Defining the Roles of the Microtubule-Associated Protein TACC in Drosophila Synapse Development

Defining the Roles of the Microtubule-Associated Protein TACC in Drosophila *synapse development*

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ABSTRACT

The development of the synapse, the essential functional unit of the nervous system, is a highly regulated process underlying neuronal circuitry and plasticity. Following axon pathfinding, complex signaling networks coordinate development of precise connections between pre- and postsynaptic compartments to establish a stable junction. Among the major effectors of these signaling networks are the actin and microtubule (MT) cytoskeletons. Despite comprehensive studies of the neuronal cytoskeleton in the postsynaptic context, a molecular understanding of the presynaptic cytoskeleton is still developing, and presynaptic MTs remain particularly enigmatic. Pioneering studies at the *Drosophila* neuromuscular junction (NMJ) have revealed critical roles for several MT-associated proteins (MAPs); however, many outstanding questions remain, such as how MT assembly/stabilization are coupled to morphological changes, and the potential significance of plus-end dynamic instability at the synapse.

In my dissertation research, I have aimed to address these questions through studies of the MAP dTACC, a novel regulator of synaptogenesis in *Drosophila*. TACC-family proteins are primarily known for regulating spindle MTs in mitotic cells, but have also been studied in interphase systems, including non-synaptic neurons. Here, I report that presynaptic dTACC is a negative regulator of bouton addition, and therefore synapse expansion. Consistent with known

roles, dTACC localizes to the presynaptic MT lattice and is required to maintain the integrity of higher-order MT architecture in motor axon terminals. I furthermore describe the development of a live-imaging and automated analysis strategy to investigate the effect of dTACC on synaptic MT dynamics, and the resultant finding that dTACC regulates both pre- and postsynaptic plus-end dynamics. This is consistent with previously described roles for dTACC and moreover raises the possibility that dTACC acts as a plus-end tracking protein (+TIP) at the synapse. Taken together, these findings support a model of synaptogenesis where a precise equilibrium of NMJ expansion and restriction is modulated by changes to the underlying cytoskeleton, which is under the control of a complex network of MAPs and other factors. Future research will focus on elucidating the biochemical mechanisms underlying the function of synaptic dTACC as well as the roles of dTACC in the postsynaptic compartment.

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Quod est inferius est sicut quod est superius.

Et quod est superius est sicut quod est inferius,

ad perpetranda miracula rei unius

That which is below is like that which is above

and that which is above is like that which is below

to do ye miracles of one only thing. – Emerald Tablet

Κατά τον δαίμονα εαυτού

Be true to your own spirit. – Unknown

CHAPTER 1: INTRODUCTION TO SYNAPSE DEVELOPMENT AND THE SYNAPTIC CYTOSKELETON

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Chapter Contribution

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Synapses: The Fundamental Units of the Nervous System

The birth of modern neuroscience can be traced back to a period of seminal research in the 19th and early 20th centuries. Among the field's foremost pioneers was Santiago Ramón y Cajal, who postulated in 1888-1889 that the animal nervous system was comprised of autonomous nerve cells, subsequently termed "neurons" by Heinrich Wilhelm von Waldeyer in 1891 (Glickstein, 2006; López-Muñoz et al., 2006). Key to Cajal's theory was that despite the physical separation between neurons, they are connected through "protoplasmic kisses," or contact points, and capable of intercellular communication. This notion was further developed by Charles Sherrington, who postulated in 1897 that neuron-to-neuron communication occurs by way of specialized junctions known as "synapses," thereby enabling the emergence of complex, unified behaviors through coordination of individual neurons (Levine, 2007). Remarkably, the basic principles of early neuron doctrine endure today: we continue to regard synapses as fundamental contact sites and functional units of the nervous system. Given that the human brain contains approximately 100 billion neurons (Herculano-Houzel, 2009; Saleeba et al., 2019) that are connected through trillions of synapses (Colón-Ramos, 2009; Comer et al., 2019), understanding synaptogenesis—the process by which synaptic connections develop and mature, and thereby form circuits that underlie all cognition and behavior—remains a monumental task.

Among the foremost motivations for achieving a better understanding of synaptogenesis is that it is essential to proper neurodevelopment, which in turn is critical to normal functions such as learning and memory. Neurodevelopmental abnormalities can lead to a diverse range of disorders with clinical presentations that range across, but are not limited to, cognitive, emotional, and behavioral domains (Mitchell, 2011; Thapar et al., 2017). Neurodevelopmental disorders typically onset during in pre-pubescent children (Thapar et al., 2017) and can arise from defects in synaptogenesis (Melom and Littleton, 2011), as well as other steps such as

neuronal migration (Valiente and Marín, 2010) and axon guidance (Engle, 2010). Due to the phenotypic variability and complexity of many neurodevelopmental disorders as well as the influence of non-hereditary factors (Homberg et al., 2016; Mitchell, 2011; Thapar et al., 2017), it has been challenging to establish a precise genetic etiology for many neurodevelopmental conditions. However, progress in the past several years has helped to implicate specific genes in specific diseases. For instance, mutations in the synaptic cell adhesion protein Neurexin-1α have now been identified in patients with a wide spectrum of neurodevelopmental disorders, including autism, schizophrenia, intellectual disability, language delay, epilepsy, as well as the neuromuscular condition hypotonia (Ching et al., 2010; Glessner et al., 2009; Guilmatre et al., 2009; Kirov et al., 2008). Neurodevelopmental disorders continue to be prevalent, particularly in low- and middle-income countries, with few effective therapies (Boivin et al., 2015; Davidson et al., 2015; Homberg et al., 2016; Thapar et al., 2017). In the coming years, understanding of the underlying mechanisms of processes such as synaptogenesis may contribute to interventions that address patient needs as well as relieve socioeconomic burdens associated with disease.

Overview of Synaptogenesis

Formally, chemical synapses are specialized asymmetric junctions that link a presynaptic neuron and a postsynaptic target (Chia et al., 2013; Jessell and Kandel, 1993). In humans, synaptogenesis begins primarily during embryonic development (Südhof, 2018). Synaptogenesis follows the process of axon pathfinding, whereby axons navigate a complex and dynamic environment to their proper targets by responding to a series of extracellular guidance cues (Cammarata et al., 2016; Comer et al., 2019; Lowery and Van Vactor, 2009). Once axons arrive at their destinations, synapse formation is initiated through adhesive interactions via cell-adhesion molecules (CAMs) and bi-directional signaling between the pre- and postsynaptic compartments (Chia et al., 2013; Südhof, 2018). As the synapse matures, the

presynaptic axon assembles the machinery that mediates neurotransmitter release via membrane fusion of synaptic vesicles (SV), which occurs within specialized regions known as active zones (AZ) upon an influx of Ca^{2+} ions via voltage-gated channels (Ackermann et al., 2015; Jin and Garner, 2008; Südhof, 2012, 2018; Zhai and Bellen, 2004). Concurrently, the postsynaptic compartment, which may be the dendrite of another neuron or another cell type, accumulates neurotransmitter receptors within specialized regions that form in precise alignment with the presynaptic release sites (Scannevin and Huganir, 2000; Sheng and Kim, 2011, 2002). Synaptogenesis involves numerous other cellular and biochemical events, including local cytoskeletal arrangements (Bodaleo and Gonzalez-Billault, 2016; Goellner and Aberle, 2012; Gordon-Weeks and Fournier, 2014; Nelson et al., 2013) and long-range cytoskeletal transport (Kapitein and Hoogenraad, 2011; Pack-Chung et al., 2007). Throughout synaptogenesis, conserved signaling pathways such as BMP (Bayat et al., 2011; Keshishian and Kim, 2004), Wnt/Wingless (Wg) (Koles and Budnik, 2012; Packard et al., 2002; Speese and Budnik, 2007), Fibroblast Growth Factor (FGF) (Sen et al., 2011), and LAR receptor protein tyrosine phosphatase (LAR-RPTP) (Han et al., 2016; Johnson et al., 2006; Um and Ko, 2013) coordinate the complex interplay of cellular and molecular events.

Synaptogenesis is not a singular event but rather a dynamic process. While the most intense period of synaptogenesis in humans occurs during embryonic and early postnatal stages, nervous system development persists throughout adolescence and even into the early 30s (Petanjek et al., 2011). Such plastic changes encompass not only the addition of new synapses but also their “pruning” or removal; it is estimated that half of the synapses within the prefrontal cortex of human newborns are removed by adulthood (Petanjek et al., 2011). Crucially, neuronal structural plasticity forms the basis to learning and memory and reflects the ability of synapses to respond not only to baseline developmental cues but to acute external stimuli (Bailey et al., 2015; Jessell and Kandel, 1993; Kandel, 2001). This remarkable property

of neurons is predicated on reciprocal bidirectional signaling and precisely orchestrated assembly and function of both the pre- and postsynaptic compartments (Bailey et al., 2015; Jessell and Kandel, 1993; Kandel, 2001), emphasizing the importance of understanding the synapse as an integrated whole.

In the remainder of Chapter 1, I will discuss in greater detail the major events in synaptogenesis that I have outlined above. Emphasis will be placed on the molecules and processes that are relevant to the work described in Chapters 2-4. Since my dissertation is devoted to *Drosophila* neurobiology, I will draw largely on work that has been performed in invertebrates, although vertebrate studies will be discussed as well. I will specifically focus on the *Drosophila* larval neuromuscular junction (NMJ), a powerful model synapse which bears both structural and biochemical similarities to the excitatory glutamatergic synapses of vertebrate central nervous system (CNS) (Collins and DiAntonio, 2007; Rushton et al., 2009; Van Vactor and Sigrist, 2017). Moreover, the fly NMJ can be manipulated and characterized with a diverse experimental toolkit encompassing sophisticated genetic, light imaging, ultrastructural, and electrophysiological techniques, among others. This technical versatility, combined with the biological simplicity of the system, has made the *Drosophila* NMJ invaluable to gaining a cellular- and molecular-level understanding of synaptic development and neurotransmission.

Synaptic Specification: Finding the Right Target

Upon the physical contact of a growth cone with its target, the specification and alignment of the correct pre- and postsynaptic partners is coordinated by various CAMs (Giagtzoglou et al., 2009; Hagler and Goda, 1998; Südhof, 2018; Thalhammer and Cingolani, 2014). CAMs act throughout synapse development, and beyond their roles in adhesion, are involved in processes ranging from SV organization, receptor clustering, and structural and functional plasticity

(Giagtzoglou et al., 2009; Hagler and Goda, 1998; Thalhammer and Cingolani, 2014). CAMs are divided into two classes: those that engage in homophilic interactions with another molecule of the same type, and those that engage in heterophilic interactions with a different CAM or the extracellular matrix (Sun and Xie, 2012; Thalhammer and Cingolani, 2014). It is now understood that CAMs are not necessarily limited to a single mode of binding. For instance, SynCAM (Biederer et al., 2002; Fogel et al., 2007; Frei et al., 2014) and the type II classical cadherins (Brasch et al., 2018) display both homophilic and heterophilic binding *in vivo*, while type I classical cadherins show both forms of binding *in vitro* (Ounkomol et al., 2010; Prakasam et al., 2006), although only homophilic interactions are observed *in vivo* (Basu et al., 2015).

Conventionally homophilic CAMs include NCAM/CD56 (Lüthi et al., 1994; Muller et al., 1996) and the related *Drosophila* protein Fasciclin II (Beumer et al., 2002; Davis et al., 1997; Schuster et al., 1996); DSCAM (Agarwala et al., 2000, 2001); SynCAM (Biederer et al., 2002; Fogel et al., 2007; Frei et al., 2014); and the type I and II classical cadherins (Brigidi and Bamji, 2011; Hirano and Takeichi, 2012; Seong et al., 2015; Suzuki and Takeichi, 2008). Of the homophilic CAMs, the type I cadherin N-cadherin/Cadherin 2 is the most highly expressed as well as best studied in the context of the CNS. N-cadherin localizes within (Yamagata et al., 1995) and at the periphery (Uchida et al., 1996) of the synaptic cleft and redistributes in response to activity (Yam et al., 2013). Beyond providing adhesive connections, N-cadherins are involved in neurotransmission (Vitureira et al., 2012), presynaptic short-term plasticity (Jüngling et al., 2006), dendritic spine morphogenesis (Abe et al., 2004; Togashi et al., 2002), activity-dependent plasticity and stabilization of dendrites (Mendez et al., 2010; Okamura et al., 2004), and long-term potentiation (Bozdagi et al., 2000, 2010). Other homophilic CAMs have similarly diverse functions, including neuronal morphogenesis (Ashley et al., 2005; Hutchinson et al., 2014; Wang et al., 2002a; Yu et al., 2009), homeostatic (Spring et al., 2016) and activity-dependent plasticity (Beumer et al., 2002; Muller et al., 1996), organization of synaptic

architecture (Biederer et al., 2002; Kohsaka et al., 2007), and long-term potentiation (Lüthi et al., 1994; Mayford et al., 1992).

Heterophilic interactions at the synapse are mediated by CAMs such as Dpr and DIP in *Drosophila* (Morey, 2017; Zinn and Özkan, 2017), Teneurins (Mosca, 2015; Tucker and Chiquet-Ehrismann, 2006), and Neurexins and Neuroligins (Bottos et al., 2011; Craig and Kang, 2007; Knight et al., 2011; Südhof, 2008); these latter two molecules are perhaps the best-characterized synaptic CAMs of all. Neurexins include three vertebrate members and a single fly ortholog, Neurexin-1 (Dnrx), while vertebrate Neuroligins 1-4 each have a corresponding *Drosophila* ortholog (Dnlg1-4) (Bottos et al., 2011; Knight et al., 2011); additional orthologs of both Neurexin and Neuroligin are present across taxa. Neurexins and Neuroligins are both exclusively synaptic, and expression of either is sufficient to induce synaptic differentiation *in vitro* (Graf et al., 2004; Scheiffele et al., 2000). Loss of either Neurexins or Neuroligins results in severe, or even lethal, defects in synapse formation and function (Chen et al., 2010, 2017; Etherton et al., 2009; Li et al., 2007; Varoqueaux et al., 2006), reflecting their central role in promoting the formation of stable junctions between pre- and postsynaptic components across organisms (Banerjee et al., 2017; Banovic et al., 2010; Chen et al., 2012; Chih et al., 2005; Chubykin et al., 2007; Owald et al., 2012; Varoqueaux et al., 2006). Work at the *Drosophila* NMJ indicates that the postsynaptic targets of Dnrx-Dnlg1 include neurotransmitter receptors (Owald et al., 2012) and the WAVE regulatory complex, which modulates actin cytoskeletal dynamics (Xing et al., 2018); presynaptic targets of Dnrx-Dnlg1 include core AZ components (Owald et al., 2012) and BMP pathway receptors and effectors (Banerjee et al., 2017). Incidentally, *Drosophila* also expresses a protein known as Neurexin IV (Baumgartner et al., 1996) that is more properly classified as a contactin-associated protein (CASPR)/paranodin (Bellen et al., 1998; Peles et al., 1997). Unlike “true” Neurexins, Neurexin IV does not have

known roles at the synapse, although it does regulate axon-glia interactions (Bellen et al., 1998; Bhat et al., 2001) and axon pathfinding (Banerjee et al., 2010).

The trans-synaptic binding and communication between CAMs, in concert with signaling cues, orchestrates membrane contacts between pre- and postsynaptic partners. Following the formation of this stable junction, the presynaptic axon takes on a distinct morphology which varies across cell/tissue types and organism. For instance, at the vertebrate NMJ, presynaptic axons form an individual terminal structure that houses all release sites, such that a given motor neuron innervates a muscle at a single site (Shen and Cowan, 2010; Ziv and Garner, 2004). By contrast, in the vertebrate CNS, synaptic release sites are distributed in varicosities, or "boutons," along the length of interneuron axons, which are thus said to form synapses *en passant* (Shen and Cowan, 2010; Ziv and Garner, 2004). *En passant* synapses are widely observed in invertebrate nervous systems, including at the *Drosophila* NMJ where detailed imaging studies have revealed that the motor axon terminal forms an elaborate arborized structure (Rushton et al., 2009; Van Vactor and Sigrist, 2017; Zito et al., 1999). Expansion of the fly NMJ occurs through the Wnt/Wg-dependent addition of new boutons (Ataman et al., 2008). Possible modes of bouton addition include asymmetric budding, similar to yeast cell division; symmetric bouton division; or *de novo* formation from the axon shaft (Zito et al., 1999). Besides bouton addition, bouton elimination or pruning is also critical to refinement of synaptic structure and preventing overexuberant growth (Rushton et al., 2009; Van Vactor and Sigrist, 2017). Intermediary structures such as presynaptic "debris" or filopodia-like synaptopods are also observed during synaptic expansion, although unlike boutons, such structures are only observed very transiently (Ataman et al., 2008; Fuentes-Medel et al., 2009; Rushton et al., 2009; Van Vactor and Sigrist, 2017). These sequential morphological processes of bouton addition, followed by expansion to full size and if necessary, pruning, are modulated by baseline

and/or activity-dependent signaling cues to achieve a synaptic size and structural that facilitates proper connectivity and strength.

Rapid Initiation of Presynaptic AZ Assembly

The membrane-level changes from bouton addition, expansion, and pruning are coordinated with progressive assembly of the subcellular molecular machinery necessary for synaptic function and maturation (**Figure 1.1**). Numerous light imaging experiments in *Drosophila* have revealed that upon forming, boutons initially lack pre- and postsynaptic specializations (Ataman et al., 2006, 2008; Vasin et al., 2014). Subsequent ultrastructural studies in flies have shown that these immature “ghost” boutons are highly transient, as AZ precursors and SV docking become visible within minutes, although postsynaptic specializations are slower to form (Vasin et al., 2014). Work in *C. elegans* indicates that certain components of the AZ cytomatrix may aggregate into precursor structures within 5 minutes of the arrival of the growth cone (Lipton et al., 2018). However, work in *Drosophila* indicates that it may take hours for all core AZ components to arrive (Fouquet et al., 2009; Owald et al., 2010). These processes of bouton addition and maturation can still observed in cut axons, albeit at a lower frequency than in intact preparations, indicating local machinery is sufficient to support at least some level of synaptic expansion in the absence of protein synthesis (Vasin et al., 2014). At the fly NMJ, rapid synaptic expansion from embryos through the third instar larval stage ultimately results in a ~100-fold increase in synaptic connections (Rushton et al., 2009), with mature boutons each housing ~10 AZs (Collins and DiAntonio, 2007).

At least five core presynaptic AZ components are conserved across multiple taxa: RIM/Unc-10, Munc13/Unc13, RIM-Binding Protein (RBP), Liprin-α/SYD-2, and ELKS/CAST/ERC/Bruchpilot (Brp) (Ackermann et al., 2015; Südhof, 2012). In addition to these highly conserved components, vertebrate AZs include Bassoon and Piccolo

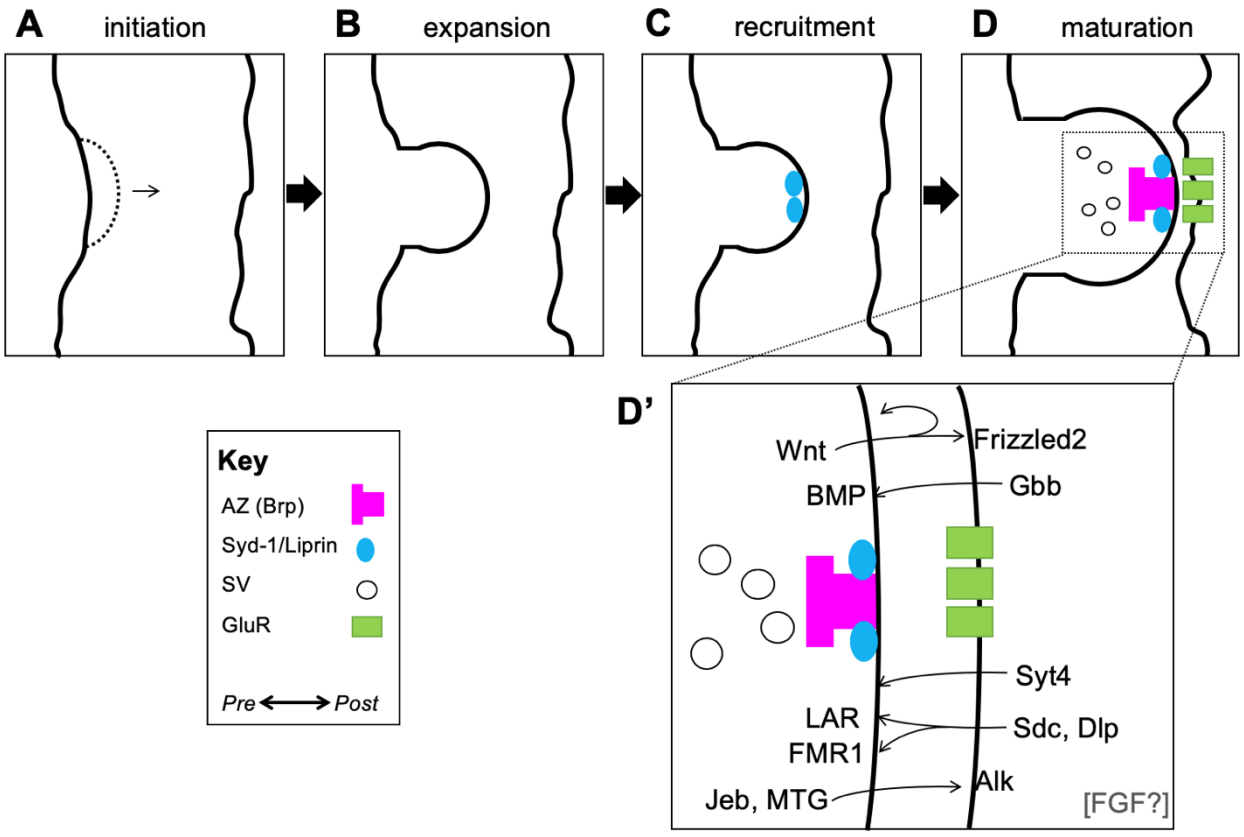

Figure 1.1. Overview of bouton growth and synaptic maturation. Addition of boutons is initiated by membrane outgrowth (**A**), followed by size expansion (**B**). Within minutes, AZ precursors are formed as early components such as Syd-1 and Liprin-α accumulate (**C**), followed by maturation (**D**) as the remaining AZ components, SVs, and glutamate receptors (GluRs) are recruited. Inset (**D**') shows bidirectional trans-synaptic pathways that are known to be important for synaptic development. Major pathways include anterograde Wnt and retrograde BMP signaling, while pathways mediated by Synaptotagmin 4 (Syt4), LAR, Fragile X Mental Retardation protein 1 (FMR1), Jelly Belly (Jeb), and Mind-The-Gap (MTG) have also been described. The FGF pathway has also been reported, but pathways details, e.g. directionality, have not been defined.

(Gundelfinger et al., 2016; Waites et al., 2005; Ziv and Garner, 2004), the latter of which has a putative *Drosophila* ortholog Fife (Bruckner et al., 2012, 2017), while invertebrate AZs also include Syd-1 (Dai et al., 2006; Owald et al., 2010; Patel et al., 2006), which may be related to mouse MSYD1A (Wentzel et al., 2013). Together, these core architectural components form an AZ structure that facilitates the release of neurotransmitter-filled SVs and the organization of Ca^{2+} channels (Ackermann et al., 2015; Owald et al., 2010; Südhof, 2012). In terms of geometry, the precise spatial arrangement of the AZ machinery produces a characteristic 3-dimensional structure that is closely apposed to the postsynaptic cytomatrix (**Figure 1.2**).

Despite the existence of local assembly mechanisms (Vasin et al., 2014), in general, AZ development relies on trafficking of AZ components, as well as SV precursors (SVPs) and other materials, via long-range motor transport (Goldstein et al., 2008; Hurd and Saxton, 1996; Maeder et al., 2014; Pack-Chung et al., 2007; Pilling et al., 2006), although mechanisms such as diffusion may also contribute (Miller and Heidemann, 2008; Popov and Poo, 1992). Several models exist for how AZ and SV components are organized into functional structures at release sites. *Ex vivo* studies have suggested the possibility that specialized dense core vesicles (DCVs) known as Piccolo/Bassoon Transport Vesicles (PTVs) traffic components in a unitary/quantal manner, such that each PTV contains a pre-assembled AZ “packet” (Shapira et al., 2003; Zhai et al., 2001). It has been proposed that AZ and SV materials are co-transported in aggregates of 1-2 PTVs and 5-6 SVPs that can very quickly form a functional AZ upon delivery (Bury and Sabo, 2011; Tao-Cheng, 2007; Waites et al., 2005; Wu et al., 2013; Ziv and Garner, 2004). However, the existence of PTVs and ready-to-go AZs packets has not been conclusively established through *in vivo* studies (Lipton et al., 2018; Petzoldt et al., 2016).

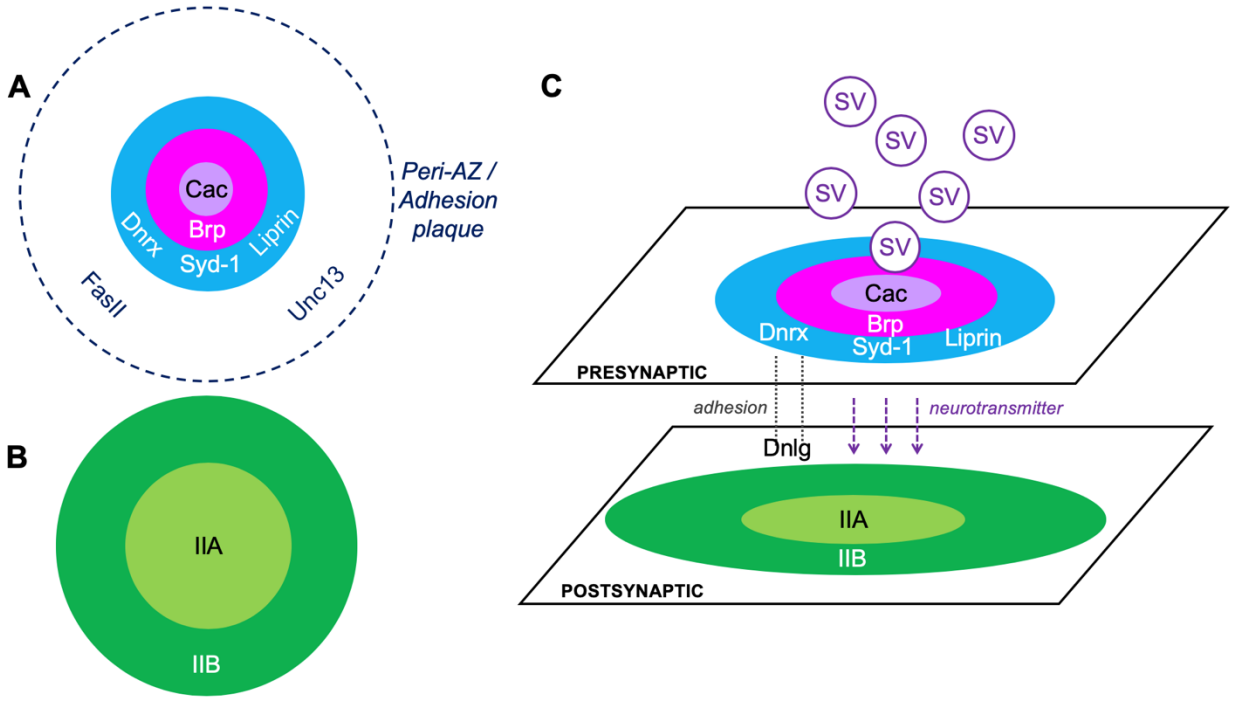

Figure 1.2. Schematic of the concentric organization of the presynaptic AZ and postsynaptic receptor clusters at the *Drosophila* NMJ. The presynaptic AZ (**A**) consists of a central cluster of the Ca^{2+} channel subunit Cacophony (Cac), which is contained within a funnel-like Brp structure. Surrounding the Brp/Cac core are proteins such as Dnrx, Syd-1, and Liprin-α. Additional proteins, such as Fasciclin II (FasII) and Unc13, are found in the peripheral AZ (peri-AZ) region. The peri-AZ region also includes adhesion plaque structures that mediate membrane interactions. In the postsynaptic specialization (**B**), receptor clusters are organized with GlurIIA-enriched receptors occupying the center, and GluRIIB-enriched receptors in a surrounding ring. This reflects the preferential recruitment of GluRIIA in early synapses, followed by GluRIIB as the synapse matures. Precise alignment of pre- and postsynaptic specializations (**C**) facilitate neurotransmission across the synaptic cleft. This interaction is regulated by CAMs such as Dnrx and Dnlg, which bind trans-synaptically (dotted lines).

Early AZ Assembly: Syd-1, Liprin-α, and Unc-13

In vivo studies in invertebrates suggest that while AZ assembly occurs very rapidly, a sequence of steps can still be distinguished: the earliest arriving AZ components "seed" the recruitment of additional core architectural molecules, followed by SV accumulation (Lipton et al., 2018). In support of hierarchical AZ assembly, ample studies in *C. elegans* and *Drosophila* indicate that two of the earliest components to be recruited to presynaptic AZs are the scaffolding proteins Syd-1 and Liprin-α/SYD-2 (*sy*napse-*d*efective) (Astigarraga et al., 2010; Dai et al., 2006; Fouquet et al., 2009; Li et al., 2014; Owald et al., 2010, 2012; Patel et al., 2006; Spinner et al., 2018; Stigloher et al., 2011; Zhen and Jin, 1999); evidence in flies suggests that these components may precede other AZ proteins by hours (Fouquet et al., 2009; Owald et al., 2010). In both worms and flies, Syd-1 and Liprin-α have close spatial as well as functional relationships in driving early AZ assembly (Owald et al., 2010; Patel et al., 2006) as well as SV organization (Li et al., 2014; Stigloher et al., 2011). In flies, loss of *syd-1* and *liprin-α* results in reduced NMJ size as well as increased AZ size as observed by light and electron microscopy, respectively (Kaufmann et al., 2002; Owald et al., 2010), indicating potential defects in AZ organization. Furthermore, loss of either *syd-1* or *liprin-α* in *Drosophila* leads to decreased neurotransmission (Kaufmann et al., 2002; Owald et al., 2010).

The Syd-1 scaffold protein localizes to presynaptic terminals and appears to be nervous-system specific in worms (Dai et al., 2006; Hallam et al., 2002; Patel et al., 2006), where it was first identified, as well as in flies (Owald et al., 2010). Early studies established that Syd-1 has PDZ, C2 and RhoGAP-like domains (Hallam et al., 2002), although the RhoGAP activity of Syd-1 was long disputed and was only recently discovered to be required for the clustering of ELKS/Brp (Spinner et al., 2018). Syd-1 is potentially one of the earliest AZ components to be recruited, as Syd-1 is upstream of Liprin-α in *C. elegans* (Dai et al., 2006; Patel et al., 2006) as well as in *Drosophila*, where Syd-1 is required for proper Liprin-α accumulation (Owald et al.,

2010). Syd-1 also interacts with the Dnrx-Dnlg trans-synaptic complex via direct binding to Dnrx to regulate postsynaptic receptor clustering (Owald et al., 2012). To date, no unambiguous mammalian orthologs of Syd-1 have been identified, although mouse MSYD1A has been suggested as a possible ortholog on the basis of partial sequence similarity and comparable roles in SV docking and synaptic transmission (Wentzel et al., 2013).

In contrast to Syd-1, the Liprin family member Liprin-α is highly conserved across metazoans, with ~50% similarity between the human and *C. elegans* orthologs, and is widely expressed in many tissues in addition to the nervous system (Miller et al., 2017; Spangler and Hoogenraad, 2007). Structurally, Liprin-α contains an N-terminal coiled-coil region that mediates homo- and hetero-multimerization and an C-terminal Liprin homology (LH) region containing three SAM (sterile-α-motif) domains (Kaufmann et al., 2002; Serra-Pagès et al., 1995, 1998). The SAM domains of the LH region, in particular, are thought to mediate the interactions of Liprin-α with Syd-1 and at least a dozen other proteins involved in synaptic development and/or function (Miller et al., 2017; Spangler and Hoogenraad, 2007). Indeed, Liprin-α was first identified from its interactions with a member of the LAR-RPTPs (Serra-Pagès et al., 1995, 1998); while these initial observations were at the focal adhesions of non-neuronal cells, studies of LAR as well as Liprin-α have since focused on their diverse functions in the nervous system. LAR, in particular, has critical roles in axon guidance, neurite extension, as well as synapse assembly, formation, and plasticity (Han et al., 2016; Um and Ko, 2013).

