewarding part of this experience.

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Abstract

Mechanisms of dendrite and axon patterning are important in both neurotypical development and neurodevelopmental disorders. This dissertation aimed to address two open questions concerning mechanisms of subcellular development: (1) How do multiple upregulated genes in Down syndrome (DS) models interact to cause aberrant axon morphology; (2) In response to changes in circuitry, how does the Wnd/DLK pathway coordinate dendritic signaling with changes at the axon terminal?

Both projects employed *Drosophila* genetics, confocal microscopy, and biochemical techniques to interrogate these uncertain molecular mechanisms. Moreover, to test for changes in axon and dendrite morphology, these works utilized larval **C**lass **IV** **d**endritic **a**rborization (C4da) neurons.

In the first half of this dissertation, I will describe how multiple DS-related genes interact to establish axon morphology. We found that **A**myloid **p**recursor **p**rotein-**l**ike (Appl) upregulates **D**own **s**yndrome **c**ell **a**dhesion **m**olecule (Dscam) to promote axon terminal growth in C4da neurons. Furthermore, we found that the post-transcriptional regulation of Dscam by Appl may occur through altered Rab5 signaling. This shows a novel, developmental mechanism of two DS-related genes interacting to establish aberrant axon morphology.

The second half of this dissertation interrogates the coordination of axon-localized events to dendritic signaling. We found that ablation of second order neurons (SONs) within the C4da nociceptive pathway resulted in decreased expression of nuclear Knot, a dendritic growth regulator. This phenocopies activation of the Wnd/DLK pathway. Moreover, Wnd/DLK required retrograde transport to repress nuclear Knot expression. This lays the foundation for determining how the Wnd/DLK pathway may coordinate axonal events to dendritic growth to maintain functional circuitry in response to changes at the axon terminal.

Overall, this work offers insight into basic and DS-relevant mechanisms of axon-dendrite patterning in development. Future works may further these findings in mammals and consider the implications of DS-gene interactions as well as the impact of axonal changes on dendritic structures for therapeutic interventions in neurodevelopmental disorders.

Chapter 1 Introduction

During development, neurons establish axon-dendrite patterning consistent with their function. As a result, thousands of neuronal morphologies have been identified with diverse subcellular structures. For example, Purkinje cells in the mammalian cerebellum have large, complex dendritic arbors with a single, long axon, while basket cells from the same region have far simpler dendritic arbors and shorter, branching axons (Purves et al., 2001). The diversity of neuronal cell types has led to the ongoing question of how a cell establishes distinct subcellular compartments during development to generate mature and functional axons and dendrites.

Indeed, axon and dendrite patterning during neurodevelopment has significant impacts on circuit function. Neurite morphology alters signaling within a circuit and atypical compartmental patterning in development has been observed in many neurodevelopmental disorders such as autism, Angelman's syndrome, Rett's syndrome, fragile X syndrome, and Down syndrome, amongst others (Martínez-Cerdeño, 2017). This dissertation discusses mechanisms of subcellular development in modelling Down syndrome and other disruptions to neural circuits.

1.1 Mechanisms of atypical subcellular development in Down syndrome models.

Down syndrome (DS) occurs at a prevalence of 1 in every 732 lives births in the United States (Canfield et al., 2006). Caused by full or partial trisomy of human chromosome 21

(HSA21), DS results in increased RNA and protein expression of many of the triplicated genes in DS patients (Olmos-Serrano et al., 2016; Cheon et al., 2003). This widescale genetic dysregulation causes a range of well-characterized signs and symptoms including broadened foreheads, poor muscle tone, and intellectual disability, as well as frequent co-morbidities with congenital heart defects and **A**lzheimer's **d**isease (AD) (Ahktar and Bokhari, 2021). Indeed, intellectual disability and AD have especially high prevalence in DS. 99% of DS individuals present with intellectual disability and 95-98% develop amyloid beta (Aβ) plaques, a pathophysiological marker of AD, by age 40 (Korbel et al., 2009; Coppus A, et al. 2006). Because DS is caused by full or partial trisomy of HSA21, which genes are triplicated can vary greatly between patients. Variability in presentation and severity of signs, symptoms and co-morbidities in DS depends partly on gene triplication, dosage, and penetrance (Gardiner et al., 2004).

1.1.1 Interactions between upregulated HSA21 genes in Down syndrome.

By age 60, AD-related harm is the leading cause of mortality in DS patients (Coppus A et al., 2006). The high comorbidity between AD and DS presents a challenge to the affected community and raises the question as to whether a genetically-derived developmental disorder enhances the likelihood of AD onset.

Amyloid **P**recursor **P**rotein (APP) is among the HSA21 genes associated with neurodegeneration (Doran et al., 2017). Indeed, studies have shown increased lymphocytic APP protein expression in DS patients compared to age-matched, neurotypical controls (Pallister et

al., 1997). Meanwhile, increased APP results in higher levels of **a**myloid-**β** (Aβ) and the formation of extracellular Aβ plaques which, while contested as a marker versus causal component of general AD, are thought to causally contribute to DS-AD pathology (Tosh et al., 2021). Because AD has only been observed in DS patients with triplication of APP (Doran et al., 2017; O'Doherty et al., 2005) and because AD severity varies significantly between patients (Salehi et al., 2006; Ahktar and Bokhari, 2021), this raises the question as to how other HSA21 genes aside from APP contribute to or worsen AD progression.

Current evidence implicates genetic interactions in determining DS-AD severity. TC1 mice are DS model mice which carry a third copy for most of mouse chromosome 16, the homologous chromosome to HSA21 (O'Doherty et al., 2005). When TC1 mice are crossed to maintain triplication of the HSA21-like genomic region but only have two functional copies of APP, they lack AD pathophysiology (Wiseman et al., 2018). Meanwhile, while APP gain-of-function mice show modest neurodegeneration, addition of HSA21 trisomy worsens neurodegenerative phenotypes, suggesting that genetic interactions with APP determine phenotypic severity in DS-AD models (Wiseman et al., 2018). These prior works suggest that while APP is necessary for DS-AD, interactions of APP with other HSA21 genes may contribute to DS-AD severity.

Because HSA21-gene dysregulation occurs throughout development in DS and because prior work suggests that APP interacts with other genes in DS-AD pathogenesis, this dissertation examined the interactions of APP and HSA21 homologues during development.

1.1.2 Atypical axon and dendrite patterning in Down syndrome.

DS models consistently show altered subcellular patterning. Various works have shown atypical dendritic arbors and spines, decreased synaptic density, and atypical synapse morphology in DS fetuses (Becker et al., 1986; Becker et al., 1991; Marin-Padilla, 1976; Suetsugu and Mehraein, 1980; Benavides-Piccione et al., 2004). Similarly, in the Ts1Cje and TsDn65 DS mouse models, both hippocampal and neocortical regions show lower spine density and increased spine volume (Belichenko et al., 2004; Belichenko et al., 2007; Cramer and Galdzicki, 2012). Note, Ts1Cje mice and TsDn65 mice both model DS with smaller triplications of mouse chromosome 16 than TC1 mice; Between Ts1Cje mice and TsDn65 mice, the TsDn65 mice have a larger triplication which includes the entire length of the Ts1Cje triplication in addition to a region containing APP (Rachidi and Lopes, 2007). Work from the Ye Lab has also shown that interneurons known as chandelier cells, which inhibit the axon initial segment (AIS) of many pyramidal neurons (Inan and Anderson, 2014), show increased synaptic bouton number and axon terminal length in the anterior cingulate cortex (ACC) of Ts65Dn DS model mice (Liu et al, 2020).

These morphological changes are thought to contribute to changes in cellular activity including the disruptions of circuit plasticity and regional excitatory-inhibitory balances in DS. Human DS patients have shown decreased motor cortex plasticity (Battaglia et al., 2008). **L**ong **T**erm **P**otentiation (LTP) and **L**ong **T**erm **D**epression (LTD) are well-established mechanisms of plasticity, memory formation, and cognition (Cramer and Galdzicki, 2012). Across DS mouse models, altered LTP / LTD and decreased performance on cognitive tasks has been observed. For

example, enhanced LTD is observed in TsDn65 hippocampi and can be reversed with an NMDA receptor antagonist that also improves the cognitive performance of Ts65dn mice (Siarey et al., 1999; Scott-McKean and Costa, 2011; Rueda et al., 2010; Lockrow et al., 2011). Meanwhile, regionalized excess inhibition may contribute to the observed suppression of hippocampal LTP in TsDn65 and Ts1Cje DS-mouse models (Siarey et al., 1997; Kleschevnikov et al., 2004; Costa et al., 2005; Costa et al., 2008; Siarey et al., 2005; Beklichenko et al., 2007; Cramer and Galdzicki, 2012). In the Ye Lab's work, increased inhibition of pyramidal cells was observed in the TsDn65 mouse ACC (Liu et al, 2020). Like in the models described above, neuron morphology and the resulting changes in cellular function may have significant implications for DS pathology.

Results from this dissertation show than an interaction between two upregulated HSA21 homologues promote axon overgrowth. The *Drosophila* APP homologue **A**myloid **p**recursor **p**rotein-**l**ike (Appl) upregulates another HSA21 homologue, **D**own **s**yndrome **c**ell **a**dhesion **m**olecule (Dscam) to drive axon terminal growth. This work tested the developmental interactions of Dscam and Appl in axon terminal patterning.

1.2 Coordination of neuronal compartment patterning in development.

Most neurons consist of three major subcellular regions: the dendrites which receive input, the axon which carries signals to synaptic partners, and the soma where the nucleus is housed. As discussed, a mature neuron achieves a morphology tailored to its function within circuits. For example, *Drosophila* C4da neurons have expansive, non-overlapping dendritic branches to allow for topographic encoding of noxious stimuli and long axons for carrying this peripheral stimulus information to the central nervous system (Fig 1). Proper development and maturation of these neuronal compartments is essential for achieving functional circuitry and behavioral output. Below I provide a brief overview of some currently understood mechanisms of subcellular development, dividing axon/dendrite regulators between those that are compartment-dedicated and those that are compartment-generalized.

1.2.1 Compartment-dedicated regulators of axon or dendrite growth.

The first set of mechanisms which regulate neuronal compartments can be described as compartment-dedicated. These factors preferentially affect either axons or dendrites. Logically, these regulators can thus be broken down into two categories: 1) dendrite-dedicated and 2) axon-dedicated.

Dendrite-dedicated regulators preferentially affect dendrite growth in neurotypical development.

Dendrite-dedicated regulators promote dendritic growth through both centralized and localized mechanisms. For example, BMP-7, NeuroD, and Dar1 alter transcription to promote dendritic growth. Meanwhile Dar2, Dar3 and Dar6 accomplish similar dendritic growth by modulating ER-Golgi transport.

Bone **m**orphogenetic **p**rotein growth factor **7** (BMP-7), promotes dendritic growth through transcriptional regulation. BMP-7, also known as osteogenetic protein-1 (OP-1), is part of the transforming growth factor superfamily (TGF-β) which is expressed throughout the nervous system (Sampath TK, et. al. 1992). Three tiers of evidence implicate BMP-7 as a dendrite-specific growth factor. First, BMP-7 initiates the growth of dendrites but not axons in cultured rat sympathetic neurons (Guo et al., 1998; Lein et al., 1995). Second, the addition of BMP-7 to cultured rat sympathetic neurons results in increased dendrite, but not axon, number (Lein et al., 1995). Finally, in cultured cerebellar and hippocampal rat neurons BMP-7 increases dendritic complexity by increasing dendritic length and the total number of higher order dendritic branches after initial growth (Le Roux et al., 1999; Withers et al., 2000). While these findings would benefit from *in vivo* replication, this suggests that BMP-7 drives dendrite-specific growth across several models.

BMP-7 promotes dendritic growth by indirectly altering transcriptional programs. Ligands which bind BMPs generally result in the phosphorylation of SMAD1/5 which in turn form transcriptional complexes (Massagué and Chen, 2000). Supporting this mechanism, the addition of the transcriptional inhibitor actinomycin-D with BMP-7 to cultured rat sympathetic

neurons prevents the dendritic growth observed with BMP-7 treatment alone (Garred et al., 2011). Furthermore, microarray analysis in these same neurons after BMP-7 treatment shows upregulation of transcriptional repressors in the **I**nhibitor of **D**NA (Id) family; This upregulation can be blocked with actinomycin-D but not with the translational blocker cycloheximide, supporting that BMP-7 transcriptionally upregulates these transcriptional repressors (Garred et al., 2011). BMP-7 also increases the expression of **m**icrotubule **a**ssociated **p**rotein 2 (MAP2), which is important for expanding the dendritic cytoskeleton (Guo et al., 1998). Thus BMP-7 alters transcriptional programing to promote dendritic, but not axonal, growth.

Several other dendrite-dedicated growth factors induce transcriptional changes, including Dar1 and NeuroD. Research has demonstrated that Dar1 promotes microtubule-based dendritic growth in dendritic arborization neurons of *Drosophila* (Ye et al., 2007; Ye et al., 2011). Meanwhile, in granule cells from both primary culture and cerebellar slices RNAi knock-down of NeuroD was found to impair dendritic growth without impacting axon development (Gaudillière et al., 2004).

Another set of dendrite-dedicated factors modulate ER-Golgi transport to increase dendritic growth. Unlike the cell-centralized transcriptional mechanisms previously described, the regulation of Golgi outposts offers a local mechanism by which factors can promote growth of dendrites without affecting distant axons. The ER-to-Golgi transport factors include Dar2, Dar3, and Dar6 and correspond to human homologues Sec23, Sar1 and Rab1 of the same function (Ye et al., 2007). Single-cell *dar3* loss of function mutants display aberrant Golgi structures and significantly shortened dendrites but show typical axon structure (Ye et al., 2007).

This effect on dendrites was also observed with a knock-down of the homologous Sar1 in cultured mammalian hippocampal neurons (Ye et al., 2007).

Axon-dedicated mechanisms preferentially affect axon growth in neurotypical development.

This section describes examples of axon preferential regulators, including Rac1- which modulates the actin cytoskeleton- and SnoN-p300 -which alters transcription.

Rac1 is a small GTPase belonging to the Rac / Rho / Cdc42 subfamily which regulates the actin cytoskeleton (Luo et al., 1994). Both dominant-negative and constitutively-active mutations of DRac1, the *Drosophila* homologue of Rac1, result in a loss of axonal projections between the dorsal and lateral clusters of the peripheral nervous system (Luo et al., 1994). Experiments in Purkinje cells of transgenic mice corroborate the axon-dedicated function of Rac1 demonstrating that constitutively active Rac1 reduces axonal, but not dendritic, growth (Luo et al., 1996). It is worth noting that in Purkinje cells the number of dendritic spines, though not general growth, increases with constitutively active Rac1, likely due to F-actin dysregulation which functions in both dendritic spine and axons (Luo et al., 1996).

The SnoN-p300 transcriptional complex also promotes axonal, but not dendritic, growth. TGF-β signaling is transcriptionally repressed by **S**ki-related **no**vel protein **N** (SnoN) (Luo et al., 2004). Studies have shown that SnoN is necessary and sufficient for axon growth. In primary cerebellar granule cells cultured from P6 rats, RNAi knock-down of SnoN inhibited axonal growth while either expression of a degradation-resistant SnoN or overexpression of wildtype SnoN resulted in increased axon length (Stegmüller et al., 2006). Genetic profiling in rat

cerebellar granule neurons further revealed significant downregulation of altered genes with SnoN knockdown; considering the established interaction of SnoN with the transcriptional coactivator p300, evidence supports that the SnoN-p300 complex activates the transcription of genes relevant to axonal growth (Ikeuchi et al., 2009). This is further supported by the fact that knockdown of p300, like knockdown of SnoN, disrupts axon, but not dendrite, growth (Su et al., 2019).

1.2.2 Compartment-generalized mechanisms of growth.

Compartment-generalized regulators affect both axon and dendrite growth. These regulators can either have a uniform effect- and regulate axon-dendrite growth in the same direction- or a nonuniform effect- and oppositely regulate axons and dendrites.

Uniform compartment-generalized regulators promote general neuronal growth in both axons and dendrites. This includes BMP (Bond et al., 2012) and various neurotrophic factors (Huang and Reichardt, 2001). While these factors are crucial for typical neuronal development, factors promoting general growth are insufficient to explain how neurons form subcellular structures which can undergo growth at different rates.

Nonuniform compartment-generalized regulators could explain how axon and dendrite growth could be coordinated through differential control. These offer a potential explanation as to how neurons can respond to activity while establishing functional morphology yet maintain the relative proportions of axonal and dendritic size or complexity.

Axon-restricting and dendrite-promoting regulators.

The first set of nonuniform compartment-generalized regulators described below promote dendritic growth while restraining axonal growth.

Semaphorin 3A (Sema 3A) modulates axonal and dendritic growth through modulation of cAMP and cGMP. Sema3A differentially regulates axonal versus dendritic growth both during initial development and in mature cells (Shelly et al., 2011; Polleux et al., 2000). Sema3A suppresses axon initiation while promoting dendrite development (Shelly et al., 2010). In cultured hippocampal neurons, cAMP activates threonine / serine kinase LBK1 which activates **p**rotein **k**inase **A** (PKA) to drive axonal growth (Barnes et al., 2007; Shelly et al., 2011). Research in cultured hippocampal neurons suggests Sema3A inhibits cAMP expression and promotes cGMP expression (Shelly et al., 2011). cAMP in turn promotes axon initiation and blocks dendrite formation (Shelly et al., 2010) providing a neat mechanism for differential regulation of neuronal compartments by Sema3A.

In the cortex, Sema3A promotes apical dendrite formation while restricting cortical axon growth by acting as a chemoattractant and chemorepellent respectively (Polleux et al., 2000). This supports a conserved role of Sema3A in differential regulation of initial axonal and dendritic growth. This differential regulation continues with maturation, with an increase in dendritic complexity observed with exposure of neurons to Sema3A and cGMP; this effect was further found to be dependent on **p**rotein **k**inase **G** (PKG) activity (Shelly et al., 2011). Thus, Sema3A promotes dendritic formation and growth while repressing axonal growth by inhibiting cAMP, promoting cGMP, and modulating downstream effectors such as PKG, PKA, and LKB1.

A far less understood nonuniform compartment-generalized regulator is **CL**IP-**as**sociated binding **p**rotein **2** (CLASP2). In cultured cortical neurons, shRNA knockdown of CLASP2 results in axonal over-branching while reducing dendritic growth (Hur et al., 2011). CLASPs cooperate with **C**ytoplasmic **li**nker **p**roteins (CLIPs) and promote microtubule stability by binding to the plus end of microtubules (Galjart et al., 2005). Indeed, regulation of microtubules by CLIPs and CLASPs may play a role in the distinct organization of axons compared to dendrites and to general plasticity (Conde and Cáceres, 2009). However, the mechanism of differential axon-dendrite regulation by CLASP2 remains unknown (Galjart et al., 2005; Wittmann et al., 2005). Further work is needed to verify that the inverse regulation of dendritic and axonal extension by CLASP2 occurs through microtubule regulation rather than an alternative pathway.