A recent study in *Drosophila* has shown that Unc13A, one of the two fly isoforms of Unc13, a central regulator of neurotransmitter release, may be co-recruited with Syd-1 and Liprin-α (Böhme et al., 2016). Unc13 was first identified in *C. elegans* (Maruyama and Brenner, 1991), with subsequent studies demonstrating its role in SV docking, priming, and fusion (Augustin et al., 1999; Brose et al., 1995). Unc13 primes the SNARE/SM machinery for exocytosis (Lai et al., 2017; Palfreyman and Jorgensen, 2017) and regulates the kinetics of

release (Böhme et al., 2016). Through its regulation of SV release, Unc13 is also involved in diverse forms of plasticity, including short-term augmentation and long-term potentiation and depression (Rosenmund et al., 2002; Xu et al., 2017). Super-resolution microscopy has revealed that the two *Drosophila* isoforms, Unc13A and Unc13B, occupy distinct localization patterns relative to other core AZ components and to Ca^{2+} channels, suggesting that different isoforms may act via independent pathways to tune and optimize SV release (Böhme et al., 2016). Interestingly, the shift from recruitment of Unc13A to Unc13B that occurs as AZ assembly progresses is reminiscent of a model where distinct receptor subunits in the postsynaptic compartment are recruited in a sequential manner (**Figure 1.2**) (Owald et al., 2012), perhaps reflecting separate roles of individual molecular isoforms at various stages of synapse maturation.

Downstream AZ Assembly: RIM, RIM-BP, and Brp

Following recruitment of Syd-1 and Liprin-α (Dai et al., 2006; Fouquet et al., 2009; Owald et al., 2010; Patel et al., 2006), and the Unc13A isoform (Böhme et al., 2016), the remaining AZ components are localized, including Unc13B, RIM/Unc-10, RIM-BP, and ELKS/Brp. Like Unc-13, RIM (*R*ab3-*i*nteracting *m*olecule) was first discovered as a regulator of SV release and neurotransmission (Mittelstaedt et al., 2010; Wang et al., 1997), and was later found to promote SV priming by monomerizing Unc-13 from autoinhibitory homodimeric complexes (Deng et al., 2011; Kaeser, 2011; Lu et al., 2006). RIM also has roles in both short- and long-term plasticity (Castillo et al., 2002; Schoch et al., 2002) and Ca^{2+} channel localization to the AZ (Graf et al., 2012; Han et al., 2015b; Kaeser, 2011; Kaeser et al., 2011). In particular, the regulation of Ca^{2+} channels by RIM is mediated by its interactions with RIM-BP (Mittelstaedt and Schoch, 2007; Wang et al., 2000). Together, RIM and the RIM-BP scaffold protein form a complex that interact with Ca^{2+} channels (Hibino et al., 2002; Kaeser et al., 2011). The importance of RIM-BPs has

been further demonstrated in *Drosophila,* where mutations in *rim-bp* results in defects in Ca^{2+} channel clustering as well as disruptions in Brp distribution, AZ ultrastructure, and synaptic release (Liu et al., 2011).

In addition to Unc-13, RIM, and RIM-BP, the scaffold ELKS/Brp is also recruited several hours following initiation of AZ assembly by Syd-1 and Liprin-α (Fouquet et al., 2009). While primarily enriched in the nervous system, Brp was initially identified for its role in carcinomas under the name *ELKS* (Nakata et al., 1999), subsequent studies of ELKS/Brp proteomic interactions identified it as an AZ component, and the protein was renamed CAST (Ohtsuka et al., 2002) and ERC (Wang et al., 2002b) in accordance with its newly-discovered roles. The *Drosophila* ortholog was later named *bruchpilot*, or German for *crash pilot,* to reflect the unsteady flight of *brp* mutants (Kittel et al., 2006; Wagh et al., 2006). The many names of Brp, which have resulted from multiple rediscoveries in different contexts, reflect its diverse functions across systems. Consistent with this, direct proteomic interactions of Brp and its orthologs have been observed with multiple other core AZ components, including but not limited to Liprin-α, Unc-13, RIM, Piccolo/Fife and Bassoon (Held and Kaeser, 2018; Hida and Ohtsuka, 2010). Of the core conserved AZ components, Brp remains arguably the most puzzling, as many of its precise functions and mechanisms remain a mystery (Hamada and Ohtsuka, 2018; Held and Kaeser, 2018; Hida and Ohtsuka, 2010; Südhof, 2012); further studies of Brp are expected to reveal fundamental insights into the AZ.

Detailed work in *Drosophila* have provided compelling clues to the functions of Brp at the AZ. *Drosophila* Brp is expressed in two isoforms, both of which are necessary at the synapse (Matkovic et al., 2013). Immunofluorescence has revealed that Brp forms distinct puncta in the presynaptic terminal of the NMJ (Kittel et al., 2006; Wagh et al., 2006) as well as in other nervous system cells, such as R8 photoreceptors (Sugie et al., 2015). Importantly, Brp is an essential structural component of the AZ and is a major component of the electron-dense T-bar

visible by electron microscopy (Fouquet et al., 2009), with loss of Brp resulting in the complete elimination of T-bars (Kittel et al., 2006; Wagh et al., 2006). Super-resolution STED microscopy has revealed that Brp puncta are in fact donut-shaped structures that represent the top half of a funnel-shaped Brp complex which is attached to the membrane of synaptic release sites (Fouquet et al., 2009; Kittel et al., 2006), and that the two *Drosophila* Brp isoforms alternate in a circular pattern (Matkovic et al., 2013). Further work combining dSTORM super-resolution with electrophysiology demonstrated that AZs consist of approximately 137 rod-like Brp proteins organized into about 15 heptameric structures, and that proper neurotransmission relies on a precise maintenance of the stoichiometry and organization of this multimeric structure (Ehmann et al., 2014). In addition to its role as a key AZ structural component, Brp is thought to regulate synaptic release by controlling the size of the readily releasable pool (RRP) of SVs (Matkovic et al., 2013). Brp is also necessary for short-term plasticity and Ca^{2+} channel clustering (Kittel et al., 2006), consistent with observations that the *Drosophila* Ca^{2+} channel subunit Cacophony (Cac) is recruited to the AZ contemporaneously (Fouquet et al., 2009).

The discussion in this chapter of AZ composition, architecture, and function addresses only a modest fraction of the expansive body of work on this highly complex and vital structure. The cytomatrix of the presynaptic AZ encompasses many other proteins, including: the scaffolds Piccolo/Fife and Bassoon; the Ras superfamily GTPases, such as Rab3 and Rac1, and their respective GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs); membrane trafficking machinery such as the SNARE proteins, synapsin, and synaptotagmin; the tripartite CASK/Mint1/Veli complex; developmental signaling pathways; and the cytoskeletal network (Ackermann et al., 2015; Held and Kaeser, 2018; Kaeser and Regehr, 2017; Sudhof, 2004; Südhof, 2012). Furthermore, despite significant progress in the field as a whole, many questions about the AZ remain. Ongoing and future studies will address topics such as the precise ultrastructure/nanostructure of the AZ, how the AZ is involved in both short-

and long-term plasticity, and how the AZ senses and regulates synapse stability and integrity (Ackermann et al., 2015; Südhof, 2012).

The Postsynaptic Cytomatrix

In contrast to the presynaptic terminal, which is always neuronal (Südhof, 2018), the postsynaptic target cell is most commonly neuronal but can also be of another cell type, such as glia (Bergles et al., 2000; Ge et al., 2006; Jabs et al., 2005; Lin and Bergles, 2004b, 2004a) or effector organs, as in the case of the *Drosophila* NMJ. Early ultrastructural studies of dendritic spine of excitatory synapses detected a 3-dimensional structure known as the postsynaptic density (PSD) directly underneath the neuronal membrane (Gray, 1959; Kennedy, 2000; Palay, 1956). The dendritic spine PSD, and analogous cytomatrix structures in other postsynaptic cell types (Zhai and Bellen, 2004), is rich in diverse protein types, including neurotransmitter receptors as well as scaffolding, signaling, and cytoskeletal molecules (Scannevin and Huganir, 2000; Sheng and Kim, 2011, 2002). Glutamate receptors (GluR), the most prevalent receptor type, are present at excitatory glutamatergic synapses that predominate in the vertebrate CNS as well as the invertebrate NMJ (DiAntonio, 2006; Scheefhals and MacGillavry, 2018). GluRs are classified as ionotropic GluRs (iGluRs), an abundant subtype that mediate fast synaptic transmission on millisecond timescales, and as metabotropic GluRs (mGluRs),which are slower-acting and less frequently occurring (DiAntonio, 2006; Scheefhals and MacGillavry, 2018). iGluRs can be further subdivided into AMPA-, NMDA-, and kainate-type receptors, with AMPA and NMDA receptors being most common (Scheefhals and MacGillavry, 2018). At the *Drosophila* NMJ, only AMPA/kainate receptors have been identified on the basis of genomic sequencing (DiAntonio, 2006; Littleton, 2000). However, more recent work has suggested that the iGluRs at the fly NMJ may be distinct from the classical vertebrate iGluR subtypes, as they display divergent structural and neurotransmitter-binding properties (Han et al., 2015a).

At the *Drosophila* NMJ, GluRs are heterometric tetramers composed of three invariant subunits, GluRIII/GluRIIC, GlURIID, and GluRIIE, as well as one of either GluRIIA or GluRIIB (DiAntonio et al., 1999; Featherstone et al., 2005; Marrus and DiAntonio, 2004; Petersen et al., 1997; Qin et al., 2005; Schuster et al., 2019). Recruitment of GluRs to the postsynaptic cytomatrix is thought to occur after the arrival of early presynaptic components Syd-1 and Liprin-α but prior to the recruitment of Brp and Cac (**Figure 1.1**) (Fouquet et al., 2009). GluRIIA and GluRIIB differ in their single channel properties, and they are responsible for large and small glutamate-gated currents, respectively (DiAntonio et al., 1999; Sigrist et al., 2002). The ratio of GluRs containing the GluRIIA versus GluRIIB subunits changes throughout the lifespan of a synapse, with younger synapses preferentially incorporating GluRIIA, followed by GluRIIB incorporation as the synapse matures (Schmid et al., 2008). GluRIIA recruitment is thought to be an essential driver of synapse formation and its incorporation into the GluR tetramer is nearly irreversible (Rasse et al., 2005; Schmid et al., 2008; Sigrist et al., 2002); this latter property, along with the tendency for GluRIIA incorporation to precede GluRIIB, likely accounts for the concentric arrangement of GluRs, where GluRIIB-rich receptors form a ring around GluRIIA-rich core (**Figure 1.2**) (Owald et al., 2012). The proper temporal sequence of subunit recruitment, as well as relative spatial arrangement of GluRIIA- and GluRIIB-associated receptors is thought to be necessary not just for the initiation synaptogenesis but also subsequent stabilization and maturation (Owald et al., 2012).

Aside from neurotransmitter receptors, the postsynaptic cytomatrix is also highly abundant in scaffold proteins. Many of these scaffolds contain PDZ domains, which are ~90 amino acid sequences that mediate interactions with other proteins and the synaptic membrane (Scannevin and Huganir, 2000; Sheng and Kim, 2011). The best-studied PDZ protein is PSD-95/SAP90 (Cho et al., 1992; Kistner et al., 1993) and its *Drosophila* ortholog Discs Large (Dlg), which was first identified as a tumor suppressor (Woods and Bryant, 1991) and shortly after

discovered to be important to synaptic growth and structural plasticity (Budnik et al., 1996; Guan et al., 1996; Lahey et al., 1994). Dlg belongs to the membrane-associated guanylyl kinases (MAGUKs) family of PDZ proteins (Scannevin and Huganir, 2000; Sheng and Kim, 2011) and regulates the clustering of Shaker K^+ ion channels (Tejedor et al., 1997) and of Fasciclin II (Thomas et al., 1997). Dlg is also necessary for clustering of GluRIIB-containing receptors (Chen and Featherston, 2005), while vertebrate PSD-95 regulates the clustering of NMDA receptors (Rao et al., 1998). While Dlg is a primarily postsynaptic protein, it is also present at lower levels in the presynaptic AZ (Budnik et al., 1996). Recently, this presynaptic population has also been shown to regulate the localization of Cac, with effects on synaptic release probability and short-term plasticity (Astorga et al., 2016).

Various factors expressed in the postsynaptic cytomatrix of *Drosophila* play additional roles in synaptic maturation and stabilization. The *Drosophila* ortholog of the p21-activated kinase (PAK) (Civiero and Greggio, 2018; Zhao and Manser, 2012), dPak, is required for GluR clustering, normal Dlg levels, muscle ultrastructure, and the formation of the subsynaptic reticulum (SSR), a postsynaptic system of tubular-lamellar membrane folds that envelops the presynaptic bouton (Lee and Schwarz, 2016; Parnas et al., 2001; Sone et al., 2000; Wan et al., 2000). Another key regulator of SSR expansion is Syndapin, a member of the F-BAR family of membrane-sculpting proteins (Kumar et al., 2009a; Quan and Robinson, 2013). Syndapin does not regulate presynaptic membrane trafficking as might be expected (Kumar et al., 2009b) and instead appears to exclusively postsynaptic; it likely mediates SSR development by regulating the muscle actin cytoskeleton (Kessels and Qualmann, 2002; Oh and Robinson, 2012). In addition, the essential postsynaptic protein Neto recruits dPak and GluRs to synaptic sites and also modulates the balance of GluRIIA/GluRIIB at the NMJ, thereby promoting postsynaptic stabilization (Kim et al., 2012, 2015; Ramos et al., 2015) in a manner similar to its vertebrate orthologs Neto1 and 2 (Ng et al., 2009; Zhang et al., 2009).

Trans-synaptic Communication Between Compartments

Despite the distinction between pre- and postsynaptic compartments, effective bidirectional communication is essential to the coordinated assembly, maturation, and function of the entire synapse (**Figure 1.2**). Key orchestrators of trans-synaptic communication include signal transduction pathways, in particular the Wnt/Wg pathway, which is the primary driver of anterograde signaling (Koles and Budnik, 2012; Packard et al., 2002; Speese and Budnik, 2007), and the BMP pathway, which is the major contributor to retrograde signaling (Bayat et al., 2011). At the fly NMJ, Wg is secreted from the presynaptic terminal and binds DFrizzled2 receptor embedded in the postsynaptic membrane, thereby enabling anterograde signaling (Mathew et al., 2005; Packard et al., 2002). Presynaptic Wg ligand also mediates a divergent pathway that regulates the presynaptic cytoskeleton; this glycogen synthase kinase (GSK3)/Shaggy- dependent signaling is thought to occur through either a retrograde or local pathway (Franco et al., 2004; Gögel et al., 2006; Miech et al., 2008). By contrast, the BMP ligand Glass Bottom Boat (Gbb) is secreted by the muscle and binds with tetrameric receptors containing Wishful Thinking (Wit), Thickveins (Tkv), and Saxophone (Sax) in the presynaptic membrane (Aberle et al., 2002; McCabe et al., 2003, 2004).

Besides Wnt/Wg and BMP, numerous other signal transduction pathways of consequence have been described at the fly NMJ. Multiple studies have established roles of retrograde signaling by Synaptotagmin 4 (Syt 4) (Barber et al., 2009; Yoshihara et al., 2005) and LAR (Leukocyte common antigen related) (Johnson et al., 2006; Kaufmann et al., 2002). Syt 4 is a postsynaptic Ca^{2+} sensor that regulates presynaptic SV fusion and activity-dependent structural plasticity in response to postsynaptic Ca^{2+} influx (Barber et al., 2009; Yoshihara et al., 2005). Presynaptic neuronal morphology and AZ assembly and function are also regulated by LAR, a receptor protein tyrosine phosphatase (RPTP) that interacts with the muscle-localized heaparan sulfate proteoglycans (HSPGs) Dallylike (Dlp) and Syndecan (Sdc) (Johnson et al.,

2006; Kaufmann et al., 2002). Dlp and Sdc have more recently been found to also act via the Fragile X Mental Retardation 1 (FMR1) protein in a retrograde manner in order to interface with the Wnt and Jelly Belly-Anaplastic Lymphoma Kinase (Jeb-Alk) signaling pathways (Friedman et al., 2013). The Jeb-Alk pathway is itself an anterograde trans-synaptic signaling pathways that regulates presynaptic morphology and neurotransmission (Rohrbough et al., 2013) under the control of Mind-The-Gap (MTG), a presynaptically secreted molecule that modulates the extracellular environment of the synaptic cleft (Rohrbough and Broadie, 2010; Rohrbough et al., 2007; Rushton et al., 2009, 2012). Interestingly, the two *Drosophila* FGF receptors, Heartless (Htl) and Breathless (Btl), have been reported to interact with the spinal muscular atrophy-associated (SMA) protein Survival Motor Neuron (SMN) to regulates NMJ morphogenesis, consistent with the roles of FGF at vertebrate synapses (Fox et al., 2007; Muha and Müller, 2013; Sen et al., 2011). Muscle-expressed Htl is itself required for presynaptic morphology, although the precise trans-synaptic mechanisms by which this is achieved remain to be defined (Sen et al., 2011).

Trans-synaptic communication at the fly NMJ also occurs through complexes formed by CAMs, including Dprs and DIPs (Morey, 2017; Zinn and Özkan, 2017), Teneurins (Mosca, 2015; Tucker and Chiquet-Ehrismann, 2006), and the previously described Dnrx and Dnlg proteins (Bottos et al., 2011; Craig and Kang, 2007; Knight et al., 2011; Südhof, 2008). Dprs/DIPs comprise a network of about 30 members (Carrillo et al., 2015; Cosmanescu et al., 2018; Özkan et al., 2013; Tan et al., 2015) with well-established roles in the synaptic connectivity of the fly visual system (Menon et al., 2019; Xu et al., 2018). While Dpr11 and DIP-γ both appear to be expressed on both sides of the NMJ synapse, tissue-specific expression has confirmed that Dpr11 and DIP-γ form trans-synaptic complexes; these Dpr11-DIP-γ complexes are required to prevent the abnormal clustering of small satellite boutons, possibly in concert with BMP signaling (Carrillo et al., 2015). Similarly, the two *Drosophila* Teneurins, presynaptic Ten-a and

postsynaptic Ten-m, form a trans-synaptic complex that regulates synaptic specification, neuronal morphology, synaptic architecture, and neurotransmission (Mosca et al., 2012). Notably, loss of neuronal *ten-a* and muscle *ten-m* produces defects in not only their respective compartment but also in the apposing cell: *ten-a* phenotypes include reduction in postsynaptic spectrin and SSR, while *ten-m* phenotypes include disruptions in presynaptic MTs, SVs, and T-bar morphology (Mosca et al., 2012). Such bidirectional CAM-mediated functions are further exemplified by the Dnrx-Dnlg1 complex. For instance, while the AZ component Syd-1 is exclusively presynaptic, it binds Dnrx directly and can therefore act via Dnrx-Dnlg1 to regulate GluRs composition and clustering (Owald et al., 2012); this illustrates one mechanism by which the core AZ machinery can organize their postsynaptic counterparts. Moreover, loss of muscle Dnlg1 results in defects in both presynaptic morphology and AZ assembly, indicating a reciprocal mechanism by which the postsynaptic apparatus can regulate release sites (Owald et al., 2012). Interestingly, Dnlg1 also regulates BMP pathway receptor subunits Wit/Tkv and the Mad (Banerjee et al., 2017), in a retrograde manner, further underscoring the bidirectional nature of Dnrx-Dnlg1 function.

Many of the molecules and pathways involved in the communication mechanisms described above have well-established roles in regulating both membrane morphogenesis and AZ assembly, raising the possibility that these two processes are coupled. In fact, the morphological and AZ phenotypes associated with numerous Wg, BMP, and LAR pathway components are consistent with an inverse relationship between the size of the AZ and the size presynaptic terminal as determined by bouton number. For instance, within the LAR pathway, this correlation is true for the LAR receptor itself as well as for Liprin-α (Kaufmann et al., 2002), the HSPG ligand Dlp (Van Vactor et al., 2006), the kinase Abelson (Abl) (Lin et al., 2009), the Rho-GEF Trio (Pawson et al., 2008; Spinner et al., 2018), and the F-actin assembly molecule Ena (unpublished data). The negative correlation between AZ and NMJ size is also observed for

Syd-1, and its downstream effectors Dnrx and Dnlg1; while Syd1 and Dnrx-Dnlg are not confirmed LAR interactors, they potentially interfaces with the LAR pathway through the interaction of Syd-1 and Liprin-α (Owald et al., 2010, 2012). While causative relationships and underlying mechanisms cannot yet be concluded, the accumulated evidence suggests that the regulatory machinery of bouton growth and AZ assembly are not fully independent. The coordination of morphogenesis and AZ formation may ensure the proper allocation of a finite pool of biological material: conceivably, restricting AZ size ensures that all boutons are populated with release sites even as membrane expansion continues. While such models can neither be confirmed nor rejected at present, the continued progress in elucidating a more thorough picture of synapse development might one day yield concrete evidence of a common upstream regulator of membrane morphogenesis and AZ assembly, and the biological rationale for the existence of such a mechanism.

The Dendritic Cytoskeleton: A Dynamic System

The morphological expansion of the neuron occurs during development and is followed by continual modifications and refinements thereafter (**Figure 1.1**). In mammals including humans, such changes have now been observed well into adulthood (Lee et al., 2006; Petanjek et al., 2011), in defiance of the long-held belief that the adult brain has limited capacity for structural plasticity. That the resultant neuronal structure is inextricably linked to its function is a fundamental tenet of neurobiology that has been demonstrated both theoretically and empirically (Jack et al., 1976; Kasai et al., 2003; Miller and Jacobs, 1984; Rall, 1977); for instance, *in silico* and experimental studies indicate that bouton size has considerable impacts on SV release probability (Knodel et al., 2014; Kurdyak et al., 1994; Li et al., 2002). The close structure-function ties in neurons, combined with the plastic nature of the nervous system, necessitates a system of molecules capable of dynamically supporting and modulating synaptic

architecture, and therefore function. Providentially, the cytoskeletal network provides such a backbone, in both a physical and biochemical sense. In *Drosophila,* the neuronal cytoskeleton includes elements such as actin, microtubules (MTs), and cortical spectrin (Goellner and Aberle, 2012), while additional polymers such as neurofilaments are present in some organisms (Kurup et al., 2018; Yuan et al., 2017). Collectively, the various polymers of the neuronal cytoskeleton network play indispensable roles in the development, maturation, and function of the synapse (Bodaleo and Gonzalez-Billault, 2016; Long and Van Vactor, 2013). Here, I will focus on the two most prominent cytoskeletal networks in neurons, actin and MTs (Goellner and Aberle, 2012), and the biochemical properties that make them singularly well-suited to driving multiple facets of synapse development and function.

Actin and MTs display rapid, polarized assembly and disassembly at their barbed and plus ends, respectively. Both actin and MTs are capable of self-regulated polymerization, although in physiological contexts, they typically assemble under the control of a wide array of molecules (Akhmanova and Steinmetz, 2008, 2015; Galjart, 2010; Pollard, 2007, 2016; Pollard et al., 2000). Key proteins associated with actin dynamics and organization include profilin, ADF/cofilin, Arp2/3, WASP, Scar/WAVE, WASH, formins, Rho GTPases, and capping protein (Pollard, 2007, 2016). MTs display the remarkable property of dynamic instability, in which they rapidly shift between states of assembly and disassembly (Cassimzeris et al., 1988; Desai and Mitchison, 1997; Howard and Hyman, 2003; Mitchison and Kirschner, 1984); this intrinsic behavior can also be modulated by MT-associated proteins (MAPs), such as plus-tip tracking proteins (+TIPs) (Akhmanova and Steinmetz, 2008, 2015; Galjart, 2010). Notably, polymerization of both actin and MTs is thought to generate protrusive forces that promote membrane deformation and rearrangements (Cooper, 2013; Etienne-Manneville, 2013; Pollard and Cooper, 2009). Furthermore, MTs are thought to engage in a “search-and-capture” mechanism that facilitates interactions between membrane, organelles, and other molecules

(Kirschner and Mitchison, 1986; Mimori-Kiyosue, 2003); in neurons, this exploratory behavior likely promotes efficient cellular responses to signaling cues (Baas et al., 2016). such as plus-tip tracking proteins (+TIPs) (Akhmanova and Steinmetz, 2008, 2015; Galjart, 2010). Taken together, both actin and MT dynamics have been well-established in the motility, migration, and morphogenesis of neurons and other cell types (Cooper, 2013; Etienne-Manneville, 2013; Kessels et al., 2011; Pollard and Cooper, 2009; Vitriol and Zheng, 2012). More specific properties of the synaptic cytoskeleton, and their specific roles in synaptogenesis, are described in the following sections. To reflect this historical sequence and research progression, I will proceed to first discuss findings relating to the dendritic actin and MT cytoskeletons and then summarize the current understanding of the presynaptic cytoskeleton.

Towards the end of the 19th century, Ramón y Cajal discovered that dendrites make synaptic contacts through numerous micron-sized filopodia-like protrusions, which are now known as spines (Glickstein, 2006; Nimchinsky et al., 2002). Modern studies have since shown that at excitatory synapses, dendritic spines house the PSDs and are thus essential sites of signal input; moreover, spine morphogenesis is driven by a dense actin network (Bertling and Hotulainen, 2017; Bosch and Hayashi, 2012; Dent et al., 2011; Hotulainen and Hoogenraad, 2010; Shirao and González-Billault, 2013; Spence and Soderling, 2015). Precise modulation of the equilibrium between globular-actin (G-actin) and filamentous-actin (F-actin) has significant effects on actin assembly rates and therefore actin-driven spine morphogenesis (Hotulainen and Hoogenraad, 2010; Spence and Soderling, 2015). While certain features of the dendritic cytoskeleton resemble the machinery of other motile and migratory structures, they also have unique features. For instance, while filopodia typically contain uniform-polarity F-actin bundles, dendritic F-actin forms mixed-polarity branched networks as well as other non-linear structures such as rings (Bertling and Hotulainen, 2017; Dent et al., 2011). F-actin in dendritic spines is thought to form at least three distinct pools: a dynamic, treadmilling pool at the spine tip; a

largely stable pool at the spine base; and a broadly-distributed pool that drives spine enlargement in a glutamate-dependent manner (Honkura et al., 2008). Collectively, these actin behaviors are important to spine morphogenesis and also stabilize the PSD and mediate activity-dependent structural plasticity in mature dendrites (Hotulainen and Hoogenraad, 2010). The continuous remodeling of dendritic actin is modulated by numerous factors also found in systems such as filopodia and lamellipodia; these include but are not limited to Rho family GTPases such as RhoA, Rac1, and Cdc42; the Arp2/3-WASP and WAVE complexes; profilin and cofilin; and capping protein (Hotulainen and Hoogenraad, 2010; Spence and Soderling, 2015).

While an abundant actin network in dendritic spines has been recognized for decades, dendritic spine MTs were only recently confirmed to exist. After many attempts over the years to detect and visualize spine MTs (Dent et al., 2011), multiple independent *ex vivo* imaging studies finally demonstrated conclusively that MTs invade the spines in an activity-dependent manner (Gu et al., 2008; Hu et al., 2008; Jaworski et al., 2009; Penzes et al., 2009). These and subsequent studies (Hu et al., 2011; Kapitein et al., 2011; McVicker et al., 2016; Merriam et al., 2011; Wagner et al., 2011) demonstrated that at a given time, MTs are only present in ~9% of dendritic spines and that each invasion lasts only minutes; the rarity and transience of these events accounts for the difficulty in detecting spine MTs. Dendritic spine MT dynamics are likely mediated by +TIPs: for instance, the end-binding protein 3 (EB3) is known to affect spine morphology (Jaworski et al., 2009), possibly through crosstalk with actin-associated proteins such as drebrin (Dent, 2017; Geraldo et al., 2008). Present evidence aside, many other questions remain about the mechanisms and functional significance of MT spine invasions, particularly in comparison to the current understanding of dendritic spine actin. Nonetheless, there is ample motivation to address these questions. For instance, MT invasions into dendritic spines are thought to play key roles in spine enlargement (Merriam et al., 2011) as well as the

maturation of spines from thin-type to mushroom-type structures (Gu et al., 2008; Jaworski et al., 2009). Moreover, dynamic MT spine invasions are also thought to be critical to activity-dependent structural plasticity of cultured neurons, and may be fundamental to brain functions such as learning and memory (Dent, 2017; Dent et al., 2011).

MTs are also a major component of the main dendritic and axon shaft, where they are essential to neuronal structure, organization, and long-range motor transport (Kevenaar and Hoogenraad, 2015; Maeder et al., 2014; Van De Willige et al., 2016). A central feature of MTs within these major neuronal structures is their polarity; this property is thought to be a major determinant of function. Axonal MTs display uniform plus-end out orientation while dendritic MTs are characterized by mixed polarity (Kapitein and Hoogenraad, 2011; Kevenaar and Hoogenraad, 2015; Matamoros and Baas, 2016; Rolls, 2011). Throughout vertebrate systems, plus- and minus-end out dendritic MTs occur in approximately equal numbers (Baas et al., 1988; Burton, 1988; Yau et al., 2016); dendritic MTs in *C. elegans* (Maniar et al., 2012; Stepanova et al., 2003; Yan et al., 2013) and *Drosophila* (Hill et al., 2012; Rolls, 2011; Rolls et al., 2007; Stone et al., 2008) are also mixed, but are predominantly (~90%) minus-end out. This orientation of MTs is thought to contribute to processes such as cargo sorting or the development of distinct subcellular identities between different neuronal compartments (Kapitein and Hoogenraad, 2011; Rolls, 2011). While the mechanisms of MT orientation remains largely unexplained, work in *Drosophila* dendrites has suggested that, as in the dendritic spine, +TIPs are likely to be critical; specifically, the +TIPs EB1 and APC are thought to recruit kinesin-2 to establish polarity (Chen et al., 2014; Mattie et al., 2010).

The Presynaptic Cytoskeleton

As in postsynaptic dendrites and other neuronal compartments, actin and MTs are important determinants of the development and function of the presynaptic terminal. At the motor axon

terminal of the fly NMJ, actin and MT networks are regulated, both directly and indirectly, by classic developmental signaling molecules, such as Wg, BMP, and LAR-Abl (Luchtenborg et al., 2014; Pawson et al., 2008; Piccioli and Littleton, 2014; Van Vactor and Sigrist, 2017). A comprehensive, molecular-level understanding of how the presynaptic cytoskeleton functions downstream of these signaling pathways is still maturing, with many remaining puzzles. For instance, how does actin and MT polymerization drive morphogenesis and structural plasticity, and what are the functional consequences of these membrane changes? How do actin and MTs interface with other components of the presynaptic machinery, and how are these interactions coordinated? I will describe current progress in addressing these questions, beginning with studies of the better-characterized actin cytoskeleton and proceeding to presynaptic MTs, which remains a largely open frontier.

The presence of F-actin throughout the presynaptic terminal was established through a combination of biochemical (Morciano et al., 2009; Stevens et al., 2003), functional (Morales et al., 2000), and ultrastructural studies (Landis et al., 1988); related studies also revealed the presence of F-actin at the AZ (Bloom et al., 2003; Hirokawa et al., 1989; Phillips et al., 2001). Consistently, actin localizes to puncta within the presynaptic boutons of the *Drosophila* NMJ (Pawson et al., 2008). The assembly and organization of presynaptic actin is mediated by interactions with CAMs, such as immunoglobulin receptors (Besse et al., 2007; Stavoe and Colón-Ramos, 2012) and Cadherins (Sun and Bamji, 2011). In cultured cells, F-actin recruitment has also been found to depend on the HSPG Sdc, which was furthermore required for formation of *in vitro* synapses (Lucido et al., 2009). Further studies have indicated that at the *Drosophila* NMJ, presynaptic actin is regulated by a diverse set of molecules, including Nervous Wreck (Nwk) (Coyle et al., 2004), Dap160/Intersectin (Koh et al., 2004; Marie et al., 2004), Cyfip (Zhao et al., 2013), Enabled (Ena; unpublished), and Diaphanous (Pawson et al., 2008). Loss of

these molecules results in NMJ morphogenesis defects, once again underscoring the importance of actin and its associated regulatory proteins in proper synaptic development.

Collectively, these and other studies have revealed a role for actin as an AZ scaffold and SV organizer (Cingolani and Goda, 2008a; Dillon and Goda, 2005; Long and Van Vactor, 2013; Nelson et al., 2013; Rust and Maritzen, 2015; Sankaranarayanan et al., 2003). For instance, in *C. elegans,* colocalization of F-actin and the neurabin ortholog NAB-1 is required to drive association of NAB-1 with Syd-1 and Liprin-α and subsequent AZ assembly (Chia et al., 2012). Similarly, the *Drosophila* adaptor protein Nwk, which directly binds the Arp 2/3 interactor WASP, has a peri-AZ localization and is required for normal AZ density and synaptic transmission (Coyle et al., 2004) as well as SV clustering and endocytosis (Rodal et al., 2008). Other actin-regulatory proteins, including the adaptor protein Dap160 (Koh et al., 2004; Marie et al., 2004), the WAVE complex-interactor Cyfip (Zhao et al., 2013), and direct actin interactor Ena (unpublished data) have similarly been shown to regulate SVs at release sites in flies. Similar roles for actin in SV organization been reported in lampreys (Bloom et al., 2003; Shupliakov et al., 2002). It is thought that actin may regulate SV organization by physically tethering SVs in a synapsin-dependent manner (Bleckert et al., 2012; Bloom et al., 2003; Fdez and Hilfiker, 2006).

As with dendritic spine MTs, the localization of presynaptic MTs has remained more contentious than that of actin despite many attempts to resolve the issue. Early ultrastructural evidence in lampreys (Schmitt, 1968), combined with biochemical work (Feit et al., 1971; Gozes and Littauer, 1979), suggested the presence of MTs not only in presynaptic terminals but in close proximity to AZs and SV release sites (Bird, 1976; Gray, 1975). However, other ultrastructural studies reported MTs and MAPs might be absent from axon terminals (Matus et al., 1981, 1975). Given that presynaptic MTs are now known to be labile and highly sensitive to fixation conditions (Bodaleo and Gonzalez-Billault, 2016; Matamoros and Baas, 2016), these discrepancies can be attributed to differences across experimental protocols and model

systems. The field has converged over time, and multiple ultrastructural and light imaging studies in systems including *C. elegans*, *Drosophila*, and vertebrates have established that MTs are present in presynaptic terminals (Bartlett and Banker, 1984; Caceres et al., 1986; Cumming et al., 1983; Paysan et al., 2000; Roos et al., 2000) and are moreover proximal to the AZ (Lepicard et al., 2014; Perkins et al., 2010; Stigloher et al., 2011)

Considerable evidence for the roles of presynaptic MTs has emerged from genetic studies of MAP phenotypes at the *Drosophila* NMJ (Bodaleo and Gonzalez-Billault, 2016; Long and Van Vactor, 2013). Perturbations in direct MT-interactors such as *futsch/map1b* (Lepicard et al., 2014; Roos et al., 2000), *stathmin* (Graf et al., 2011), and the formins *diaphanous* (Pawson et al., 2008) and *DAAM* (Migh et al., 2018) all produce NMJ undergrowth. Mutations in additional factors that regulate MTs, such as *DVAP-33A* (Pennetta et al., 2002), *dapkc* (atypical protein kinase C) (Ruiz-Canada et al., 2004), and the giant Ankyrin2 isoform *ank2-XL* (Stephan et al., 2015) also result in undergrowth. Orthologs of these molecules have been implicated in disease, and additional links are likely to be discovered in the future. For instance, mutations in both *futsch* and *DVAP-33A* have been associated with amyotrophic lateral sclerosis (ALS) (Coyne et al., 2014; Nishimura et al., 2004; Ratnaparkhi et al., 2008), and mutation of *stathmin* is associated with SMA (Wen et al., 2010). In addition, the human *dapkc* ortholog is potentially implicated in the pathology of Alzheimer's (Crary et al., 2006), and formins are linked to multiple diseases (Kawabata and Kengaku, 2019). These observations, along with broader findings that MTs are critical to neuronal health (Lasser et al., 2018; Matamoros and Baas, 2016), underline the importance of proper MT regulation and of sufficient synaptic growth and size.

In addition to the clear requirement for MAPs and other factors in promoting synaptic expansion, there is also compelling evidence for the importance of preventing of overexuberant synapse growth via presynaptic MT regulation. For instance, significant NMJ overgrowth results from mutation of *spastin,* the most frequently affected locus in hereditary spastic paraplegias

(HSP) (Sherwood et al., 2004), and from mutation of *dfxr* (homolog of FMR1), which regulates Futsch expression and is associated with Fragile X syndrome, one of the most common forms of inherited intellectual disability (Penagarikano et al., 2007; Zhang et al., 2001). Overgrowth phenotypes are also observed for loss of *katanin*, which is associated with microcephaly (Bartholdi et al., 2014; Mao et al., 2014); *spichthyin* (homolog of NIPA1 and ichthyin), which is associated with HSP (Wang et al., 2007); and tubulin-specific chaperone E (*tbce*), which is associated with hypoparathyroidism-intellectual disability-facial dismorphism, a fatal condition (Jin et al., 2009). Loss of *pavarotti/MKLP1/KIF23*, a kinesin that cross-links MTs and is necessary for vertebrate neuron development (Yu et al., 1997, 2000) also results in overgrowth (McLaughlin et al., 2016). Collectively, the under- and overgrowth phenotypes observed upon MT or MAP perturbation at the fly NMJ suggest that a maintaining a precise equilibrium of both synapse expansion and restriction is imperative and that MTs have critical roles in this process.

These studies at the fly NMJ established important roles for presynaptic MTs while raising questions about underlying mechanisms. Perhaps one of the most pressing issues is the significance of MT polymerization and plus-end behaviors: it is largely unknown how these unique MT properties might drive presynaptic development, but the importance of MT assembly in dendritic spines and growth cones strongly suggests comparable roles in the presynaptic compartment (Akhmanova and Steinmetz, 2008, 2015; Dent, 2017; Dent and Gertler, 2003; Jaworski et al., 2008; Lowery and Van Vactor, 2009; Vitriol and Zheng, 2012). An interesting explanation emerges when one also considers the indisputable role of MTs in providing physical tracks for motor transport through presynaptic terminal (Goldstein et al., 2008; Kapitein and Hoogenraad, 2011; Maeder et al., 2014). Could plus-end assembly and disassembly provide an additional layer of local, fine-grained control over the delivery of cargos to critical regions, such as the AZ? Such a possibility is consistent with demonstrations at the fly NMJ of a distinct sub-population of exploratory presynaptic MTs (Pawson et al., 2008), similar to pioneer MTs in the

neuronal growth cone. Conceivably, these pioneer-like MTs may facilitate a search-and-capture mechanism (Kirschner and Mitchison, 1986; Mimori-Kiyosue, 2003) that allows for the rapid distribution and rearrangements of components, such as AZ machinery or SVs, perhaps in cooperation with the role of AZ-localized presynaptic actin (Cingolani and Goda, 2008a; Dillon and Goda, 2005; Nelson et al., 2013). To date, the presynaptic roles of MT +TIPs have not yet been defined in the *Drosophila* NMJ or other comparable presynaptic systems (Akhmanova and Steinmetz, 2008, 2015), but if MT plus-end dynamics do mediate presynaptic organization, the +TIP network would almost certainly be one of the major regulatory mechanisms that would warrant further investigation.

Within the presynaptic terminal, MT assembly and dynamics might also regulate the membrane rearrangements that result in morphological changes, such as bouton growth and arborizations. Specifically, could the forces produced by MT polymerization induce membrane deformations that are necessary during bouton growth? Given that MT invasions promote enlargement and morphogenesis of dendritic spines (Gu et al., 2008; Jaworski et al., 2009; Merriam et al., 2011), it is interesting to imagine that the presynaptic pioneer MTs (Pawson et al., 2008) described above produce similar “invasions” that define new sites of bouton addition and enlargement. A further question concerns how the fundamental properties of MT assembly and stabilization might contribute to the overall neuronal morphology: for instance, does a more substantial and stable MT network imply greater NMJ expansion? It is well known that Futsch (Roos et al., 2000) and Spastin (Sherwood et al., 2004) have opposite roles in stabilizing and severing MTs, and also have opposite roles in promoting and restricting NMJ size, respectively, which is indeed consistent with a positive correlation between synaptic size and MT assembly. However, the collective evidence over the years hints at a more complex relationship between MT and NMJ expansion. Assuming that a given neuron has finite resources, formation of a very extensive MT network would eventually exhaust the pool of available tubulin; if the neuron were

still expanding, some MT disassembly and severing would likely be necessary to generate the necessary materials for further MT outgrowth (Roll-Mecak and Vale, 2006). Such explanations of how MT assembly relates to NMJ expansion remain thus far largely speculative, but the growing body of work on presynaptic MTs, bolstered by key technical and experimental advances, may begin to offer answers in the near future.

Overview of Dissertation

My dissertation research crystallized through a desire to unravel some of the many mysteries surrounding the roles and regulation of presynaptic MTs. While I considered each of the questions discussed in the previous paragraph throughout my studies, the focal points of my work became (1) the potential relationships between presynaptic MT assembly and stability and synaptic expansion and (2) the significance of presynaptic MT plus-end dynamics, potentially by +TIPs. In the course of investigating these questions, I have gravitated towards the protein dTACC, the *Drosophila* ortholog of the highly conserved TACC family of MAPs (Ding et al., 2017; Hood and Royle, 2011; Peset and Vernos, 2008; Thakur et al., 2013). dTACC drew initial interest as a novel regulator of synaptic morphogenesis, and understanding its function in the broader context of presynaptic MT biology and synaptogenesis is the crux of my dissertation. The following chapters collect several independent dTACC storylines, which each form the basis for a completed or emerging manuscript.

In Chapter 2, I describe our lab's initial, unexpected discovery that dTACC is a negative regulator of synapse growth despite being a MT-stabilizing protein in other contexts. In the remainder of Chapter 2 and in Chapter 3, I discuss the subsequent light-imaging work that helped define roles for dTACC in regulating presynaptic MTs at the developing synapse. Chapter 2 focuses on immunofluorescence studies that investigated the effect of dTACC on overall MT architecture, while Chapter 3 focuses on the design and application of a timelapse

imaging strategy to understanding the effect of dTACC on MT plus-end behaviors and is consistent with the possibility that dTACC has +TIP functions at the synapse. Thus, Chapters 2 and 3 use complementary static and dynamic methods to elucidate dTACC function. More broadly, our lab's work on dTACC is motivated by the broader question of how MT stability/polymerization and synapse expansion are related. While the simple model where MT stability and synapse growth correlate is attractive to consider, the reality is undoubtedly far less elegant, given that synaptogenesis is a mutable and complex amalgamation of different cellular and subcellular events that themselves are in constant flux. We thus aim to provide some perspectives into how cytoskeletal and morphological changes are potentially related.

Finally, in Chapter 4, I summarize my findings and describe ongoing work on postsynaptic dTACC. While our lab's work has ostensibly focused on presynaptic dTACC, we serendipitously discovered a postsynaptic population of dTACC that is closely associated with the presynaptic AZ. Our results are still highly preliminary and clear roles for postsynaptic dTACC have not yet emerged; thus, I will focus chiefly on potential future directions.

Chapter 1. Introduction to synapse development and the synaptic cytoskeleton.
This chapter provides a general survey of the synaptogenesis literature and the overarching motivation and key findings of my dissertation.

Chapter 2. dTACC restricts bouton addition and regulates microtubule organization at the *Drosophila* neuromuscular junction.
This chapter is a reprint of the Chou V.T., Johnson, S., *et al.* (2020) manuscript published in *Cytoskeleton* and describes the identification of dTACC as a novel regulator of synaptogenesis and of presynaptic MT architecture.

Chapter 3. A new 3D particle tracking tool for noninvasive in vivo analysis of synaptic microtubule dynamics in dendrites and at the neuromuscular junction of *Drosophila*.

This chapter is a reprint of the Chou V.T., Yesilyurt H.G., *et al.* manuscript that is forthcoming in the *Journal of Visualized Experiments* and describes the use of an *in vivo* imaging strategy to investigate the role of dTACC in both pre- and post-synaptic MT plus-end dynamics.

Chapter 4. Conclusions and future directions.

This chapter summarizes Chapters 2 and 3 and describes the unexpected identification and potential future investigation of a postsynaptic dTACC population.

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CHAPTER 2: DTACC RESTRICTS BOUTON ADDITION AND REGULATES MICROTUBULE ORGANIZATION AT THE *DROSOPHILA* NEUROMUSCULAR JUNCTION

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Chapter Contribution

This is a reprint of a manuscript that has been published as Chou, V. T., Johnson, S., *et al.* (2020). *Cytoskeleton (Hoboken)* 77, 4-15, doi:10.1002/cm.21578. Experiments using first-instar larvae and Western blotting were performed by Seth Johnson. The design, execution, analysis, and interpretation of all other experiments were led by Vivian Chou with assistance from Seth Johnson, Jennifer Long and Maxime Vounatsos. Vivian Chou drafted the manuscript and collaborated with Seth Johnson on revisions and edits.

ABSTRACT

Regulation of the synaptic cytoskeleton is essential to proper neuronal development and wiring. Perturbations in neuronal microtubules are associated with numerous pathologies, yet it remains unclear how changes in microtubules may be coupled to synapse morphogenesis. Studies have identified many microtubule regulators that promote synapse growth. However, less is known about the factors that restrict growth, despite the potential links of synaptic overgrowth to severe neurological conditions. Here, we report that dTACC, which is implicated in microtubule assembly and stability, prevents synapse overgrowth at the *Drosophila* neuromuscular junction by restricting addition of new boutons throughout larval development. dTACC localizes to the axonal microtubule lattice and is required to maintain tubulin levels and the integrity of higher-order microtubule structures in motor axon terminals. While previous reports have demonstrated the roles of microtubule-stabilizing proteins in promoting synapse growth, our findings suggest that in certain contexts, microtubule stabilization may correlate with restricted growth.

INTRODUCTION

Synapses are the essential functional units of the nervous system. Formation of intricate synaptic geometry, which involves complex arborization of cell processes and cell-cell connections, is critical to the function and plasticity of neural circuits. Following axon pathfinding, signaling pathways coordinate synaptic morphogenesis and the formation of stable junctions between pre- and postsynaptic compartments (Goda and Davis, 2003; Collins and DiAntonio, 2007; Van Vactor and Sigrist, 2017). A major target and effector of these signaling networks is the presynaptic microtubule (MT) cytoskeleton (Broadie and Richmond, 2002; Ruiz-Cañada and Budnik, 2006; Rushton et al., 2009). MTs have been linked to numerous neurodevelopmental and neurodegenerative disorders (Bodaleo and Gonzalez-Billault, 2016; Goellner and Aberle, 2012; Lasser et al., 2018; Matamoros and Baas, 2016). However, despite the clear importance of synaptic MTs, our understanding of their regulation and function still lags behind our comprehension of the upstream signaling pathways that orchestrate synapse development.

Despite limited mechanistic understanding, synaptic morphogenesis has been well characterized at the phenomenological level through studies at the *Drosophila* neuromuscular junction (NMJ) (Jan and Jan, 1976). In this system, a motor axon contacts its target muscle during late embryogenesis and transitions from a motile, sheet-like growth cone into a branched structure decorated with synaptic varicosities (“boutons”) (Yoshihara et al., 1997). Throughout larval development, the NMJ rapidly expands through the addition of new immature boutons (Schuster et al., 1996; Zito et al., 1999), which then recruit presynaptic active zone components and postsynaptic receptors as they mature (Rushton et al., 2009; Vasin et al., 2014). In response to both developmental cues and neural activity (Budnik et al., 1990; Chklovskii et al., 2004; Van Vactor and Sigrist, 2017), the NMJ undergoes continuous remodeling via both bouton addition and removal (Eaton et al., 2002; Fuentes-Medel et al., 2009). These processes are modulated by numerous signaling pathways, such as BMP (Bayat et al., 2011; Keshishian and Kim, 2004), FGF

(Sen et al., 2011), LAR (Han et al., 2016; Um and Ko, 2013), and Wnt/Wg (Packard et al., 2002; Park and Shen, 2012; Speese and Budnik, 2007).

While the downstream MT-related targets of developmental signaling pathways remain largely unknown, several components of the Wnt/Wg pathway directly regulate the MT cytoskeleton by binding MTs themselves and/or with MT-associated proteins (MAPs) (Salinas, 2007). One such target of Wnt/Wg signaling is the MAP Futsch (homolog of MAP1B), which is phosphorylated by glycogen synthase kinase 3 (GSK3)/Shaggy (Sgg) in both mammals and flies (Cohen and Frame, 2001; Franco et al., 2004; Gögel et al., 2006). At the *Drosophila* NMJ, Futsch promotes MT stability and synaptic expansion (Lepicard et al., 2014; Roos et al., 2000), while inhibition of Futsch by Sgg restricts synapse size (Franco et al., 2004). These findings suggest a model where increased stabilization of MTs is associated with increased NMJ expansion. Additional factors that are associated with MT stability, such as the formins Diaphanous (Pawson et al., 2008) and DAAM (Bartolini and Gundersen, 2010; Migh et al., 2018), have also been found to promote NMJ expansion.

As a counterbalance to MT-stabilizers, MT destabilizers/severing proteins, such as Spastin (Sherwood et al., 2004) and Katanin (Mao et al., 2014) restrict NMJ size. Consistently, repression of Futsch mRNA levels by Dfxr (homolog of FMR1) prevents NMJ overgrowth (Zhang et al., 2001), further supporting the notion that MT stability correlates with NMJ expansion. Interestingly, mutation of human *spastin* is the most frequent cause of hereditary spastic paraplegias (HSP) (Solowska and Baas, 2015), while mammalian *katanin* has been associated with behavioral deficits and intellectual disability (Banks et al., 2018; Bartholdi et al., 2014). Similarly, *dfxr* is associated with Fragile X syndrome, one of the most common forms of inherited intellectual disability (Penagarikano et al., 2007). The synaptic phenotypes and disease relevance of genes such as *spastin, katanin*, and *dfxr* has led to the understanding that excessive synaptic growth is highly detrimental. Collectively, these findings suggest that a complex set of factors is

responsible for maintaining a precise balance of both synapse expansion and restriction to ensure neurological function and health.

Here, we report a new negative regulator of synapse growth, the *Drosophila* homolog of the highly conserved TACC (transforming acidic coiled coil) family (Peset and Vernos, 2008; Hood and Royle, 2011; Thakur et al., 2013; Ding et al., 2017). Early studies of *Drosophila* and mammalian TACC-family proteins showed that these proteins are often concentrated near MT minus ends and have roles in regulating MTs and spindle function during mitosis (Gergely et al., 2000b, 2000a), in cooperation with the MT polymerase ch-TOG/XMAP215/Minispindles (Msps) (Akhmanova and Steinmetz, 2008, 2015; Brouhard et al., 2008; Lee et al., 2001). Similar observations have since been reported across phyla (Bellanger and Gönczy, 2003; Le Bot et al., 2003; Peset et al., 2005; Samereier et al., 2011; Sato et al., 2004; Srayko et al., 2003). TACC can also localize at the MT plus-end, where it is thought to regulate MT assembly dynamics (Long et al., 2013; Lucaj et al., 2015; Nwagbara et al., 2014; Rutherford et al., 2016; Samereier et al., 2011; Srayko et al., 2003). However, TACC localization to the MT lattice has been observed in multiple settings (Gergely et al., 2000b; Peset et al., 2005; Sato et al., 2004; Thadani et al., 2009). Altogether, these studies strongly suggest that TACC proteins serve as conserved mediators of both the assembly and stability of MTs (Peset and Vernos, 2008; Hood and Royle, 2011; Thakur et al., 2013; Ding et al., 2017).

Given (1) prior studies suggesting that increased MT stability correlates with growth and (2) the established roles of TACC proteins in MT assembly and stability, dTACC would naturally be expected to promote synapse growth. Surprisingly, we discovered instead that presynaptic dTACC negatively regulates the growth of the larval NMJ by limiting addition of synaptic boutons during development. We also found that within the motor axon terminal, dTACC associates abundantly along the lattice of MTs and regulates both the integrity and higher-order organization

of MTs. Our results suggest that in certain contexts, assembly and/or organization of MTs by proteins such as dTACC may restrict NMJ expansion.

RESULTS AND DISCUSSION

Presynaptic dTACC is required to restrict NMJ size

The NMJ has a highly stereotyped morphology consisting of a branched motor axon terminal that is decorated by numerous presynaptic boutons (**Fig. 2.1A**). In this system, boutons are often quantified as a measure of overall NMJ size. To determine the effect of dTACC on synapse morphogenesis, we initially counted mature type I boutons at the muscle 6/7 NMJ in late-stage third instar *dtacc* mutant larvae labeled with the neuronal membrane marker anti-horseradish peroxidase (HRP) (Jan and Jan, 1982). To generate a strong *dtacc* loss background, we raised transheterozygotes using two independently derived alleles: $dtacc^{592}$ ($d\text{-}tacc^{stella}$), a complete null (Lee et al., 2001), and $dtacc^{1}$ ($d\text{-}TACC^{1}$), which has been previously described as a very strong allele (Gergely et al., 2000b). We combined $dtacc^{592}$ and $dtacc^{1}$ over *Df(3R)110*, a deletion at the locus (**Fig. 2.2A**) and confirmed that these animals showed phenotypes comparable to $dtacc^{592}/dtacc^{1}$ transheterozygotes (**Fig. 2.1**). However, $dtacc^{592}$/*Df(3R)110* and $dtacc^{1}$/*Df(3R)110* animals were very weak, suggesting that haploinsufficiencies uncovered by the deletion contributed to pleiotropy. Thus, we focused our remaining analysis on $dtacc^{592}/dtacc^{1}$ transheterozygotes to avoid additional phenotypes resulting from deletion of flanking genes.

Our analysis showed that $dtacc^{592}/dtacc^{1}$ animals display striking NMJ overgrowth compared to genetically matched w^{1118} controls (**Fig. 2.1B,C,H**); this phenotype was reminiscient of mutations in MT destabilizers such as *spastin* (Sherwood et al., 2004) and *katanin* (Mao et al., 2014). We also found that dTACC is haploinsufficient, as $dtacc^{592}$/+ heterozygotes showed a significant and reproducible 1.35-fold increase in bouton number compared to controls (**Fig. 2.1H**), revealing that the NMJ is highly sensitive to levels of dTACC. As expected, $dtacc^{592}/ dtacc^{1}$ animals displayed an even more dramatic but qualitatively comparable phenotype, including a

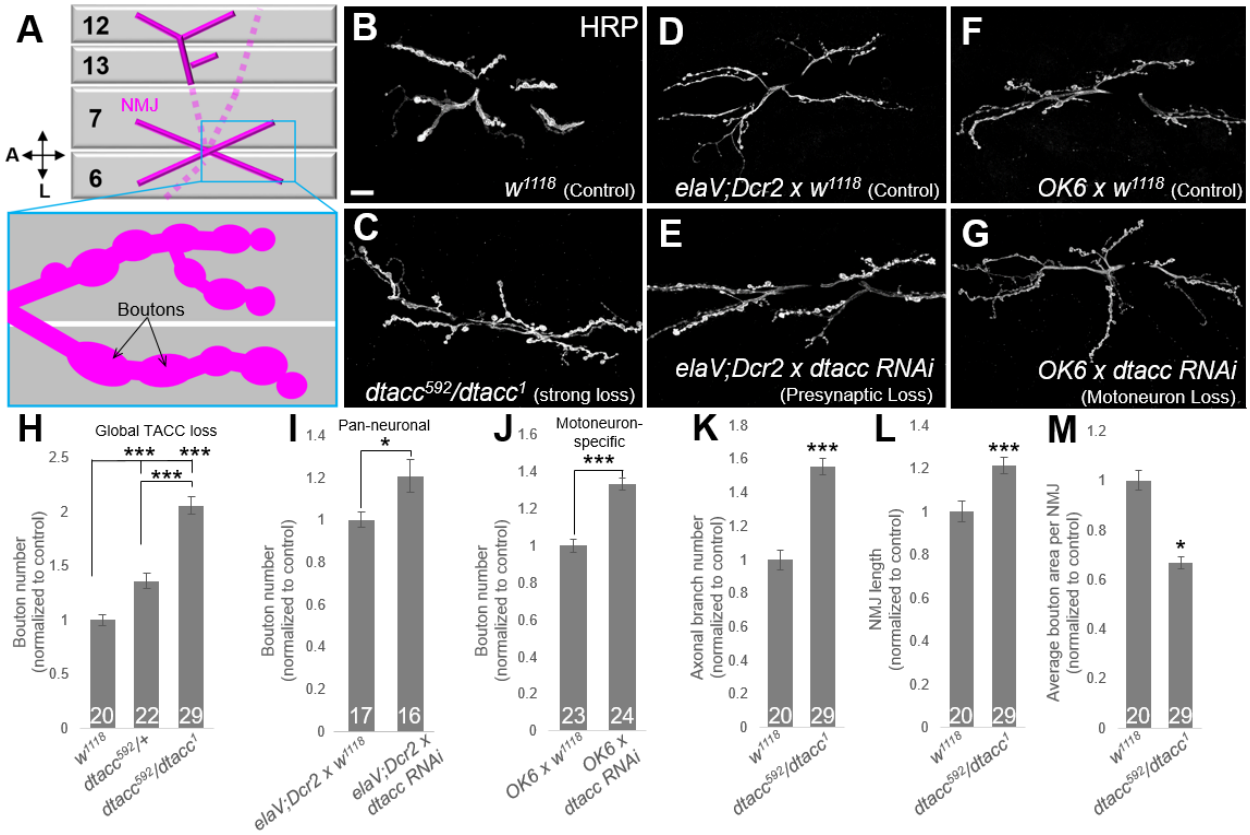

Figure 2.1. dTACC is a negative regulator of NMJ size. ***A***, Schematic of larval ventral musculature and nerve innervation pattern. For clarity, only select structures are depicted. Inset shows the morphology of the NMJ, including varicosities or "boutons." ***B-J***, Loss of dTACC results in NMJ overgrowth. ***B-G***, Images show third instar NMJs stained with the neuronal membrane marker α-HRP. In contrast to w^{1118} controls (**B**), $dtacc^{592}/dtacc^{1}$ flies (**C**) showed increased NMJ size, as did $elaV^{C155}$*; Dcr2 x dtacc-RNAi* (**E**) and *OK6 x dtacc-RNAi* (**G**) animals compared to their respective $elaV^{C155}$*; Dcr2 x* w^{1118} (**D**) and *OK6 x* w^{1118} (**F**) controls. Quantification of bouton number (**H**) indicates that dTACC is haploinsufficient, as $dtacc^{592}$/+ heterozygotes show a 1.35-fold increase in bouton number, while $dtacc^{592}/dtacc^{1}$ flies show a more severe, albeit qualitatively indistinguishable, 2.05-fold increase. $elaV^{C155}$*; Dcr2 x dtacc-RNAi* (**I**) and *OK6 x dtacc-RNAi* (**J**) animals showed 1.21 and 1.33-fold increase, respectively, in bouton number, comparable to $dtacc^{592}$/+ heterozygotes. Relative to controls, $dtacc^{592}/dtacc^{1}$ also showed increased axonal

Figure 2.1 (continued). branch number (**K**) and NMJ length (**L**). ***M***, *dtacc^{592}/dtacc^{1}* NMJs showed a decrease in average bouton area. Raw bouton counts: *w^{1118}*, 96.6; *dtacc^{592}*/+, 167.6; *dtacc^{592}/dtacc^{1}*, 198.6; *elaV^{C155}; Dcr2 x w^{1118}* , 144.8; *elaV^{C155}; Dcr2 x dtacc-RNAi* , 174.9; *OK6 x w^{1118}* , 127.1; *OK6 x dtacc-rnai* , 169.4. * P < 0.05, *** P < 0.001, t-test; error bars indicate ± s.e.m; number of NMJs quantified indicated on graph; scale bar, 20 μm.

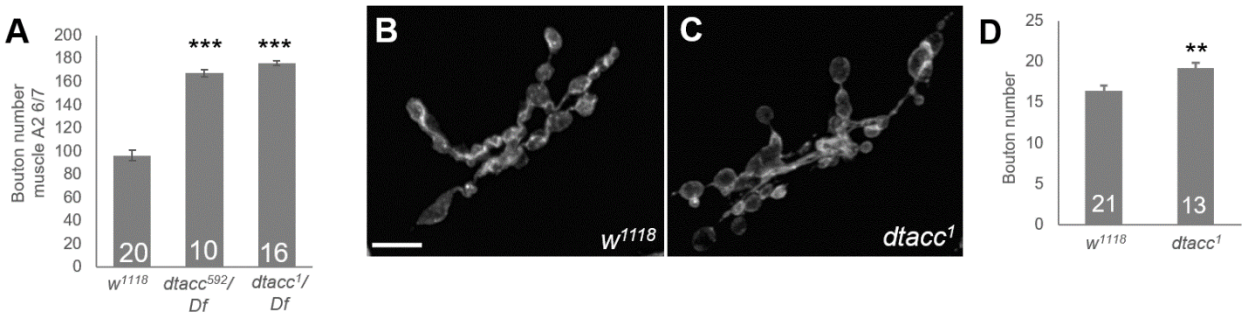

Figure 2.2. Confirmation of TACC null alleles and first instar phenotype. *A*, *dtacc*^{592}*/Df(3R)110* and *dtacc*^{1}*/ Df(3R)110* flies were generated. Both individual TACC alleles produced overgrowth compared to *w*^{1118} controls. ***B-D***, *dtacc* phenotype in first-instar animals. Compared to controls (**B**), *dtacc*^{1} animals (**C**) showed significant overgrowth, as confirmed by quantification (**D**). *** P < 0.001, determined by Student's t-test; error bars indicate ± s.e.m; number of NMJs quantified indicated on graph; scale bar, 5 μm.

2.05-fold increase in bouton number compared to controls (**Fig. 2.1B,C,H**). There is thus a proportional relationship between dTACC levels and bouton number, suggesting that dTACC expression or activity could modulate NMJ expansion. Notably, the *dtacc*^{592}*/ dtacc*^{1} overgrowth phenotype was apparent as early as in first-instar larvae (**Fig. 2.2B-D**), indicating a continuous requirement for dTACC throughout the span of NMJ development. To determine if the change in neuronal structure reflects a presynaptic requirement for dTACC, we drove pan-neuronal and motoneuron-specific RNAi knockdown of a *UAS-dtacc-RNAi* construct using *elaV*^{C155} and *OK6*-GAL4 drivers, respectively. Both *elaV*^{C155} (**Fig. 2.1D,E,I**) and *OK6* (**Fig. 2.1F,G,K**) driven RNAi showed significant overgrowth, indicating that dTACC is required presynaptically, and, more specifically, in motoneurons. The fold-increase in bouton number in *dtacc-RNAi* animals was comparable to that observed in *dtacc*^{592}/+ heterozygotes (**Fig. 2.1H**), likely reflecting the partial efficacy of the RNAi-knockdown.