Axon-promoting and dendrite-restricting regulators.

Rit is a Ras GTPase (Lee et al., 1996) like the previously discussed axon-dedicated Rac1. Overexpression of a dominant-negative Rit mutant in cultured hippocampal neurons resulted in reduced axon length and increased dendritic length, while overexpression of a constitutively active Rit mutant yielded the inverse effect (Lein et al., 2007). The mechanistic understanding of this differential regulation is limited. Prior work suggests constitutively active Rit requires MEK1; This implies that the **e**xtracellular signal **r**egulated **k**inase **1/2** (ERK1/2) is required for Rit to promote axon growth and restrict dendrite growth (Lein et al., 2007).

Finally, **d**ual **l**eucine zipper **k**inase (DLK) is a **m**itogen **a**ctivated **p**rotein **k**inase **k**inase **k**inase (MAPKKK) that promotes axonal growth and restricts dendritic growth (Ghosh-Roy et al., 2010). Indeed, the DLK pathway is well-established in regulating axon growth, regeneration, and degeneration (Collins et al., 2006; Hammurlund et al., 2009; Nakata et al., 2005.; Lewcock et al., 2007.; Tedeschi et al., 2013.; Watkins et al., 2013; Xiong et al 2010.; Yan et al., 2009; Xiong et al., 2012) as well as organizing the presynaptic structures of axon terminals (Klinedinst et al., 2013). Upstream of DLK, **P**AM/**H**ighwire/**R**PM-1 (PHR) is an E3 ubiquitin ligase that targets DLK for degradation (Collins et al., 2006; Nakata et al., 2005).

Axon terminal overgrowth occurs with both overexpression of DLK and inhibition of PHR in a variety of neuron types and species including *C. Elegans*, *Drosophila*, and various mammals (Wang et al., 2013; Collins et al., 2006; Lewock et al., 2007; Zhen et al., 2000; Wan et al., 2000; Wu et al., 2005). Conversely, loss of DLK prevents new axon outgrowth after nerve injury (Hammurlund et al., 2009; Watkins et al., 2013; Xiong et al., 2012; Klinedinst et al., 2013; Shin et al., 2012).

Overexpressing Wallenda (Wnd), the fly homologue of DLK (Collins et al., 2006), both promoted axonal growth and restricted dendritic branching in *Drosophila* C4da neurons (Wang et al., 2013). This effect was observed both with Wnd overexpression and loss-of-function for highwire (Hiw), the fly PHR homologue (Wan et al., 2000).

To achieve opposite regulation of axons and dendrites Wnd regulates Dscam and Knot. Prior work has demonstrated that Wnd upregulates Dscam to promote axon growth by increasing Dscam protein expression (Kim et al., 2013). Wnd neither binds to nor changes the whole cell

transcript levels of Dscam (Kim et al., 2013). This suggests that Wnd post-transcriptionally regulates Dscam. Meanwhile Wnd represses the transcription factor Knot to restrict dendrite development (Wang et al., 2013; Kim et al., 2013). In *Drosophila* dendritic arborizations neurons Class I-III, which lack Knot, the DLK/Wnd pathway only regulates axons (Wang et al., 2013).

The bimodal function of the DLK/Wnd pathway offers potential translational Applications as DLK/Wnd expression increases in fly and mouse models after nerve crush (Xiong et al., 2012; Watkins et al., 2013) and thus would provide an explanation for the restriction of dendritic growth while regeneration occurs as a mechanism of preserving functional morphology.

This dissertation Applied the Wnd/DLK pathway to examine how a cell differentially regulates compartments in response to changes in the circuit. Specifically, I examined how an axon-localized event in C4da neurons altered Wnd/DLK signaling to dendrites of the same cell.

1.3 Scope of this Dissertation

This dissertation interrogates two aspects of subcellular development in both neuro-atypical and neurotypical development.

First, Chapter 2 tests how genetic interactions between the DS-related genes *Appl* and *Dscam* alter axonal patterning in modelling DS. We show that Appl post-transcriptionally promotes Dscam expression possibly through modulation of the endosomal factor Rab5. This

models the upregulation of multiple DS-genes seen in DS patients and investigates the impact on axon patterning in development.

Second, Chapter 3 examines how cells coordinate axon and dendrite development within the same cell. We test how an axon-localized event, ablation of second order neurons within nociceptive circuitry, alters signaling to dendrites through the Wnd/DLK pathway.

These works Apply *Drosophila* genetics to interrogate several molecular mechanisms of axon / dendrite patterning in development. Both projects utilize C4da neurons (Fig 1) to examine changes in axons and / or dendrites.

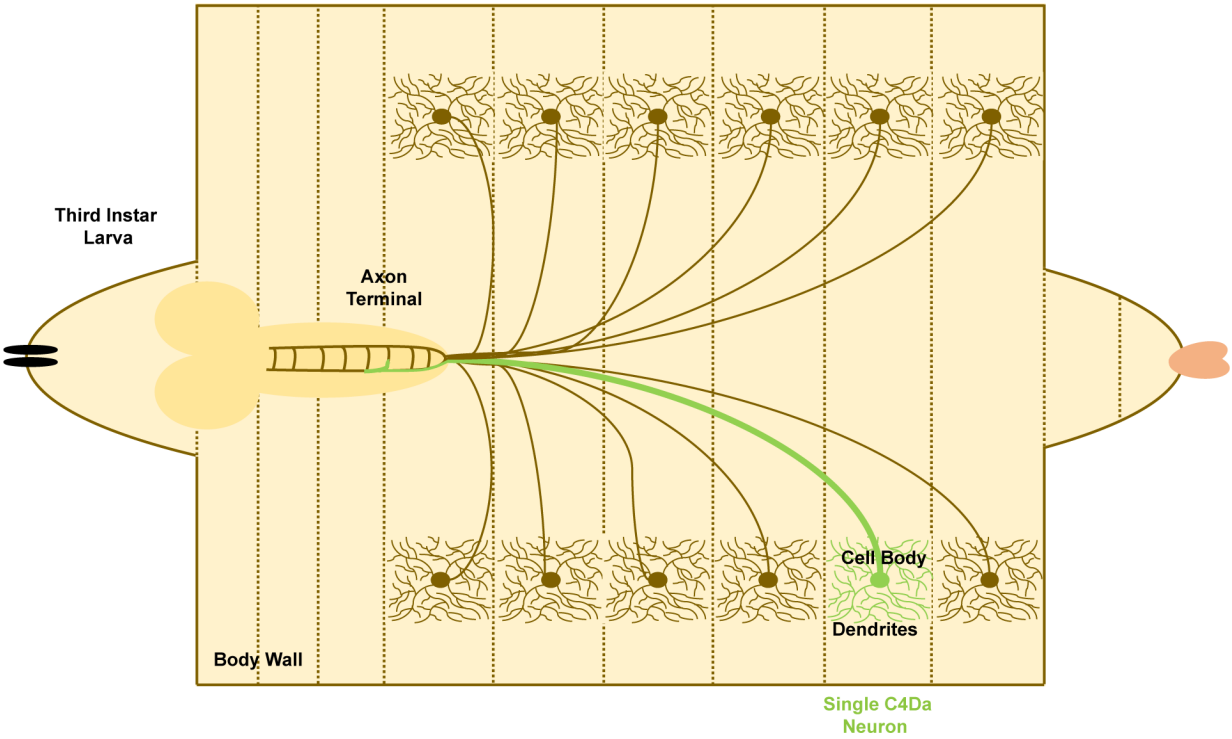

Figure 1: Class IV Dendritic Arborization (C4da) neurons as a model for dendrite and axon development.

The figure above shows the titling of C4da dendrites on the *Drosophila* larva body wall. As shown by the highlighted single C4da neuron, these nociceptors project into the ventral nerve chord (VNC) of the central nervous system (CNS). There, C4da axon terminals synapse onto second order neurons, the axons forming a ladder-like structure with other C4da neurons.

Chapter 2 *Appl* Promotes the Expression *Dscam* to Drive Axon Terminal Growth During Development.

2.1 Abstract

We interrogated how multiple Down Syndrome (DS) -related genes interact to establish axon morphology. This project employed *Drosophila* genetics, confocal microscopy, and biochemical techniques. We found that **A**myloid **p**recursor **p**rotein-**l**ike (Appl) upregulates **D**own **s**yndrome **c**ell **a**dhesion **m**olecule (Dscam) to promote axon terminal growth in **C**lass **IV** **d**endritic **a**rborization (C4da) neurons of *Drosophila*. Furthermore, we found that the post-transcriptional regulation of Dscam by Appl occurs independently of Appl cleavage and intracellular domains. Finally, my work interrogated how Appl potentially regulates transmembrane expression more broadly by modulating Rab5 expression and endosomal processing. We showed a novel, developmental mechanism of two DS-related genes interacting to establish aberrant axon morphology. Such mechanisms offer a key consideration for future therapeutic interventions in aberrant connectivity in DS and the related persistence of DS-caused intellectual disability.

2.2 Introduction

Gene mapping in Down syndrome (DS) has identified dose-dependent genes which impact disorder severity. DS is a genetic disorder caused by partial or full trisomy of human chromosome 21 (HSA21). Accordingly, many of the triplicated genes show increased RNA and protein expression in DS patients (Olmos-Serrano et al., 2016; Cheon et al., 2003). This

widescale genetic dysregulation causes a range of well-characterized signs, symptoms, and co-morbidities (Ahktar and Bokhari, 2021). Studies of partial trisomy DS patients have allowed for the mapping of some DS features to causal genes, such as the <2 Mb region responsible for determining the type and severity of DS-specific heart disease (Korbel et al., 2009).

Unlike other DS features, intellectual disability (ID) has not been isolated to a single causal region, despite its presence in about 99% of DS patients (Korbel et al., 2009). This begs the question as to whether HSA21 genes interact with one another to cause aberrant neurodevelopment and / or act independently on converging neurodevelopmental pathways.

We aimed to test genetic interactions between HSA21 genes using the powerful genetic tools of *Drosophila melanogaster*. While DS mouse models have successfully triplicated large sections homologous to HSA21 (O'Doherty A, 2005; Wiseman et al., 2018), these result in wide-scale genetic dysregulation which makes examining gene-interactions difficult. Furthermore, introducing multiple gain-of-function mutations in rodent models poses a significant technical challenge whereas the *Drosophila* Gal4/*uas* system (Brand and Perrimon, 1993) allows for overexpression of multiple HSA21 genes with spatial-, temporal-, and dosage- control. We used *Drosophila* to mimic the gain-of-function seen in DS individuals while isolating two HSA21 genes to test for molecular interactions during development. Our work elucidated that HSA21 homologues ***A****myloid* ***p****recursor* ***p****rotein-****l****ike* (*Appl*) and ***D****own* ***s****yndrome* ***c****ell* ***a****dhesion* ***m****olecule* (*Dscam*) interact to cause atypical axon patterning during development.

Appl is the highly conserved fly homologue to human ***A****myloid* ***P****recursor* ***P****rotein* (*APP*) (Rosen et al., 1989). APP is triplicated and has increased expression in DS patient neurons

(Oyama et al., 1994; Matsui et al., 2007; Rovelet-Lecrux et al., 2006; Sleegers et al., 2006; Rovelet-Lecrux et al., 2007; Kasuga et al., 2009; Sun et al., 2006; Wu et al., 2015; Wu et al., 2016) and alters both synapse formation and axon patterning during development (Hoe et al., 2012). Indeed, in *Drosophila*, APP and Appl induce axon terminal growth (Leyssen et al., 2005). Supporting prior literature, in this dissertation work we showed that *Appl* gain-of-function causes C4da axon terminal overgrowth during development.

As might be expected with the well-known AD-related processing of APP into Aβ, 98% of DS patients develop Aß plaques, by the age of 40 (Coppus et al. 2006). While the role of APP in general AD pathogenesis is debated, a DS-patient case study shows that a rare DS patient without APP triplication did not develop AD (Doran et al., 2017) reinforcing the importance of APP in DS-AD pathology. Accordingly, when TC1 DS model mice are crossed to maintain the triplicated genomic region but only have two functional copies of APP, they lack AD pathophysiology (O'Doherty et al., 2005; Wiseman et al., 2018). Prior works have shown that while APP gain-of-function mice show modest Aβ plaque formation, addition of HSA21-like trisomy worsens the phenotype despite HSA21-like trisomy without APP triplication causing no Aβ plaque formation; this supports genetic interactions determining phenotypic severity in DS-AD models (Wiseman et al., 2018). With these prior works showing the interactions of APP with other HSA21 genes in DS-AD pathophysiology, we examined the interactions of APP and another HSA21 homologue during development.

As shown in this dissertation, Dr. Gabriella Sterne and Dr. Macy Veling, two former graduate students in the Ye Lab, found that *Appl* promotes axon terminal growth by upregulating

the expression of Dscam, a highly conserved fly homologue to human DSCAM (Schmucker et al., 2000). DSCAM, known to contribute to DS-heart disorders, is also increased in DS patient neurons (Baumann et al., 2007). Prior works have demonstrated the importance of Dscam in establishing both dendritic and axonal patterning during development (Grueber et al., 2003; Jan et al., 2003; Sugimura et al., 2003; Wu et al., 2005; Zhen et al., 2000). Specifically, Dscam promotes C4da axon terminal overgrowth in a dose-dependent manner (Kim et al., 2013), which reflects the C4da phenotype we observed with overexpression of Appl.

In my study, I found that Appl may increase Dscam expression through the modulation of Rab5. This work elucidates a secretion- and intracellular domain-independent mechanism of Appl modulation of axon growth. Furthermore, I found that Appl increased the expression of an exogenous transmembrane protein as well, suggesting a broader effect. This finding suggests that interactions between triplicated HSA21 genes contribute to atypical neurodevelopment. Moreover, modulation of Rab5 endosomal signaling by Appl may affect other triplicated HSA21 genes on the membrane.

2.3 Materials and Methods

2.3.1 Drosophila genetics

uas-LacZ::GFP.nls (Shiga et al., 1996), w^{118} (Pastink et al., 1988), $Appl^{d}$ (Torroja et al., 1999), *uas-DscamTM2::GFP* #2 3.36.25 (Wang et al., 2004) , *uas-mCD8::GFP* (Lee et al., 1999), $Dscam^{18}$ (Wang et al., 2002), $hsFLP^{122}$ (Campbell et al 1993), FRT^{19A} (Xu and Rubin,

1993), *FRT^{G13}* (Lee et al., 1999), *ppk-RFP* (Han et al., 2011), *ppk-tdtomato* (Grueber et al., 2003), *ppk-Gal4* (Grueber et al., 2003) , yw;; nsyb-Gal4 (Flybase), *uas-APP695::myc* (Fossgreen et al, 1998), *uas-APP695ΔCT::myc* (Fossgreen et al., 1998); *uas-ApplΔE2* (Torroja et al., 1999) ; *uas-ApplΔE1* (Torroja et al., 1999), *yw ;P{UASp-YFP.Rab5}02* (Zhang et al., 2007), *yw ;P{UASp-YFP.Rab5.S43N}01* (Zhang et al., 2007)

2.3.2 Generation of DNA Constructs and Fly Lines

Dr. Macy Veling generated the *pUASTattB-Appl::V5* transgenic fly by isolating the *Appl* sequence from *w^{118} Drosophila* and subcloning it into the pUASTattB-V5 vector using the InFusion cloning system protocol (Clontech, Mountain View, California). She then injected the construct into *w^{118}* embryos (Veling, 2019).

Dr. Macy Veling also generated constructs for S2 Cell transfection by inserting the *Appl*, *CD8*, and *Dscam* sequences into the pAc5.1-V5/His or the pAc5.1-GFP plasmid backbone. I generated *E1-CD8::GFP* by cloning the endogenous Appl E1 domain into the pAc5.1_CD8::GFP construct. Plasmids were generated using the InFusion cloning system.

2.3.3 Labeling Presynaptic Terminals with Mosaic Analysis with Repressible Marker (MARCM)

Single presynaptic terminals were visualized with MARCM as previously described (Kim et al., 2013). Unlike the original protocol, *FRT^{G13} and FRT^{19A}* lines were heat-shocked for 15

minutes and no HRP staining was performed. Axon terminals were measured using Neurolucida software, and branches under 5 μm were excluded.

ppk-Gal4, hsFLP, UAS-mCD8::GFP; tub-Gal80, FRT^{G13} were mated with: 1) *uas-Appl::V5, FRT^{G13}, uas-lacZ::GFP.nls, FRT^{G13}* ; *2) uas-Appl::V5, uas-lacZ::GFP.nls , FRT^{G13}* ; *3) uas-DscamTM2::GFP, uas-lacZ::GFP.nls , FRT^{G13}* ; *4) uas-Appl::V5, uas-DscamTM2::GFP, FRT^{G13}; 5) Dscam^{18}, FRT^{G13}; 6) Dscam^{18}, uas-Appl::V5, FRT^{G13}* ; or *7) FRT^{G13}*.

ppk-Gal4, hsFLP, UAS-mCD8::GFP; tub-Gal80, FRT^{19A} were mated with: 1) *Appl^{d}, FRT^{19A}* ; 2) *FRT^{19A} ; 3) uas-DscamTM2::GFP, FRT^{19A}* ; *4) Appl^{d}, FRT^{19A}* ; or *5) Appl^{d},uas-DscamTM2::GFP, FRT^{19A}*.

2.3.4 Immunohistochemistry and Confocal Microscopy

Immunostaining of third-instar larvae is described in previous work (Ye et al., 2011). Antibodies used include chicken anti-GFP (Aves, Tigard, Oregon), rabbit anti-RFP (Rockland, Limerick, Pennsylvania), and mouse anti-Myc (Sigma Aldrich). After staining, fillets were dehydrated through a series of ethanol and xylene washes and were then mounted with DPX mounting media (Electron Microscopy Sciences, Hatfield, Pennsylvania). Confocal imaging was performed on a Leica SP5 confocal system equipped with a resonant scanner, 20X oil-immersion lens, and 63X oil-immersion lens. Images were collected and quantified as previously described in Kim et al., 2013.