In the *dtacc*^{592}/*dtacc*^{1} background, we also quantified branching within the motor axon terminal, overall length of the NMJ, and bouton size; both branch number and NMJ length confirmed highly significant increases of NMJ size in *dtacc*^{592}/*dtacc*^{1} compared to control (**Fig. 2.1K,L**). We found that *dtacc*^{592}/*dtacc*^{1} animals display a ~33% decrease in average bouton area (**Fig. 2.1M**). This bouton size phenotype, along with the *dtacc* overgrowth phenotype, raised the question of which step(s) of bouton formation may require dTACC. During normal NMJ expansion (Zito et al., 1999), baseline bouton addition is controlled by developmental signaling cues (**Fig. 2.3Ai**), which coordinate and balance neuronal expansion with muscle growth [reviewed by Van Vactor and Sigrist, 2017]. Bouton addition can also be induced acutely by activity-dependent cues in response to stimuli (**Fig. 2.3Aii**) (Budnik et al., 1990; Chklovskii et al., 2004). Immediately following baseline or activity-induced addition, nascent boutons lack pre- and postsynaptic markers and thus have a “ghost”-like appearance (**Fig. 2.3Ai,ii,** black triangles) (Ataman et al., 2006). However, this is a highly transient state, as components required to form the presynaptic

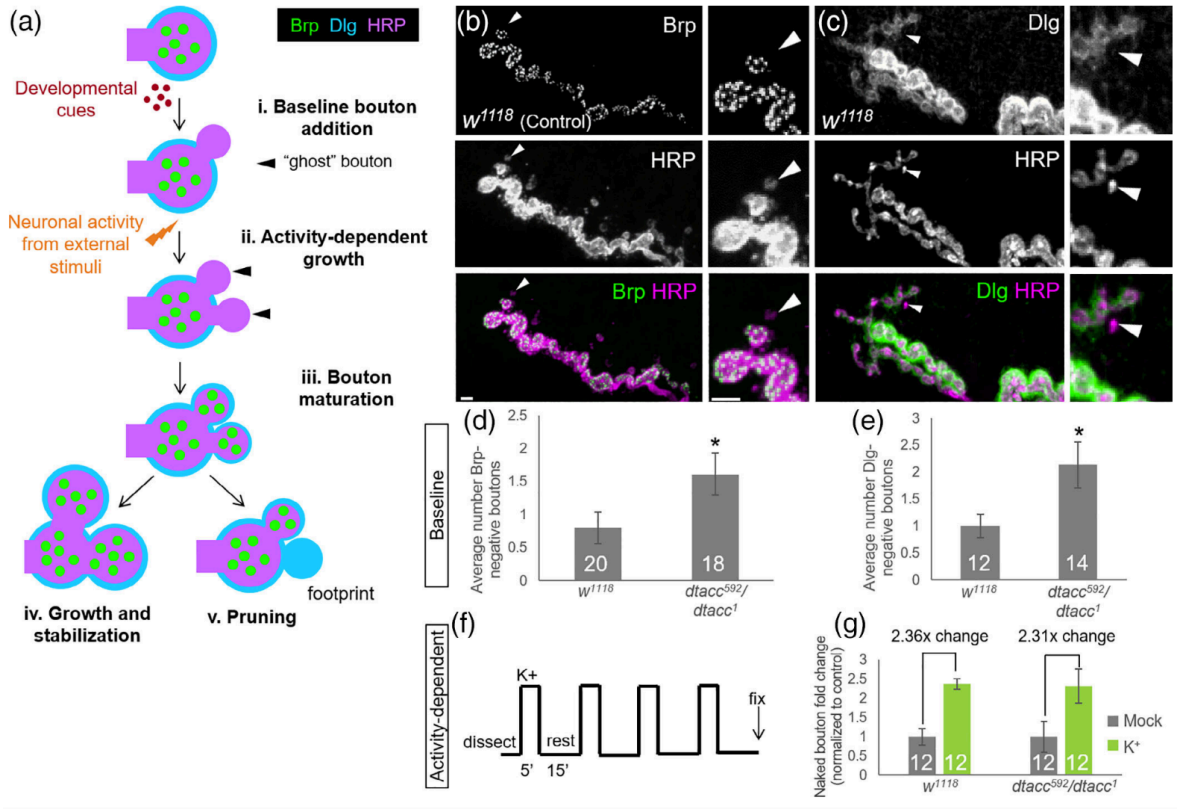

Figure 2.3. dTACC regulates bouton addition during development. ***A,*** Schematic showing the steps of bouton formation, including addition of boutons in response to (i) baseline or (ii) activity dependent growth, followed by (iii) bouton maturation via recruitment of pre- and postsynaptic components. Boutons may continue to (iv) grow to full size and stabilize or (v) retract instead, leaving "footprints" of post-synaptic material. ***B,C***, NMJ stained α-HRP and counterstained with with α-Brp (Nc82; **B**) or α-Dlg (4F3; **C**). Nascent boutons (triangle) can be identified by the lack of post-synaptic markers such as Dlg. ***D, E,*** *dtacc*^{592}*/dtacc*^{1} flies showed a 2-fold increase in Brp-negative boutons (**D**) and in Dlg-negative boutons (**E**) compared to *w*^{1118} controls. ***F***, spaced stimulation paradigm used to induce rapid activity-dependent bouton budding. ***G***, controls and *dtacc*^{592}*/dtacc*^{1} showed nearly identical fold-changes (2.36 and 2.31-fold, respectively) in the number of nascent boutons following activity compared to mock-treated controls. * $P < 0.05$, t-test; error bars indicate ± s.e.m; number of NMJs quantified indicated on graph; scale bar, 1 μm.

active zone and postsynaptic cytomatrix begin to accumulate within ~30 minutes of new bouton formation (**Fig. 2.3Aiii**) (Vasin et al., 2014). Following maturation, boutons may continue to grow to full size and stabilize (**Fig. 2.3Aiv**). Alternatively, boutons can be removed or pruned, occasionally leaving visible "footprints" of postsynaptic material, such as the scaffold protein Discs Large (Dlg; **Fig. 2.3Av**) (Eaton et al., 2002).

We investigated the potential involvement of dTACC in each of these steps. We considered the possibility that dTACC promotes bouton pruning (**Fig. 2.3Av**) but did not find changes in Dlg footprints in *dtacc* animals, making this explanation unlikely (see Materials and Methods). Furthermore, we found no striking defects in the overall distributions of synaptic cytomatrix antigens in *dtacc* animals, such as the core active zone component Bruchpilot (Brp; **Fig. 2.4A,B**) or Dlg (**Fig. 2.4C,D**), suggesting that there were no catastrophic effects on initial bouton maturation (**Fig. 2.3Aiii**). However, the ~33% decrease in average bouton area (**Fig. 2.1E**) did suggest a defect at the stage where maturing boutons grow to full size (**Fig. 2.3Aiv**). Reduction in bouton growth could reflect some compensation for the effects of overgrowth. Alternatively, this could arise because synthesis and/or transport may not increase at the same rate as bouton number, thus making materials too sparse to form normal-sized boutons. In either scenario, the defects in bouton growth and number raised the possibility that there might be upstream defects in bouton initiation.

dTACC regulates bouton initiation in response to baseline developmental cues

To investigate the potential role of dTACC in bouton addition, we looked for changes in the incidence of nascent boutons, which can be identified by the lack of maturation markers such as Brp (**Fig. 2.3B**) or Dlg (**Fig. 2.3C**). Compared to w^{1118} controls (mean = 0.8 boutons/NMJ), $dtacc^{592}/dtacc^{1}$ animals showed a 2-fold increase in the number of Brp-negative nascent boutons (mean = 1.6 boutons/NMJ, p = 0.04; **Fig. 2.3D**). Consistent with the Brp presynaptic marker, Dlg

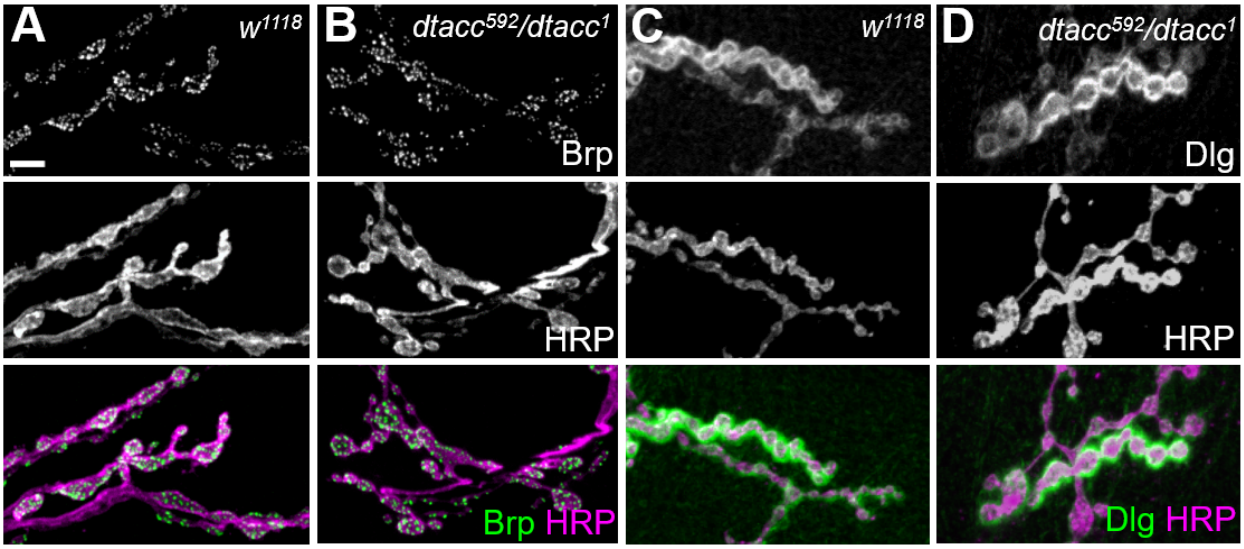

Figure 2.4. *dtacc* animals show normal accumulation of pre- and postsynaptic markers. *w*^{1118} and *dtacc*^{592}/*dtacc*^{1} animals were co-stained with the neuronal membrane marker α-HRP and the presynaptic active zone marker α-Brp (**A,B**) or the postsynaptic marker α-Dlg (**C,D**). Compared to *w*^{1118} (**A,C**) animals, the distribution of markers in *dtacc*^{592}/*dtacc*^{1} (**B,D**) animals was grossly normal. Scale bar, 5 μm

staining revealed that compared to controls (mean = 1 bouton/NMJ), *dtacc* animals also showed a 2-fold increase in small nascent boutons lacking postsynaptic specializations (mean = 2.1 boutons/NMJ, p = 0.008; **Fig. 2.3E**). Collectively, these results revealed an increased frequency of "ghost" boutons in *dtacc* animals, which suggested a greater rate of bouton initiation.

The increased bouton addition in *dtacc* animals observed through end-point analysis could occur in response to baseline developmental cues (**Fig. 2.3Ai**) and/or to neural activity from external stimuli (**Fig. 2.3Aii**). To evaluate these scenarios, we tested the requirement of dTACC in acute activity-dependent growth using a spaced-stimulation paradigm (**Fig. 2.3F**) that induces rapid budding of "ghost" boutons (Ataman et al., 2008; Nesler et al., 2013; Piccioli and Littleton, 2014; Vasin et al., 2014). Both w^{1118} and $dtacc^{592}$/ $dtacc^{1}$ animals showed an increase in Dlg-negative nascent boutons upon stimulation, and the fold increase was indistinguishable between controls and *dtacc* animals (2.36- and 2.31-fold, respectively; **Fig. 2.3G**). These results indicate that dTACC is not required for acute activity-dependent bouton initiation. This suggests that the greater frequency of nascent boutons in *dtacc* animals likely reflects an increase in baseline bouton addition in response to developmental cues. This potential role of dTACC as a negative regulator of baseline bouton addition is consistent with the observation that significant NMJ overgrowth can be observed throughout the span of development (**Fig. 2.2B-D**). Moreover, the 2-fold increase in putative nascent boutons in *dtacc* animals (**Fig. 2.3**) is equal to the 2-fold increase we previously observed in mature bouton number (**Fig. 2.1**). This doubling of both nascent and total bouton numbers further supports the model that increased bouton number in *dtacc* mutants is due to increased baseline bouton addition.

dTACC colocalizes with the lattice of synaptic MTs.

To better understand how dTACC might affect NMJ growth, we asked if dTACC associates with MTs at the NMJ. While TACC has been most frequently reported to localize to MT minus- or plus-

ends (Ding et al., 2017; Hood and Royle, 2011; Peset and Vernos, 2008; Thakur et al., 2013), dTACC puncta have been observed along the lattice of spindle MTs (Gergely et al., 2000b). Furthermore, *Xenopus* TACC3, the TACC isoform most highly expressed in the *Xenopus* embryonic nervous system (Rutherford et al., 2016; Tessmar et al., 2002), abundantly decorates the length of MTs in egg extracts (Peset et al., 2005), and the *S. pombe* TACC homolog Alp7/Mia1p is found along MTs both *in vivo* (Sato et al., 2004) and *in vitro* (Thadani et al., 2009).

We investigated the precise nature of dTACC protein localization at the NMJ to distinguish between potential modes of MT interaction *in vivo*. We generated and validated a novel monoclonal antibody against dTACC (see Materials and Methods). Quantification of dTACC intensity and Western blotting confirmed loss of signal in *dtacc* mutants (**Fig. 2.5A,B**). Using this antibody, we found that the majority (~90%) of dTACC signal in controls was found in polymer lattice-like structures within the axon terminal (**Fig. 2.6A**; solid triangles) which strongly resemble the MT lattice at the core of the axon, consistent with prior reports that *C. elegans* TAC-1 localizes to the axons of sensory neurons (Chen et al., 2015). A smaller fraction of dTACC intensity (~10%) was found in punctate bouton-associated structures (**Fig. 2.6A**; hollow triangles). When we used our antibody on $dtacc^{592}/\ dtacc^{1}$ animals, dTACC signal was dramatically reduced with the minor punctate fraction virtually abolished and the major lattice-like staining markedly decreased (**Fig. 2.6B**). Although dTACC was not detectable on a Western blot with our antibody (**Fig. 2.5A**), there was small residual immunohistochemical signal in the $dtacc^{592}/\ dtacc^{1}$ mutant (**Fig. 2.5B**) that may reflect residual expression (as much as 5% in $dtacc^{592}/\ dtacc^{1}$) predicted by previous characterization of the $dtacc^{1}$ background (Gergely et al., 2000b). Motivated by our finding that dTACC is required throughout early NMJ development (**Fig. 2.2B-D**), we also examined dTACC distribution in first instar larvae (**Fig. 2.5C**) and found, as expected, that dTACC is highly expressed in the ventral nerve cord (VNC) of the CNS (**Fig. 2.5D**) and throughout motor/sensory axon tracts (**Fig. 2.5E**).

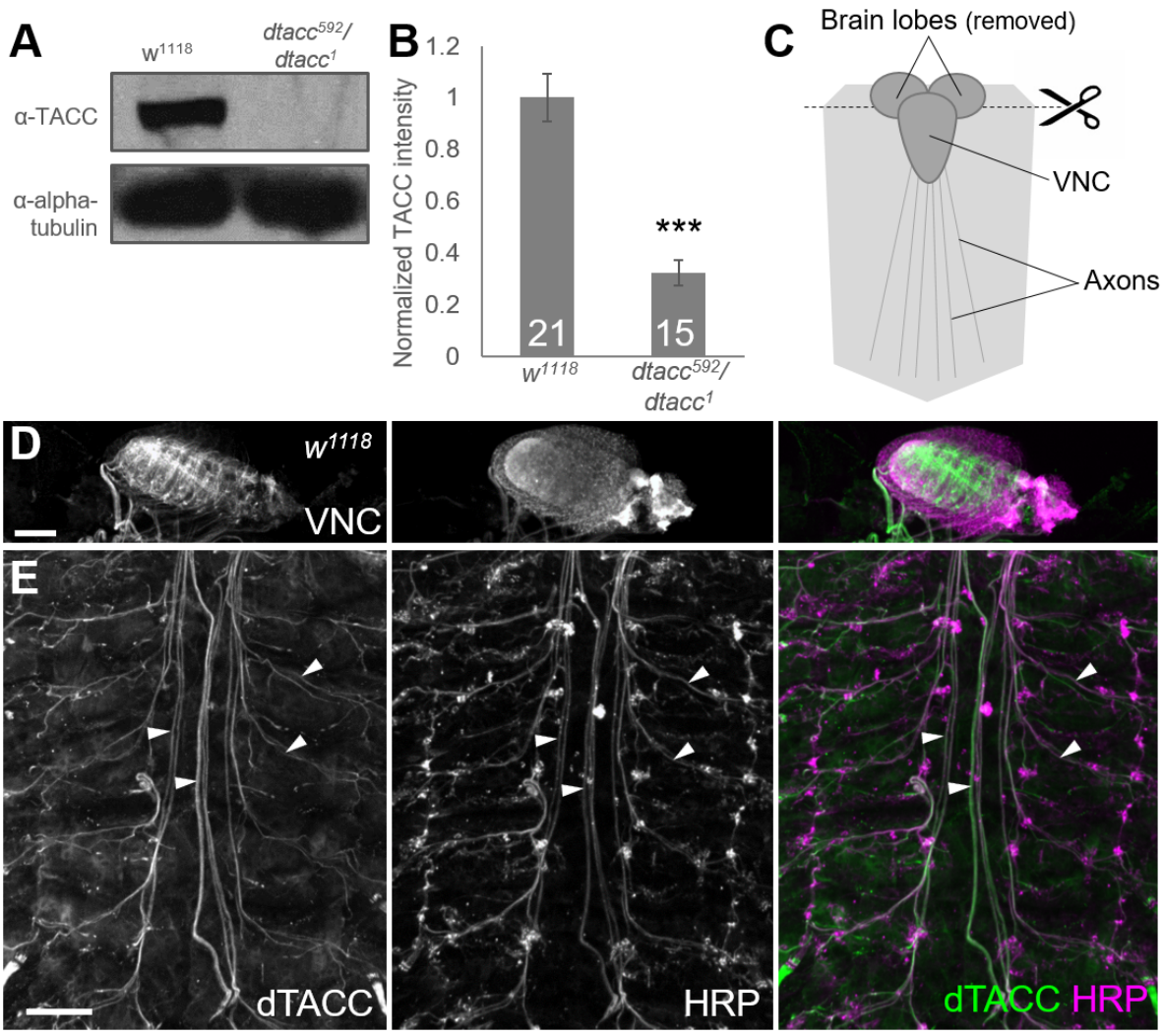

Figure 2.5. Validation of the TACC antibody. ***A*,** Western blotting showed complete reduction of dTACC antibody signal in *dtacc*^{592}/*dtacc*^{1} null animals. α-alpha-tubulin (Ab7291) was used as a loading control. ***B***, dTACC staining intensity was significantly reduced in *dtacc* null flies by ~68%. *** P < 0.001, determined by Student's t-test; error bars indicate ± s.e.m; number of NMJs quantified indicated on graph. ***C-E***, First instar *w*^{1118} animals were co-stained with α-dTACC and α-HRP. ***C***, Schematic showing dissection technique of first instars, which removes the brain lobes but leaves the ventral nerve cord (VNC) intact. Strong dTACC staining was observed in the VNC (**D**) and throughout the motor and sensory axon tracts (**E**; triangles), along with some muscle staining. Scale bars, 50 μm.

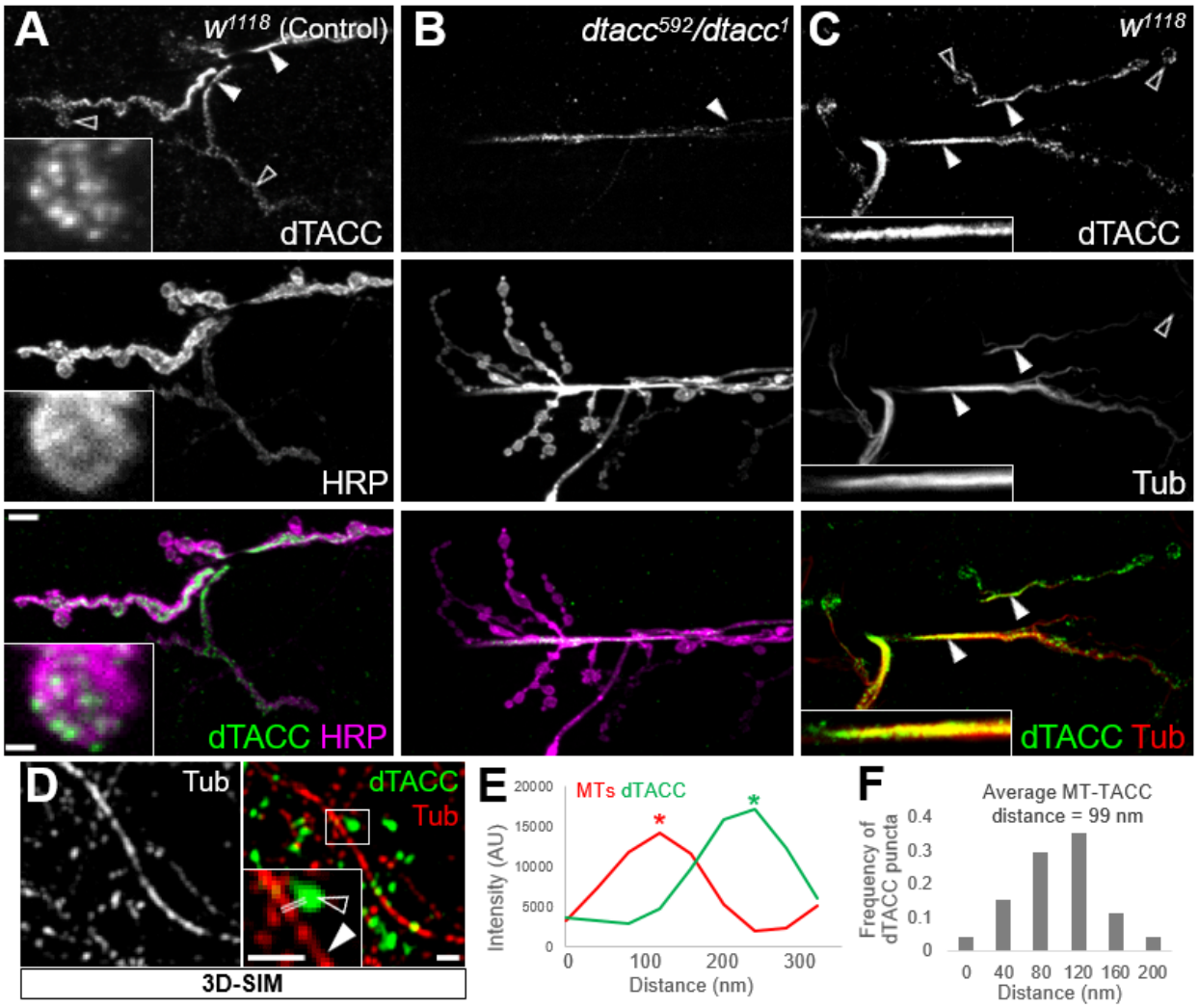

Figure 2.6. TACC colocalizes with tubulin at the synaptic terminal. ***A, B,*** Validation of dTACC antibody in NMJs stained with α-dTACC and α-HRP. Compared to control w^{1118} flies (**A**), $dtacc^{592}/dtacc^{1}$ flies showed reduced dTACC staining (**B**). In controls (**A**), the majority of TACC showed a filamentous distribution highly reminiscent of the MT lattice within motor axon terminals (solid triangles), while a smaller TACC population formed puncta (hollow triangles; see high magnification inset). In $dtacc^{592}/dtacc^{1}$ animals (**B**), punctate TACC was virtually absent, and filamentous TACC was dramatically reduced. ***C,*** Colocalization of α-dTACC and α-alpha-tubulin (Ab15246) in the axon terminal. The distribution of filamentous TACC was similar to the distribution of tubulin, while punctate TACC appeared spatially distinct from tubulin. The Manders'

Figure 2.6 (continued). overlap coefficients were M1 = 56.3% ± 3.7 (percent TACC colocalizing with tubulin) and M2 = 58.6% ± 6.8 (percent tubulin colocalizing with TACC; n=15 NMJs). Scale bar, 5 μm for main panels, 1 μm for insets. ***D-F***, Spatial relationships between punctate dTACC and MTs were examined through 3D-SIM of samples stained with α-dTACC and α-alpha-tubulin (**D**). Double white lines (**D**) represent positions from which intensity profile plots were drawn. ***E***, Representative intensity profile plot showing separation between the peaks of α-dTACC and α-tubulin staining. ***F***, Quantification of mean distance between peaks of α-dTACC and α-tubulin staining measured from intensity profile plots (n=71 puncta). Mean dTACC-MT distance was found to be 98.6 nm. Scale bar, 500 nm.

Importantly, we tested the colocalization of dTACC with MTs by staining for alpha-tubulin (Ab15246 for all tubulin immunohistochemistry) and found that the distribution of lattice-like TACC and alpha-tubulin was highly coincident (**Fig. 2.6C**). The Manders' overlap coefficient M1 (percent dTACC colocalizing with tubulin) was found to be 56.3% ± 3.7, while M2 (percent tubulin colocalizing with dTACC) was 58.6% ± 6.8 (n=15 NMJs) (Manders et al., 1993). We also considered the possibility that the punctate fraction of dTACC might be associated with MT plus-ends within the motor terminal. To test this idea, we used 3-dimensional structured illumination microscopy (3D-SIM) to measure the average distance between dTACC and MTs based on a published methodology (Lepicard et al., 2014). Compared to confocal microscopy, 3D-SIM improves resolution by two-fold in all three dimensions and can thus resolve objects with up to eight-fold smaller volume (Gustafsson et al., 2008; Gustafsson MG, 2000; Schermelleh et al., 2010). At this improved resolution, we noted that dTACC puncta appeared visually distinct from synaptic MTs (**Fig. 2.6D**). To confirm this observation, we generated intensity profile plots (**Fig. 2.6E**) of dTACC and tubulin staining (**Fig. 2.6D,** double lines show sample line scans) and found that the mean distance between the dTACC and tubulin peaks was 98.6 nm (**Fig. 2.6F**). Although this method may not be sensitive enough to detect single MTs or MTs that are highly dynamic and/or labile, we were unable conclude that the distal puncta of dTACC in boutons are closely associated with MTs.

Overall, our results suggested that in the NMJ arbor, the majority of dTACC is spatially localized with the lattice of MTs, similar to prior observations in *Xenopus*, *Drosophila* and fission yeast (Gergely et al., 2000b; Peset et al., 2005; Sato et al., 2004; Thadani et al., 2009). Interestingly, purified yeast Alp7 localizes to regions of overlap between adjacent (parallel or anti-parallel) MTs where it is thought to mediate cross-linking of bundled MTs, thereby promoting the assembly and stability of linear MT arrays both *in vitro* and *in vivo* (Thadani et al., 2009). A role in cross-linking kinetochore MTs is also observed for TACC3 in HEK293 cells (Booth et al., 2011).

Given that neuronal MTs are organized into polarized bundles that resemble the MT arrays found in *S. pombe* (Baas et al., 2016; Bartolini and Gundersen, 2006; Hoogenraad and Bradke, 2009), it seemes possible that dTACC serves a similar function in regulating synaptic MT organization. Interestingly, this possibility would be consistent with findings that Pavarotti, a kinesin that cross-links MTs and stabilizes the mitotic spindle, is also a negative regulator of NMJ size (McLaughlin et al., 2016).

dTACC is required for normal levels and organization of synaptic MTs.

The MT-lattice localization of dTACC at the NMJ is consistent with previous studies of TACC function in MT organization and stability. In *Drosophila* embryos, loss of dTACC results in short astral and spindle MTs (Gergely et al., 2000b), and similar roles in regulating both mitotic and interphase MTs have been observed for TACC proteins in a variety of systems (Peset and Vernos, 2008; Hood and Royle, 2011; Thakur et al., 2013; Ding et al., 2017). Thus, the close association of dTACC with the MT lattice suggested a specific role in regulating MTs within motor axon terminals.

To investigate if dTACC regulates synaptic MTs, we compared the staining intensity and distribution of tubulin in $dtacc^{592}/dtacc^{1}$ animals to w^{1118} controls (**Fig. 2.7A,B**). Due to the fragility of MTs, we used a specifically optimized fixation protocol (see Materials and Methods). Control tubulin staining was clear and robust, with distinct filamentous structures (**Fig. 2.7A**). There was a clear concentration of tubulin in the main axon shaft, with thinner filaments leading out into the branches of the synaptic terminal where bouton addition is more frequent. In contrast to previous demonstrations (Jin et al., 2009; Mao et al., 2014; Sherwood et al., 2004; Trotta et al., 2004) of robust muscle MT staining, we observed comparatively weaker post-synaptic MT signal. This likely reflects differences in our protocol, which was specifically optimized to target the labile, unstable presynaptic MT population.

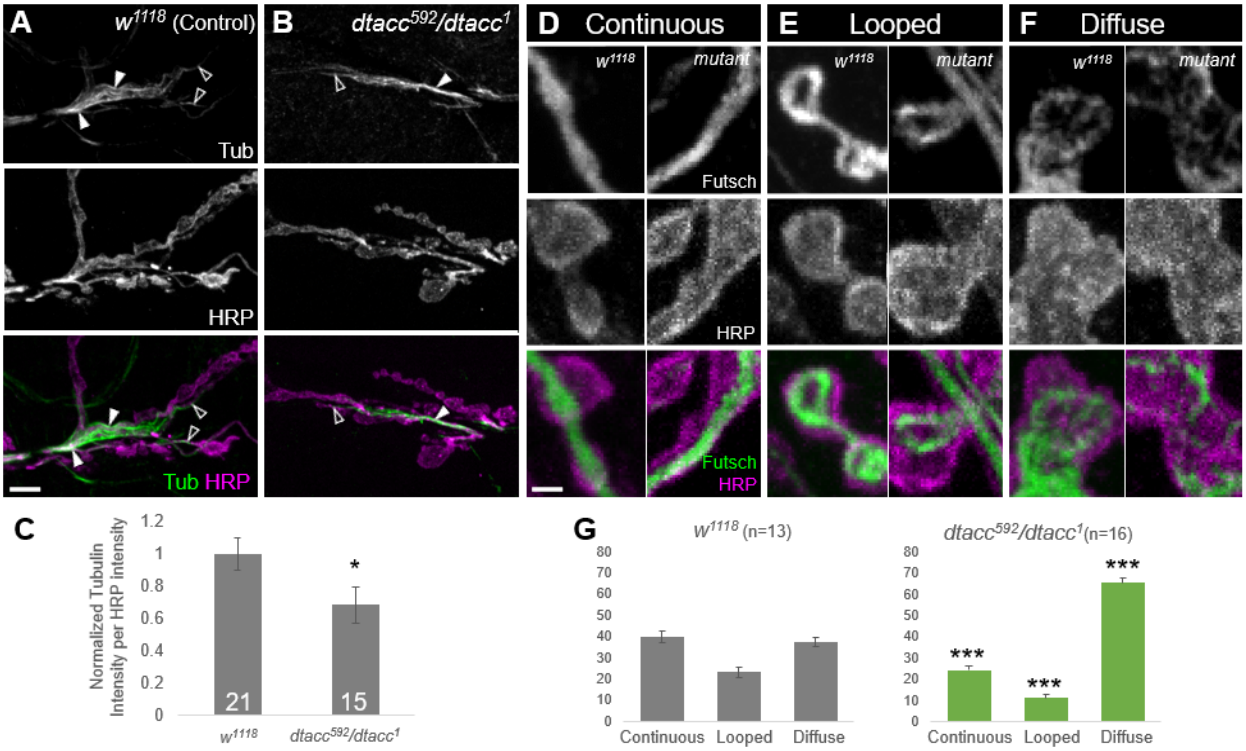

Figure 2.7. TACC is regulates the architecture and higher-order organization of synaptic MTs. ***A-C****,* Comparison of tubulin intensity in control and *dtacc*^{592}*/dtacc*^{1} NMJs stained with α-tubulin (Ab15246) and α-HRP. Control animals (**A**) showed robust tubulin staining with clear filamentous structures (triangles). Tubulin staining was most concentrated in the main axonal shaft (solid triangles), while thinner tubulin filaments were observed in terminal branches (hollow triangles). In *dtacc*^{592}*/dtacc*^{1} flies (**B**), tubulin staining appeared weaker and was often undetected in branches. Quantification (**C**) indicates that detectable tubulin intensity was significantly reduced *dtacc*^{592}*/dtacc*^{1} flies. Scale bar, 10 μm. ***D-G***, Analysis of Futsch-labeled MT arrangements in control and *dtacc*^{592}*/dtacc*^{1} NMJs labeled with α-Futsch (22C10) and α-HRP. Futsch-decorated MTs in the three terminal boutons of each branch were categorized into continuous, looped, and diffuse patterns (**D-F**). Side-by-side comparison of boutons showed that Futsch MT structures are clearly reproducible between controls and *dtacc*^{592}*/dtacc*^{1} mutants. Quantification (**G**) shows that in *dtacc*^{592}*/dtacc*^{1}, the distribution of different MT structures is altered, with the frequency of

Figure 2.7 (continued).continuous and looped structures very significantly decreased and diffuse staining very significantly increased. * P < 0.05, *** P <0.001, t-test; error bars indicate ± s.e.m; number of NMJs quantified indicated on graph; scale bar, 1 μm.