2.3.5 Western Blotting

Blotting was performed as described previously (Kim et al., 2013). Samples were prepared to a final concentration of 1X SDS with beta-mercapto ethanol (BME) and run on 8% acrylamide gel on a BioRad Mini-Protean Tetra Cell system. Primary antibodies included rabbit anti-GFP (gift from Dr. Yang Hong, Hong et al., 2003), mouse anti- V5 (Invitrogen), mouse anti-a-Tubulin (Developmental Studies Hybridoma Bank), rat anti- Elav (Developmental Studies Hybridoma Bank), mouse anti- Dscam Exon18 (gift from Dr. Tzumin Lee, Shi et al., 2007), and rabbit anti-Rab5 (Abcam). Secondary antibodies included mouse anti- HRP, rabbit anti-HRP, and rat anti-HRP (all by Cayman Chemical Company). Chemiluminescence was detected using the ABC-HRP Kit (Vector Laboratories) and a BioRad Chemidoc for imaging. Pixel intensity was measured using Fiji ImageJ software to determine the total arbitrary units under the curve for a given band. Chemiluminescence for all quantified protein bands was normalized to that of a housekeeping protein (α-Tubulin or Elav).

2.3.6 RT qPCR (Real Time quantitative polymerase chain reaction)

RT-qPCR was performed as described previously (Kim et al., 2013). *Chmp1* was used as the reference gene. Two sets of primers were used to catch all known forms of *Dscam* isoforms. Analysis was performed using QuantStudio 5 Real-Time PCR Systems with Design and Analysis software 2.5 (Thermo Fisher). See primer sequences below (Kim et al., 2013):

Chmp1
5’-AAAGGCCAAGAAGGCGATTC-3’ and 5’-GGGCACTCATCCTGAGGTAGTT-3’

*Dscam*3Q
5’-CTTACGATTGTGCTCATTACTC-3’ and 5’CAGTTTCGATTTGTTCTGTTGG-3’

*Dscam*5Q
5’-ATCGAAACTGTTCAATGCAC-3’ and 5’-CTT GAGTGTATCTGTGTTTCGG-3’

2.3.7 S2 Cell Culture and Cycloheximide assays

Drosophila Schneider 2 (S2) Cells were cultured in S2 media with 10% heat-inactivated fetal bovine serum (FBS) at 28°C. 500 ul of cells were plated at a density of 0.8 x 10^6 24 hours before transfection with home-made polycation polyethylimine (PEI). DNA was mixed with Opti-Mem Solution (Thermo Fisher) and PEI was used at a ratio to DNA of 1:5.

48 hours after transfection, wells of S2 cells were collected with ice-cold 1X PBS, centrifuged at 200 g for 2.5 minutes to remove media, and suspended in 50 uL 2x SDS with 2-mercaptoethanol. Cells were mechanically disrupted and sonicated prior to western blotting.

For the cycloheximide (CHX) assays, cycloheximide was dissolved in a minimal amount of DMSO and added to each well at T0 at the same concentration (0.5ng/uL). Wells were collected individually as described above at the corresponding 4-hour time increment and frozen immediately at -25°C to prevent degradation.

2.3.8 Experimental Design and Statistical Analysis

All statistical analysis was performed using GraphPad. Analysis was performed double-blind. Normality was assessed for all groups. For normal data, two-group comparisons were

made using an unpaired two-tailed t-test and multiple group comparisons were made using one-Way ANOVA with Tukey multiple comparisons post hoc analysis. For non-normal data, two-group comparisons were made using two-tailed Mann-Whitney U Test and multiple group comparisons were made using Kruskal-Wallis with Dunn's multiple comparisons post hoc analysis. For normal data compared to a theoretical value, such as rations compared to 1, one-sample t-tests were Applied. For all statistical analyses: ns for $p>0.05$, * for $p<0.05$, ** for $p<0.01$, *** for $p<0.001$, **** for $p<0.0001$.

2.4 Results

C4da neurons serve as a classic model for examining neuronal morphology (Grueber et al., 2003; Jan et al., 2003; Sugimura et al., 2003; Wu et al., 2005; Zhen et al., 2000). In *Drosophila*, these nociceptors have cell bodies and dendrites located on the larval body wall and axons that project into the ventral nerve chord (VNC) in the central nervous system (CNS) (Fig 1). This allows for clear visualization of axon morphology separate from dendritic structures. The consistency of C4da axon terminal morphology allows for reliable assessment of changes. We used this model to examine the morphological impacts of altering the DS-related *Drosophila* homologues *Appl* and *Dscam.*

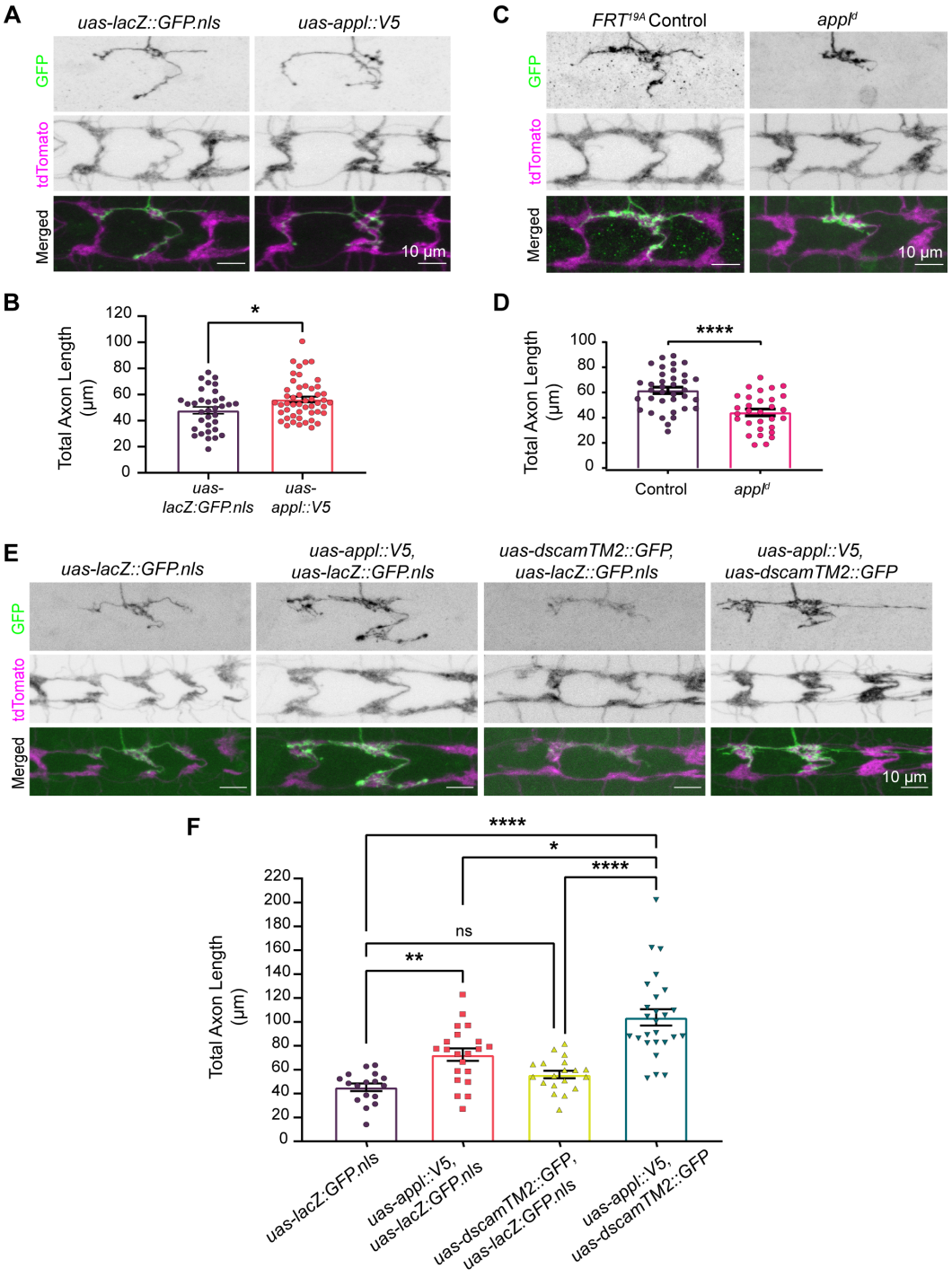

Figure 2: *Appl* loss- and gain-of-function alters C4da axon terminal length.

A,C,E) Representative images of the axon terminals of single C4da MARCM clones (GFP) contextualized in total C4da neuropil (tdTomato).

A) Images compare a single axon terminal with overexpression of *uas-Appl::V5* compared to a negative control with overexpression of *uas-lacZ::GFP.nls* both on an FRT^{G13}*, uas-mCD8::GFP* background (Veling, 2019).

B) Quantification of (A) shows a significant increase in the length of single C4da axon terminals with overexpression of *uas-Appl::V5*. Two-tailed Mann-Whitney U Test, $U = 614$, $p = 0.0424$, $n = 34$ cells for the control and $n = 49$ cells for Appl overexpression.

C) Images compare a single axon terminal on $Appl^{d}$ background compared to a wildtype *Appl* background (control) both with a FRT^{19A} *, uas-mCD8::GFP* background (Veling, 2019).

D) Quantification of (C) shows a significant decrease in the length of single C4da axon terminals with *Appl* loss-of-function. Unpaired two-tailed t-test, $p < 0.001$, $n = 36$ cells for the control and $n = 30$ cells for $Appl^{d}$.

E) Images compare a single axon terminal with overexpression of 1) *uas-Appl::V5 and uas-lacZ::GFP.nls* ($n = 21$ cells), 2*) uas-DscamTM2::GFP* and *uas-lacZ::GFP.nls* ($n = 19$ cells), and 3) *uas-Appl::V5* and *uas-DscamTM2::GFP* ($n = 26$ cells) compared to the control overexpression of *uas-lacZ::GFP.nls* ($n = 17$ cells) all on an FRT^{G13}*, uas-mCD8::GFP* background (Veling, 2019).

F) Quantification of (E) shows a significant increase in the length of single C4da axon terminals with co-overexpression of *DscamTM2::GFP* an *Appl::V5* compared to all other groups. One-Way ANOVA with Tukey multiple comparisons post hoc analysis.

2.4.1 Gain and loss of Appl during development respectively promotes and restricts C4da axon terminal length in Drosophila larvae.

With the developmental up-regulation of APP in DS well-established (Cataldo et al., 2008), we aimed to test how increasing *Appl* expression during development affected axon morphology. Prior work has shown that *Appl* loss-of-function impairs axon growth and synaptogenesis (Klinedinst et al., 2013). To test the developmental impact of *Appl* gain-of-function, we used the yeast-derived Gal4/*UAS* system (Brand and Perrimon, 1993), to achieve C4da-specific overexpression of a V5-tagged *Appl* construct (*uas-Appl*::V5) under the *pickpocket*

(*ppk*) promoter in late-third instar larvae (Grueber et al., 2003). To model an increased expression more biologically representative to DS than the artificially high expression that some *UAS* constructs achieve, Dr. Macy Veling generated a weaker-expressing *uas-Appl::V5* transgene (Veling, 2019).

Dr. Gabriella Sterne and Dr. Macy Veling examined single C4da axon terminals using Mosaic Analysis with Repressible Cell Marker (MARCM) (Lee and Luo, 1999) to compare *Appl* gain-of-function with overexpression of a control construct (*uas-lacZ::GFP.nls*) on the same genetic background. Overexpression of *Appl::V5* caused a 17% increase in C4da axon terminal length compared to the negative control (Fig 2A&B). This demonstrates that increasing *Appl* expression during development is sufficient to promote C4da axon terminal growth.

Dr. Gabriella Sterne and Dr. Macy Veling next examined if *Appl* loss-of-function caused a decrease in axon terminal length, to ensure that the overgrowth phenotype was not merely an artifact of overexpression. Using an *Appl* deletion mutant that results in no functional peptide ($Appl^{d}$) (Torroja et al., 1999), we again examined single C4da axon terminals with MARCM. Loss of *Appl* function caused a 28% decrease in axon terminal length compared to the control (Fig 2C&D). Thus, *Appl* is necessary to establish typical axon terminal length in C4da neurons during development.

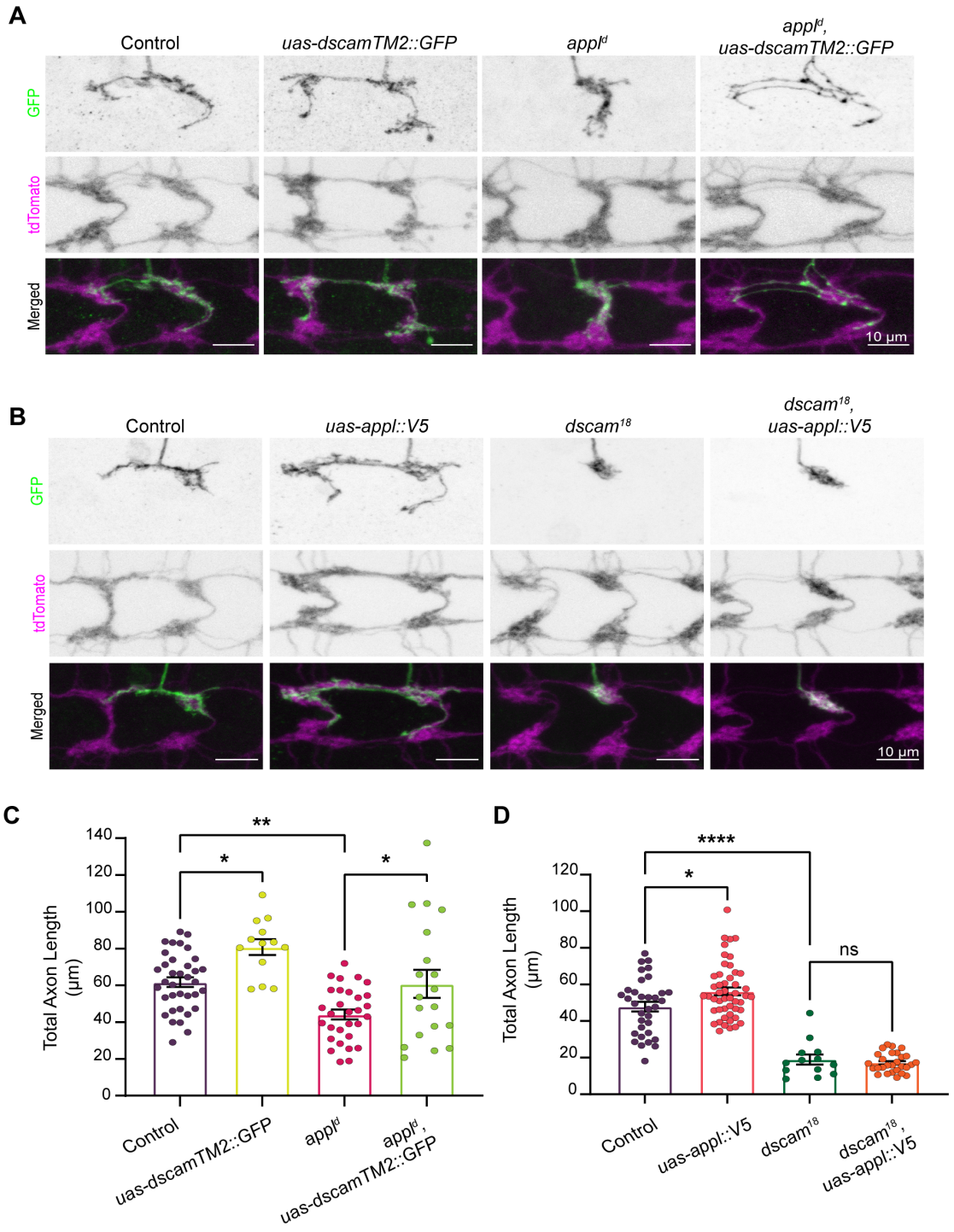

Figure 3: *Appl* requires *Dscam* to drive single C4da axon terminal growth but not the inverse.

A,B) Representative images of the axon terminals of single C4da MARCM clones (GFP) contextualized in total C4da neuropil (tdTomato). All overexpression is driven with *ppk-Gal4* and all backgrounds contain *uas-mCD8::GFP* for clone selection.

A) Images compare a single axon terminal of 1) *uas-DscamTM2::GFP* (n = 13), 2) $Appl^{d}$ (n = 30), and 3) $Appl^{d}$ and *uas-DscamTM2::GFP* (n = 19) to the control (n =36) all on an FRT^{19A} background (Veling, 2019).

B) Images compare a single axon terminal of 1*) uas-Appl::V5* (n = 49), 2) $Dscam^{18}$ (n = 13), and 3) $Dscam^{18}$ and *uas-Appl::V5* (n = 28) to the control *uas-mCD8::GFP* (n = 34) all on an FRT^{G13} background (Veling, 2019).

C) Quantification of (A) shows a significant increase in the length of single C4da axon terminals with overexpression of *DscamTM2::GFP* in the absence of functional *Appl*. One-Way ANOVA with Tukey multiple comparisons post hoc analysis.

D) Quantification of (B) shows no significant increase in the length of single C4da axon terminals with overexpression of *Appl::V5* in the absence of functional *Dscam*. One-Way ANOVA with Tukey multiple comparisons post hoc analysis.

2.4.2 Appl requires functional Dscam to promote C4da axon terminal growth.

We noted that the effect of *Appl* overexpression on C4da axon terminal growth reflected the effect of *Dscam* overexpression in the same cell-type observed in prior literature (Kim et al, 2013). Prior literature has shown that *Dscam* drives axon terminal growth during development (Wang et al., 2013; Bruce et al., 2017; Liu et al., 2020) and dose dependent *Dscam* expression drives proportional amounts of axon terminal growth (Kim et al., 2013). Like *Appl*, loss of *Dscam* restricts total axon terminal length (Kim et al., 2013).

Because of these comparable phenotypes, Dr. Macy Veling next tested if *Dscam* and *Appl* interact to establish axon terminal length during development. Through MARCM, she compared single-C4da axon terminals of: 1) a negative control group, 2) an *Appl::V5*-only

overexpressing group, 3) a *Dscam::GFP*-only overexpressing group, and 4) a group co-overexpressing *Appl::V5* and *Dscam::GFP*.

Note, the same number of *uas* sites was present in each experimental group to control for *uas*-site competition for Gal4 binding which would otherwise significantly alter expression levels for these dose-dependent genes. All groups expressed a cell membrane marker for labeling axon terminals (*uas-mCD8::GFP*). The negative control group, *Appl::V5*-only group, and *Dscam::GFP*-only group also express a control construct (*uas- LacZ::GFP.nls)*. This additional *uas* site in the *Dscam::GFP*-only overexpressing group, dilutes *Dscam* expression levels enough to not drive changes to the axon terminal length, as would be expected based on prior literature demonstrating the dose-dependent effect of *Dscam* expression on axon terminal length (Kim et al., 2013). By titrating *Dscam::GFP* expression to a non-phenotypic level, we tested if co-expression with *Appl::V5* could drive more axon growth than expression of either construct independently.

Co-overexpression caused a longer axon terminal than expressing either construct independently (Fig 2E&F). This suggests three possible hypotheses of *Dscam* and *Appl* promoting C4da axon terminal growth: 1) *Dscam* could act upstream of *Appl*; 2) Appl could act upstream of *Dscam*; or 3) *Dscam* and *Appl* could act on converging molecular pathways.

To address these hypotheses, we tested if *Dscam* could increase axon terminal length in the absence of functional *Appl*. Again, with MARCM, Dr. Macy Veling examined single C4da axon terminal length, this time on an *Appl* loss-of-function background (Fig 3A&C). As a positive control, *Dscam* overexpression caused the expected increase in axon terminal length

compared to a negative control. Similarly, loss of *Appl* caused an expected decrease in axon terminal length compared to the control. Importantly, Dscam caused an increase in axon terminal length without the presence of functional *Appl* compared to the Appl-null group. Thus, *Dscam* can drive axon terminal growth without *Appl* and *Appl* is not downstream of *Dscam*. Note, as this is not a complete normalization of the overgrowth phenotype, there are likely other, Dscam-independent pathways by which loss of *Appl* restricts axon terminal growth.

Dr. Macy Veling and I next tested if *Appl* could increase axon terminal length in the absence of functional *Dscam*. As a positive control, *Appl* overexpression caused an expected increase in axon terminal length compared to the control. Similarly, loss of *Dscam* caused an expected decrease in axon terminal length compared to the control. However, *Appl* did not cause an increase in axon terminal length in the absence of functional *Dscam* (Fig 3 B&D). Thus, *Appl* requires *Dscam* to drive axon terminal growth in C4da neurons. This suggests that *Dscam* is downstream of *Appl*.

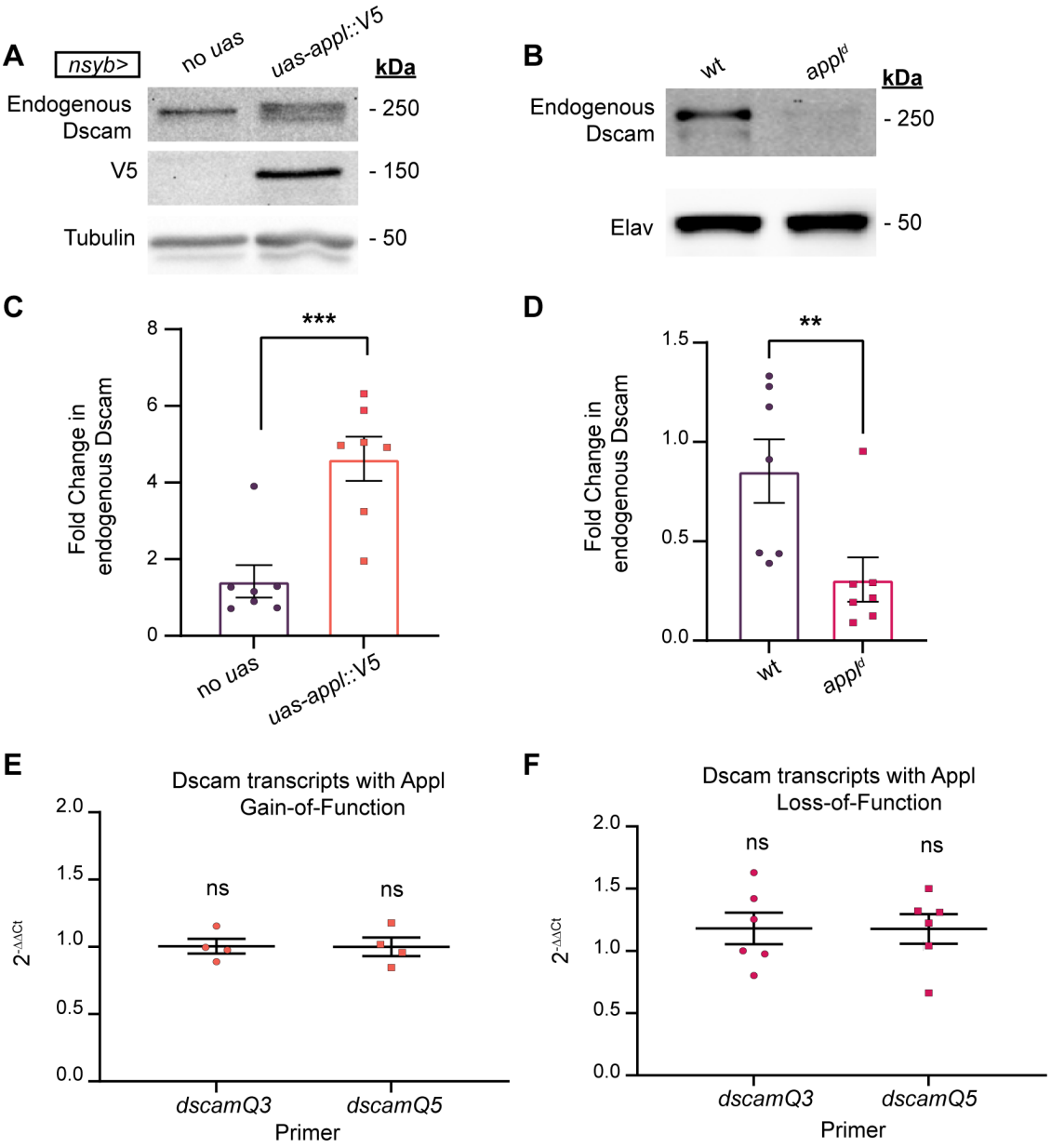

Figure 4: *Appl* modulates Dscam protein but not mRNA expression.

A) Western blot staining for endogenous Dscam (250 kDa), Appl::V5 (150 kDa), and Tub (50 kDa, housekeeping control for normalization) with Appl overexpression.

B) Western blot staining for endogenous Dscam (250 kDa) and Elav (50 kDa, housekeeping control for normalization) with loss of Appl (Veling, 2019).

C) Quantification of (A) shows significant increase of endogenous Dscam expression with pan-neuronal (*nsyb>Gal4*) overexpression of *uas-Appl::V5* compared to a "no *uas*" control (*w;;*). Two-tailed Mann-Whitney U Test, U = 2 , n = 7 groups of 20 larval CNS per genotype, p = 0.0023.

D) Quantification of (C) shows a significant decrease in endogenous *Dscam* expression with loss of *Appl* (*Appl*^{d}) compared to a wildtype control (*w;;*). Two-tailed Mann-Whitney U Test, U = 4, n = 6 groups of 20 larval VNCs per genotype, p = 0.0070.

E & F) Quantification of fold change in endogenous *Dscam* from RT-qPCR. No change was detected in endogenous *Dscam* transcript expression with gain or loss of *Appl*. Transcript expression was normalized internally to *chmp* (housekeeping gene). Two separate primers (*dscamQ3* and *dscamQ5*) were used to catch all known *Dscam* isoforms. Significance was assessed for each genetic manipulation and primer, (Veling, 2019).

E) $2^{-\Delta\Delta Ct}$ of *Dscam* transcripts for pan-neuronal overexpression of *Appl* (*nsyb>Appl::v5*) was calculated using control (*nsyb>mCD8::GFP*) to determine the fold change of transcripts. One-sample t-test, n = 4 biological replicates per genotype, p = 0.9493 and 0.9998 left to right.

F) $2^{-\Delta\Delta Ct}$ of *Dscam* transcripts with total loss *Appl* (*Appl*^{d}) was calculated using the control (w;;) to determine the fold change of transcripts. One-sample t-test, n = 6 biological replicates per genotype, p = 0.2132 and 0.1998 left to right.

2.4.3 Appl post-transcriptionally promotes Dscam protein expression.

To directly test the effect of Appl on Dscam, Dr. Macy Veling and I examined the impact of *Appl* loss- and gain-of-function on Dscam protein and RNA. To examine endogenous Dscam/*Dscam* expression, we used early-third instar larvae for protein and RNA collection since endogenous Dscam expression is higher and thus more easily detectable at this developmental timepoint. We used a pan-neuronal-driver (*nsyb*) to overexpress *Appl::V5* and assessed protein extracted from larval CNS via western blotting. When staining for endogenous Dscam we saw an increase in Dscam levels with *Appl::V5* overexpression compared to the no *uas* control (Fig

4A&C). Note, a double band of Dscam was also more consistently detected with *Appl* overexpression. Conversely, on an *Appl* null background the amount of endogenous Dscam decreased to one-third the amount observed in the wildtype control (Fig 4B&D). These data show that *Appl* regulates Dscam protein expression.

Next, Dr. Macy Veling and I tested if the same positive regulation of *Dscam* by *Appl* was present at the transcript level. We extracted RNA from the early- 3rd instar larvae CNS and determined transcript levels through real-time quantitative polymerase chain reaction (RT-qPCR). Two separate primers (*Dscam3qf* and *Dscam5qf*) were used against *Dscam* transcripts to catch all known isoforms. We detected no change in *Dscam* transcript levels with pan-neuronal *Appl* overexpression (Fig 4E) or whole-body loss-of-function (Fig 3F). This suggests that *Appl* regulates Dscam expression post-transcriptionally.

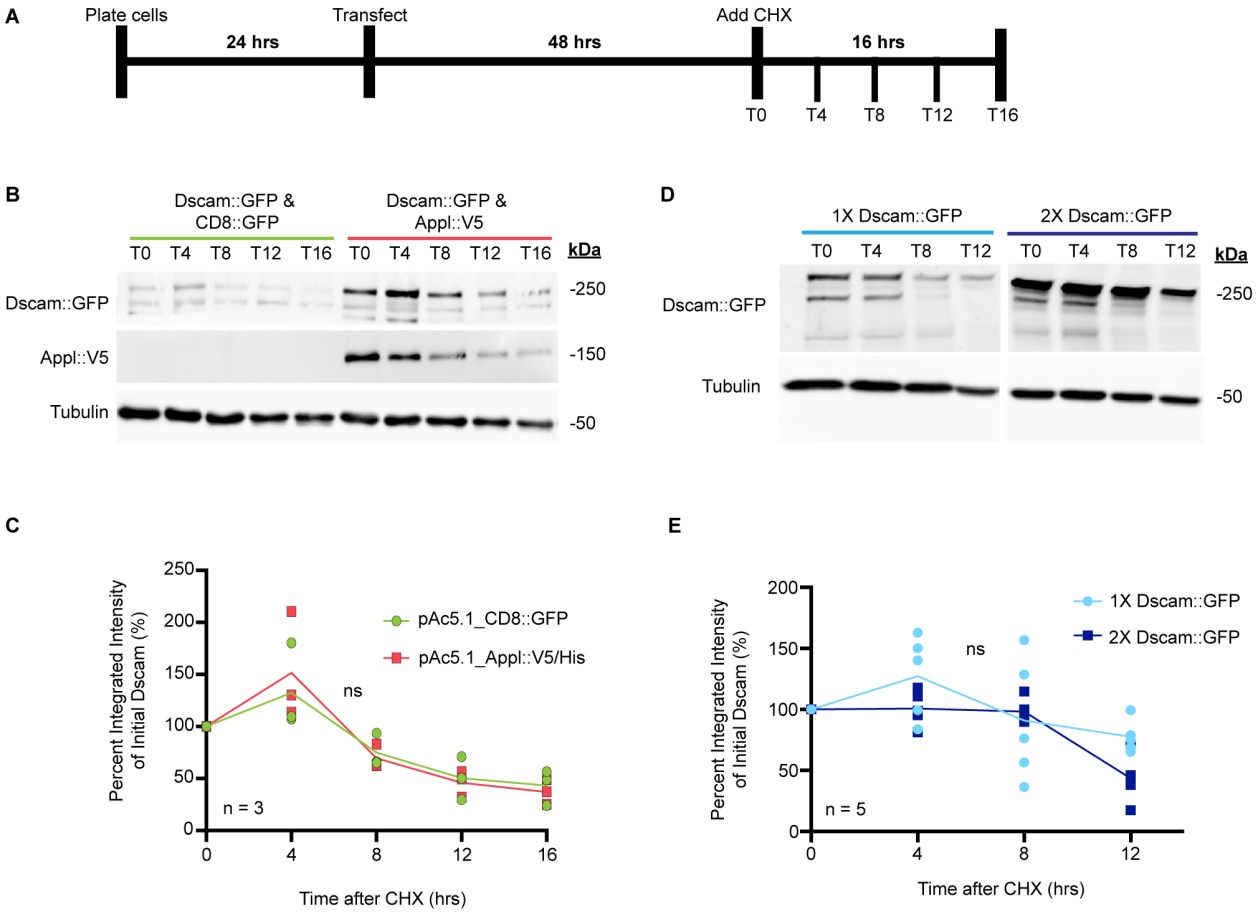

Figure 5: Gain of Appl causes no detectable change in the rate of Dscam protein degradation.

A) Experimental timeline for the cycloheximide assay to assess the rate of protein degradation in S2 Cells after the addition of the translational inhibitor cycloheximide (CHX).

B) A representative western blot shows the amount of Dscam::GFP detected from S2 Cell cultures collected at different timepoints after CHX treatment.

C) Quantification of (B) using Holm-Šídák Multiple t-tests. No difference was detected in the presence and absence of Appl::V5 when comparing the amount of Dscam::GFP relative to the initial timepoint.

D) A representative western blot shows the amount of Dscam::GFP detected from S2 Cell cultures collected at different timepoints after CHX treatment.

E) Quantification of (D) using Two Stage Set-Up Multiple t-tests (Benjamini, Krioger, & Yekutieli). No difference was detected between the low Dscam::GFP concentration group and the high Dscam::GFP concentration group when comparing the amount of Dscam::GFP relative to the initial timepoint.

2.4.4 Gain of Appl causes no detectable change in the rate of Dscam protein degradation.

Because Appl alters Dscam protein expression without affecting *Dscam* transcript expression, I aimed to identify the post-transcriptional regulatory mechanism. Because Appl and Dscam are both transmembrane proteins located on the cell membrane and could theoretically interact directly to form a stable complex, I first tested if Appl decreased Dscam protein turnover.

Using S2 cell culture, I co-expressed Dscam::GFP and either Appl::V5/His or an exogenous membrane protein (CD8::GFP) as a negative control. I then used the translational inhibitor cycloheximide (CHX) to prevent further protein synthesis (Fig 5A). By then sampling the cells in four hour increments, I tested if Dscam::GFP took longer to degrade in the presence of Appl::V5/His. I detected no difference in the rate of Dscam::GFP degradation in the absence and presence of Appl::V5/His (Fig 5B&C).

Note, transfection of Appl::V5/His with Dscam::GFP increases the initial amount of Dscam::GFP protein present, resembling the phenotype observed *in vivo* (Fig 4B&C). However, this caused cells expressing Appl::V5/His to have a higher initial amount of Dscam::GFP than the control. To ensure that the amount of initial Dscam does not affect the rate of Dscam::GFP degradation, I performed another cycloheximide experiment and varied the amount of

Dscam::GFP transfected. A control group transfected with *Dscam::GFP* (1X Dscam::GFP) was compared to a group with double the amount of *Dscam::GFP* (2X Dscam::GFP) (Fig 5 D&E). While Dscam::GFP was increased in the 2X Dscam::GFP group, I saw no difference in the rate of degradation between the two groups, suggesting that the initial concentration of Dscam::GFP did not affect the degradation rate (Fig 5 D&E). These findings suggested that Dscam protein turnover was not the most likely post-transcriptional mechanism to pursue.

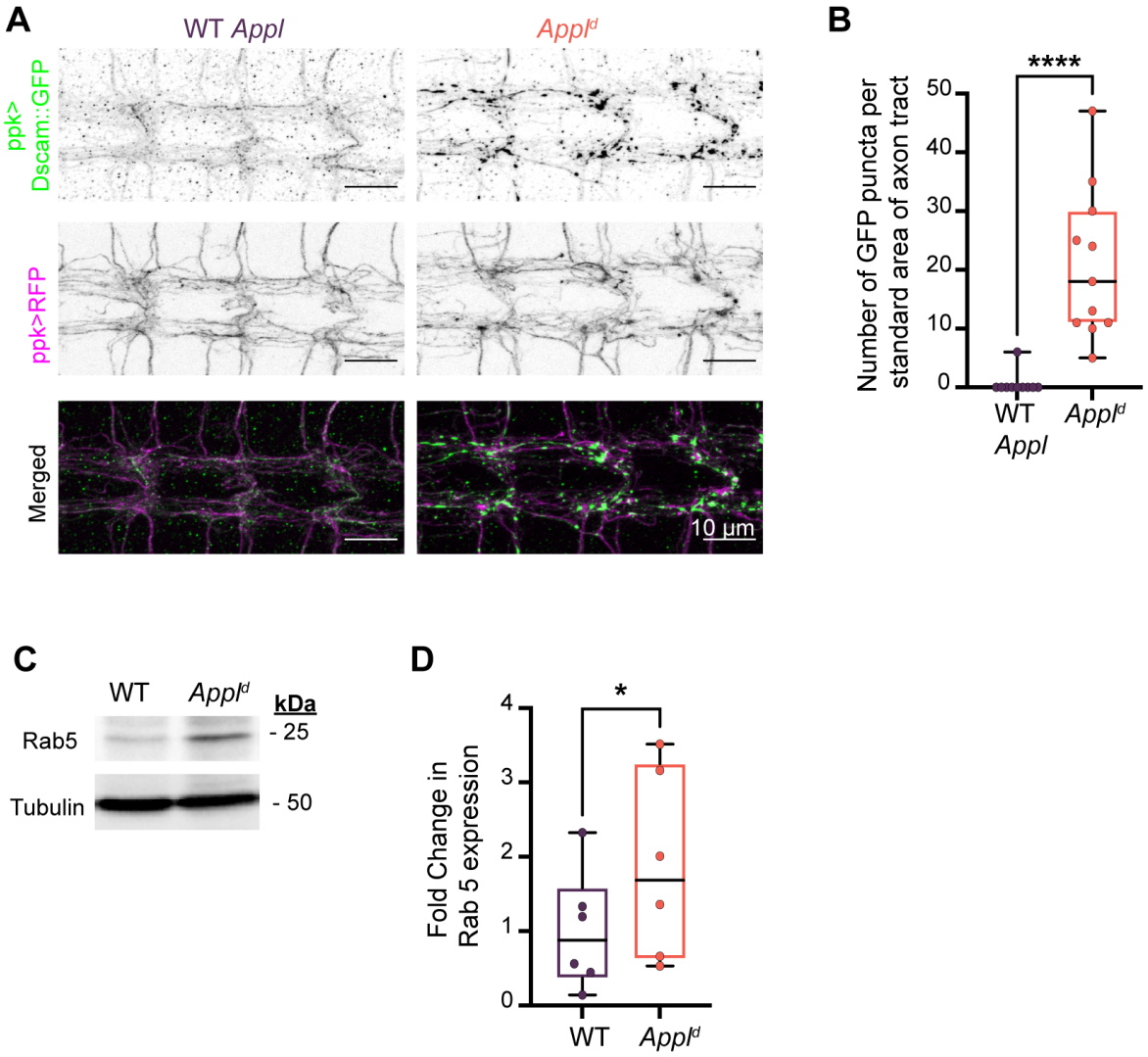

Figure 6: *Appl* modulates Rab5 expression levels.