Compared to controls, *dtacc* animals displayed diminished or undetectable tubulin staining in terminal branches, where the majority of bouton addition events occur. While tubulin was still detected in the main axon shaft, this population was also visibly reduced (**Fig. 2.7B**). Consistently, quantification indicated a ~32% overall decrease in tubulin intensity in *dtacc* animals (**Fig. 2.7C**). This reduction in staining could reflect a decrease in total tubulin mass, however, no obvious decrease in alpha-tubulin (Ab7291) was detected on our Western blots of *dtacc* mutant larvae (**Fig. 2.5A**). This suggested that total MT polymer must be reduced in *dtacc* mutants. Alternatively, since single MTs may be difficult to resolve with *in vivo* light microscopy compared to bundled MT arrays, a change in the spatial organization of MTs could also reduce detectable tubulin staining. This encouraged us to seek an additional histological probe for MTs and their *in situ* organization.

To further understand correlation between the NMJ size and MT phenotypes, we next tested the effect of dTACC on the formation of higher-order MT structures that have been associated with different states of bouton growth and division (Conde and Cáceres, 2009; Roos et al., 2000; Ruiz-Cañada and Budnik, 2006). Most non-dividing, *en passant* boutons are traversed by a continuous Futsch-decorated MT bundle, while non-dividing boutons on the ends of branches (i.e. terminal boutons) often display Futsch-labeled MT loops. Actively growing or dividing terminal boutons display reorganization of loops into dispersed structures, which can appear as diffuse or punctate staining (Conde and Cáceres, 2009; Roos et al., 2000; Ruiz-Cañada and Budnik, 2006). Given that Futsch binds a subpopulation of stabilized MTs (Roos et al., 2000) and is not known to bind individual tubulin dimers, the diffuse appearance of dispersed/splayed MTs likely reflects structures such as short MT fragments, or longer individual MTs that are splayed from the main bundle, as opposed to free tubulin.

We investigated the effects of *dtacc* loss on Futsch-labeled MTs structures previously categorized as “continuous”, “looped”, or “diffuse” (Jin et al., 2009; Sherwood et al., 2004). These structures are clearly and reproducibly distinguished in both w^{1118} controls and in $dtacc^{592}/dtacc^{1}$

animals (**Fig. 2.7D-F**). We focused on the three most terminal boutons of each branch, as branch ends are the sites of most active growth (Zito et al., 1999). Loss of *dtacc* produced consistent, measurable changes to Futsch-MT structures: compared to controls, mutants showed very significantly decreased frequency of both continuous (40% vs. 24%) and loop structures (23% vs. 11%; **Fig. 2.7G**). In contrast, the majority of *dtacc* boutons (65%) showed diffuse staining, in contrast to controls (37%) (**Fig. 2.7G**). Our findings thus suggest that dTACC promotes the organization of MTs into stable continuous and looped structures in wild-type, whereas *dtacc* loss nearly doubles the number of boutons containing dispersed/splayed MT structures.

Collectively, our findings suggest that dTACC serves to restrict NMJ overgrowth and regulate MT organization and/or assembly. These studies support the notion that MT regulaton is vital to controlling synapse expansion, as implied by studies of several other MT-associated factors. Interestingly, the frequency of diffuse Futsch staining is increased by 1.8-fold in *dtacc* mutants (**Fig. 2.7G**). This is reminiscent of the 2-fold increase in the number of both mature (**Fig. 2.1**) and nascent boutons (**Fig. 2.3**) in *dtacc* animals, and is thus consistent with the possibility that bouton addition and MT organization are linked. A parallel correlation between MT reorganization and membrane growth has been well-established in the axonal growth cone: MTs are splayed/dispersed in migrating growth cones, and shift to bundled and looped distributions in paused growth cones (Dent et al., 1999; Kalil et al., 2000; Tanaka and Kirschner, 1991; Tanaka et al., 1995). Given the resemblance of the MT organizations we observe at the NMJ (**Fig. 2.7D-F**) to the distributions of MTs at the growth cone, and the correlation of different MT structures to different growth states in both systems, it seems plausible that the growth cone and synapse share common mechanisms of coupling membrane growth and MTs reorganization despite differences in structure and dynamics.

Our result that the *dtacc* overgrowth phenotype (**Fig. 2.1**) correlates with a reduction in detectable tubulin (**Fig. 2.7A-C**), while surprising, is consistent with the loss-of-function

phenotypes of other MT regulators. For instance, loss of tubulin-specific chaperone E (*tbce*), which facilitates the folding of α-tubulin, results in NMJ overgrowth, along with a decrease in both presynaptic Futsch staining and the postsynaptic MT network (Jin et al., 2009). Intriguingly, the overgrowth phenotype of *spastin* mutants is also accompanied by a reduction in both tubulin and Futsch staining, despite the function of Spastin as a MT-destabilizer (Sherwood et al., 2004). It has been proposed that the MT severing activity of Spastin generates seeds that nucleate the growth of new MTs (Roll-Mecak and Vale, 2006), thus explaining the attenuated MT network of *spastin* mutants (Sherwood et al., 2004). Collectively, our findings in *dtacc* mutants, as well as the previous studies of *tbce* and *spastin*, suggest that NMJ growth may not be correlated solely with MT stabilization and levels, but may also be related to the organization of MTs. This possibility is consistent with prior studies that have shown a correlation of displayed/splayed MT structures with actively growing or dividing boutons (Conde and Cáceres, 2009; Roos et al., 2000; Ruiz-Cañada and Budnik, 2006). Indeed, both *tbce* and *spastin* mutants show increases in diffuse Futsch-MT staining concurrent with NMJ overgrowth (Jin et al., 2009; Sherwood et al., 2004).

In conclusion, we demonstrate that dTACC is a negative regulator of bouton addition during the development of the NMJ and that dTACC associates with and regulates the stability and organization of synaptic MTs. We provide evidence that dTACC promotes MT structures associated with paused bouton growth and division. Further studies may investigate the functional partners of dTACC at the NMJ, and how the roles of dTACC may relate to the roles of factors such as TBCE and Spastin, which show similar overgrowth and MT organization phenotypes.

MATERIALS AND METHODS

Drosophila Genetics

Stocks were raised at 25°C according to standard procedures. The w^{1118}, $elaV^{C155}$-GAL4, *UAS-Dcr2*, *OK6*-GAL4, and *Df(3R)110* stocks were obtained from the Bloomington Stock Center

(Bloomington, IN, USA). The *UAS-dtacc-RNAi* stock was obtained from the Vienna *Drosophila* Resource Center (Vienna, Austria). To enhance *dtacc-RNAi* expression, $elaV^{C155}$-GAL4 was also used to express *UAS-Dcr2*, an endonuclease that promotes processing of long dsRNAs to siRNAs. The previously described $msps^{P}$ (Cullen et al., 1999), $dtacc^{1}$ (Gergely et al., 2000b), and $dtacc^{592}$(Lee et al., 2001) stocks were provided by Jordan Raff.

Antibody production and purification

dTACC sequence containing amino acids 146-327 was His-tagged, bacterially expressed, and purified. dTACC antibody was raised in mice against and purified by PrimmBiotech, Inc. (Cambridge).

Immunohistochemistry

First instars and wandering third instars were dissected in Ca^{2+}-free saline and fixed in 4% paraformaldehyde in PBS for 10 min, except for tubulin immunostaining, where larvae were dissected in Brinkley Buffer 1980 (80mM PIPES, 1mM MgCl2, 1mM EGTA, pH 6.8) and fixed in 4% paraformaldehyde in PBS with 5mM EGTA. Primary antibodies obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA, USA) include: mouse anti-Brp NC82 (1:50), mouse anti-Dlg 4F3 (1:50), and mouse anti-Futsch (1:50). The following primary antibodies were also used for immunohistochemistry: mouse anti-dTACC (1:50) and rabbit anti-alpha-tubulin (1:200; Ab15246; Abcam). Secondary antibodies conjugated to AlexaFluor 488 and 594 were used (1:200; Invitrogen). Anti-HRP antibodies conjugated to AlexaFluor 594 and 647 were used (1:200; Jackson Immunoresearch).

Activity assay

The spaced-stimulation paradigm was adapted from published protocols (Ataman et al., 2008; Nesler et al., 2013; Piccioli and Littleton, 2014). Larvae were semi-dissected in HL3 (in mM): 70 NaCl, 5 KCl, 0.2 $CaCl_2$, 20 $MgCl_2$, 10 $NaHCO_3$, 5 trehalose, 115 sucrose, 5 HEPES-NaOH, pH 7.2. Relaxed fillets were pulsed with four 5 min 25ºC incubations with high K^+ solution (in mM): 40 NaCl, 90 KCl, 1.5 $CaCl_2$, 20 $MgCl_2$, 10$NaHCO_3$, 5 Trehalose, 5 sucrose, and 5 HEPES-NaOH, pH 7.2, spaced by 15 min in 25ºC HL3. After the fourth high K^+ pulse, larvae were allowed to recover in HL3 solution for 15 min, stretched, and then fixed. Nascent boutons were identified by lack of Brp or Dlg staining.

Western blotting

30 wandering third-instar larvae of each genotype were dissected in ice-cold dissection buffer (PBS, 1 mM EGTA, 1× cOmplete, Mini Protease Inhibitor Cocktail; Roche), leaving body wall musculature. Dissected pelts were homogenized in lysis buffer (dissection buffer, 0.5% Tween-20). Homogenates were loaded onto 4-15% SDS-PAGE gels (BioRad). Protein was transferred to PVDF membrane (BioRad) and immunoblotted using standard protocols and exposed using chemiluminescence reagents (Thermo Scientific). The following antibodies were used for blotting: mouse anti-TACC (1:50), mouse anti-alpha tubulin (1:5000; Ab7291; Abcam) goat anti-mouse HRP (1:1000; Cell Signaling Technology).

Image acquisition and analysis

Synaptic arbors of muscle 6/7 in the abdominal segment A2 were used for all analyses. Imaging was performed on a Nikon A1R point scanning confocal and a Nikon Yokogawa spinning disc confocal with a Hamamatsu ORCA-R2 cooled CCD camera. 3D-SIM was performed on a DeltaVision OMX Blaze microscope (GE Healthcare Life Sciences) with a PCO sCMOS camera.

Lasers were adjusted to prevent oversaturation. Images were processed and analyzed with ImageJ and/or MATLAB. Bouton number and size were counted and traced by hand. An HRP mask was used to restrict analysis to neuronal signal for intensity analysis, and MATLAB scripts were used to quantify dTACC and tubulin signals relative to HRP. Line scans were used to create intensity profiles to distinguish different Futsch structures.

Statistics

All comparisons were done using Welch's t-test for unequal variances using Graphpad.

KEYWORDS

Drosophila melanogaster; microtubules; synapse; transforming acidic coiled coil protein.

AUTHOR CONTRIBUTIONS

Conceptualization: V.T.C., S.A.J., J.B.L., D.V.V.; Methodology: V.T.C., D.V.V.; Formal Analysis: V.T.C., S.A.J.; Investigation, V.T.C., S.A.J., J.B.L., M.V.; Resources: V.T.C., S.A.J., J.B.L., D.V.V.; Data Curation: V.T.C.; Writing – Original Draft: V.T.C., D.V.V.; Writing – Review & Editing: V.T.C., S.A.J., D.V.V.; Visualization: V.T.C., D.V.V.; Funding Acquisition: V.T.C., D.V.V.; Supervision: D.V.V.

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CHAPTER 3: A NEW 3D PARTICLE TRACKING TOOL FOR NONINVASIVE IN VIVO ANALYSIS OF SYNAPTIC MICROTUBULE DYNAMICS IN DENDRITES AND AT THE NEUROMUSCULAR JUNCTION OF *DROSOPHILA*

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Chapter Contribution

This is a reprint of a forthcoming methods manuscript that has been accepted as Chou, V. T., Yesilyurt, H.G., Lai, H., *et al.* by *Journal of Visualized Experiments.* Vivian Chou performed analysis of raw data in collaboration with Hoyin Lai, who oversaw the development of the computational tracking algorithm. The design, execution, analysis, and interpretation of all experiments was led by Vivian Chou, with assistance from Gizem Yesilyurt. Vivian Chou drafted, revised, and edited the manuscript.

ABSTRACT

Microtubules (MTs) play critical roles in neuronal development, but many questions remain about the molecular mechanisms of their regulation and function. Furthermore, despite progress in understanding postsynaptic MTs, much less is known about the contributions of presynaptic MTs to neuronal morphogenesis. In particular, studies of in vivo MT dynamics in *Drosophila* sensory dendrites have yielded significant insights into polymer-level behavior. However, the technical and analytical challenges associated with live imaging of the fly neuromuscular junction (NMJ) have limited comparable studies of presynaptic MT dynamics. Moreover, while there are many highly effective software strategies for automated analysis of MT dynamics in vitro and ex vivo, in vivo data often necessitates significant operator input or entirely manual analysis due to the inherently less favorable signal/noise ratio and complex cellular morphology. To address this, we collaborated with DRVision to optimize a new software platform, Aivia, for automated and unbiased in vivo particle detection. We performed multiparametric analysis of live time-lapse confocal images of EB1-GFP labeled MTs in both dendrites and the NMJ of *Drosophila* larvae, finding striking differences in MT behaviors. We furthermore analyzed MT dynamics following knockdown of the MT-associated protein (MAP) dTACC, a key regulator of *Drosophila* synapse development, and identified statistically significant changes in MT dynamics compared to wild-type. We therefore demonstrate a novel strategy for the automated multiparametric analysis of both pre- and postsynaptic MT dynamics at the polymer level that significant reduces human-in-the-loop criteria. We furthermore show the utility of our method in detecting distinct MT behaviors upon dTACC-knockdown, indicating a possible future application for functional screens of factors that regulate MT dynamics in vivo. Future applications of this method may also focus on elucidating cell type and/or compartment-specific MT behaviors, and multi-color correlative imaging of EB1-GFP with other cellular and sub-cellular markers of interest.

INTRODUCTION

Morphogenesis is the process by which cells organize to form functional structures through the coordination of intra- and intercellular changes. A remarkable example of morphogenesis is the development of the highly specialized neuronal structure. Neurons display remarkable polarization in which they extend two structurally and functionally distinct types of processes, dendrites and axons (Craig and Banker, 1994; Rolls, 2011), which can achieve immense lengths. The complexity of neuronal development arises not only from the sheer size of dendrites and axons but also from the difficulty in forming their intricately branched geometries (Jan and Jan, 2010; Lewis and Polleux, 2012; Lewis et al., 2013; Whitford et al., 2002). The challenges of neuronal morphogenesis, and its consequences for learning and memory (Kandel, 2001), motivate the ongoing investigation of both its genetic control and the underlying cell biological mechanisms. Such mechanisms include, but are not limited to, the intracellular transport of membrane (Witte and Bradke, 2008) and the many cytoskeletal (Hoogenraad and Bradke, 2009; Jan and Jan, 2010; Kapitein and Hoogenraad, 2011; Lewis and Polleux, 2012; Lewis et al., 2013; Rolls, 2011; Whitford et al., 2002) rearrangements needed for changes in neuronal morphology.

Studies of neuronal morphogenesis have leveraged a variety of advanced visualization techniques. Static methods, such as electron microscopy or fluorescence microscopy of fixed probes, have been widely used to perform high-resolution morphological and structural analysis. However, besides the artifacts that are inevitable to any preservation method, static visualization, by definition, cannot capture the dynamic changes that underpin morphogenesis. Thus, many pivotal insights originated from time-lapse fluorescence microscopy of living tissues. Early work by Lichtman and colleagues (Kerschensteiner et al., 2005; McCann and Lichtman, 2008; McCann et al., 2008; Misgeld et al., 2007; Turney and Lichtman, 2008; Turney et al., 2012) utilized in vivo imaging of the mammalian nervous system to investigate axon

regeneration/degeneration, organization of synaptic components, and long-range axonal transport. Furthermore, seminal studies in primary neuronal explants were critical to establishing the importance of microtubule (MT) dynamics to axonal elongation and motility (Tanaka and Kirschner, 1991; Tanaka et al., 1995). Crucially, early neuronal explant studies established the use of fluorescently-tagged end-binding family proteins (EBs) to gain invaluable insights into MT plus-end dynamics in developing neurons at the level of individual MT polymers (Stepanova et al., 2003). These studies drew upon observations that the EB family member EB1 preferentially localizes to MT plus ends (Tirnauer and Bierer, 2000) in *S. cerevisiae* (Schwartz et al., 1997; Tirnauer et al., 1999) and in cultured cells (Juwana et al., 1999; Mimori-Kiyosue et al., 2000). Since then, EB1 and other plus tip tracking proteins (+TIPs) (Akhmanova and Hoogenraad, 2005; Akhmanova and Steinmetz, 2008, 2015; Galjart, 2010; Schuyler and Pellman, 2001; Vaughan, 2005) have been widely used in in vivo studies of MT dynamic instability (Desai and Mitchison, 1997; Howard and Hyman, 2003; Mitchison and Kirschner, 1984), including in the context of neuronal development (Bearce et al., 2015; Voelzmann et al., 2016; Van De Willige et al., 2016).

Drosophila has emerged as a powerful model for in vivo imaging studies of MT dynamics during neuronal development due to the vast genetic and imaging toolkit available in the system (Bier, 2005; Rebollo et al., 2014; del Valle Rodríguez et al., 2012; Venken and Bellen, 2005) as well as the similarities in structure and function between *Drosophila* and vertebrate neurons (Rolls, 2011). A key early study at the neuromuscular junction (NMJ) of *Drosophila* larvae performed repeated noninvasive imaging of a fluorescent membrane marker through the translucent cuticle of intact animals to document presynaptic terminal morphogenesis (Zito et al., 1999). Using a similar method to image whole, live *Drosophila* larvae, our lab provided an initial demonstration of subcellular, particle-level analysis of processive movement of motor cargos in the axons (Miller et al., 2005). More recently, meticulous studies by Rolls and

colleagues in the sensory dendrites of intact *Drosophila* larvae (Hill et al., 2012; Mattie et al., 2010; Rao et al., 2016; Rolls et al., 2007; Stewart and McLean, 2004; Stone et al., 2008, 2010) have characterized postsynaptic MT plus-end dynamics by performing particle tracking and analysis of green fluorescent protein (GFP)-tagged EB1. Such studies in *Drosophila* (Hill et al., 2012; Mattie et al., 2010; Rao et al., 2016; Rolls et al., 2007; Stewart and McLean, 2004; Stone et al., 2008, 2010) and other systems(Hu et al., 2008, 2011; Jaworski et al., 2009; Merriam et al., 2011, 2013; Yau et al., 2016) have significantly advanced our understanding of single-polymer behavior of MT plus ends in the dendrites of developing neurons (Dent, 2017; Dent et al., 2011; Gu and Zheng, 2009; Hoogenraad and Akhmanova, 2010; Kapitein et al., 2010; Shirao and González-Billault, 2013; Svitkina et al., 2010).

Despite the impressive in vivo studies of postsynaptic MT dynamics (Hill et al., 2012; Hu et al., 2008, 2011; Mattie et al., 2010; Merriam et al., 2011, 2013; Rao et al., 2016; Rolls et al., 2007; Stewart and McLean, 2004; Stone et al., 2010, 2008; Yau et al., 2016), there have been far fewer comparable studies of presynaptic MT dynamics at the developing axon terminal. MT dynamics at the *Drosophila* larval NMJ have been studied using fluorescent speckle microscopy (FSM) and fluorescence recovery after photobleaching (FRAP) (Yan and Broadie, 2007); these techniques evaluated the overall tubulin kinetics but not the behavior of individual MT plus ends. As of this writing, there has been one sole investigation of individual MT plus ends at the *Drosophila* NMJ; this study combined live time-lapse imaging with manual analysis of kymographs to characterize a population of dynamic, EB1-GFP labeled “pioneering MTs” that appeared distinct from a broader population of stabilized MTs (Pawson et al., 2008). This paucity in research on presynaptic MT dynamics may be due at least in part to anatomy: while it is relatively straightforward to obtain images of dendrites due to their proximity to the larval cuticle, NMJs are obstructed by other tissues, making it challenging to acquire images of sufficient signal/noise ratio for particle-level analysis. Nonetheless, given the well-established

importance of the presynaptic MTs to synaptic morphogenesis and stabilization (Broadie and Richmond, 2002; Ruiz-Cañada and Budnik, 2006; Rushton et al., 2009), as well as their links to neurodevelopmental and neurodegenerative disorders (Bodaleo and Gonzalez-Billault, 2016; Goellner and Aberle, 2012; Lasser et al., 2018; Matamoros and Baas, 2016), bridging this gap between our understanding of pre- and postsynaptic MTs is likely to yield invaluable insights.

An additional challenge to analysis of in vivo MT dynamics in general, in contrast to in vitro or ex vivo analysis, is the limited automated software tools that can extract dynamics parameters from in vivo data. Presently, one of the most popular and powerful techniques for analysis of +TIP-labeled MT plus ends is plusTipTracker (Applegate et al., 2011; Matov et al., 2010), a MATLAB-based software that allows automated tracking and analysis of multiple dynamics parameters. Notably, plusTipTracker measures not only MT growth but also shrinkage and rescues: while +TIP labels such as EB1-GFP only associate with growing plus ends, plusTipTracker can algorithmically infer shrinkage rates and rescue events. However, while plusTripTracker has been very successfully applied to many contexts, including our lab's previous multiparametric analysis of ex vivo MT dynamics in *Drosophila* S2 cells (Long et al., 2013), plusTipTracker is not optimal for analysis of in vivo data given their lower signal/noise ratio. As a result, in vivo studies of plus-end dynamics at dendrites (Hill et al., 2012; Mattie et al., 2010; Rao et al., 2016; Rolls et al., 2007; Stewart and McLean, 2004; Stone et al., 2008, 2010) and at the NMJ(Pawson et al., 2008) of *Drosophila* have relied on manual generation and analysis of kymographs using software such as ImageJ (Schneider et al., 2012), or on semi-automated strategies that involve numerous human-in-the-loop components.

Here, we present an experimental and analytical workflow that reduces the experimental and analytical overhead required to perform noninvasive polymer-level analysis of presynaptic MT dynamics in both sensory dendrites and the motor axon terminal of *Drosophila* third-instar larvae. We describe a method to immobilize intact larvae and therefore avoid injuries known to

trigger stress responses as well as other non-physiological conditions that might perturb in vivo MT dynamics. To label dynamic MT plus-ends, we expressed EB1-GFP pan-neuronally using the GAL4/UAS system (Brand and Perrimon, 1993), allowing visualization of MTs at both dendrites and NMJ with a single driver. While some early steps are inevitably subject to human decision-making, such as the selection of animal specimens and identification of regions to image, we sought to automate the steps following data acquisition. Crucially, we optimized a new software, Aivia, to perform automated, unbiased analysis requiring minimal human input. While other particle-tracking methods are available (Cheezum et al., 2001; Ma et al., 2019; Manzo and Garcia-Parajo, 2015; Shen et al., 2017; Zwetsloot et al., 2018), we chose to optimize Aivia, which is now available to users for a variety of applications, because it was algorithmically well-suited to address the particular challenges of our data. Specifically, the use of coherence-enhancing diffusion filtering (Weickert, 1999) was very effective in automated segmentation and background removal, and Aivia furthermore implements custom algorithms specifically designed to automate particle detection and tracking. We found that Aivia was well-suited to handle the low signal/noise ratio inherent to our data, as well as other challenges, such as movement of EB1-GFP comets through different focal planes. While it is not feasible to exhaustively test the performance of Aivia against all other particle analysis software, we found that the performance of Aivia equaled or approached our benchmark of human performance. Furthermore, to our knowledge, there has been no other software specifically trained on in vivo data from sensory dendrites and the presynaptic terminal. Given that the performance of image-analysis algorithms is often highly specific to the data they were designed for, and that generalized computer vision is not yet possible, we reasoned that training Aivia to the specific in vivo data of interest would be the most algorithmically sound approach.

Given the extensive work on dendritic MTs (Hill et al., 2012; Mattie et al., 2010; Rao et al., 2016; Rolls et al., 2007; Stewart and McLean, 2004; Stone et al., 2008, 2010) as well as the

consistent quality of data that can be acquired from this system, we first validated our image acquisition and Aivia-based analysis strategy in *Drosophila* sensory dendrites. Importantly, we showed in dendrites that the use of different neuronal Gal4 drivers, even in otherwise identical wild-type backgrounds, results in significant differences in EB1-GFP dynamics due to differences in expression of the EB1 construct, emphasizing the importance of using a single Gal4 driver for consistent results. We next used our strategy for multiparametric analysis of EB1-GFP dynamics at the presynaptic terminal of the NMJ. To further illustrate the investigative value of our method, we applied our imaging and software strategy to assess both pre- and postsynaptic EB1-GFP dynamics following knockdown of dTACC, the *Drosophila* homolog of the highly conserved TACC (transforming acidic coiled coil) family (Ding et al., 2017; Hood and Royle, 2011; Peset and Vernos, 2008; Thakur et al., 2013). Our prior work in *Drosophila* S2 cells (Long et al., 2013), as well as work by Lowery and colleagues in the Xenopus growth cone (Lucaj et al., 2015; Nwagbara et al., 2014; Rutherford et al., 2016), has shown that TACC family members regulates MT plus-end dynamics. Furthermore, we recently reported evidence from confocal and super-resolution immunofluorescence imaging that dTACC is a key regulator of presynaptic MTs during neuronal morphogenesis (Chou et al., 2020), raising the question of whether dTACC regulates live MT dynamics. Here, we show that we can detect differences in MT behaviors upon dTACC knockdown using our current method. We thus present an in vivo method that can effectively identify and characterize key regulators of MT dynamics, particularly in the presynaptic compartment, within the developing neuron.

PROTOCOL

1. Generation of *Drosophila* specimens.
    1.1. Select a suitable MT plus-end marker. This study utilized GFP-tagged EB1, a well-characterized plus-end marker with a strong, clear signal (Mimori-Kiyosue et al., 2000;

Schwartz et al., 1997; Tirnauer and Bierer, 2000; Tirnauer et al., 1999). Alternatives include other +TIPs such as EB3 (Juwana et al., 1999; Stepanova et al., 2003), CLASP/Orbit (Lee et al., 2004; Maiato et al., 2003), and CLIP-170 (Komarova et al., 2002; Perez et al., 1999).

1.2. Obtain or generate flies with MT marker under control of UAS promoter, e.g. *UAS-EB1-GFP.*

1.3. Choose the appropriate tissue-specific Gal4-driver. This study used the pan-neuronal driver *elaV-Gal4* to drive expression in both sensory dendrites and at the NMJ.

1.4. Perform crosses to generate flies to express the MT plus-end marker in the desired cells/tissues. NOTE: For any Gal4-driver and UAS-transgene combination, the experimental design should include proof-of-concept and validation experiments to characterize the system and avoid artifacts from overexpression.

2. Equipment setup.

2.1. Setup a workstation, including the steromicroscope and illumination source, close to the confocal microscope to minimize the time spent between sample preparation and imaging to prolong the health and viability of the larvae.

2.2. Prepare the anesthetic by mixing a 9% chloroform mixture (0.1 mL chloroform + 1.0 mL halocarbon oil) in an 1.5mL Eppendorf tube. To avoid separation, mix well by inverting the tube prior to each new slide.

2.3. Prepare the glass slide: Cut four strips of double-sided tape (~15 mm wide). Line up two of the pieces on the glass slide, leaving a space of ~5 mm in between the strips. Layer the remaining two pieces on top of the first two to double the thickness of the tape.

2.4. Add a large drop (~100 microliters) of chloroform/oil mixture onto the glass slide in the 5mm space between the tape pieces.

3. Preparation of larval samples for imaging.

    3.1. Fill a container, e.g. a 6-well plate, with 1X PBS.

    3.2. Collect 3rd instar larva from the fly vial using a probe or tweezers. Identify larva at the proper stage by their crawling behavior and by the presence of 9-12 prominent, serrated mouth-hooks. Use stereomicroscope to assist in staging larva.

    3.3. Place larva in the PBS and move it gently to wash off any remains of food or other debris. Dry the larva gently on a KimWipe.

    3.4. Anesthetize the larva by placing it into chloroform/oil drop on the slide from Step 2.

    3.5. Place #1.5 coverslip on top. Adhere the coverslip to the tape by applying gentle pressure, thus immobilizing the larva without damaging it.

    3.6. Seal the chamber with Vaseline or nail polish if preferred.

4. Time-lapse confocal imaging of live samples.

    4.1. Prepare confocal microscope and the 60x objective lens with oil immersion. Place the sample on the stage.

    4.2. Use the acquisition software, e.g. Metamorph, to configure experiments. Set the time-lapse duration to 30 seconds at an interval of 2 seconds, for a total of 16 frames. Set laser exposure and intensity to ensure sufficient signal while avoiding saturation and photobleaching. For EB1-GFP imaging, the 488nm laser was set to an exposure time of 100 ms and intensity of 30%.

    4.3. Use the eyepieces of the microscope to find the larva in widefield-green illumination. Find the dendrites or NMJs by adjusting the stage slowly. Do not expose larva to illumination (widefield or confocal) for any longer than necessary.

        4.3.1. Dendrites appear as thin bright-green webs of nerves easily distinguishable from thick long axon bundles.

4.3.2. NMJs appear as groups of bright-green individual boutons, approximately 5μm in diameter, at the ends of thick long axon bundles that diverge from the nerve cord.

4.4. Using the live camera feed, quickly focus on region of interest using 488nm illumination. Immediately stop illumination once proper focus is found to avoid phototoxicity.

4.5. Initiate image acquisition. EB1 comets are recognizable as bright, motile punctae.

5. Image processing and analysis with Aivia software.

5.1. Analyze each video file individually. Within Aivia, select File>Import>Image Sequence and drag the TIF files in the box that appears. Preview the video within Aivia.

5.2. Under the Detection Parameters menu, tune the software parameters to ensure detection of only clearly visible punctae and avoid detection of spurious objects. For instance, reducing particle intensity results in greater sensitivity of software to punctae but increases potential false-positives. The precise values of the parameters will vary empirically.

5.3. Use the cursor to select the EB1 puncta detected in the previous step. Multiple EB1 puncta can be selected and analyzed simultaneously using Ctrl+Select. NOTE: Depending on the project aims and application, additional heuristics may be used to filter the puncta. For instance, punctae with a lifetime of fewer than 8-10 seconds (4-5 frames) might be omitted because they do not present sufficient information about the entire growth event. The need for such heuristics will vary empirically.

5.4. Run the Neuron Particle Tracking software recipe for the selected puncta. Aivia will output results for the tracking parameters listed in Table 3.1. For ease of later analysis and interpretation, the results can be stored in Excel or similar spreadsheet software.

RESULTS

We raised flies from stable stocks that constitutively express the *UAS-EB1-GFP* transgene either pan-neuronally (*elaV-Gal4; UAS-EB1-GFP*) or in sensory neurons (*221-Gal4; UAS-EB1-GFP*). EB1 was chosen for this study because of it specifically localizes to growing ends and dissociates immediately upon pause and shrinkage (Akhmanova and Steinmetz, 2008, 2015; Vaughan, 2005), and has been shown through multiple studies, including in *Drosophila* (Hill et al., 2012; Mattie et al., 2010; Pawson et al., 2008; Rao et al., 2016; Rolls et al., 2007; Stewart and McLean, 2004; Stone et al., 2008, 2010), to be a robust marker that does not have significant detrimental effects on underlying biology of the organism. We performed imaging of wandering third-instar larvae on an inverted spinning-disc confocal microscope (**Fig. 3.1A, B**). Larvae were staged based on behavior (active crawling along vial walls) and the presence of large, extended mouth hooks with 9-12 teeth (**Fig. 3.1C**). Sensory dendrites superficially located near the larval cuticle (**Fig. 3.1D**) were imaged to provide comparisons with published data, while NMJs located at deeper image planes on the surface of body wall muscle within the animal (**Fig. 3.1E**) were imaged to define parameters characteristic of presynaptic MT dynamics.