A) Total C4da neuropil labeled by RFP fluorescence. *Pickpocket* (*ppk*) drives *uas-Dscam::GFP* expression in both an *Appl* loss-of-function (*Appl*^{d}) and a wildtype Appl (*w*$^{-}$*;;*) background. Puncta of Dscam::GFP are only visible on the loss-of-function background (Sterne, 2016).

B) Quantification of the Dscam puncta seen in (A). The number of puncta were counted in ImageJ with a consistent threshold to remove background noise outside of the neuropil. Two-tailed Mann-Whitney U Test, $p < 0.0001$, $n = 10 - 11$ larvae per genotype.

C) Western blotting for Rab5 and a housekeeping protein (Tubulin) in an *Appl* loss-of-function (*Appl*^{d}) and a wildtype (*w;;*) background. Loss of *Appl* significantly increases Rab5 expression in *Drosophila* CNS extracts.

D) Quantification of the fold change in Rab5 expression in (C). Unaired two-tailed t-test, p = 0.0372, n = 6 biological replicates per genotype composed of 15 CNS per replicate.

2.4.5 Appl may increase Dscam protein expression by modulating Rab5.

To isolate the post-transcriptional mechanism by which *Appl* regulates *Dscam,* Dr. Gabriella Sterne and I next examined the localization of Dscam with loss of *Appl*. Since our phenotype was axonal, we examined total C4da neuropil to assess if the patterning of Dscam on the membrane was altered by loss of *Appl*. Since loss of *Appl* reduces Dscam expression, we expected to see uniformly dimmer Dscam::GFP expression in the *Appl*^{d} mutant compared to a control with a wildtype *Appl* background. In both genotypes *ppk* drove *uas-Dscam::GFP* expression. Unexpectedly, loss of *Appl* resulted in the formation of Dscam::GFP puncta along the C4da neuropil not seen in the control (Fig 6A&B). In response to loss of *Appl*, we had expected to see a general decrease in Dscam::GFP signal. Surprisingly, unlike the uniform distribution of Dscam::GFP along the neuropil as seen in the control lines, Dscam::GFP appeared to clump in the absence of functional *Appl*.

APP in DS has been well-characterized for its activation of endosomal pathways through Rab5 signaling (Xu et al., 2016; Cataldo et al., 2003; Cattaneo and Calissano, 2012; Salehi et al., 2006). Since these Dscam puncta occurred in the same condition that caused a decrease in the total amount of Dscam, we next interrogated if *Appl* manipulation affected Rab5 expression.

Prior literature has established that increased APP expression increases the amount of activated Rab5 protein and the resulting size of early endosomes (Xu et al., 2016; Cataldo et al., 2003; Cattaneo and Calissano, 2012; Salehi et al., 2006). However, my western blotting shows that loss of *Appl* results in an increase of total Rab5 expression in the *Drosophila* CNS (Fig 6C&D).

Because this effect differs between that observed in current literature, how Appl modulates Rab5 and endosomal activity requires further testing. One possible hypothesis is that the observed changes in Rab5 may indicate compensatory modulation of expression based on altered Rab5 activity. APP promotes Rab5 activity to cause enlarged endosomes with altered function, which in turn disrupts trafficking from the membrane (Xu et al., 2016; Cataldo et al., 2003; Cattaneo and Calissano, 2012; Salehi et al., 2006). Potentially, Appl increases Rab5 activity, resulting in higher Rab5 turnover and lower total expression; the increased Rab5 activity then could increase Dscam expression due to failed trafficking from the membrane. This hypothesis requires direct testing. Please see Chapter 4 for discussion on future testing of this hypothesis.

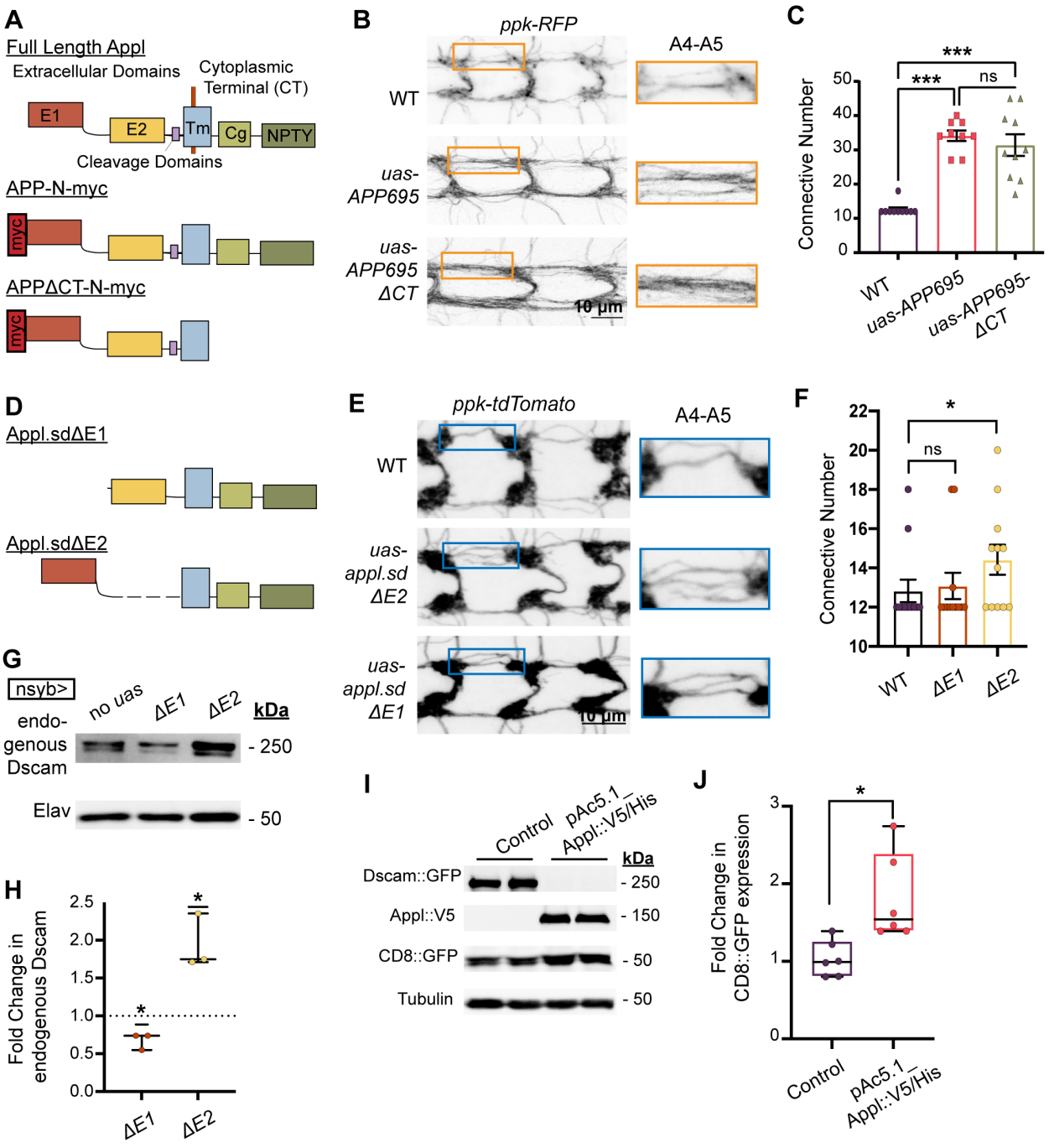

Figure 7: *Appl* may generally affect transmembrane protein expression through a secretion- and intracellular domain-independent mechanism.

A) Schematic of the domains in full length Appl and the APP mutants used in B&C.

B) Total C4da neuropil labeled by RFP fluorescence with: 1) *ppk*>no overexpression (wildtype, *w;;*), 2) *ppk>uas-APP695::myc,* and 3) *ppk>uas-APP695ΔCT::myc*. The orange box indicates the magnified segment to the right for viewing the axon tracts which were individually counted (Sterne, 2016).

C) Connective Number describes the total number of visible axon tracts between segments A4-A6. Quantification of (B) shows a significant increase in the number of axon tracts with C4da-specific overexpression of APP695 (*uas-APP695::myc*) and APP695 lacking the cytoplasmic domain (*uas-APP695DCT::myc*) compared to the control. Kruskal-Wallis with Dunn's Multiple comparisons post hoc analysis, n = 9-10 per genotype.

D) Schematic of the domains in the Appl mutants used in E&F.

E) Total C4da neuropil labeled by tdTomato fluorescence with 1) *ppk*>no overexpression (wildtype, *w;;*), 2) *ppk>uas-ApplΔE2*, and 3) *pp*k>*uas-ApplΔE1*. The blue box indicates the magnified segment to the right for viewing the axon tracts which were individually counted.

F) Quantification of (E) shows a significant increase in the number of axon tracts with C4da-specific overexpression of *Appl* lacking the E2 domain (*uas-Appl.sdΔE2*) but not with *Appl* lacking the E1 domain (*uas-Appl.sdΔE1*). Two-tailed Mann-Whitney U Test, U = 45 (top) and 66 (bottom), p = 0.0391(top) and p = 0.3975 (bottom), n = 12 larvae per genotype.

G) Western blot staining of endogenous Dscam (250 kDa) and housekeeping protein Elav (50 kDa) with pan-neuronal (*nsyb*) overexpression of nothing (no uas, *w;;*)**,** *uas-Appl.sdΔE2*, or *uas-Appl.sdΔE1*.

H) Quantification of (G) shows that Appl overexpression still increases endogenous Dscam expression even in the absence of the E2 domain and secretase binding sites but not in the absence the E1 domain. Two-tailed One-Sample t-test, p = 0.0096 (left)and 0.0186 (right).

I) Western blotting from S2 cell co-transfection of *pAc5.1_CD8::GFP* with either *pAc5.1_Dscam::GFP* (Control) or *pAc5.1_Appl::V5/His.*

J) Quantification of (I) shows a significant increase in the fold change of CD8::GFP in the presence of full length Appl. Unpaired two-tailed t-test p = 0.0107, n= 6 biological replicates per genotype across 2 sets of transfections.

2.4.6 Appl and its human homologue promote axon growth through an intracellular domain-independent pathway.

With Appl shown to modulate Rab5 expression, not just activity as seen in literature, we next sought to understand which domains of Appl/APP were required to drive axon growth. Prior works in DS mouse models and human cell cultures have shown that, in mature neurons, APP triplication results in enlarged endosomes through upregulation of Rab5 (Xu et al., 2016; Cataldo et al., 2003; Cattaneo and Calissano, 2012; Salehi et al., 2006). This promotion of Rab5 by APP depends on beta cleavage of APP and the resulting expression of C99 product derived from the intracellular APP cytoplasmic domain (Xu et al., 2016).

First, we aimed to test if human APP had the same effect on C4da axon terminals as the fly homologue. Human APP695 is the most commonly expressed isoform in neurons (Rohan de Silva et al., 1997; Kang and Muller-Hill, 1990). Prior work generated a *uas* line driving Myc-tagged human *APP695* (Fig 7A) (Mhatre et al., 2014). Dr. Gabriella Sterne and I quantified the connective number, or total number of visible axon projections between segments 4-6 in the CNS, to assess C4da axon overgrowth. Overexpression of the human APP construct showed about 3 times the connective number than the wildtype control (Fig 7B&C). Thus, *Drosophila Appl* and the human homologue both produce axon overgrowth when overexpressed in C4da neurons.

To determine if the intracellular domains were required for *APP* promotion of C4da growth, Dr. Gabriella Sterne expressed a construct lacking the intracellular domains of APP. Note, APP-Rab5-endosome signaling requires the C99 fragment derived from the intracellular, C-terminus domain of APP (Xu et al., 2016). We expressed a *uas* line driving Myc-tagged

human *APP-695* lacking the cytoplasmic domain (*uas-APP-695ΔCT*) (Fig 7A) (Fossgreen et al., 1998). I quantified overexpression of the human APP with or lacking the intracellular domain in C4da and saw that both cause a robust overgrowth phenotype when compared to the control (Fig 7 B&C). This shows that the cytoplasmic domains are not required for APP to increase C4da axon terminal growth. This suggests a C99-independent pathway for APP-to-axon growth signaling.

To directly test if the Appl-axon growth phenotype requires cleavage of Appl I next tested two mutants that lack the secretase binding sites. APP/Appl have two highly conserved extracellular domains - Extracellular Domain 1 (E1) and Extracellular Domain 2 (E2) (Rosen et al., 1989). Dr. Gabriella Sterne and I examined axon overgrowth when expressing *Appl* transgenes with excision of either extracellular domain. Both constructs were secretion deficient (.sd) and lacked the required binding sites for all known cleavage enzymes (Fig 7D) (Torroja et al., 1999). Transgenes were deletion mutants that produced peptides localized to the cell membrane but not the associated cleavage products in the extracellular matrix (Fig 7D) (Luo et al., 1992). If either construct still caused overgrowth in the absence of the excised domain, we could conclude that neither the missing domain nor cleavage is required for Appl to promote axon terminal growth. Note, constructs could not be directly compared to one another because they were not inserted into the same genomic locations and thus likely varied in the amount of construct expression. Overexpression of the Appl construct lacking Extracellular Domain 2 (uas-*ApplΔ E2*) still caused mild overgrowth in C4da axon terminals (Fig 7 E&F). By contrast, overexpression of the Appl construct lacking Extracellular Domain 1 (*uas-ApplΔ E1*) caused no

detectable overgrowth phenotype. This suggests that Appl does not require the E2 domain to drive C4da axon growth. Moreover, Appl can drive the observed axon overgrowth phenotype without cleavage.

I next tested if the same phenotypes were observed with selective extracellular domain loss-of-function when examining Dscam protein expression. Reflective of the axon phenotypes, pan-neuronal overexpression of *Appl.sdΔ E2* caused an increase in endogenous Dscam as shown via western blot (Fig 7 G&H). Meanwhile, pan-neuronal overexpression of *ApplΔ E1* caused a consistent mild decrease in endogenous Dscam expression (Fig 7 G&H). These data show that neither the Appl-E2-Domain nor Appl cleavage is necessary to cause C4da axon overgrowth or the required increase in Dscam.

Since Appl may alter endosomal signaling through Rab5 to increase Dscam, we suspected that Appl would affect transmembrane protein expression more broadly, rather than this being a unique pathway from Appl to Dscam. To test this, we transfected *Drosophila* S2 cells with an exogenous transmembrane protein derived from the immune system (CD8::GFP) and tested its relative expression in the absence and presence of Appl via western blot. Note, S2 cells do not express innate Appl (Soba et al., 2005), so without transfection the control cells entirely lacked Appl. We found that Appl significantly increased the expression of CD8::GFP (Fig 7I & J), suggesting that Appl more broadly regulates transmembrane protein expression. This broader regulation could also explain the remaining restriction of axon terminal length with *Dscam* overexpression in the absence of functional *Appl* (Fig 3A&C), which could be caused by other transmembrane proteins at the axon terminal downregulated in the absence of *Appl*. While

this result supports a broader effect of Appl, Appl-driven axon growth still requires functional *Dscam*. Please see Chapter 4 for further discussion.

2.5 Discussion

We showed that in development, Appl promotes axon terminal growth through upregulation of another HSA21 homologue, Dscam. Prior work showed that non-amyloidergic processing of APP and its homologues is important in neuronal development (Chow et al., 2010). For example, sAPPα causes increased proliferation of embryonic and adult neural stem cells (Ohsawa et al., 1999; Caillé et al., 2004) and acts as a growth factor in epidermis-derived cells (Herzog et al., 2004; Siemes et al., 2006). Moreover, outside of the context of DS, APP has been shown to be expressed early in development. In fetal mice, APP was detected by immunocytochemistry in radial glial cells (Trapp and Hauer, 1994; Nicolas and Hassan, 2014) and *App* mRNA was detected at the peak of neurite outgrowth and neural differentiation (Embryonic day 9.5) (Salbaum and Ruddle,1994). Indeed, in the *Drosophila* neuromuscular junction (NMJ), Appl regulates synaptogenesis, with a loss of *Appl* decreasing synaptic bouton number and overexpression of *Appl* increasing the synaptic bouton number (Torroja et al., 1999). This reflects the neurite patterning observed with the same manipulation in the fly mushroom body (Li et al., 2004; Soldano et al., 2013). Even in higher organisms, APP regulates synaptic growth with APP undergoing rapid axonal transport to synaptic sites (Koo et al., 1990) and with APP present in vesicular elements of dendrites and axons (Schubert et al., 1991). These works

are consistent with the Dscam-dependent axon terminal phenotypes that we observed with *Appl* loss- and gain-of-function.

Perhaps most surprising in this study was that the highly conserved *Drosophila* homologue of APP may repress total Rab5 expression, not described in prior literature. Works have shown that in DS models, increased APP expression causes an increase in Rab5 activation (Xu et al., 2016). This in turn causes enlarged early endosomes which are thought to contribute to disease progression and neurodegeneration in DS-AD (Xu et al., 2016; Cataldo et al., 2003; Cattaneo and Calissano, 2012; Salehi et al., 2006). Possibly, the effect of Appl on Rab5 that we saw could reflect compensation for the same increased Rab5 activation described in prior literature. Alternatively, differences in species or developmental timepoint could explain the discrepancy. The effect of Appl on Rab5 activity and the effect of Rab5 activity on Dscam requires further testing.

Rab5 activation by APP has been described as dependent on β-secretase cleavage of APP into secreted APP beta (sAPPβ) and C99 (Xu et al., 2016). Rab5 and endosomal activity have been further shown to be modulated by γ-secretase cleavage of sAPPβ into Amyloid beta (Aβ) (Bhattacharyya et al., 2022). However, in *Drosophila* both APP and Appl are rarely cleaved by γ- or β- secretase but rather are cleaved by α-secretase into the secreted amyloid precursor protein alpha (sAPPα) (Tan and Azzam, 2017). We showed that Dscam increases with expression of a secretion deficient Appl mutant, suggesting that the interrogated molecular pathway is independent of secreted products despite affecting Rab5 signaling. Rather than

contradicting prior literature, this finding begs the question as to in which cell types and at what developmental timepoints triplicated APP promotes or represses endosomal activity.