Following image acquisition as described in the protocol above, we performed automated, unbiased analysis of the EB1-GFP comets using Aivia software (**Fig. 3.2**), producing measurements for nine dynamics parameters (**Table 3.1**). Statistical analysis, including exploratory data analysis and hypothesis testing, was performed in MATLAB. We noted through data visualization and the Anderson-Darling test that our data contained non-normally distributed values. Thus, to avoid making assumptions about the underlying distribution of the data, all hypothesis testing was performed using the non-parametric Wilcoxon-Mann-Whitney test. We compared EB1-GFP dynamics under the control of both the *elaV-Gal4* and *221-Gal4* drivers in otherwise equivalent wild-type backgrounds (**Fig. 3.3**). Interestingly, we

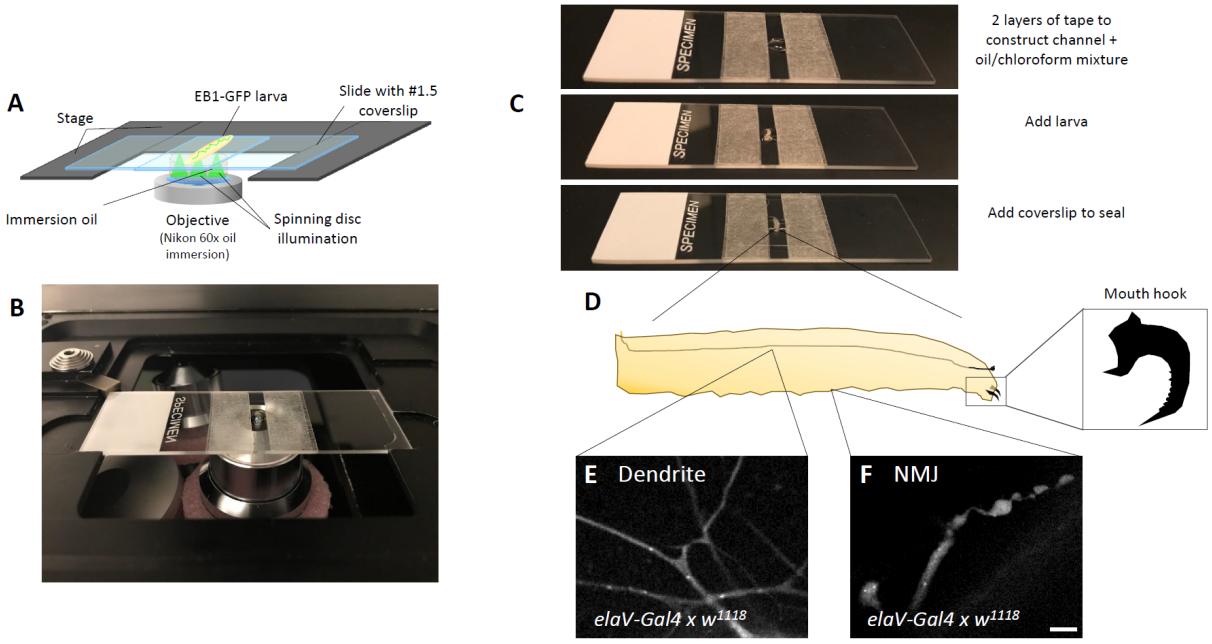

Figure 3.1. Experimental setup. ***A,B,*** Schematic (**A**) and actual example (**B**) of imaging setup. Anesthetized, whole-mount larvae were imaged on an inverted spinning disc confocal. ***C,*** Example of slide preparation using third-instar larvae. ***D,*** Larvae were staged by their crawling behavior and by the presence of 9-12 prominent, serrated mouth-hooks. Imaging was performed on (**E**) sensory neuron dendrites, which have a relatively superficial location close to the outer cuticle, and (**F**) the presynaptic terminal of the NMJ, which is located deeper within the animal. Scale bar, 2 μm.

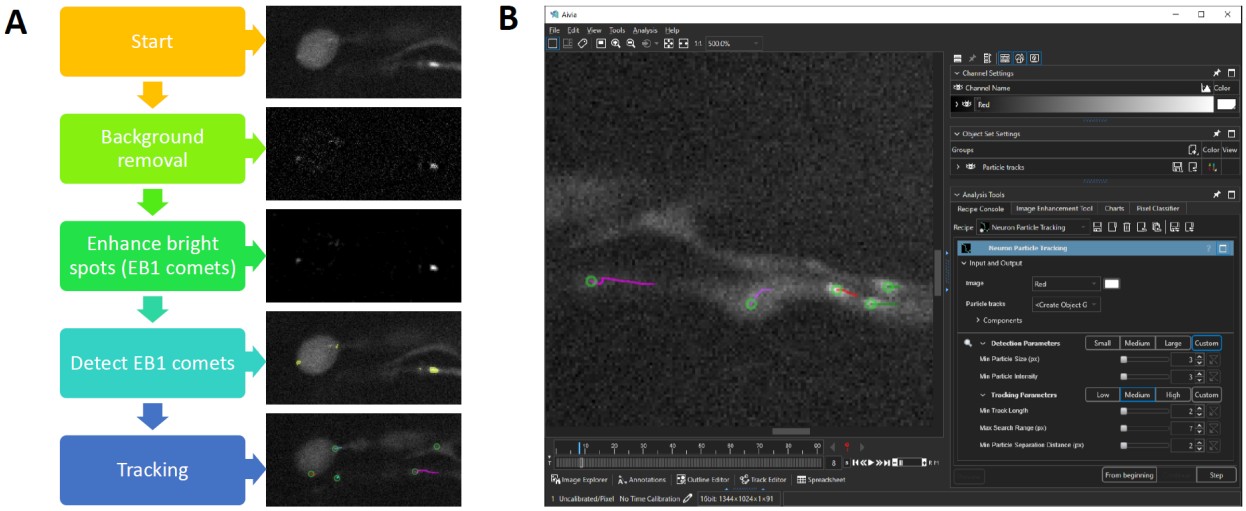

Figure. 3.2. Demonstration of dendrite and NMJ analysis with Aivia. ***A***, Summary of the Aivia automated analysis processing pipeline. A common issue of typical morphological approaches to background removal is the enhancement of image signal along the edges of small and narrow structures (e.g. dendrites). To address this, we applied a coherence-enhancing diffusion filter (Weickert, 1999) in Aivia to the raw image to extract the whole dendrite / NMJ structure as background and to isolate the EB1 comets on the image. This approach enables Aivia to identify and track the comets even where the contrast between the background structure and the EB1 comet is low. ***B***, Workflow integration by the Aivia software interface that allows the user to 1) optimize analysis parameters for a given image and 2) review the analysis results.

Table 3.1. Plus-end dynamic parameters analyzed with Aivia.

Tracking Parameter	Description
Mean Comet Velocity	average of the detected track velocity (scalar) over the lifetime of the track
Max Comet Velocity	highest value of track velocity (scalar) detected over the lifetime of the track
Straight Line Velocity	growth length divided by the growth lifetime
Mean Acceleration	average of the rate of change of detected track velocity (scalar) over the lifetime of the track
Sinuosity	growth length divided by path length
Mean Square Displacement	sum of the particle displacement squared at all time points divided by growth lifetime
Growth Length	straight line distance between the starting frame position and ending frame position of the track
Path Length	total distance traveled by the track
Growth Lifetime	total length (in time) of the detected track

found highly significant differences ($P < 0.005$) in several measured parameters (mean acceleration, sinuosity, growth length). While EB1 is not generally expected to perturb native MT biology to an adverse degree (Komarova et al., 2002; Piehl and Cassimeris, 2003; Stepanova et al., 2003), MTs are nevertheless highly sensitive to EB1 expression levels (Damme et al., 2004; Mathur et al., 2003; Yang et al., 2017; Zhang et al., 2009). Our results indicate clear differences in MT dynamics in response to different EB1 dosages. To avoid artifacts from differences in genetic expression, we carried out all further experiments in both dendrites and at the NMJ using only the elaV-Gal4 pan-neuronal driver.

We first validated our method in sensory dendrites (**Fig. 3.4**) and repeated the protocol at the NMJ (**Fig. 3.5**). To assess the potential of our strategy for investigating the role of specific molecules on MT dynamics, we compared EB1-GFP dynamics of wild-type control animals to animals expressing *UAS-dtacc-RNAi*. We chose to test dTACC because it is a known regulator of MT plus-end dynamics (Long et al., 2013; Lucaj et al., 2015; Nwagbara et al., 2014; Rutherford et al., 2016) in other systems, and also based on our recent evidence that dTACC regulates presynaptic MTs at the *Drosophila* NMJ (Chou et al., 2020). To enhance *dtacc-RNAi* expression, *elaV-GAL4* was also used to express *UAS-Dcr2*, an endonuclease that promotes processing of long dsRNAs to siRNAs. Upon reduction of dTACC expression to ~50% (Chou et al., 2020), we find significant changes in EB1-GFP dynamics in both dendrites (**Fig. 3.4**) and at the NMJ (**Fig. 3.5**). Notably, the effects of dTACC knockdown in dendrites closely resembles the effects of dTACC knockdown we previously observed in S2 cells (Long et al., 2013). In contrast, we observed some striking differences between dendrites and the NMJ upon dTACC knockdown. While loss of dTACC affects seven parameters in dendrites, and three parameters at the NMJ, all but one of the parameters (sinuosity) are unique to either dendrites or NMJ. Furthermore, while sinuosity is affected by dTACC loss in both contexts, the effect is opposite between dendrites (increase) and the NMJ (decrease). We therefore show that our protocol

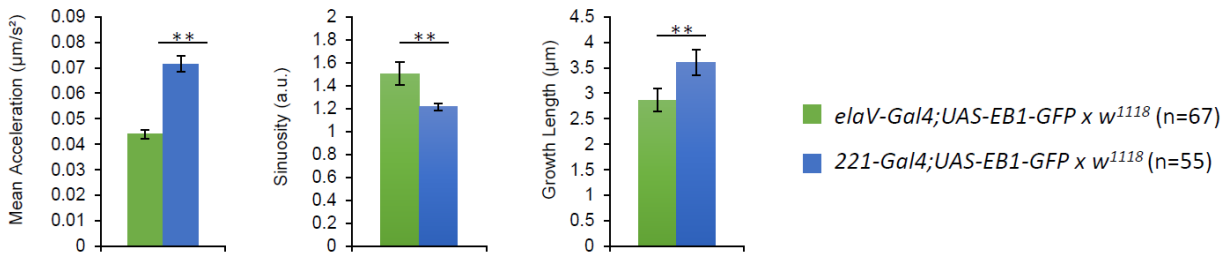

Figure. 3.3. Comparison of elaV- and 221-Gal4 drivers in wild-type control dendrites. To determine the effects of Gal4-dependent *UAS-EB1-GFP* expression levels on EB1-GFP dynamics, *elaV-Gal4; UAS-EB1-GFP* and *221-Gal4; UAS-EB1-GFP* were expressed in a w^{1118} control background. Highly significant differences were observed in mean acceleration, sinuosity, and growth length. ** P < 0.005, Wilcoxon-Mann-Whitney-test; error bars indicate ± s.e.m; number of NMJs quantified indicated on graph.

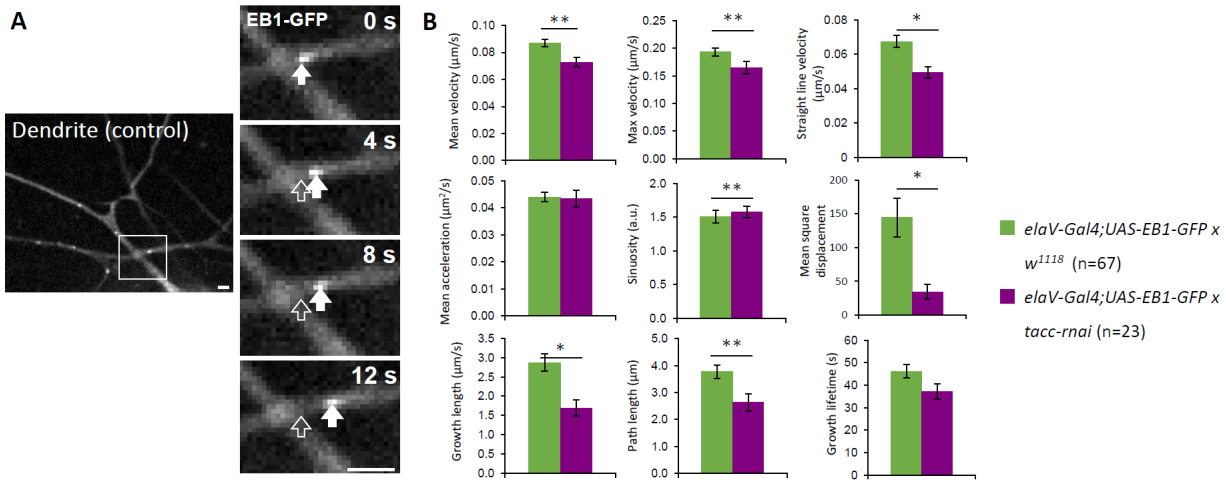

Figure. 3.4: Neuronal RNAi-knockdown of TACC affects EB1-GFP dynamics in sensory dendrites. ***A***, Representative time-lapse images of EB1-GFP comet dynamics in control *elaV-Gal4; UAS-EB1-GFP; UAS-Dcr2 x* w^{1118} sensory dendrites. Image series on right shows detailed view of region indicated by the box in the left-hand image. In each panel, the solid white arrow indicates the position of the EB1-GFP comet at the most recent timepoint, while the hollow arrow indicates the original position of the comet at t=0s. ***B***, comparison of EB1-GFP dynamics in *elaV-Gal4;UAS-EB1-GFP; UAS-Dcr2 x* w^{1118} and *elaV-Gal4; UAS-EB1-GFP; UAS-Dcr2 x UAS-tacc-rnai* dendrites. Knockdown of dTACC significantly affects all dynamics parameters other than mean acceleration and growth lifetime. * P < 0.05, ** P < 0.005, Wilcoxon-Mann-Whitney-test; error bars indicate ± s.e.m; number of NMJs quantified indicated on graph; scale bar, 1 µm.

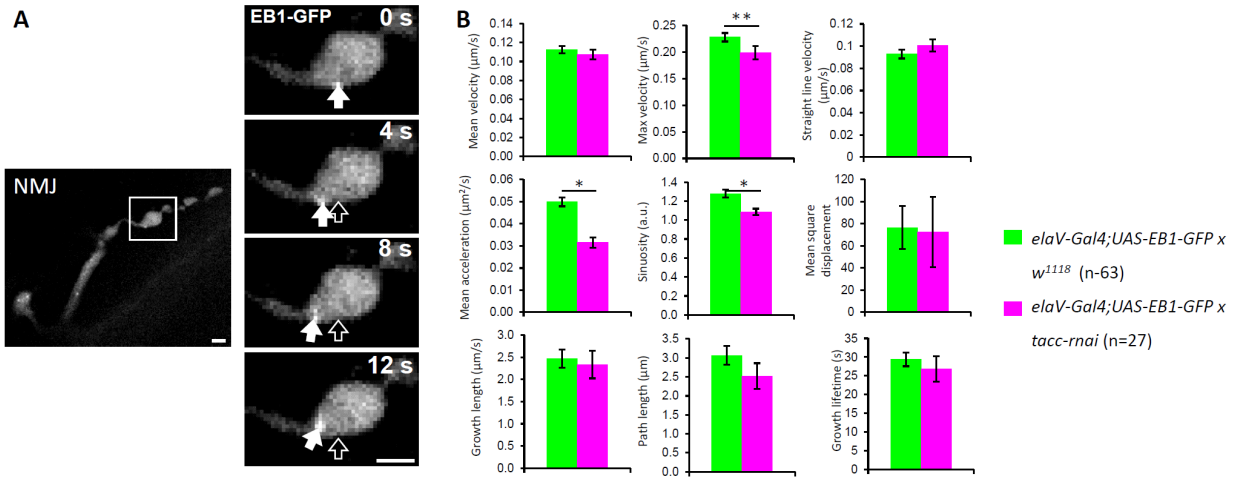

Figure. 3.5: Neuronal RNAi-knockdown of TACC affects EB1-GFP dynamics at the NMJ.

A, Representative time-lapse images of EB1-GFP comet dynamics at the presynaptic terminal of control *elaV-Gal4; UAS-EB1-GFP; UAS-Dcr2 x* w^{1118} NMJs. Image series on right shows detailed view of region indicated by the box in the left-hand image. In each panel, the solid white arrow indicates the position of the EB1-GFP comet at the most recent timepoint, while the hollow arrow indicates the original position of the comet at t=0s. B, comparison of EB1-GFP dynamics at *elaV-Gal4; UAS-EB1-GFP; UAS-Dcr2 x* w^{1118} and *elaV-Gal4; UAS-EB1-GFP; UAS-Dcr2 x UAS-tacc-rnai* NMJs. Knockdown of dTACC significantly affects max velocity, mean acceleration, and sinuosity. * $P < 0.05$, ** $P < 0.005$, Wilcoxon-Mann-Whitney-test; error bars indicate ± s.e.m; number of NMJs quantified indicated on graph; scale bar, 1

can not only identify significant differences in MT dynamics between genetic backgrounds but can also demonstrate distinct roles for a single MT regulator in different contexts.

DISCUSSION

In this paper, we discuss a protocol to perform noninvasive intravital imaging of MT dynamics in the dendrites and at the NMJ of *Drosophila* during development. Human input is required during the experimental steps, such as in selecting animals to image, and may introduce bias in the data collection process that cannot be reasonably removed. Thus, we sought to minimize bias by performing automated analysis with Aivia, which was optimized to handle the low signal/noise ratio inherent to our in vivo data. The algorithms used by Aivia allows machine-based particle detection, kymograph generation, and track analysis, reducing the need for human input compared to traditional methods. We note that our analysis with Aivia is not completely equivalent to that which is possible with plusTipTracker: while it is possible to infer shrinkage and rescue events with plusTipTracker, the current Aivia algorithms cannot perform such measurements. Nonetheless, given the considerable constraints on data quality that are inherent to in vivo data, our Aivia-based method makes progress towards achieving automated, reproducible data analysis in vivo.

We observed significant effects on multiple MT dynamics parameters upon knockdown of dTACC in both dendrites and at the NMJ. We thus demonstrate that our method may be a potential screening tool for regulators of synaptic MT dynamics; moreover, we identified a potential role for dTACC in dendrites. While we have recently established the role of presynaptic dTACC in the development of the motor axon terminal, the roles of postsynaptic dTACC are unknown. Thus, future studies by our research group may focus on role of postsynaptic dTACC, either in sensory dendrites and/or in the muscle of the NMJ.

We noted key differences in the effects of dTACC-knockdown on MT dynamics in sensory dendrites and the NMJ, indicating clear biological differences between the two contexts. This raises the question of whether MT dynamics differ between neuronal types, between distinct compartments of a single neuron, or both. The differences we observed between dendrites and the NMJ might reflect differences between sensory and motor neurons, but could also indicate differences between dendritic and axonal compartments, independent of the neuronal type. Because our focus in the present study was on developing a robust methodology rather than comprehensive characterization of neuronal MT dynamics, we have not performed analysis of motor neuron dendrites or the axon terminals of sensory neurons. Due to their less accessible location within the animal, these structures are more challenging to image and analyze, compared to the structures we discuss presently. We anticipate that with our optimized protocol will enable efficient and informative future studies of compartment- and cell-type differences in MT dynamics.

We envision that our in vivo imaging and analysis strategy will be of value to researchers who are interested in detailed understanding of MT dynamics behaviors during critical stages of neuronal development. A key future innovation would be multi-color imaging through co-expression of EB1-GFP with other markers, such as those that label the cell membrane (CD8 (Lee and Luo, 1999), myristol (Resh, 1999)), the actin cytoskeleton (moesin (Edwards et al., 1997), LifeAct (Riedl et al., 2008)), and other structures of interest. This would allow correlative analysis of the spatiotemporal interactions of MTs with other key cellular structures. While such multi-color imaging has been used to study MT-actin interactions in the neuronal growth cone (Dent and Kalil, 2001; Schaefer et al., 2002; Suter and Forscher, 2000), it has not been demonstrated in dendrites or the presynaptic axon terminal. Thus, developing a comparable method for in vivo *Drosophila* studies will be a significant addition to the imaging toolkit for understanding the role of MTs in the broader context of neuronal development.

KEYWORDS

Drosophila melanogaster; neuron; microtubule; synapse; dendrite; neuromuscular junction; time-lapse imaging; live imaging; particle tracking; quantitative analysis; transforming acidic coiled coil protein.

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DISCLOSURES

The authors Hoyin Lai, Michael Jones, Hideki Sasaki, Luciano A.G. Lucas, Sam Alworth (formerly), and James Shih-Jong Lee are employees of DRVision Technologies LLC, which produces the Aivia software used in this protocol. Vivian Chou has no financial interests to disclose and does not receive any compensation from DRVision.

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CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

Chapter Contribution

Experiments on post-translational MT modifications were performed in collaboration with Maxime Vounatsos. Jennifer Long performed dTACC pull-downs and confocal colocalization experiments with the AZ. Seth Johnson obtained results on the dTACC cell specificity phenotype, confocal colocalization of dTACC with PAK, and the effect of Liprin-α on dTACC localization. All other experiments were performed by Vivian Chou.

Summary of conclusions

The overarching motivation for my dissertation was to investigate the relatively uncharted territory of how the presynaptic MT cytoskeleton contributes to synaptogenesis. The actin cytoskeleton has well-defined postsynaptic roles in dendritic spine morphogenesis and plasticity and in presynaptic AZ structure and SV organization (Bosch and Hayashi, 2012; Cingolani and Goda, 2008; Dillon and Goda, 2005; Luo, 2002; Matus, 1999; Nelson et al., 2013; Schubert and Dotti, 2007). In recent years, MTs have also been well-studied in postsynaptic dendritic spines and in neuronal axon transport (Dent, 2017; Dent et al., 2011; Kapitein and Hoogenraad, 2011; Maeder et al., 2014). By comparison, understanding of presynaptic MTs is still maturing. Nevertheless, considerable insights have emerged from genetic studies of the *Drosophila* NMJ, beginning with pioneering work on the MT-stabilizing protein Futsch/MAP1B (Roos et al., 2000) and spanning the last two decades. When I started my dissertation in 2014, newly published findings demonstrated the role of Futsch in regulating AZs and neurotransmission (Lepicard et al., 2014); the roles of the TRPV channel Inactive in promoting NMJ growth, Futsch phosphorylation, and MT stabilization (Wong et al., 2014); and the role of the MT-severing protein Katanin in restricting synapse growth and regulating Futsch-labeled MT loop structures (Mao et al., 2014). More recent studies have revealed roles for the kinesin Pavarotti/KIF23 in preventing overgrowth (McLaughlin et al., 2016) and for the formin DAAM in promoting NMJ growth and regulating the architecture of Futsch-labeled MTs (Migh et al., 2018).

My project on the role of dTACC at the NMJ was instigated by our lab's discovery that dTACC is a novel regulator of synaptogenesis. Through my work on dTACC, I also considered broader questions of (1) how presynaptic MT assembly/stability and synaptic expansion might be related, and (2) the possible significance of presynaptic MT plus-end dynamics. In Chapter 2, I describe our finding that dTACC restricts NMJ size, specifically by suppressing bouton addition, and that this role in NMJ growth is concurrent with a role in promoting the integrity of

presynaptic MT architecture. We subsequently performed live-imaging analysis in Chapter 3 to determine the effects of dTACC on MT dynamics in both sensory dendrites and at the presynaptic terminal, finding that loss of *dtacc* disrupts MT dynamics in both compartments and/or cell types. Collectively, this work is consistent with the possibility that dTACC regulates synapse growth through regulation of MT architecture, dynamics, or both.

More broadly, our work is consistent with a model of synaptogenesis where there is a complex interplay of cytoskeletal and morphological changes, driven by a diverse array of MAPs and other molecules, that modulates a precise equilibrium of NMJ expansion and restriction. While early work on molecules such as Futsch (Roos et al., 2000) and Spastin (Sherwood et al., 2004) might have suggested a straightforward model where greater MT stability correlates with greater NMJ growth, further studies have since revealed a more complex relationship. In support of this more nuanced view, our work in Chapter 2 demonstrates that presynaptic dTACC is required to prevent NMJ overgrowth, despite the known roles of TACC proteins in MT stability. Though the underlying mechanisms remain unknown, we speculate that some degree of MT destabilization may be required for the morphological and cellular rearrangements necessary for growth, and that increased MT stabilization may serve as a "brake" during synapse expansion. Future work might investigate this idea by further developing the experimental and analytical strategy in Chapter 3 for live imaging of synaptic MT dynamics. For instance, dual-color imaging of membrane dynamics concurrent with MT dynamics could contribute insight into how MT dynamic instability correlates with morphological events such as bouton addition or elimination. Such imaging, combined with acute (e.g. pharmacological or optogenetic) perturbations to induce rapid changes in MTs or NMJ growth, may help to better dissect the causative relationships between cytoskeletal and morphological changes.

An outstanding question regarding dTACC concerns the mechanisms through which it regulates MTs. Multiple studies in mitotic cells have demonstrated that minus-end localized

TACC proteins stabilize the mitotic spindle by forming a complex with ch-TOG/XMAP215/Msps at the centrosome, thereby recruiting additional stability factors and promoting MT crosslinking (Ding et al., 2017; Hood and Royle, 2011; Peset and Vernos, 2008). Such TACC-Msps interactions have been observed in dividing cells of multiple organisms as well as in the growth cone (Lee et al., 2001; Nwagbara et al., 2014; Samereier et al., 2011; Srayko et al., 2003). However, it is not known whether Msps is required at the NMJ nor whether Msps interacts with dTACC in this context. We observed that *msps*^{P} homozygotes show larval/pupal lethality, consistent with prior reports (Cullen et al., 1999), yet we find that complete *dtacc* null flies are viable through adulthood, albeit female sterile. Thus, because Msps is essential in *Drosophila*, dTACC cannot be required for all Msps functions. Given that the fission yeast ortholog of dTACC can bind the MT lattice in a purified system lacking Msps (Thadani et al., 2009), it seems entirely possible that the MT-lattice binding of dTACC we observe at the NMJ can occur independent of Msps. Thus, a future priority will be to determine whether the role of dTACC at the *Drosophila* presynaptic terminal reflects one of its Msps-independent roles.

Interestingly, the MT plus-end dynamics phenotypes we observed in Chapter 3 upon *dtacc* reduction in both dendrites and at the presynaptic terminal are consistent with reports that dTACC tracks MT ends (Lucaj et al., 2015; Nwagbara et al., 2014; Rutherford et al., 2016; Samereier et al., 2011; Srayko et al., 2003), suggesting a possible +TIP role for synaptic dTACC. We plan future live imaging of dTACC itself to test this possibility, i.e. we will determine if dTACC tracks dynamically with EB1 or a similar +TIP marker. The effect of dTACC on MT architecture might also suggest roles in regulating post-translational modifications (PTMs) of tubulin, such as acetylation (Ac-Tub). We have not found evidence of this at presynaptic terminal (**Fig. 4.1**), although we speculate that dTACC may regulate tubulin PTM in another region, e.g. in the distal axon. Thus, future work may aim to define the precise effect, if any, of dTACC on MT PTMs.

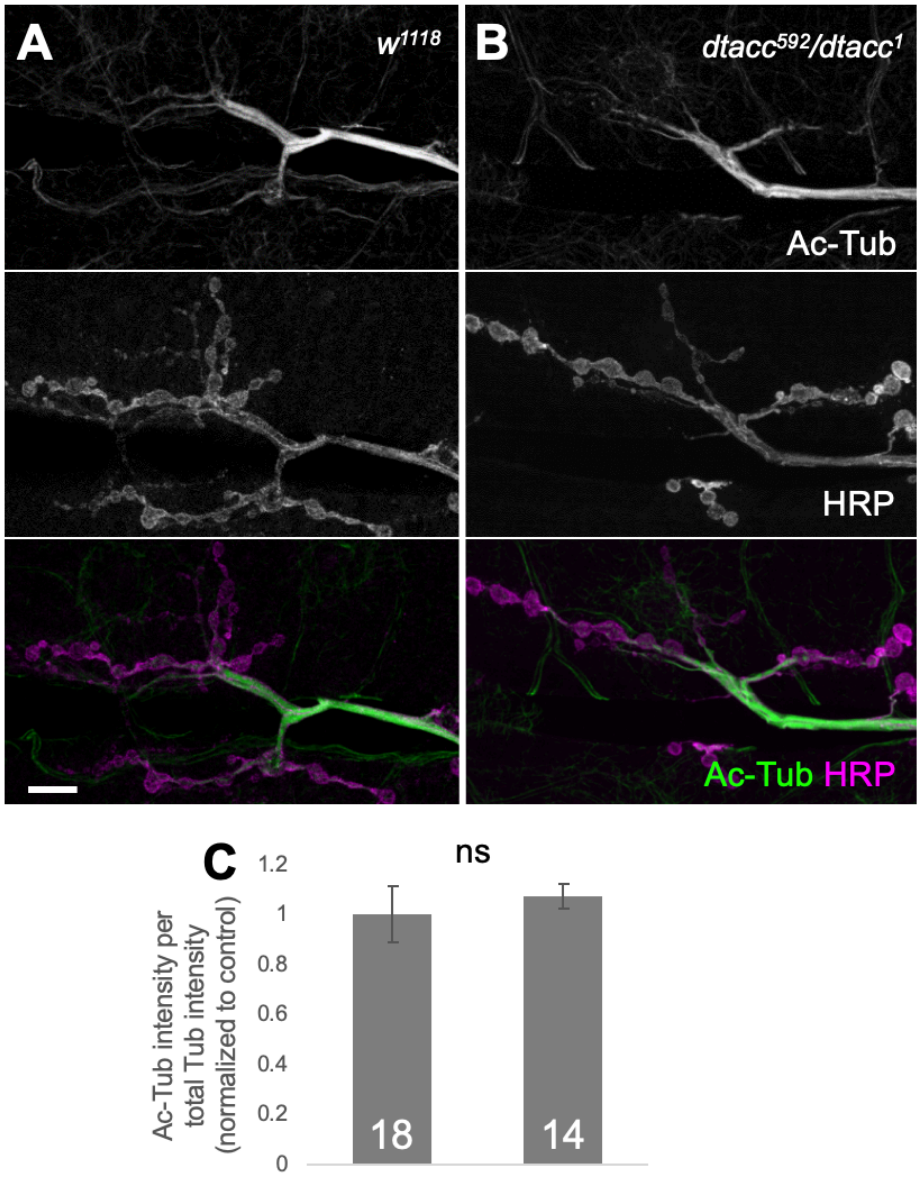

Figure 4.1. *dtacc* animals show normal acetylation of tubulin. Control w^{1118} and $dtacc^{592}/dtacc^{1}$ NMJs were stained with α-Ac-Tub (**A-C**), as well as with α-alpha tubulin to detect total tubulin and α-HRP. No difference in Ac-Tub signal per total tubulin signal was observed between (**A**) controls and (**B**) *dtacc* animals, as confirmed by quantification (**C**). ns, i.e. not significant, indicates P >=0.05, determined by Student's t-test; error bars indicate ± s.e.m; number of NMJs quantified indicated on graph; scale bar, 5 μm.

While dTACC is viewed first and foremost as a MAP, it may be worthwhile to pursue the possibility that dTACC has functional interactions with proteins outside of MTs. Notably, *Xenopus* TACC3/Maskin, the TACC isoform most highly expressed in the embryonic nervous system (Rutherford et al., 2016; Tessmar et al., 2002), interacts with the RNA-associated protein CPEB and binds eIF4E in competition with eIF4G, thereby acting as a translational repressor (Barnard et al., 2005; Cao and Richter, 2002; Stebbins-Boaz et al., 1999; Zukin et al., 2009) (**Fig. 4.2A**). Preliminary pull-down data indicate that fly dTACC also binds with eIF4E and CPEB/Orb2, as well with eIF3-S10 (**Fig. 4.2B**), suggesting that dTACC may have a similar role as a translational repressor in *Drosophila*. Consistently, work on the *Drosophila* protein interaction network (DPiM) indicates that Msps co-purifies with various translational proteins, including poly(A)-binding protein and ribosomal subunits (Guruharsha et al., 2011). While our data indicates that dTACC and Msps must have some independent functions in *Drosophila*, we speculate that dTACC may in fact interact with Msps to function as a translational repressor.