Given the developmental effects of APP and its homologues on axon patterning shown in this and prior studies and given that APP triplication in DS exists throughout development, this work further begs the question as to the significance of APP dysregulation during development in DS. Here, we show that Appl dysregulation has noticeable effects on axon patterning during development through modulation of the highly conserved Dscam protein. As we have shown in prior works, the effect of Dscam on axon patterning is conserved in Ts65dn model mice and results in altered GABAergic signaling in the anterior cingulate cortex (ACC) (Liu et al., 2020). While future studies should examine the novel pathway of Appl regulating Dscam in mammals, pharmaceutical interventions into endosomal dysregulation in DS-AD should consider the developmental and cell-type specific contributions of APP-endosomal signaling.

For discussion of limitations and future directions of this work, please see Chapter 4.

2.6 Description of Manuscript and Author's Contribution

The experiments described in this Chapter are in review for publication and can be found with the following citation:

Pizzano, S.*, Veling, M.*, Sterne G.R.*, and Ye, B. The *Drosophila* homologue of amyloid precursor protein promotes the expression of Down syndrome cell adhesion molecule to drive axon terminal growth. *Submitted.*
(*Co-First authors)

The relative contributions of each author to this work are as follows:

SP: Conceived of the project and designed the experiments. Performed MARCM for rescue of *Dscam* loss of function with overexpression of *Appl*, western blotting for *Appl* gain of function, analysis of western blotting for *Appl* loss and gain of function, analysis of qPCR for *Appl* loss and gain of function, analysis and staining for western blotting for Rab5 with *Appl* loss of function, analysis for visualizing Dscam puncta in C4da axon tracts, S2 cell transfection and western blotting for *Appl* overexpression effecting Rab5, western blotting for effect of Rab5 overexpression on endogenous Dscam, analysis of overexpression of human APP in C4da axon tracts, IHC and analysis for *Appl* mutant constructs in C4da axon tracts, western blotting and analysis for *Appl* mutant constructs, S2 cell transfection and western blotting and analysis for effect of Appl expression on CD8::GFP. Generated Figures. Wrote the paper.

MV: Conceived of the project and designed the experiments. Performed MARCM and analysis for: overexpression of *Appl*, loss of *Appl*, co-overexpression of *Appl* and *Dscam*, epistasis of *Dscam* loss of function with overexpression of *Appl*, epistasis of *Appl* loss of function with

overexpression of *Dscam*. Performed western blotting for *Appl* loss and gain of function, qPCR for *Appl* loss and gain of function, generated plasmids for S2 transfection, and generated *uas-Appl::V5* fly line.

GRS: Conceived of the project and designed the experiments. Performed total C4Da overexpression of Appl, MARCM for co-overexpression of *Appl* and *Dscam*, epistasis of *Dscam* loss of function with overexpression of *Appl*, epistasis of *Appl* loss of function with overexpression of *Dscam,* IHC for Dscam puncta in C4da axon tract, IHC for *Appl* mutant construct effect on C4da axon tracts, IHC for overexpression of APP in total C4da axon tracts.

NS: Assisted SP in experimental execution of IHC for *Appl* mutants in C4da axon tracts.

AP: Assisted SP in experimental execution of western blotting for *Appl* mutant constructs.

BY: Conceived of the project and designed the experiments, supervised project, wrote the paper.

Chapter 3 The Wnd/DLK Pathway Coordinates Axonal Events with Dendritic Signaling.

3.1 Abstract

The second half of this dissertation interrogates the coordination of axon-localized events to dendritic signaling. I Applied *Drosophila* genetics, immunohistochemical techniques, and confocal microscopy to address how an axon-localized event may activate the Wnd/DLK pathway to alter signaling to dendrites within the same neuron. I found that ablation of second order neurons (SONs) within the C4da nociceptive pathway resulted in decreased expression of nuclear Knot, a dendritic growth regulator. This mimics the phenotype observed in C4da neurons with activation of the Wnd/DLK pathway. Moreover, supporting prior literature on axon injury, the Wnd/DLK pathway required retrograde transport to repress nuclear Knot expression and to promote axon growth. This work interrogated an essential yet poorly understood mechanism by which neurons coordinate axonal events to dendritic growth to maintain functional circuitry in response to changes at the axon terminal. Such mechanisms are critical for understanding how to therapeutically intervene in disruptions to functional circuitry and how cellular coordination goes awry in establishing aberrant connectivity in neurodevelopmental disorders.

3.2 Introduction

3.2.1 Wnd/DLK signaling in coordinating dendrite-axon patterning.

Neurons develop dendrites and axons with morphologies tailored to their function within neural circuits. For example, C4da neurons have expansive, non-overlapping dendritic branches that allow for topographic encoding of noxious stimuli. These same neurons also have short, simple axon terminals for conveying this peripheral information to the central nervous system (CNS). Defects in these refined structures can lead to circuit-level dysregulation. Indeed, many intellectual disabilities, including those observed in Down syndrome (DS), are associated with aberrant axonal and dendritic morphology during development (Hall et al., 2011; Koleske et al., 2013; Kulkarni et al., 2013; Lin et al., 2010).

During an organism's development a neuron performs compartment-specific adjustments, necessitating mechanisms to separately regulate axonal and dendritic growth (Bentley et al., 1981; Gerhard et al., 2017; Kelliher et al., 2019; Truman et al 1988; Wang et al., 2014; Zwart et al., 2013). Such mechanisms can be either compartment-dedicated or compartment-generalized.

Compartment-dedicated mechanism can be further broken down into axon- and dendrite-dedicated mechanism. Prior works have elucidated axon-dedicated growth regulators like Rac1 and Sno-p300 (Ikeuchi et al., 2009; Lein et al., 2007; Luo et al., 1996; Stegmüller et al., 2006) and dendrite-dedicated regulators like Dar1 and NeuroD (Gaudillière et al., 2004; Ye et al., 2007; Ye et al., 2011).

Compartment-generalized regulators affect both axons and dendrites and also fit into one of two categories. First, uniform compartment-generalized regulators, which have the same effect on both axons and dendrites. This includes BMP which promotes general neurite outgrowth (Bond et al., 2012). Second, regulators that differentially affect the growth of axons and dendrites can be described as nonuniform compartment-generalized regulators. Wallenda (Wnd) / **d**ual **l**eucine **k**inase (DLK) fall into this final category by promoting axon growth while restricting dendrite growth (Wang et al., 2013).

Our lab previously demonstrated that the Wnd/DLK pathway differentially regulates dendritic and axonal growth in *Drosophila* (Wang et al., 2013). The *Drosophila* DLK, Wallenda (Wnd), promotes axon terminal growth and diminishes dendritic growth in C4da neurons, a cell-type widely used to study dendrite and axon development (Grueber et al., 2003; Jan et al., 2003). The Wnd/DLK pathway promotes axon terminal growth by up-regulating **D**own **s**yndrome **c**ell **a**dhesion **m**olecule (Dscam) and restricts dendritic growth by down-regulating the transcription factor Knot (Wang et al., 2013). Wnd/DLK is localized in the axon and excluded from the cell body based on prior literature (Xiong et al., 2010; Baumgardt et al., 2007; Hirai et al., 2005) and preliminary data from the Ye Lab.

An important, open question in subcellular development is whether a neuron adjusts dendrite growth in response to axonal events. For example, if a cell's axon receives less circuit feedback, does the cell adjust its dendritic shape and responsivity as well? Nonuniform compartment-generalized regulators are candidates for coordinating such adjustments. I tested if

and how changes in activity at the axon can modulate dendritic signaling within the same neuron through the established nonuniform compartment-generalized Wnd/DLK pathway.

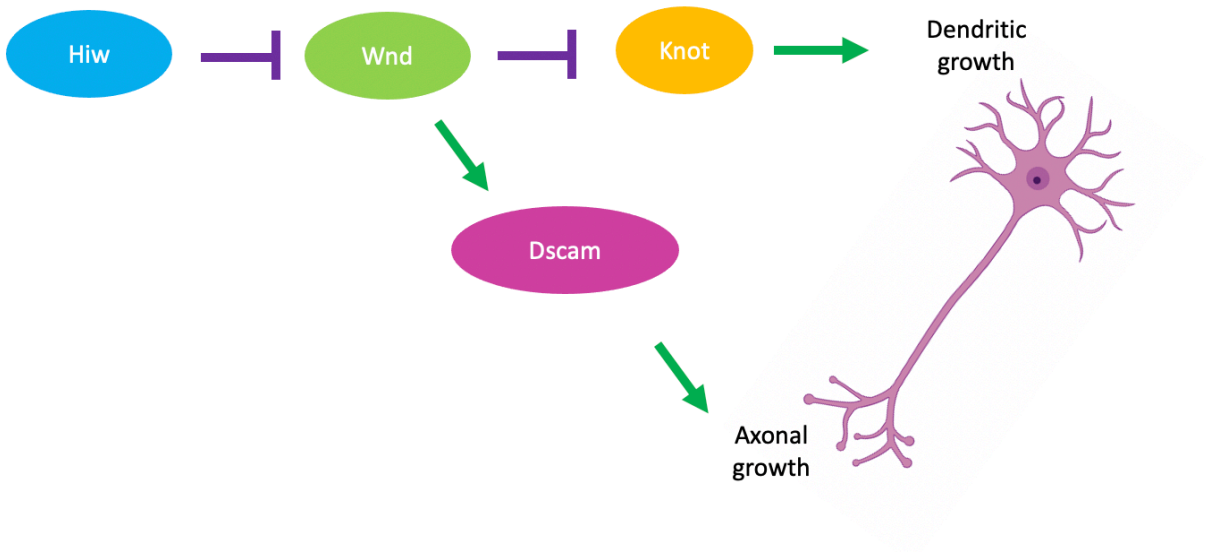

Figure 8: Established pathway for bimodal regulation of axon and dendrite growth by Wnd.

The above image summarizes the current understanding of how Wnd signaling promotes axon growth while restricting dendrite growth within the same cell. Based on work from Wang et al., 2013.

3.3 Materials and Methods

3.3.1 Drosophila Genetics

hiw$^{\Delta N}$ (Wu et al., 2005), *uas-glued*^{DN} (Allen et al., 1999), *w*^{1118} (Pastink et al., 1988), *ppk-tdtomato* (Grueber et al., 2003), *ppk-Gal4* (Grueber et al., 2007), *82E12-Gal4* (Vogelstein et al.,

2014), *72F11-Gal4* (Ohyama et al., 2015), *uas-hid::rpr* (Hsu et al., 2002) , *ppk-LexA* (Vogelstein et al., 2014), *LexAop-GFP* (Vogelstein et al., 2014), *uas-mCD8::GFP* (Lee and Luo, 1999)

3.3.2 Immunohistochemistry and Confocal Imaging

Immunostaining of early third-instar larvae is described in previous work (Ye et al., 2011). Antibodies used include chicken anti-GFP (Aves, Tigard, Oregon), rabbit anti-RFP (Rockland, Limerick, Pennsylvania), rat anti-Elav (DSHB), and guinea pig anti-Knot (Gift from Adrian Moore). Fillets were dehydrated through a series of ethanol and xylene washes and were then mounted with DPX mounting media (Electron Microscopy Sciences, Hatfield, Pennsylvania). Confocal imaging was performed on a Leica SP5 confocal system equipped with a resonant scanner, 20X oil-immersion lens, and 63X oil-immersion lens. Images were collected and quantified as previously described (Wang et al., 2013).

For images comparing fluorescent intensity, compared samples were stained in the same container and antibody solution to minimize artificial differences in fluorescent intensity. Similarly, compared samples were mounted on the same slide and imaged in one sitting after allowing the lasers to warm for a minimum of 30 minutes. For each cell, the fluorescent intensity of Knot was normalized to the fluorescent intensity of the neuronal nuclear marker Elav. This normalized expression was then compared to the average normalized expression of Knot for cells in the negative control group. This was the same procedure used in Wang et al., 2013.

For quantification of puncta, a standard threshold of puncta size was established for all images, set to the smallest size which excluded all background noise that fell outside of the HRP-

labeled tracts. Number of puncta was counted within a standard area across images which did not extend beyond segments A4-6.

3.3.3 Second Order Neuron Ablation

I achieved cell-specific ablation of either total Basin neurons or A08N neurons with the corresponding Gal4 drivers *72F11* and *82E12*, respectively. Ablation was caused genetically through *uas*-driven expression of the pro-apoptotic factors *Hidden* and *Reaper* (Hsu et al., 2002). C4da neurons were labeled with tdTomato using the *pickpocket* (*ppk*) promoter.

3.3.4 Dendritic Tracing and Scholl Analysis

To determine the total dendritic length, cell contrast was adjusted in ImageJ to ensure thin processes were visible. Images were then processed with Neurolucida software where a consistent threshold was set to automatically identify branches. Tracing of each cell was then completed manually in Neurolucida to ensure that no excess branches were identified and that no dim branches were excluded. Neurites shorter than 5 μm were excluded.

Scholl Analysis was similarly performed using Neurolucida after the same image processing in ImageJ. After automatic analysis, each cell was manually examined to ensure all branch points were identified.

3.3.5 Statistical Analysis

All statistical analysis was performed using GraphPad. Analysis was performed double-blind. Normality was assessed for all groups. For normal data, two-group comparisons were

made using an unpaired two-tailed t-test and multiple group comparisons were made using One-Way ANOVA with Tukey multiple comparisons post hoc analysis. For non-normal data, two-group comparisons were made using two-tailed Mann-Whitney U Test and multiple group comparisons were made using Kruskal-Wallis with Dunn's multiple comparisons post hoc analysis. For all statistical analyses: ns for $p>0.05$, * for $p<0.05$, ** for $p<0.01$, *** for $p<0.001$, **** for $p<0.0001$.

3.4 Results

As discussed in Chapter 2, *Drosophila* C4da neurons serve as a classic model for examining subcellular morphology because of the ease of distinguishing subcellular compartments (Grueber et al., 2003; Jan et al., 2003; Sugimura et al., 2003; Wu et al., 2005; Zhen et al., 2000). Moreover, because C4da dendrites self-avoid and tile in a non-overlapping pattern with other C4da dendrites, the dendritic structure of an entire C4da neuron can be examined without confounding overlap from other C4da neurons (Grueber et al., 2003). These combined with the consistency of C4da neurite patterning allows for consistent quantification of changes in neuronal morphology. Since prior work has shown that the Wnd/DLK pathway differentially regulates dendrites and axons in C4da neurons (Wang et al., 2013), I used this model to test if and how axonal events alter dendritic structures during development.

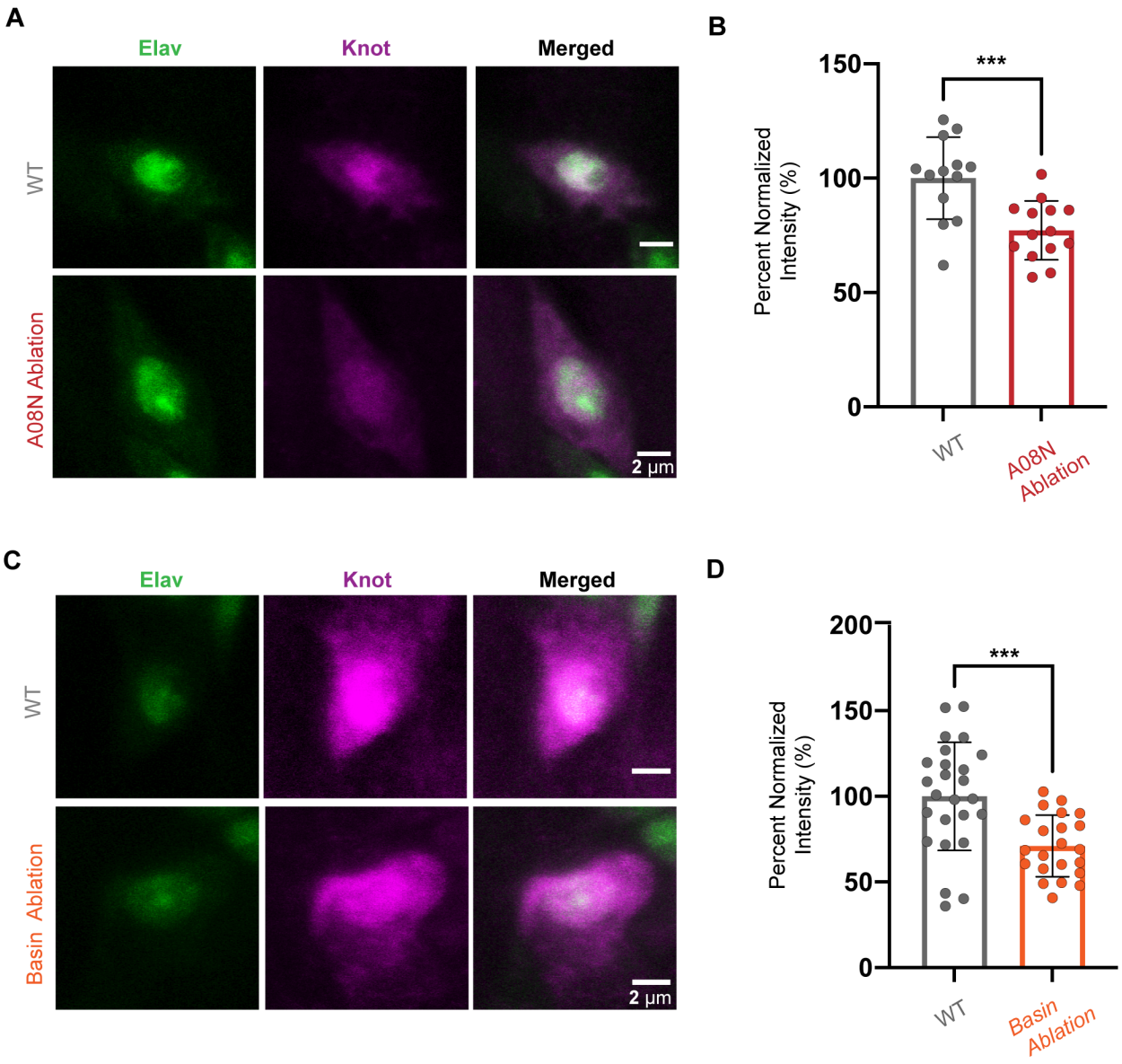

Figure 9 : SON ablation decreases nuclear Knot expression.

A) Images compare nuclear Knot expression in a wildtype control to a group with all A08N neurons ablated.

B) Quantification of (A). Fluorescent intensity for Knot expression was normalized by cell to the nuclear marker Elav. The percent normalized fluorescent intensity was then determined for each

cell compared to the average for the wildtype control. Unpaired two-tailed t-test, $p < 0.001$, n = 13 or 14 cells across 3-5 larvae.

C) Images compare nuclear Knot expression in a wildtype control to a group with ablation of all Basin neurons.

D) Quantification of (C). Fluorescent intensity for Knot expression was normalized by cell to the nuclear marker Elav. The percent normalized fluorescent intensity was then determined for each cell compared to the average for the wildtype control. Unpaired two-tailed t-test, $p < 0.001$, n = 22 or 25 cells across 3-5 larvae.

3.4.1 Ablation of second order neurons decreases nuclear expression of the dendritic growth-promoting Knot in C4da neurons.

We hypothesized that events at the axon terminal can affect dendritic structures within the same cell. To test this, I first sought a manipulation localized to the C4da axon terminal to test if events local to the axon terminal could alter dendritic signaling through the Wnd/DLK pathway.

To create an axon-localized event for C4da axon terminals, I ablated known **S**econd **O**rder **N**eurons (SONs) that are post-synaptic to C4da neurons. C4da axon terminals synapse onto both A08N and Basin neurons in the VNC as shown by the use of split-Gal4 labeling (Chun et al., 2017). Indeed, C4da signaling to Basin and A08N neurons is part of the nociceptive sensation and response pathway in *Drosophila* larvae (Ohyama et al., 2015; Hu et al., 2017; Kaneko et al., 2017). Because C4da axon morphology is activity-dependent, and because changes in A08N and Basins alter C4da-to-SON synaptic transmission through serotonergic feedback modulation at the C4da axon terminal (Kaneko et al., 2017), I expected loss of SONs to restrict growth signaling to dendrites in C4da neurons.

SON ablation was performed through genetic silencing with the Gal4/*UAS* system driving A08N- or Basin- specific expression of *Hidden* (*Hid*) and *Reaper* (*Rpr*). Hid and Rpr are pro-apoptotic factors known to induce cell death in many cell types including neurons (Goyal et al., 2000). Hid and Rpr form the RHG complex with Grim to cause caspase-dependent apoptosis by inhibiting that anti-apoptotic factor Diap1 (Clavier et al., 2014). Overexpression of only Hid and Rpr is sufficient to cause neuronal ablation (Zhou et al., 1997).

To determine if this axonal-event affects dendritic pathways, I stained for the nuclear-localized factor Knot in wildtype larvae versus SON-ablated larvae. Knot is a transcription factor, which promotes dendritic growth within the Wnd/DLK pathway (Fig 8) (Wang et al., 2013). Ablation of either A08N (Fig 9 A&B) and total Basins (Fig 9 C&D) decreased the nuclear Knot signal. This phenocopies an increase in Wnd and shows that SON-ablation at the C4da axon terminal decreases expression of a nuclear-localized dendritic-growth factor. Note, I have not yet checked if this corresponds to changes in dendritic morphology in response to SON ablations.

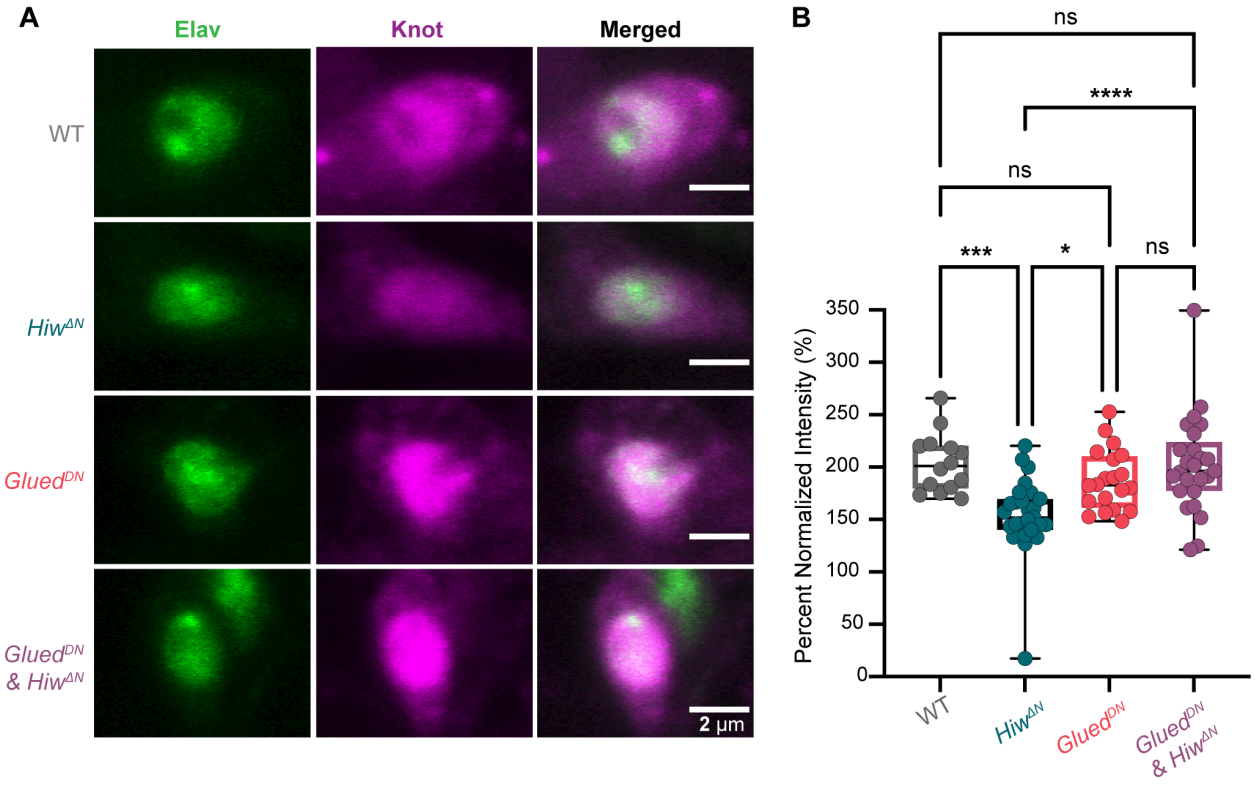

Figure 10: Loss of retrograde transport blocks Wnd from decreasing nuclear Knot expression.

A) Images compare nuclear Knot expression (magenta) in: 1) a wildtype control, 2) loss of *hiw*, 3) overexpression of *glued*^{DN}, and 4) loss of *hiw* and overexpression of *glued*^{DN}..

B) Quantification of (A). Fluorescent intensity for Knot expression was normalized by cell to the nuclear marker Elav. The percent normalized fluorescent intensity was then determined for each cell compared to the average for the wildtype control. Kruskal-Wallis test with Dunn's multiple comparisons post hoc analysis, n = 14-27 cells across 5-7 larvae.

3.4.2 Loss of retrograde transport blocks the effect of Wnd on nuclear Knot expression.

Because a nuclear factor in the Wnd/DLK pathway was decreased by an axon-localized event, I hypothesized that the inhibition of Wnd by Knot requires retrograde transport. I wanted

to test the importance of retrograde transport in the established pathway before testing the need for retrograde transport in response to decreasing Knot after SON ablation.

Prior work and preliminary evidence in the Ye Lab has shown that Wnd localizes to axons (Xiong et al., 2010; Baumgardt et al., 2007; Hirai et al., 2005). This begs the question of how an axon-localized protein can alter the expression of a transcription factor. One possible mechanism is that Wnd sends a retrograde signal to the cell body or undergoes retrograde transport, as supported by prior literature (Xiong et al., 2010). I tested if indirectly increased Wnd could decrease nuclear Knot in the absence of retrograde transport.

To achieve a modest increase in Wnd I used a *highwire* null ($hiw^{\Delta N}$) mutant shown in prior work to repress nuclear Knot expression in C4da through upregulation of Wnd (Kim et al., 2013). Hiw is a PHR protein that represses Wnd expression. By using a loss of function *hiw* mutant I modestly increased the expression of Wnd through dis-inhibition of Wnd.

To disrupt retrograde transport, I drove C4da-specific overexpression of a dominant-negative loss of function mutant for *Glued* ($Glued^{DN}$) (Allen et al., 1999). Glued is the *Drosophila* homologue of Dynactin 1 and is necessary to facilitate cargo binding to dynein for retrograde transport (Waterman-Storer and Holzbaur, 1996). Loss of Glued blocks retrograde transport.

Using *ppk-tdTomato* to mark C4da neurons, I stained for nuclear Knot in four genetic conditions to see the effect of loss of *hiw* on Knot requires retrograde transport (Fig 10). As shown in prior literature, loss of *Hiw* caused a decrease in nuclear Knot expression when compared to a negative control. Overexpression of $Glued^{DN}$ had no effect on Knot expression

compared to the same negative control. However, loss of *Hiw* failed to reduce nuclear Knot expression when retrograde transport was blocked through overexpression of $Glued^{DN}$. Thus, the Wnd/DLK pathway requires retrograde transport to modulate Knot expression.

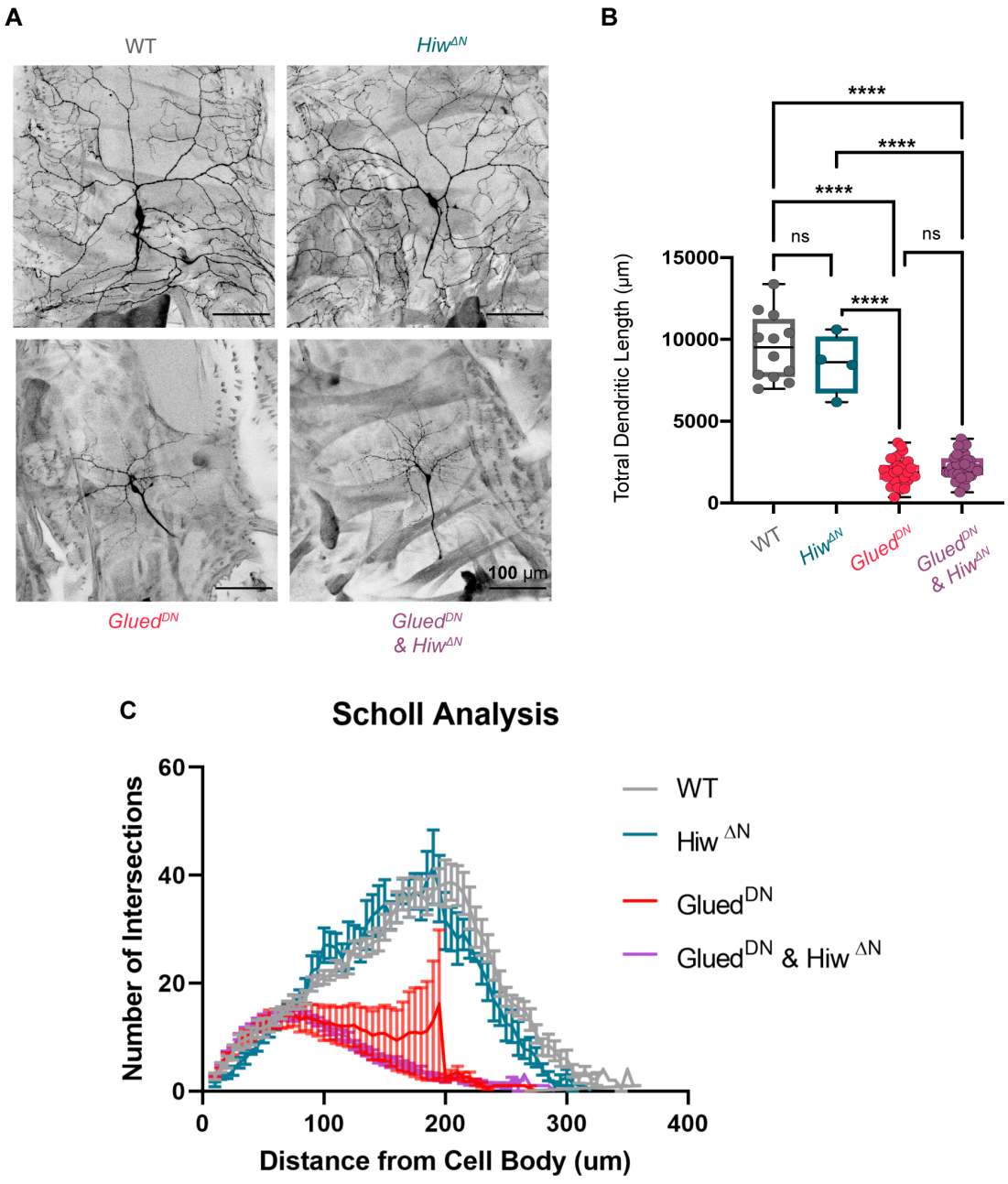

Figure 11 : Overexpression of dominant-negative *Glued* severely restricted dendritic growth.

A) Representative images of dendritic morphology of single C4da neurons with: 1) a wildtype control, 2) loss of *hiw*, 3) overexpression of *glued*^{DN}, and 4) loss of *hiw* and overexpression of *glued*^{DN}.

B) Quantification of total dendritic length based on tracing of images from (A). One-Way ANOVA with Tukey multiple comparisons post hoc analysis.

C) Scholl analysis of the images from (A) quantifies self-avoidance of dendrites in relation to distance from the cell body.

3.4.3 Loss of retrograde transport severely restricts C4da dendritic growth and patterning.

Because the Wnd/DLK pathway required retrograde transport to modulate Knot, I next tested if the corresponding regulation of dendritic growth by the Wnd/DLK pathway also required retrograde transport. Using the same genetic conditions as in Figures 9 & 10, I examined the effect of blocking retrograde transport on the Wnd/DLK pathway's regulation of dendrites.

I first measured dendritic growth by determining the total dendritic length of C4da neurons from each genotype (Fig 11A&B). I again compared: 1) a negative control (WT, no overexpression), 2) $Hiw^{\Delta N}$, 3) Overexpression of $Glued^{DN}$ and 4) $Hiw^{\Delta N}$ with $Glued^{DN}$. Loss of *Hiw* resulted in no significant difference from the total dendritic length of the wildtype, likely due to technical challenges in imaging the fine dendritic structures which Wnd most significantly restricts. Interestingly, overexpression of $Glued^{DN}$ caused a significant decrease in the total dendritic length. This is consistent with prior literature showing the importance of retrograde transport for dendritic growth and patterning during development (Zhou et al., 2012). Notably, since I saw that loss of retrograde transport had no effect on Knot expression (Fig 10), the restriction of dendritic growth caused by overexpression of $Glued^{DN}$ occurs through a Knot-independent pathway.

Note, the absence of functional *hiw* with overexpression of *Glued*^{DN} did not further decreases dendritic growth compared to the *Glued*^{DN} phenotype (Fig 11 A&B). While it is possible that this indicates that Wnd requires retrograde transport to restrict dendritic growth, this result is inconclusive because it is equally possible that overexpression of *Glued*^{DN} causes a floor effect for C4da dendritic growth where further restriction cannot be detected. In other words, the *Glued*^{DN} phenotype may create a lower limit of dendritic outgrowth which loss of *hiw* cannot further decrease.

To see if I could compare dendritic growth between the genotypes in a different manner, I next performed Scholl analysis. Here, I counted the number of branchpoints in relation to how far the branch point was from the soma. This has been used in prior works as an alternative measure to assess dendritic patterning in C4da (Tenenbaum et al., 2017). However, like dendritic length, no discernable difference could be seen in the amount of branching comparing the *Glued*^{DN} and the null *hiw* with *Glued*^{DN} phenotypes (Fig 11 A&C). More work is needed to determine if Wnd-driven restriction of dendritic outgrowth requires retrograde signaling.

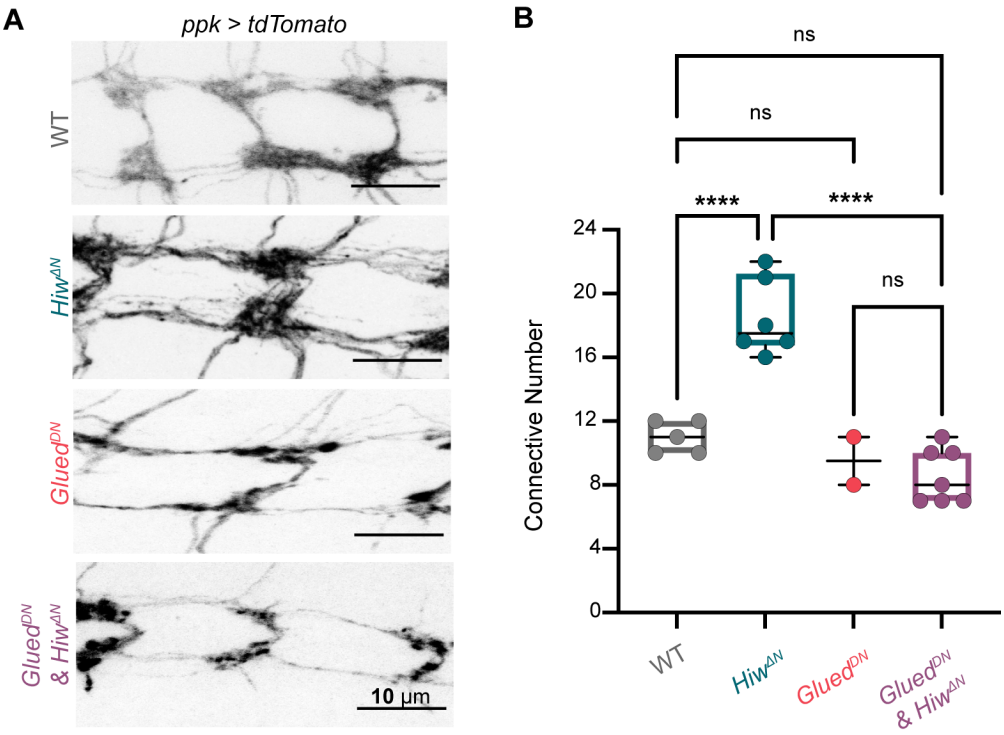

Figure 12: Loss of *hiw* fails to drive axon terminal growth in absence of retrograde transport.

A) Representative images of total C4da axon tracts with 1) a wildtype control, 2) loss of *hiw*, 3) overexpression of $glued^{DN}$, and 4) loss of *hiw* and overexpression of $glued^{DN}$.

B) Quantification of connective number for (A). One-Way ANOVA with Tukey multiple comparisons post hoc analysis.

3.4.4 Wnd-driven axon terminal growth requires retrograde transport.

I next aimed to determine if Wnd-driven axon growth requires retrograde transport. Since Wnd localizes to axons (Xiong et al., 2010; Baumgardt et al., 2007; Hirai et al., 2005), it was possible that the mechanism by which Wnd promotes axon growth was also local and independent of retrograde transport. However, since I saw that a loss of retrograde transport

impacted the signaling from Hiw to Knot, I hypothesized that the Wnd/DLK pathway to axon growth signal would also require retrograde signaling.

To measure axon growth, I examined the connective number of total C4da axon tracts in the four prior described genetic conditions: 1) a negative control (WT, no overexpression), 2) $Hiw^{\Delta N}$, 3) overexpression of $Glued^{DN}$, and 4) $Hiw^{\Delta N}$ with overexpression of $Glued^{DN}$. As a reminder, connective number is the total number of axon tracts visible between the most consistent C4da neuropil (A4-A6). Our lab and others have used this as a readout of axon terminal growth in prior work (Sterne et al., 2015).