Potential AZ role of dTACC: preliminary findings

My dissertation was largely focused on understanding the roles of dTACC in the context of the presynaptic MT cytoskeleton. In Chapter 2, I describe our finding of two distinct dTACC pools at the NMJ, i.e. “filamentous” and “punctate” dTACC. Until now, I have been primarily concerned with the filamentous population due to its colocalization with presynaptic tubulin. It has been comparatively challenging to ascertain the biological significance of the punctate dTACC population. Super-resolution analysis with 3D-SIM suggested that dTACC puncta are spatially separated from the plus ends of presynaptic MTs by ~100nm; however, we could not fully eliminate the possibility that dTACC puncta are associated with the plus ends of presynaptic MTs bundles that are too sparse or labile to detect by 3D-SIM. In the absence of definitive

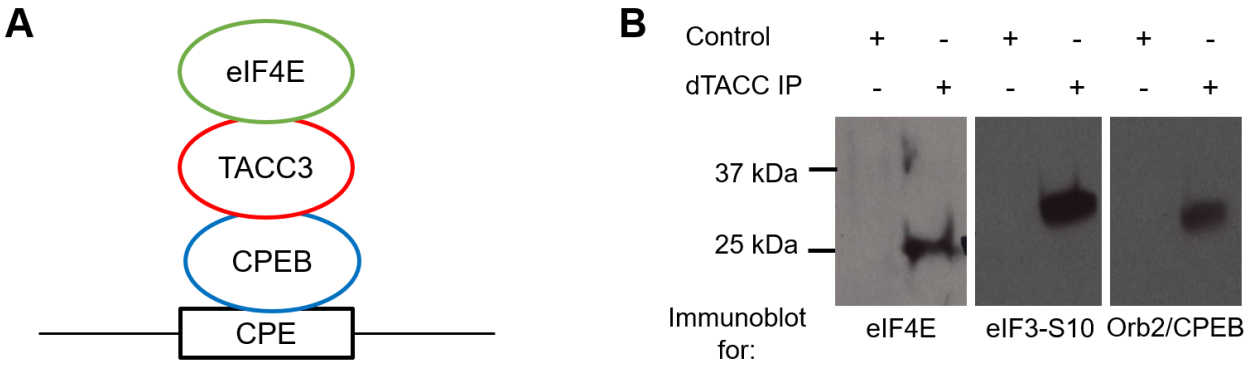

Figure 4.2. TACC proteins are translational repressors. ***A***, *Xenopus* TACC3/Maskin, which is the TACC isoform most highly expressed in the frog embryonic nervous system, interacts with the RNA-associated protein CPEB and binds eIF4E in competition with eIF-4G to repress translation. ***B***, Immunoprecipitation of dTACC in flies results in co-immunoprecipitation of the translational proteins eIF4E, eIF3-S10, and Orb2, the *Drosophila* ortholog of CPEB.

evidence for plus-end localized dTACC puncta, we considered instead that dTACC might be associated with the presynaptic AZ, given the striking resemblance of punctate dTACC staining to the core AZ component Bruchpilot (Brp) (Wagh et al., 2006). While dTACC and Brp did not overlap in a conclusive way, dTACC frequently appeared adjacent to Brp (**Fig. 4.3A**), suggesting localization peripheral to Brp. Similarly, while we did not observe clear colocalization of dTACC with the actin-regulator and SV organizer Nervous Wreck (Nwk) (Coyle et al., 2004) (**Fig. 4.3B**), dTACC was again consistently proximal. Furthermore, high-throughput data indicated proteomic interactions of dTACC with known AZ components (human Syntaxin 5 and Cadherin 1/E-Cadherin, *C. elegans* Syd-1; **Fig. 4.3C**). Thus, while our results do not support a role for dTACC as a core AZ component, the consistent spatial relationships of dTACC to AZ proteins nevertheless led to the conjecture that dTACC is a "peri-AZ" protein.

Of the predicted proteomic interactions of TACC proteins, we were particularly interested in the binding of *C. elegans* TAC-1 with Syd-1. In worms as well as in flies, Syd-1 is an essential early component that drives AZ assembly (Dai et al., 2006; Owald et al., 2010; Patel et al., 2006). At the fly NMJ, Syd-1 staining is observed in both the neuron and muscle. However, only the presynaptic population of Syd-1, which predominates in staining intensity, has been characterized; presynaptic Syd-1 forms clusters surrounding a central core consisting of Brp and Cacophony (Cac)-containing voltage-sensitive Ca^{+2}-channels (Owald et al., 2010). We proceeded to use 3D-SIM at the fly NMJ to test the colocalization of dTACC with the AZ-localized presynaptic population of Syd-1. Consistent with prior proteomic data, we found partial overlap of these proteins, in which large dTACC puncta form diffuse "rings" surrounding presynaptic Syd-1 (**Fig. 4.4A-C**). Manders' colocalization coefficients indicate that 50% of TACC overlapped with (non-aggregated) Syd-1, and 35% of Syd-1 overlapped with dTACC. This suggests a concentric arrangement of AZ components: dTACC surrounds Syd-1, which, in turn, clusters around Brp surrounding the Ca^{+2} channel core (**Fig. 4.4D**).

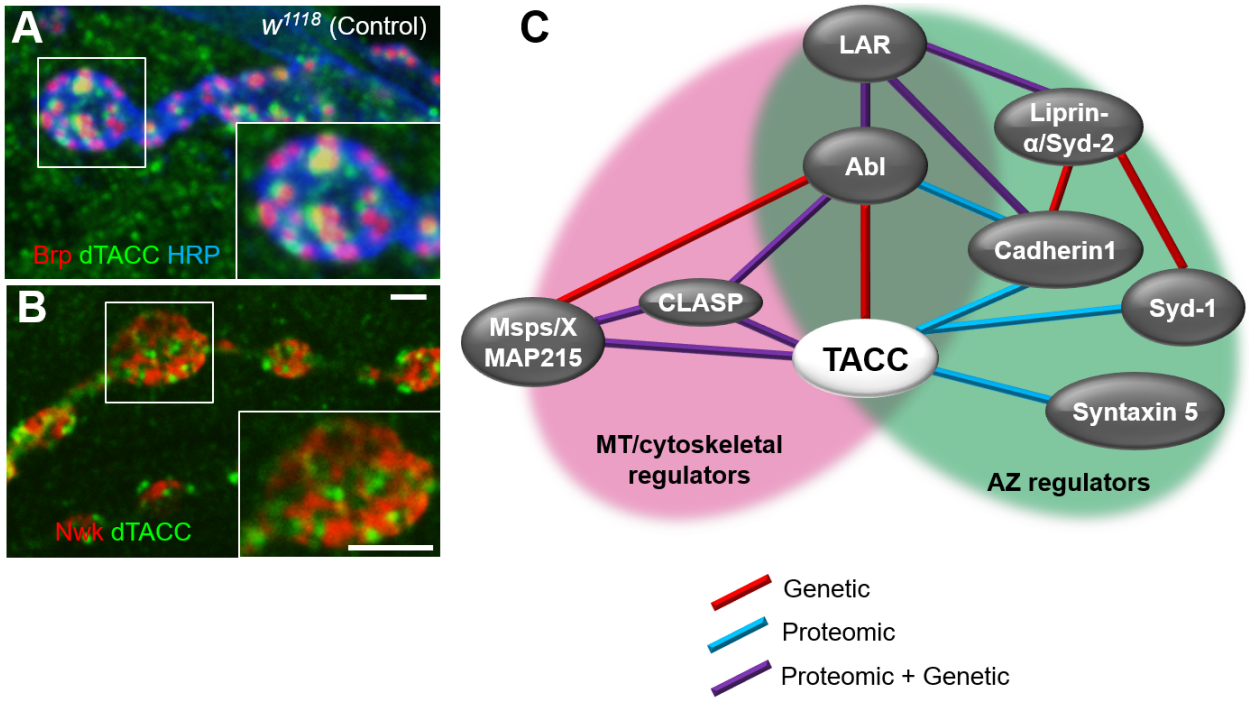

Figure 4.3. dTACC interacts with both MAPs and AZ regulators and has a peri-AZ localization. ***A, B,*** *w*^{1118} muscle 6/7 NMJs were stained with α-dTACC and either (**A**) Brp and (**B**) Nwk. Insets (**A, B**) shown for areas indicated by white boxes. ***C***, Subnetwork of dTACC interactions. Interactions with MAPs (left) are drawn from published literature, while interactions with AZ proteins (right) were extracted from the CCSB Database. Interactions shown include those found for human, *Drosophila*, and *C. elegans* TACC orthologs. Scale bar, 1μm.

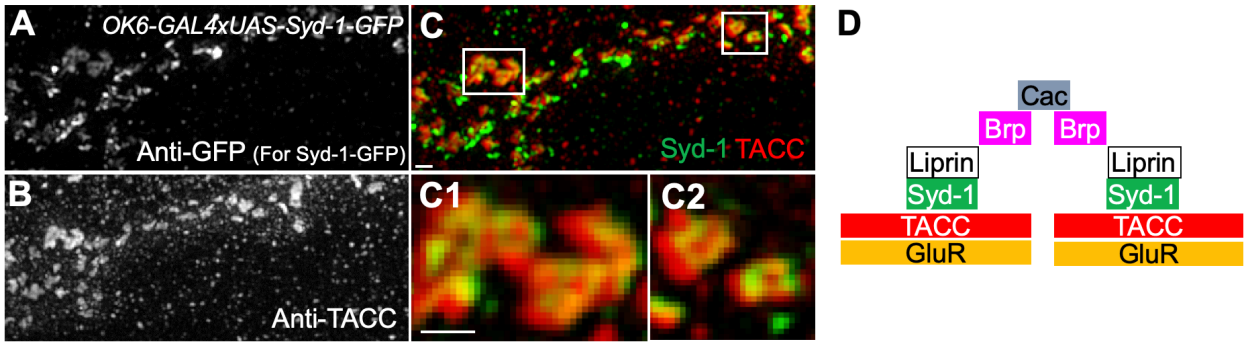

Figure 4.4. dTACC shows partial colocalization with Syd-1. Syd-1-GFP was expressed in the motor neurons of third instar larvae. NMJs were labeled with α-GFP (**A**) and α-dTACC (**B**). At super-resolution, dTACC and Syd-1 were observed to have similar but non-identical localizations (**C**). ***D***, Simplified lateral view of synapse components. Scale bar, 400 nm.

Punctate dTACC represents a postsynaptic population

During our initial investigation of punctate dTACC, we unexpectedly observed a striking cell-type specificity of dTACC puncta that provided a surprising but crucial clue to their nature. This serendipitous discovery occurred when we examined dTACC-GFP expressed under control of a ubiquitous ubiquitin (Ubi) promotor to better characterize dTACC localization in a variety of contexts. In a small number of *Ubi-dTACC-GFP* flies, we observed mosaicism in which dTACC-GFP expression was absent from a single muscle at the NMJ (**Fig. 4.5**). Anti-GFP antibodies detected no dTACC-GFP puncta on the boutons associated with these dTACC-negative muscles (**Fig. 4.5A**). However, these boutons still had filamentous, endogenous dTACC staining within the motor axon terminals (**Fig. 4.5B**). This fortuitous discovery provides invaluable context, as it reveals that dTACC is present in both pre- and postsynaptic compartments, and that that "peri-AZ" punctate dTACC staining in fact represents a postsynaptic localization. Notably, this result conclusively validates our earlier finding that dTACC puncta are not associated with presynaptic MT plus ends and furthermore explains the lack of complete overlap of dTACC with the AZ. Nonetheless, the consistently peripheral localization of dTACC with AZ markers suggests that there is a systematic spatial arrangement of postsynaptic dTACC relative to the presynaptic AZ, perhaps to facilitate trans-synaptic functional interactions.

Having already noticed consistent localization of dTACC peripheral to Brp, we performed 3D-SIM to more closely examine how these proteins are arranged in 3-dimensional space (**Fig. 4.6**). Our results provide evidence that despite being located in separate cell compartments, dTACC and Brp appear to be associated in a consistent way. In certain imaging planes, the alignment of Brp and dTACC across the synaptic cleft becomes readily apparent: Brp appears as discs that demarcate the neuronal membrane, while dTACC clouds are clearly superficial to

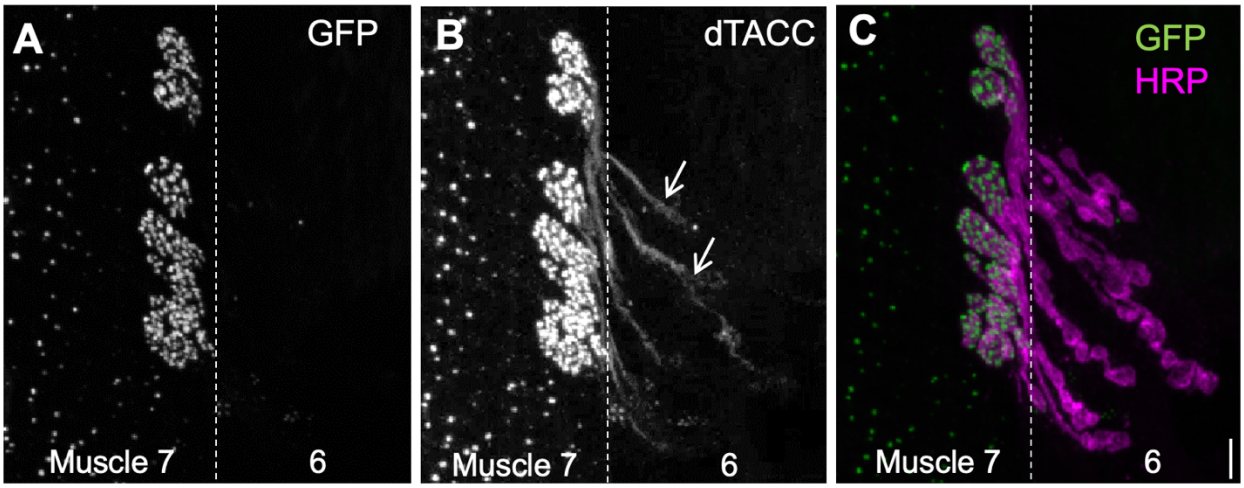

Figure 4.5. dTACC puncta are only present on boutons innervating muscles that express dTACC. A small number of *Ubi-dTACC-GFP* flies fail to express dTACC-GFP in a single muscle (here, muscle 6, in contrast to 7; **A**) due to mosaicism. In boutons that innervate GFP-negative muscle (**B, C**), dTACC puncta are absent, but filamentous dTACC is still present (arrows). Scale, 5µm.

the boundary implied by the arrangement of Brp (**Fig. 4.6Aiii**). Interestingly, the cloud-like dTACC puncta, and their arrangement relative to Brp, closely resemble the diffuse clouds of the glutamate receptor (GluR) subunit GluRIID that surround Brp (Owald et al., 2010). We performed further colocalization experiments to confirm the proximity of dTACC to the postsynaptic cytomatrix. As expected, dTACC overlaps with dPak, the *Drosophila* ortholog of the p21-activated kinase (PAK) (**Fig. 4.6B**). PAK is a serine/threonine kinase that regulates actin dynamics downstream of the Rac1 and Cdc42 Rho family GTPases (Civiero and Greggio, 2018; Zhao and Manser, 2012) and has postsynaptic roles both in dendritic spines (Harden et al., 1996; Parnas et al., 2001; Zhang et al., 2005) and at the NMJ, where it regulates receptor organization along with postsynaptic ultrastructure and maturation (Lee and Schwarz, 2016; Parnas et al., 2001; Sone et al., 2000; Wan et al., 2000). We thus propose a preliminary spatial model (**Fig. 4.6C**) where diffuse dTACC accumulations colocalize with GluR clusters, possibly regulating receptor organization, levels, and/or maturation. Future experiments will assess the colocalization of dTACC with GluR subunits and other proteins in the postsynaptic cytomatrix.

Trans-synaptic interactions of dTACC

While punctate dTACC clearly represents a postsynaptic population, potential interactions with presynaptic components would be consistent with well-established precedents for trans-synaptic communication (Bailey et al., 2015; Jessell and Kandel, 1993). For instance, the dTACC interactor Syd-1 regulates GluR clustering via direct interactions with Neurexin-Neuroligin (Dnrx-Dnlg1) (Banerjee et al., 2017; Owald et al., 2012; Xing et al., 2018). Specifically, loss of *syd-1* increases overall GluR levels as determined by GluRIID intensity and disrupts GluRIIA and GluRIIB incorporation, consistent with a defect in GluR maturation (Owald et al., 2010, 2012); these defects can be rescued by presynaptic but not postsynaptic Syd-1 expression

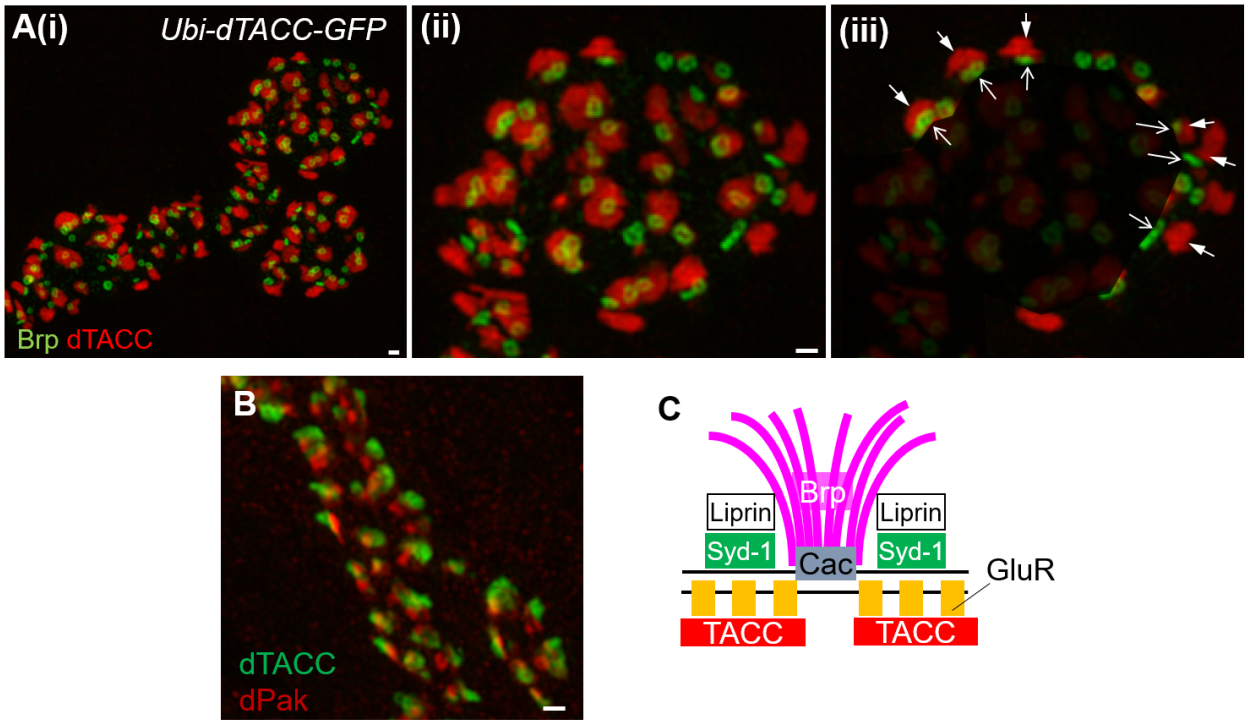

Figure 4.6. Spatial relationships of dTACC with pre- and postsynaptic proteins.

A, Immunocolocalization of Brp and ubiquitously expressed dTACC-GFP. Panels (**ii, iii**) both display insets of the top-left bouton in (**i**), with select Brp and dTACC puncta shown/omitted for purposes of illustration (**iii**). Presynaptic Brp are consistently found at the periphery of the bouton membrane (**iii**, hollow arrows), while cloud-like dTACC clusters are consistently found "outside" the Brp puncta (**iii**, solid arrows). ***B***, Immunocolocalization of dTACC with the postsynaptic marker Pak (**B**). ***C***, Proposed arrangement of pre- and postsynaptic components. Scale, 500 nm.

(Owald et al., 2010), demonstrating the trans-synaptic nature of this interaction. Given the spatial relationships of dTACC and Syd-1 (**Fig. 4.4**), we asked if Syd-1 interacts with postsynaptic dTACC by testing the effect of *syd-1*$^{ex3.4}$ mutation on dTACC distribution (**Fig. 4.7**). Compared to wild-type animals (**Fig. 4.7A**), *syd-1*$^{ex3.4}$ animals show a dramatic loss of puncta (**Fig. 4.7B**); similar effects were observed with presynaptic *syd-1-rnai* knockdown (data not shown). Loss and overexpression of the Syd-1 associated factor *liprin-α* also perturbed dTACC localization (**Fig. 4.7C**). Somewhat surprisingly, Syd-1 and Liprin-α appear to have opposite effects on dTACC: Liprin-α overexpression results in a loss of puncta similar to that observed upon *syd-1* loss (**Fig. 4.7C**), while loss of *liprin-α* results in a qualitative increase in the size and intensity of dTACC puncta compared to controls (**Fig. 4.7D**). While further experiments are necessary to confirm the interactions of Syd-1 and Liprin-α, these preliminary experiments suggest possible trans-synaptic interactions of postsynaptic dTACC with core AZ components.

Collectively, the present literature indicates a model where the Syd-1, along with Liprin-α, has functions not only in presynaptic AZ assembly but also in postsynaptic GluR organization via Dnrx-Dnlg1 (Owald et al., 2010, 2012). While it is not yet known which other postsynaptic molecules may be involved in regulation of GluRs downstream of Syd-1-Dnrx-Dnlg1, our results suggest that dTACC may be a candidate for this role. We speculate that dTACC, under control of the Syd-1-Dnrx-Dnlg1 functional complex, may regulate GluR clustering, possibly in cooperation with dPak, which is required for receptor clustering and composition (Lee and Schwarz, 2016). Future experiments will assess whether dTACC, like Syd-1, affects the distribution and/or intensity of invariant GluR subunits and therefore overall GluR levels, and will also measure the effect of dTACC on GluRIIA/GluRIIB incorporation to determine roles in receptor maturation. Furthermore, epistasis and tissue-specific knockdown experiments will be required to confirm whether postsynaptic dTACC is indeed required for the postsynaptic roles of Syd-1.

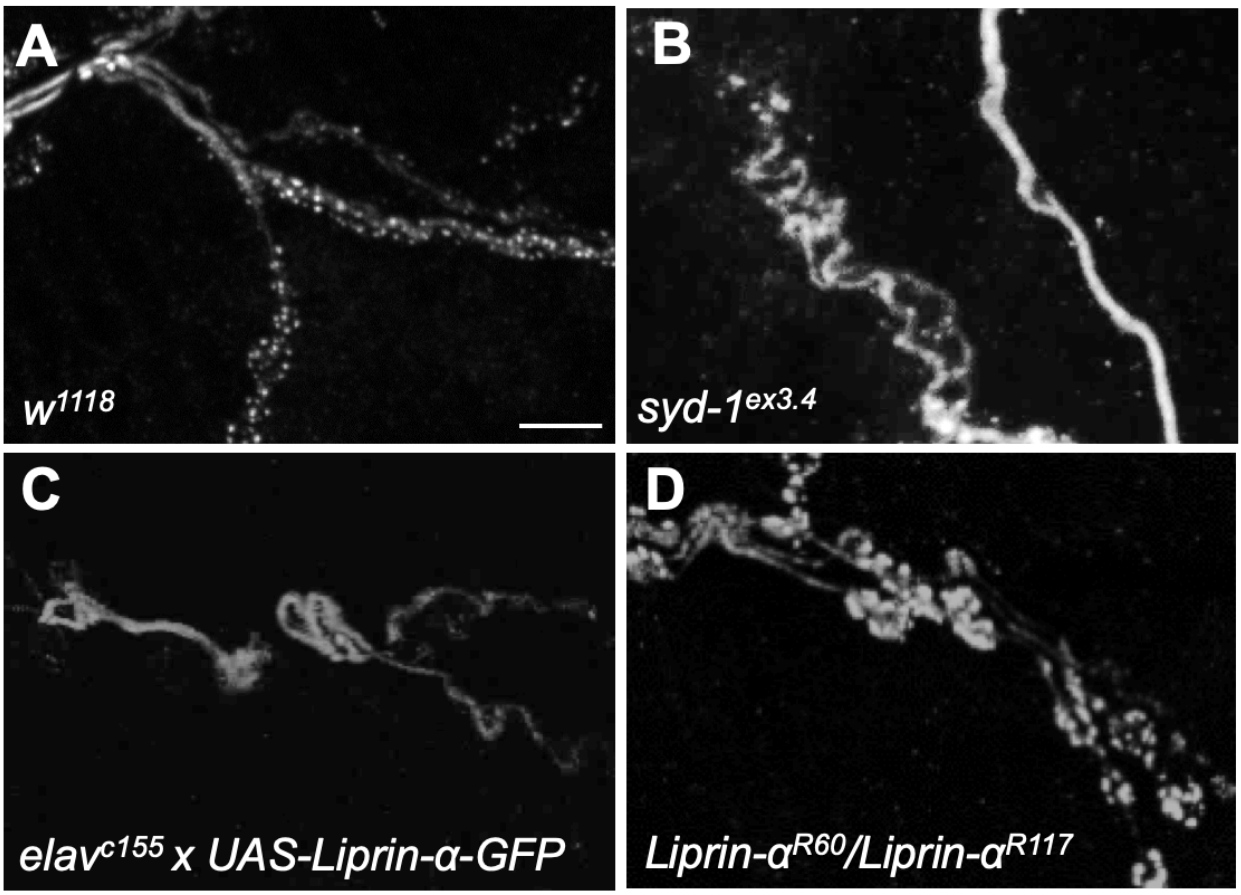

Figure 4.7. Liprin-α and Syd-1 regulate punctate dTACC localization. ***A***, Wild-type dTACC shows filamentous and punctate distributions. ***B***, *syd-1* loss reduces punctate dTACC. ***C***, Pre-synaptic Liprin-α overexpression reduces punctate TACC in a manner similar to *syd-1* loss, while *liprin-α* nulls (**D**) show larger and brighter puncta. Scale, 5μm.

Potential roles for dTACC on the core architecture of presynaptic AZ

Effective trans-synaptic communication across the synaptic cleft requires not only anterograde signals but also reciprocal retrograde signals. Such bidirectional communication establishes feedback loops between pre- and postsynaptic compartments, in spite of their physical separation, and thereby facilitates coordinated assembly, maturation, and function of the entire synapse. Given that dTACC is proximal to Syd-1 and Brp (**Fig. 4.4, 4.6**) and shows putative interactions with core AZ components Syd-1 and Liprin-α (**Fig. 4.7**), we considered that postsynaptic dTACC might be part of one such retrograde pathway that controls the presynaptic AZ. In particular, confocal microscopy has demonstrated that Syd-1 regulates Brp area and clustering (Owald et al., 2010), and ultrastructural work has demonstrated the effect of Liprin-α on AZ size and shape (Kaufmann et al., 2002). We thus sought to determine if dTACC regulates the presynaptic AZ in a similar fashion. To this end, we performed super-resolution microscopy to evaluate the effect of *dtacc* loss on the core AZ component, Brp, based on three parameters: volume, integrated density, and the number of Brp puncta per bouton (**Fig. 4.8A**). Brp volume reflects the architecture of the AZ, with increased (or decreased) volume possibly reflecting improper organization where components are not correctly distributed. Integrated density is proportional to the concentration of Brp at the AZ, and changes to this parameter might indicate over/underexpression of Brp, relative to normal levels. Finally, Brp density indicates the number of release sites present in each bouton and thus reflects the allocation of release machinery across the entire neuron.

We proceeded to measure these parameters upon loss of both pre- and postsynaptic *dtacc*. Our work on presynaptic dTACC in Chapter 2, and on both motor terminal and dendritic dTACC in Chapter 3, consistently indicates that both pre- and postsynaptic dTACC play important roles at the synapse. Thus, while the AZ-proximal population of dTACC appears to be predominantly, if not entirely, muscle-localized, we also considered the possible roles of

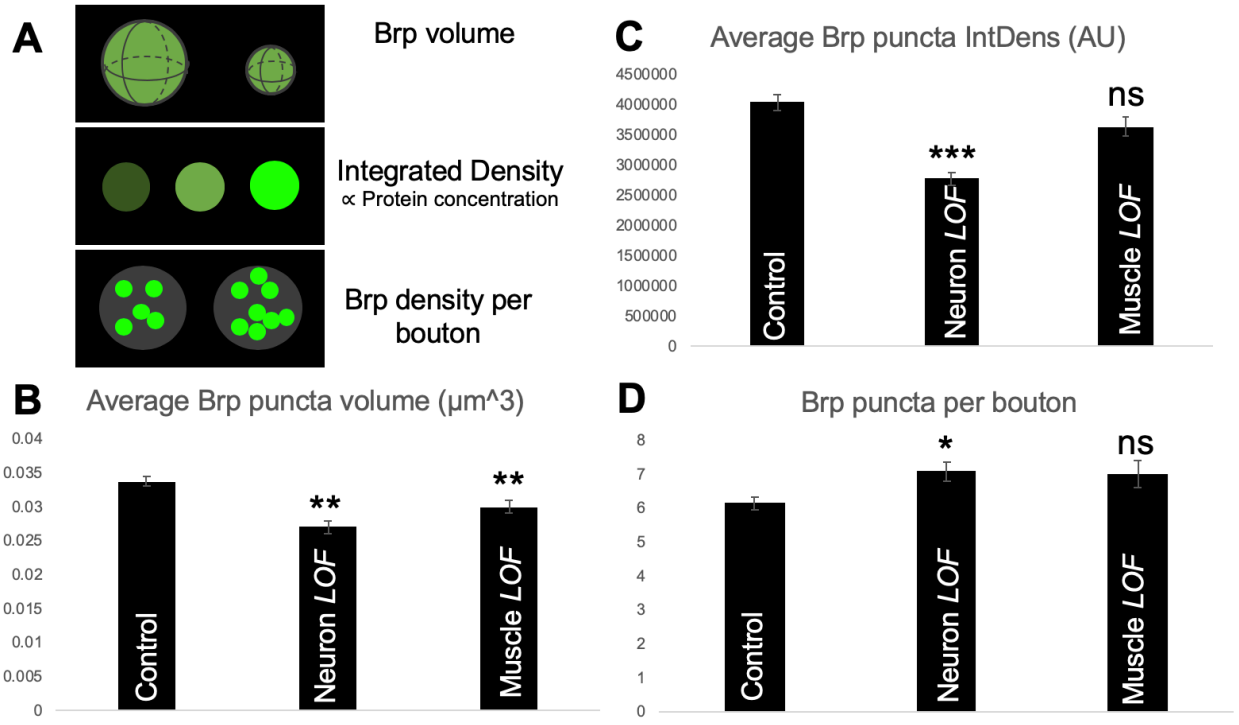

Figure 4.8. Pre- and postsynaptic loss of dTACC result in changes to Brp. (**A**) Parameters measured using super-resolution microscopy. Presynaptic *dtacc-rnai* driven by *elav*-Gal4 in the *dtacc*^{1}/+ heterozygous background resulted insignificant changes to all three parameters (**B-D**), while post-synaptic *dtacc-rnai* driven by *DMEF*-Gal4 in a wild-type background resulted in significant reduction of Brp puncta volume (**B**). * P < 0.05 ** P < 0.01 *** P < 0.001; Mann-Whitney; N = 13 control animals, 15 *elav-Gal4* x *UAS-dtacc-rnai; dtacc*^{1}/+ animals, 11 postsynaptic *elav-Gal4* x *UAS-dtacc-rnai* animals.

presynaptic dTACC on the AZ. We therefore compared the effects of *UAS-dTACC-RNAi* expression both pre- and postsynaptically using the *elaV*^{C155}- and *DMEF*-GAL4 neuronal- and muscle-drivers, respectively. Consistent with the synaptic localization of dTACC-GFP in muscle, postsynaptic *dtacc* knockdown resulted in significant reduction to Brp volume (**Fig. 4.8B**), although Brp integrated density (**Fig. 4.8C**) and puncta density (**Fig. 4.8D**) did not change significantly. Interestingly, presynaptic loss resulted in significant changes to all three parameters (**Fig. 4.8B-D**). In the future, we will conduct cell-type specific rescue of *dtacc* nulls to conclusively determine the pre- and post-synaptic roles of dTACC.