I found that loss of *hiw* did not promote axon growth in the absence of retrograde transport (Fig 12A&B). For the positive control, I saw that a loss of *hiw* caused the anticipated increase in total connective number, and thus axon terminal growth, when compared to the control. Like with dendritic growth, overexpression of $Glued^{DN}$ resulted in a decrease in axon growth when compared to the negative control. This is consistent with literature showing that retrograde transport is needed for axon growth during development (Tuttle et al., 2019). Interestingly, when $Glued^{DN}$ was overexpressed with loss of *hiw* there was no increase in connective number when compared to overexpression of $Glued^{DN}$. This suggests that retrograde transport is needed for loss of *hiw* to drive axon terminal growth. Note, another possible explanation is that the loss of retrograde transport has a stronger effect on axon growth than the Wnd pathway. While more work is needed to verify that C4da axon growth driven by Wnd requires retrograde transport in this context, this finding is consistent with prior literature testing

retrograde transport of Wnd in the context of axon injury (Xiong et al., 2010). See further discussion in Chapter 4.

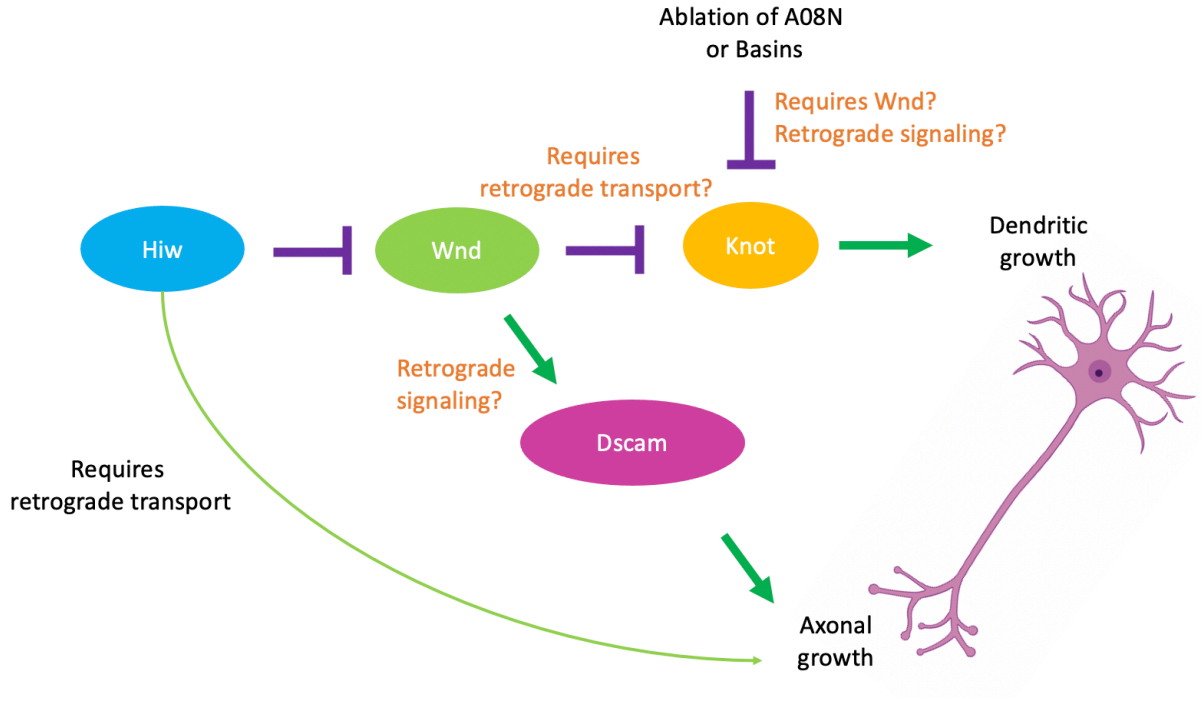

Figure 13 : Summary of how retrograde transport and axonal events affect the bimodal regulation of dendrites and axons by Wnd/DLK.

3.5 Discussion

These preliminary results show that disruption of retrograde transport eliminates the Wnd pathway's modulation of Knot, raising the question as to whether Wnd coordinates dendritic growth to axonal events. Indeed, these results show that ablation of second order neurons (SONs) during development, an event local to C4da axon terminals, reduces the levels of nuclear Knot,

phenocopying activation of the Wnd/DLK pathway. This suggests that local changes at the axon terminal alter a key regulator of dendritic growth and possibly adjust dendritic patterning within the same neuron. Because Knot and its homologues are also well-known for regulating degenerin/epithelial Na(+) channels (DEG/NaC) (Take-Uchi et al., 1998; Jinushi-Nakao et al., 2007; Crozatier et al., 2008; Zhong et al., 2010), it further begs the question of how loss of postsynaptic partners may alter ion channel expression and neuronal activity.

Moreover, the Wnd/DLK pathway would provide a novel mechanism by which cells within a circuit may adjust the structure and resulting activity of a neuron to maintain circuit function. After validation of this pathway's retrograde-dependent coordination, future work should consider how changes in the activity of SONs might affect C4da dendritic and axonal structures and if Wnd/DLK mediates these changes. This would provide insight into the cellular mechanisms of maintaining functional circuitry.

For discussion of limitations and future directions of this work, please see Chapter 4.

3.6 Contributions of Authors

The experiments described in Chapter 3 are unpublished.

Pizzano, S., Szlatcha N., and Ye, B. *The Wnd pathway coordinates axonal events to dendritic signaling.*

The relative contributions of each author are as follows:

SP: Conceived of the project, designed, and completed the experiments, wrote the summary.

NS: Assisted SP in replicating the SON ablation experiments.

BY: Conceived of the project, designed the experiments, supervised, helped write the summary.

Chapter 4 Discussion

4.1.1 Pitfalls and Limitations of Chapter 2: Appl promotes the expression Dscam to drive axon terminal growth during development.

In Chapter 2 we showed that Appl promotes Dscam expression post-transcriptionally and may modulate Rab5. Here I will discuss the pitfalls and limitations of this work.

1. *Expression levels of the same transgene may vary between groups in co-overexpression experiments (Fig 2E&F, Fig 3A-D).* The efficacy of Gal4/*UAS* driven expression can be affected by many factors including temperature, driver efficacy, and the position of the transgene in the genome (Weaver et al., 2020). We conserved the driver and temperature between compared groups for all experiments.

   In experiments where more than one transgene was overexpressed, we recognized the need control for Gal4 competition at multiple *uas* binding sites. Prior work has shown that some *uas* sites can outcompete others for binding Gal4, resulting in unequal rates of overexpression of the multiple transgenes (Vashee and Kodadek, 1995). To mitigate Gal4 binding competition, we ensured that groups with multiple *uas* sites were only compared to groups with the same number of *uas* sites. This controls for artificial dilution of gene expression. This was especially important, knowing that Dscam has dose-dependent effects on axonal growth (Kim et al., 2013).

A limitation of this control is that the multiple *uas* sites were not always in the same position in the genome. Position in the genome is a known contributor to transgene expression levels (Weaver et al., 2020; Vashee and Kodadek, 1995). Co-expression results should be viewed with this caveat in mind.

2. *MARCM epistasis experiment showed Dscam can drive C4da axon terminal growth without functional Appl but did not exclude non-cell-autonomous mechanisms (Fig 3A&B).* This experiment was performed on a total loss of *Appl* background. To test whether *Dscam* can drive axon terminal growth in the absence of functional *Appl,* loss of function of *Appl* was established by using mutation of the endogenous gene on the X chromosome. This does not change the interpretation of the results, but unlike the typical conclusions drawn from MARCM, these results do not test cell autonomy.

3. *We measured Rab5 total expression, not the amount of active Rab5 which is also important for endosomal dysfunction (Fig 6 C&D).* Prior work has shown that increased APP increases Rab5 activation but not total expression levels (Xu et al., 2016). This differs from the increase in total Rab5 expression that I saw with loss of *Appl*. While Rab5 total expression also alters endosomal signaling in DS-AD based on prior literature (Xu et al., 2016; Cataldo et al., 2003; Cattaneo and Calissano, 2012; Salehi et al., 2006), we have not yet tested the impact of Appl manipulations on active Rab5.

4. *We have not directly tested changes in endosomes in response to Appl and Dscam manipulation.* We examined Rab5 expression as a readout of endosomal signaling. This metric is supported by prior literature (Pensalfini et al., 2020). Future work should directly interrogate how Rab5 alters endosome functions in response to changes in Appl and Dscam expression. Measuring changes in endosomal function could include examining localization of Rab5 and Dscam within C4da neurons, changes in endosome trafficking, quantifying endosome size, and staining for endosomal factors downstream of Rab5. Future work should also Apply western blotting to examine if Appl loss- and gain-of-function results in changes in active Rab5 as seen in prior literature.

5. *Overexpression of the Appl mutant transgenes is not comparable between mutant groups*. For Figure 7, the *Appl.sdΔE1* and *Appl.sdΔE2* groups cannot be directly compared to one another. The position of the *uas* sites differs between the two lines and thus likely produces differing amounts of each transgene. Furthermore, these deletion mutants may or may not impair other regions of Appl. Prior work shows that these lines do not result in secreted product and are found on the cell membrane as anticipated (Torroja et al., 1999; Luo et al., 1992). However, it is unclear if these deletion mutants impact the function of other Appl domains. As a result, these mutants should only be examined in the context of if they are necessary for an observed phenotype. A negative result should be considered inconclusive. For example, overexpression of *Appl.sdΔE2* drives axon terminal growth

and an increase in Dscam protein (Fig 7D-H). Thus, the E2 domain is not required for these phenotypes. In contrast, overexpression of *Appl.sdΔE1* drives no change in axon terminal growth or Dscam protein expression (Fig 7D-H). Thus, this result is inconclusive, as E1 may be required, or another domain may be disrupted by the deleted region.

6. *Repeat the transfection of CD8::GFP with a different control than Dscam::GFP*. I saw that CD8::GFP expression was higher in the presence of Appl::V5 than in the presence of Dscam::GFP in S2 Cells (Fig 7I&J). Because I also saw an increase in Dscam::GFP co-transfected with Appl::V5 compared when Dscam::GFP is co-transfected with CD8::GFP, I interpreted this as Appl::V5 increasing CD8::GFP expression. However, this experiment should be replicated with a control other than Dscam::GFP, to ensure that Dscam::GFP does not decrease the amount of CD8::GFP instead.

4.1.2 Future Work for Chapter 2: Appl promotes the expression Dscam to drive axon terminal growth during development.

Here I will discuss future work which could build off findings of Chapter 2.

Having observed a different effect of Appl on Rab5 than seen in prior literature, future work should characterize the source of this difference. I will offer two possible hypotheses. First, Appl and APP_{695} may function differently due to species-specific differences in the protein. Because these homologues share 30% overall sequence identity and higher homology at the most

conserved domains (43% at extracellular domain 1, 36% at extracellular domain 2, and 77% at the cytoplasmic domain) (Rosen et al., 1989) this would provide interesting regions to target in APP to try to therapeutically drive Appl-like activity. Second, APP/Appl may act differently in developing neurons than in mature neurons. Much work determining the effect of APP in AD and DS-AD models examines mature neurons, since this is where AD pathology is observed. However, APP/Appl is developmentally expressed (Trapp and Hauer, 1994; Nicolas and Hassan, 2014) and may have earlier implications or provide sooner insult than previously understood. This is especially of concern in DS, where APP is highly expressed in neurons throughout development. The effect of APP on the endosome may be developmentally dependent. This would have significant implications for when therapeutics for APP-caused endosomal dysregulation should be implemented.

Moreover, since Appl may modulate Dscam through an endosomal factor, Appl/APP may have a broader effect on other DS-related genes. The interaction of APP with other genes in DS models has already been established (Wiseman et al., 2018). With our new result, examining if APP affects the endosomal processing of other DS-related genes in development would offer insight into large scale endosomal dysregulation. In DS, some triplicated genes, such as Synaptojanin (SynJ), are upregulated disproportionately higher than the amount expected from gene dosage (Cheon et al., 2003). Synaptojanin, like Dscam, is a transmembrane protein (Verstreken et al., 2003). Given the effect of Appl on the exogenous membrane protein CD8 (Fig 7I&J), a future direction will be to examine if other DS-related membrane proteins are disproportionately regulated by App/Appl through endosomal mechanisms.

Finally, we should replicate this work in higher organisms. The Ye Lab has already shown a developmental mechanism of Dscam discovered in *Drosophila* to be conserved in Ts65Dn model mice (Liu et al., 2020). Increased Dscam drives axon terminal growth in *Drosophila* C4da neurons (Kim et al., 2013). The Ye Lab showed that in TsDn65 model mice, increased axon terminal length and bouton number in chandelier cells of the anterior cingulate cortex (ACC) can be rescued by normalization of Dscam gene dosage (Liu et al., 2020). Similarly, the dissertation work detailed here should be tested in TsDn65 mice to determine 1) if during development mouse App similarly effects Rab5 and 2) if increases in Dscam expression in TsDn65 model mice can be normalized through loss of APP and or repression of Rab5.

4.1.3 Limitations and Pitfalls for Chapter 3: The Wnd/DLK pathway alters dendritic signaling in response to axonal events.

In Chapter 3, I tested if axonal events could impact dendritic signaling through the Wnd/DLK pathway. Indeed, I showed that ablation of SONs of C4da neurons phenocopies Wnd/DLK pathway activation by reducing nuclear Knot expression. Here I will discuss the pitfalls and limitations of the data described in Chapter 3.

1. *I indirectly modulated Wnd by using a null hiw mutant.* I increased Wnd to drive established phenotypes by manipulating the upstream inhibitor *hiw*, as used in prior literature (Kim et al., 2013). This was to initially avoid the dosage complications of

multiple *uas* sites, especially when using Gal4/*UAS* to drive ablation. This work should be repeated with direct manipulation of Wnd.

2. *I need to test the individual elements of the Wnd/DLK pathway*. This preliminary work leaves open questions as to if each element of the Wnd/DLK pathway requires retrograde transport in the context of development and circuit maintenance. For example, we saw that null *hiw* does not cause axon terminal growth without retrograde transport. So, does Wnd require retrograde transport to increase Dscam expression?

   Similarly, we need to test which elements of the Wnd/DLK pathway are affected by SON ablation. SON ablation reduces nuclear Knot expression by 20%. This is comparable to the decrease in Knot expression seen with Wnd overexpression which further results in decreased dendritic branching. So, does SON ablation also restrict dendritic growth? Does SON ablation alter axon terminal length? Does it increase Wnd and Dscam or decrease Hiw expression? Future work should address if SON ablation activates the Wnd/DLK pathway.

3. *Ablation of Second Order Neurons is a biologically irrelevant occurrence*. While SON ablation successfully induced an axon-specific event for C4da neurons, such an event would not likely occur naturally and may be producing an artificial cellular response. Optogenetic silencing of SONs, axon injury, and disease models should be tested in future

experiments to see if the Wnd/DLK pathway still coordinates C4da dendritic growth and responsivity with activity at C4da axon terminals.

4. *Results should be validated with alternative methods of manipulating or measuring retrograde transport.* Other mechanisms of more temporarily altering retrograde transport in conjunction with live imaging should be used validate if retrograde transport occurs and is necessary for Wnd to promote axonal growth and restrict dendritic growth in development and circuit maintenance. The current genetic manipulation introduces too strong a reduction in axonal and dendritic patterning to provide conclusive evidence.

4.1.4 Future work for Chapter 3: The Wnd/DLK pathway alters dendritic signaling in response to axonal events.

Here I will discuss future work which could build off the findings of Chapter 3.

The initial follow up work for this project should validate current findings. Alternative methods are needed for testing the role of retrograde transport in the Wnd /DLK pathway for regulating axon patterning, dendrite patterning, signaling from Wnd to Dscam, and signaling from Wnd to Knot. Furthermore, activation of the Wnd/DLK pathway should be validated with the SON ablation model by also quantifying axon patterning, dendrite patterning, Dscam expression, and Wnd trafficking in response to SON ablation.

Ideally, an alternative axon-localized event should also be used to test how Wnd/DLK coordinates dendritic structures to axonal occurrences. SON ablation is an artificial metric and could produce biologically irrelevant changes to the Wnd/DLK pathway. However, feedback serotonin signaling onto C4da axon terminals has already been shown to modulate nociceptive responses during development (Kaneko et al., 2017). We should examine how modulation of serotonin signaling at the C4da axon terminal from downstream neurons alters 1) Wnd/DLK signaling, 2) dendritic structure, and 3) behavioral response to the nociceptive stimuli that this circuit processes. This follow-up work would contribute to our understanding of cellular mechanisms that modulate circuit plasticity and the maintenance of functional circuitry.

In the long term, this work should also interrogate the need for Wnd/DLK signaling to preserve circuit function. By temporally inhibiting Wnd in response to a circuit manipulation, such as increasing serotonergic input to the axon terminal, we can test if C4da morphology and activity can still adjust as expected. If not, this experiment would show that the Wnd/DLK pathway is necessary for cellular adjustments to maintain functional circuitry.

4.1.5 The interactions of established pathways in DS-modeling and circuit manipulations.

Neurodevelopment and the maturation of functional circuitry requires cell-specific patterning of axons and dendrites. Both in the case of DS models and in cellular responses to axon-localized events, the molecular mechanisms within a cell that establish axon and / or dendrite patterning feed into other molecular pathways, circuit function, and behavioral output.

As systems neuroscience progresses in understanding how the adjustment of circuitry effects behavioral output, the field of molecular neuroscience also needs to progress in testing the interactions of molecular pathways and their impact on circuitry.

The interaction of Appl and Dscam in modeling the increased expression of multiple DS-related genes presents such an examination of how known and distinct signaling molecules interact during development in disorder-relevant contexts. Literature has thoroughly established how APP/Appl-related pathways and Dscam-related pathways impact neuronal development. The novel addition of this work is considering when both these pathways are upregulated what new cellular effects result.

Similarly, how the established Wnd/DLK pathway intersects with circuit-level changes offers insight into how intracellular molecular mechanisms impact functional circuitry and behavioral output. Ablation of cells downstream of C4da neurons impacted intracellular signaling in C4da neurons to repress Knot. Furthermore, the repression of Knot by the axon-localized Wnd required retrograde transport. This offers insight into how a known bimodal regulator of axon and dendrite growth acts within a circuit-level context in development.

Overall, therapeutic treatments for circuit impacting disorders, like Down syndrome, must consider how molecular targets impact neuronal circuits and other molecular pathways. In the instance of DS, there is a need to interrogate the impacts of factors like APP interacting with many other DS-genes. Therapeutics targeting APP should consider the likely negative externalities on other upregulated pathways and altered circuits to improve the likelihood of successful therapeutic interventions.

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