While our experiments have not found a presynaptic dTACC population at the AZ, quantification of Brp indicates that both pre- and postsynaptic dTACC regulate AZ assembly, suggesting that a transient pool of dTACC may be present at the presynaptic AZ. The possibility of a smaller, peri-AZ pool of dTACC alongside a larger postsynaptic pool is reminiscent of the localization of Discs Large (Dlg) at the *Drosophila* NMJ. At the light level, Dlg has an ostensibly postsynaptic localization (Budnik et al., 1996; Guan et al., 1996; Lahey et al., 1994). However, immuno-electron microscopy reveals a peri-AZ population of Dlg, in addition to the expected SSR-localized postsynaptic population (Lahey et al., 1994), consistent with genetic data indicating a presynaptic requirement for Dlg (Budnik et al., 1996). This dual localization of Dlg may explain its roles in both pre- and postsynaptic morphogenesis and plasticity as well as in neurotransmitter release at the *Drosophila* NMJ (Budnik et al., 1996; Guan et al., 1996). Future experiments will address the possible existence of a transient pool of dTACC at the pre-synaptic AZ by performing immunogold-labeling and electron microscopy of dTACC. To this end, we are optimizing a high pressure freezing/freeze substitution protocol (compare **Fig. 4.9A** and **B**). This improved protocol will also be used to further characterize the effects of *dtacc* loss at the ultrastructural level, along with immuno-localization of dTACC.

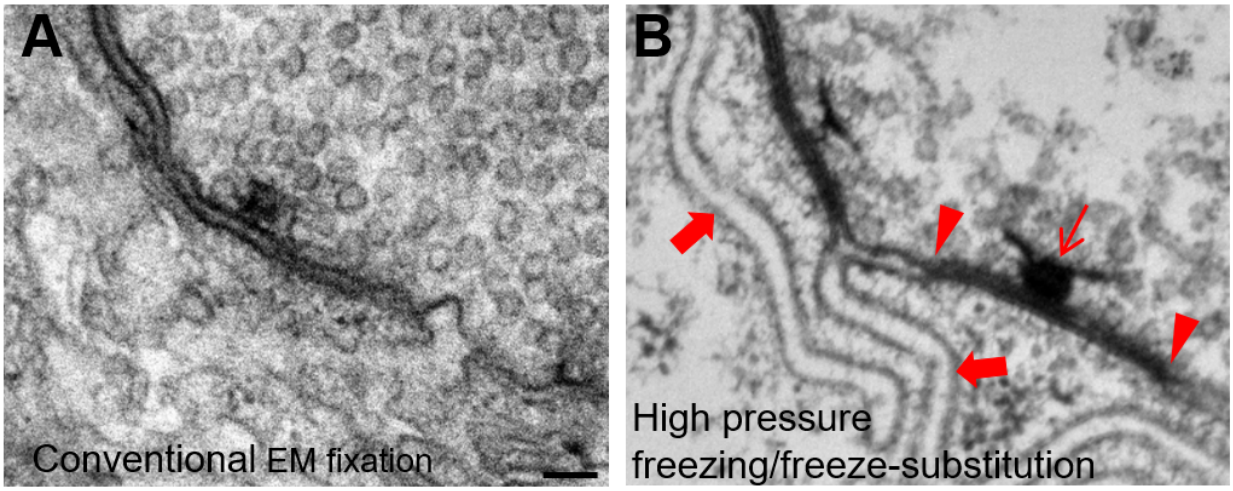

Figure 4.9. Optimization of ultrastructural analysis. For optimized preservation, flies were briefly fixed and then subjected to high pressure freezing/freeze substitution. Compared to conventional methods (**A**), the optimized protocol (**B**) improved preservation of structures, e.g. the T-bar (thin arrow), adhesion plaque (triangles), and the SSR (block arrows). Scale, 50 nm.

Incidentally, Liprin-α, like both dTACC and Dlg, not only displays pre- and postsynaptic expression but is in fact overwhelmingly postsynaptic (Miller et al., 2017; Spangler and Hoogenraad, 2007). Yet, in spite of this, Liprin-α has been studied almost exclusively in the presynaptic context. Work in dendritic spines indicates that Liprin-α interacts with the PDZ-domain protein GRIP in the PSD and regulates receptor clustering and synaptic morphology under control of LAR signaling (Dunah et al., 2005; Wyszynski et al., 2002); postsynaptic Liprin-α is also regulated by the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Hoogenraad et al., 2007). Outside of these studies, the roles of postsynaptic Liprin-α, including at the *Drosophila* NMJ, is largely a mystery. Syd-1 also displays some postsynaptic staining, although unlike Liprin-α, Syd-1 appears to be primarily presynaptic (Owald et al., 2010). As of this writing, it is not known whether Syd-1 has any postsynaptic roles. Thus, as we continue to investigate the roles of both pre- and postsynaptic dTACC, we are also interested in considering the possible roles of Liprin-α and Syd-1 on both sides of the synapse.

Functions of dendritic dTACC

The primary focus of my dissertation has been to study synaptogenesis at the *Drosophila* NMJ. As such, my discussion of postsynaptic dTACC has focused on a muscle-localized population. Nonetheless, our findings in Chapter 3 indicate that *dtacc* loss also produces defects in postsynaptic MT dynamics in dendrites. To this end, a future priority will be to determine if *dtacc* loss produces morphological phenotypes in dendrites, such as defects in arborization (Wang et al., 2019). In the longer term, our lab may investigate the specific consequences of dTACC-mediated dendritic MT dynamics. Numerous studies have established the existence of a population of highly dynamic MTs that transiently invade nascent dendritic spines, thereby regulating dendritic morphogenesis and plasticity (Gu et al., 2008; Hu et al., 2008, 2011; Jaworski et al., 2009; Kapitein et al., 2011; McVicker et al., 2016; Merriam et al., 2011; Penzes

et al., 2009; Wagner et al., 2011). The molecular regulation of MT invasions is largely unknown, although the +TIP EB3 has been implicated (Jaworski et al., 2009). Given our finding that dTACC regulates MT plus-end dynamics in dendrites, and that TACC proteins can behave as +TIPs (Lucaj et al., 2015; Nwagbara et al., 2014; Rutherford et al., 2016; Samereier et al., 2011; Srayko et al., 2003), it is tempting to speculate on a role for dTACC in regulating dynamic MT invasions of dendritic spines. Additional experiments in *ex vivo* mammalian systems suited for dendritic spine imaging would be necessary to test this possibility, as MTs have not yet been detected in *Drosophila* dendritic spines (Leiss et al., 2009). Conversely, dTACC may have a separate role in regulating non-spine MTs in the main dendritic shaft. Notably, the +TIPs EB1 and APC are required for proper MT steering and polarity in dendrites (Chen et al., 2014; Mattie et al., 2010; Van De Willige et al., 2016), which is thought to orchestrate long-range MT-based transport. Thus, while dTACC may regulate MT spine invasions, an alternative/additional role may be in MT steering, through which dTACC may regulate the distinctive orientations of dendritic MTs and thus modulate cargo sorting and transport (Kapitein and Hoogenraad, 2011; Matamoros and Baas, 2016; Rolls, 2011).

MATERIALS AND METHODS

Drosophila genetics

Stocks were raised at 25°C according to standard procedures. The w^{1118}, $elaV^{C155}$-*GAL4*, *UAS-Dcr2*, *OK6-GAL4, DMEF-GAL4*, and *Ubi-dTACC-GFP* stocks were obtained from the Bloomington Stock Center (Bloomington, IN, USA). The *UAS-dtacc-RNAi* stock was obtained from the Vienna *Drosophila* Resource Center (Vienna, Austria). To enhance *dtacc-RNAi* expression, $elaV^{C155}$-GAL4 was also used to express *UAS-Dcr2*, an endonuclease that promotes processing of long dsRNAs to siRNAs. The *liprin-α*^{R60} and *liprin-α*^{R117} were previously generated in the lab (Kaufmann et al., 2002). The previously described $msps^{P}$ (Cullen et al.,

1999), *dtacc*^{1} (Gergely et al., 2000) and *dtacc*^{592}(Lee et al., 2001) stocks were provided by Jordan Raff, while the *UAS-Liprin-α-GFP* (Fouquet et al., 2009)*, UAS-Syd-1-GFP,* and *syd-1*$^{ex3.4}$ (Owald et al., 2010) stocks were provided by Stephan Sigrist.

Immunohistochemistry

Wandering third instars were dissected in Ca^{2+}-free saline and fixed in 4% paraformaldehyde in PBS for 10 min, except for tubulin immunostaining, where larvae were dissected in Brinkley Buffer 1980 (80mM PIPES, 1mM MgCl2, 1mM EGTA, pH 6.8) and fixed in 4% paraformaldehyde in PBS with 5mM EGTA. Primary antibodies include: mouse anti-Brp NC82 (1:50; Developmental Studies Hybridoma Bank, Iowa City, IA, USA); rabbit anti-alpha-tubulin (1:200; Ab15246; Abcam); mouse anti-acetylated tubulin (1:800; 6-11B-1; Sigma-Aldrich); rabbit anti-GFP (1:500; A-6455; ThermoFisher); and mouse anti-dTACC (1:50) (Chou et al., 2020). Rabbit-anti-Pak (1:1000) was provided by Nicholas Harden (Harden et al., 1996). Rabbit-anti-Nwk antibody was provided by Avital Rodal (Rodal et al., 2008). Secondary antibodies conjugated to AlexaFluor 488 and 594 were used (1:200; Invitrogen). Anti-HRP antibodies conjugated to AlexaFluor 594 and 647 were used (1:200; Jackson Immunoresearch).

Image acquisition and processing

Synaptic arbors of muscle 6/7 in the abdominal segment A2 were used for all analyses. Imaging was performed on a Nikon A1R point scanning confocal and a Nikon Yokogawa spinning disc confocal with a Hamamatsu ORCA-R2 cooled CCD camera. 3D-SIM was performed on a DeltaVision OMX Blaze microscope (GE Healthcare Life Sciences) with a PCO sCMOS camera. Lasers were adjusted to prevent oversaturation. Images were processed and analyzed with ImageJ. An HRP mask was used to restrict analysis to neuronal signal for tubulin intensity analysis, and MATLAB scripts were used to quantify dTACC and tubulin signals relative to HRP.

Cell culture, immunoprecipitation, and Western blotting

Drosophila S2 cells were grown and maintained in Schneider's *Drosophila* medium supplemented with 10% heat-inactivated fetal bovine serum and penicillin-streptomycin. S2 cells were harvested by washing and aspirating with 1x in PBS, and 1 ml lysis buffer (with protease and phosphatase inhibitor) was added directly to the dishes. Cells were triturated, collected into a 1.5 ml tube (Eppendorf), rotated at 4°C for 30 minutes, and spun to collect the supernatant. For each immunoprecipitation, 20 µl of protein G beads were used. Tubes were washed 6 times lysis buffer, twice with Naar buffer, and twice with PBS, then combined with 40 µl of 4x Laemmli buffer, boiled for 10 minutes, and spun at 10,000 rpm for 5 minutes. The supernatant was spun again to remove all beads. 4-15% agarose gels were loaded and run at 20 mA constant per gel. Protein was transferred to PVDF membrane and immunoblotted using standard protocols and exposed using chemiluminescence reagents. The following antibodies were used for blotting: mouse anti-eIF4E(1:500, Developmental Studies Hybridoma Bank) mouse anti-eIF3-S10 (1:500, Abnova), mouse anti-Orb2 (1:500, Developmental Studies Hybridoma Bank).

Super-resolution quantification of Brp puncta

For analyzing Brp puncta at the synapse (**Fig. 4.7**), maximum intensity projections from z-stacks were quantified using the following Fiji/ImageJ protocol.

- Brp puncta volume and integrated density
    - Drag and drop.SIR.dv files into ImageJ and split channels
    - Convert to 16 bit image
    - Using the 16 bit Brp channel (Channel 1), select Analyze >> 3D objects counter
        - Auto-threshold: set lower limit 10, upper limit auto
        - Manual threshold not advised

    - Will provide number of puncta, puncta volume, puncta integrated density, map, and run info (can customize)
    - Get z-projection sum of volume map → compare to z-stack sum of original image to double-check if correct objects detected
    - Copy output to Excel and determine average → compare to control
- Brp puncta/bouton
    - Number of 3D objects found/manually counted boutons in image

Electron microscopy

Third instar larvae were pinned onto Sylgard-coated plates using 0.1 mm minutien pins (Fine Science Tools) and dissected in ice-cold Ca^{2+}-free saline. For conventional EM fixation, pelts were fixed for 2-4 hours in yellow fixed (2.5% formaldehyde, 5% glutaraldehyde 0.06% picric acid, 0.1M sodium calcodylate, and 0.06% calcium chloride), then transferred to fresh fix and stored overnight at 4°C. Fillets were then rinsed in 0.2M cacodylate buffer, pH 7.4, then unpinned and trimmed. Samples were post-fixed with osmium tetroxide and embedded according to standard protocols (Harvard Medical School Electron Microscopy).

For high pressure freezing/freeze-substitution, larvae were dissected as described and briefly fixed with a solution of 4% paraformaldehyde and generously washed with PBS. Larvae were submerged in Ca^{2+}-free saline, loaded into and frozen by a Leica EM ICE. Once frozen, samples were processed in a Leica EM AFS-2 using a standard 72 hour freeze-substitution protocol and a fixative of uranyl acetate, glutaraldehyde, water ("Giovanni's fix"). Samples were kept cold between steps by submersion in storage dewars filled with liquid nitrogen.

For all samples, sections were cut parallel to the surface of the muscle. Once a A2 6/7 muscle bouton was identified ~50-90 nm sections were taken for a total of 5 μm. Sections were

mounted on single slot grids, stained with lead and uranyl acetate, and imaged on a JEOL 1200EX – 80kV electron microscope at 6,500x, 10,000x and 25,000x magnification.

Statistics

All comparisons were done using Welch's t-test for unequal variances using Graphpad.

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CHAPTER 5: APPENDIX

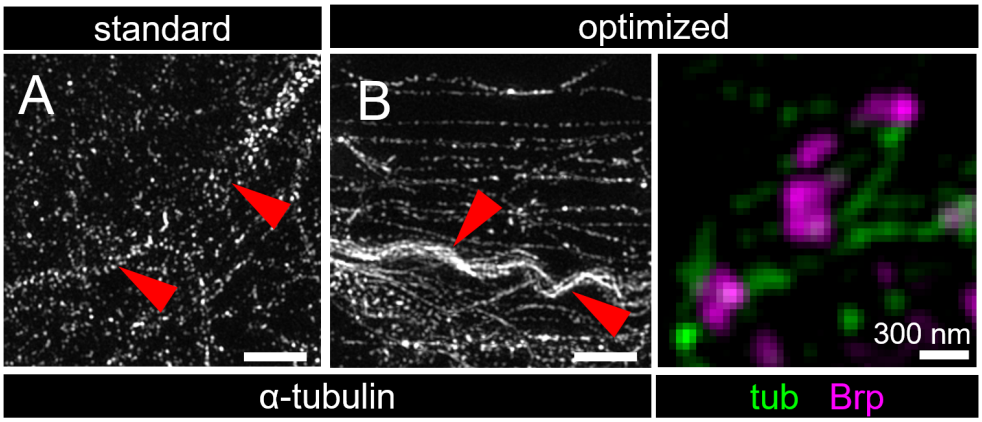

Figure supplement 5.1. Optimization of MT preservation and MT-Brp measurement. Triangles indicate synaptic MTs. Samples were prepared using (**A**) standard lab protocol or (**B**) optimized dissection/fixation and stained with α-tubulin (tub) (**A, B** scale=2 μm). To determine MT-Brp distance (**C**), α-tub and α-Brp intensity profiles were plotted to measure the distance between their peak intensities.

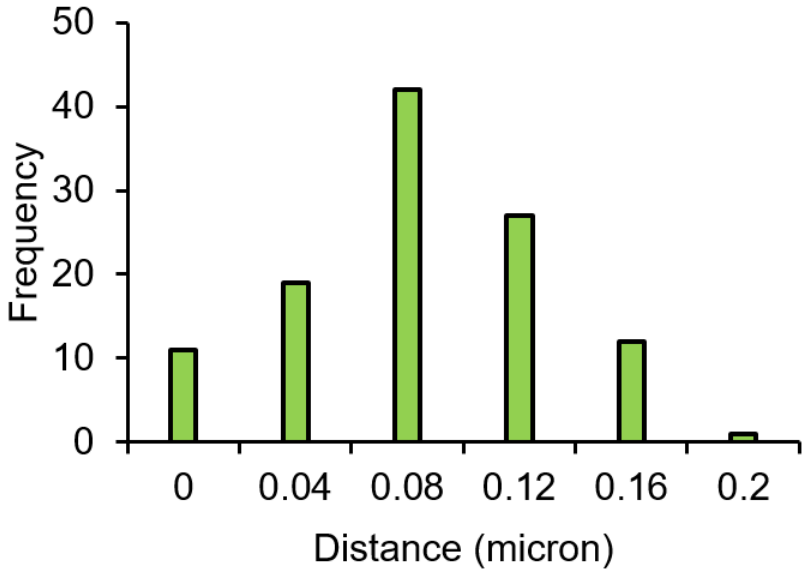

Figure supplement 5.2. Verification that MTs localize proximal to AZs. MTs and AZs were labeled with α-tub and α-Brp. Mean distance between MT and AZ peaks of staining intensity was quantified as in Lepicard *et al.,* 2014. Our replication of the published method yielded a data distribution with close resemblance to the distribution in Lepicard *et al.,* 2014. Using our data, mean MT-AZ distance was found to be 85 nm (n=112 AZs, 3 larvae); compared to published mean distance of 54 nm (n=227 AZs, 3 larvae).

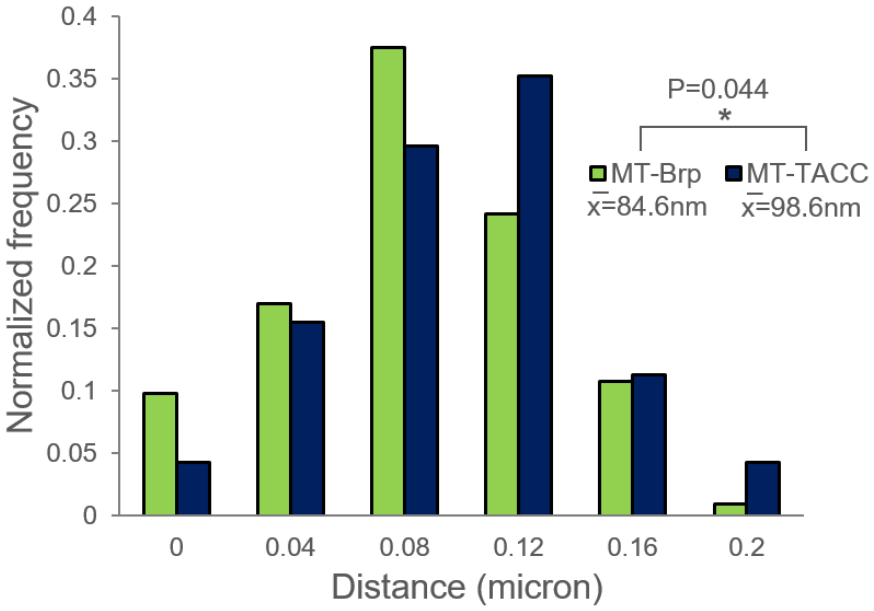

Figure supplement 5.3. Brp and dTACC distances relative to MTs indicate a dTACC-AZ offset. Using WT animals, the distance between MTs and Brp (green, mean = 84.6 nm, N=127 AZs) or MTs and dTACC puncta (blue, 98.6 nm, N=71 AZs) was measured. If dTACC were a MT-AZ anchor, we expect MT-dTACC distances to be smaller than MT-Brp distances. However, the average MT-dTACC distance was 14 nm greater than the MT-Brp distance (P = 0.044). Thus, TACC is unlikely to be between AZs and MTs. Thus, based on dTACC and AZ positions relative to MTs, it is unlikely that dTACC is a MT-AZ anchor.

Table supplement 5.1. Effect of dTACC RNAi on MT-AZ distance.

Condition		Mean distance (nm)	s.e.m.	N	
				AZs	flies
MT-Brp	WT	84.6	4.3	112	2
	dTACC RNAi	94.1	4.9	127	2
P = 0.151					

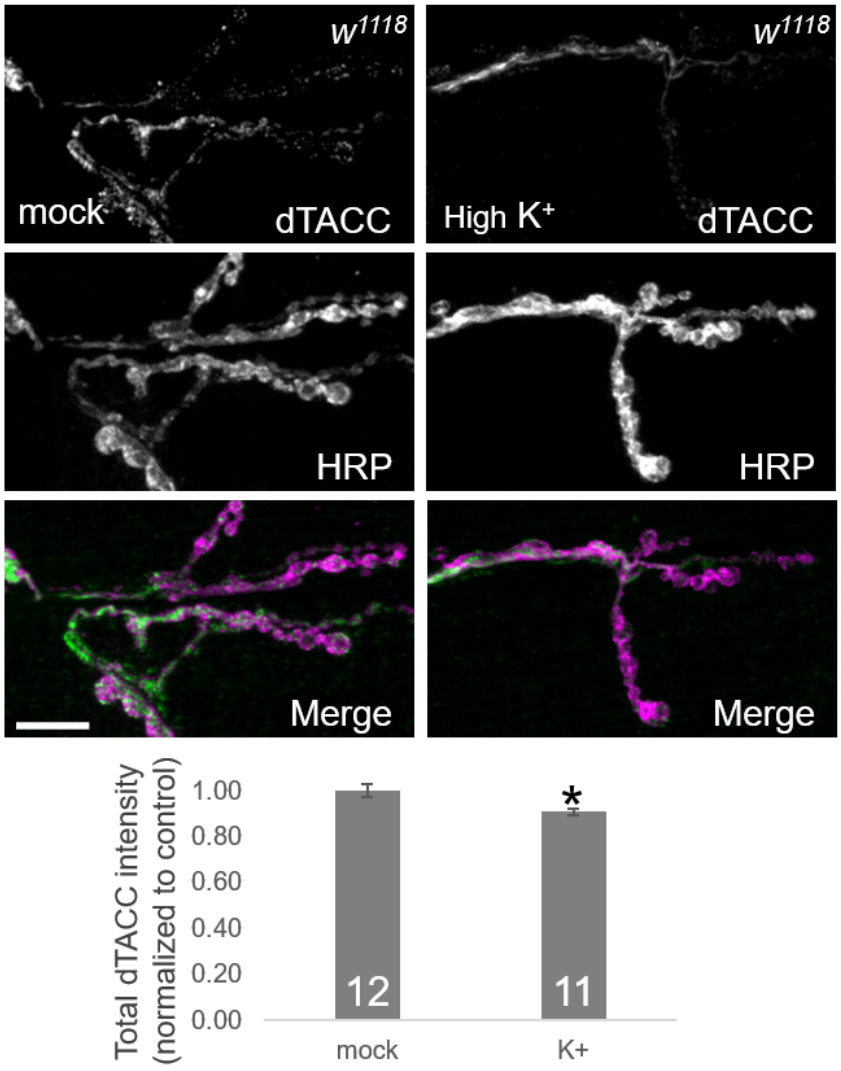

Figure supplement 5.4. Elevated activity reduces overall dTACC levels at the NMJ.

Compared to mock-stimulated controls (**A**) induction of activity with high K+ (**B**) produces a significant ~10% decrease in dTACC (**C**), with filamentous and punctate TACC affected equally.

* P < 0.05, ** P < 0.005, t-test; error bars ± s.e.m; sample size on graph; scale 10 μm.

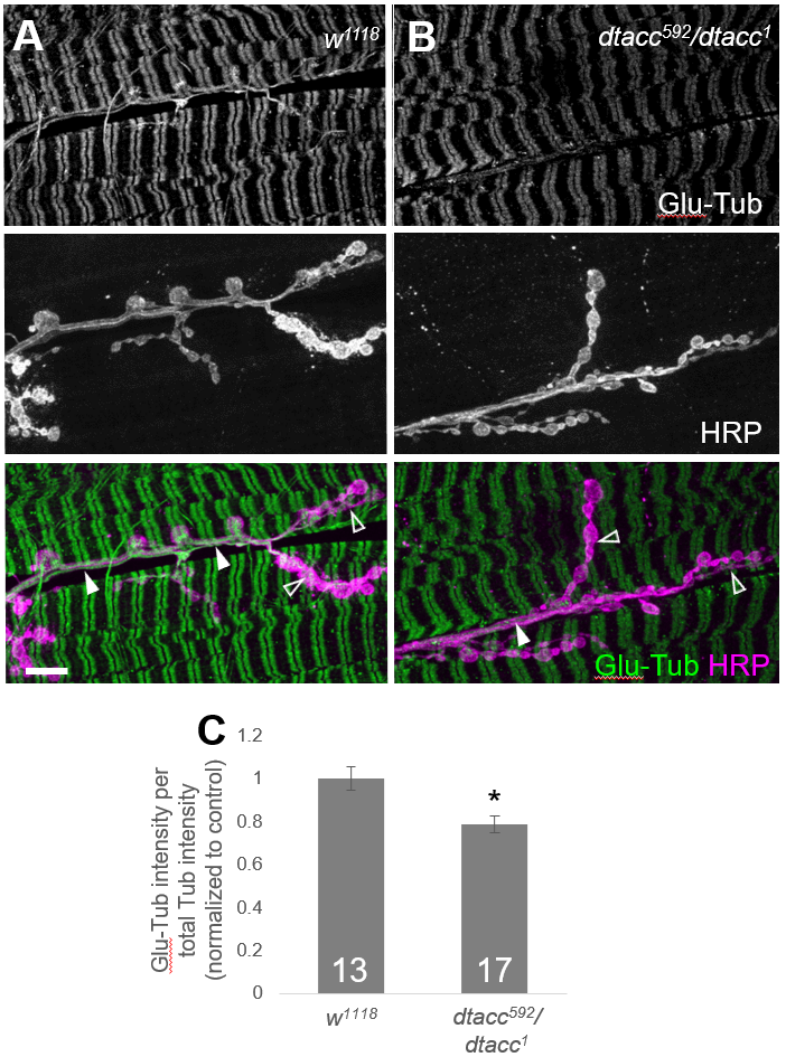

Figure supplement 5.5. *dtacc* animals show reduced polyglutamylation of tubulin. Control w^{1118} and $dtacc^{592}/dtacc^{1}$ NMJs were stained with α-Glu-Tub as well as with α-HRP and α-alpha tubulin to detect total tubulin. While controls showed clear Glu-Tub staining in both the main axon shaft (solid triangles) and in branches (hollow triangles; **A**), *dtacc* animals showed reduced/absent staining (**B**). Quantification (**C** confirmed a statistically significant reduction in *dtacc* animals of Glu-Tub staining per total tubulin. * $P < 0.05$, determined by Student's t-test; error bars indicate ± s.e.m; number of NMJs quantified indicated on graph; scale bar, 5 μm.

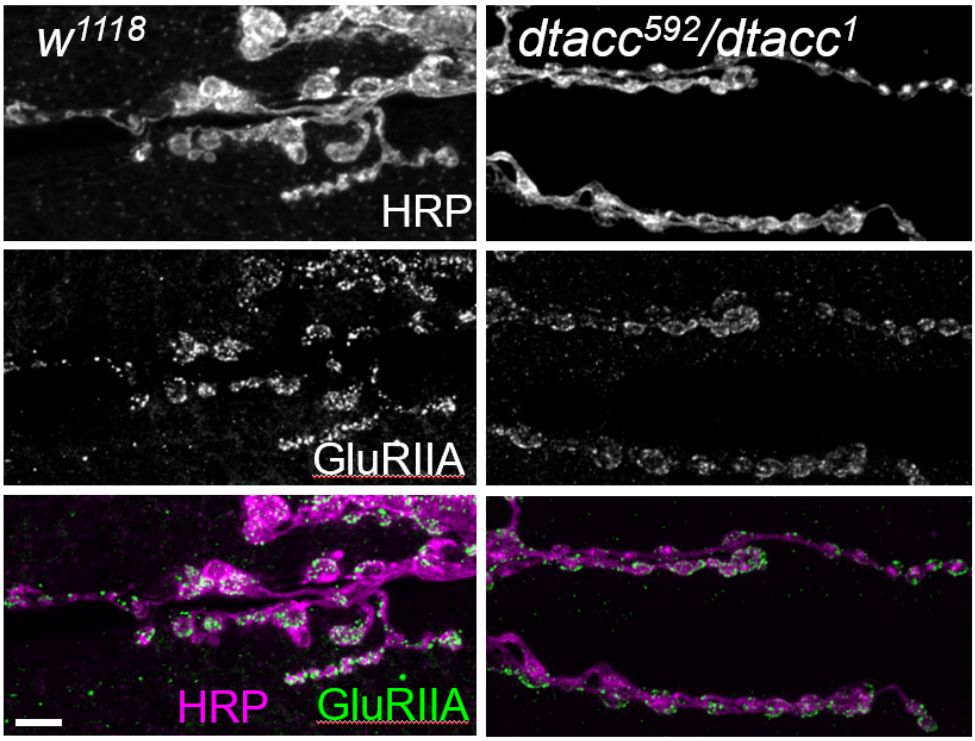

Figure supplement 5.6. *dtacc* animals show reduced GluRIIA staining. Control w^{1118} and $dtacc^{592}/dtacc^{1}$ NMJs were stained with α-GluRIIA, one of two variant subunits in fly GluR complexes, as well as with α-HRP. Compared to controls (left), *dtacc* animals showed qualitatively reduced GluRIIA levels. Scale bar, 5 μm.

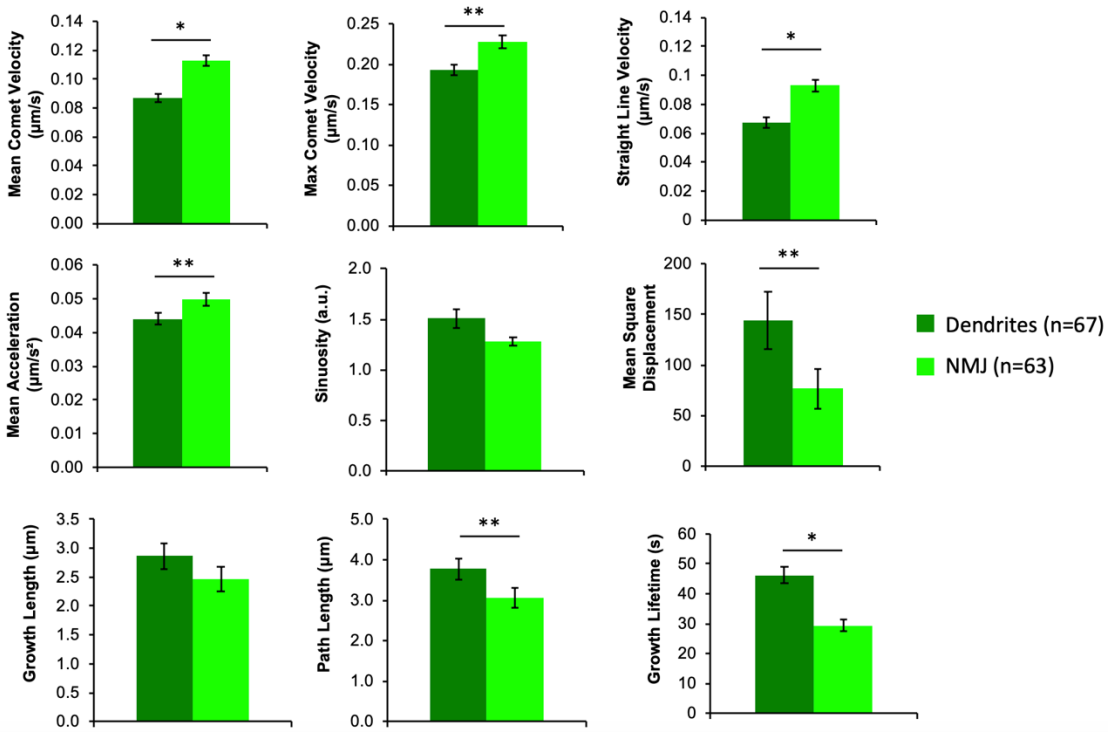

Figure supplement 5.7. Comparison of EB1 dynamics in dendrites and at the NMJs of wild-type animals. EB1-GFP dynamics in dendrites (dark green) and at the NMJ (light green) were compared in control animals of the genotype *elaV-Gal4;UAS-EB1-GFP; UAS-Dcr2 x* w^{1118}. Significant differences in multiple parameters were observed in the two different structures/cell types. * $P < 0.05$, ** $P < 0.005$, Wilcoxon-Mann-Whitney-test; error bars indicate ± s.e.m; number of NMJs quantified indicated on graph.