Spring 2018

Tao-1 and its function at the Drosophila melanogaster neuromuscular junction

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May 2018

John Carroll University

Honors Program Thesis

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ABSTRACT

Tao-1 is a gene that controls the growth of mitotic tissues in *Drosophila melanogaster* through the Hippo signaling pathway. We have found that *Tao-1* also functions independently of this pathway, in regulating the growth of synapses and formation of boutons at Drosophila neuromuscular junctions. We hypothesized that Tao-1 could be exerting its effects at the neuromuscular junction by working through a signaling pathway involving Par-1 and the microtubule associated protein, tau, to affect the microtubule cytoskeleton, which is essential for proper synaptic growth. We overexpressed or knocked down (by RNAi) the expression of each of these genes presynaptically in order to study the resultant effect on bouton number. Loss of *Tao-1*, not its overexpression, affected neuromuscular junction growth by significantly increasing bouton number. Overexpression of *par-1* and RNAi knockdown of *tau* also led to a significant increase in bouton number. Overall, this data suggests that Tao-1 could be working with Par-1 and tau to control microtubule stability and bouton formation.

INTRODUCTION

All multicellular organisms arise from a single-celled zygote. Eventually, this single cell becomes a ball of cells that differentiate into three discrete layers: the endoderm, the mesoderm, and the ectoderm, which gives rise to the nervous system (Gilbert and Barresi, 2016). Some of the cells coming from this nervous tissue become neurons, which eventually have protrusions (axons and dendrites) extending from the central cell body. These protrusions gradually grow from the

cell body of the neuron to their targets through cell-surface signaling. The growth cone at the front of the long axon grows towards attractive signals and away from repulsive ones (Dickson, 2002). When the axon has found its target, signals are sent between the neuron and target cell, and cell adhesion molecules make the connection, forming a synapse (Figure 1A; Darabid et al., 2014).

Synapses are intersections between a neuron and its target cell that transmit information between them via small chemical molecules called neurotransmitters. The presynaptic terminals cluster vesicles containing neurotransmitter at release sites. An action potential causes these vesicles to release their contents, which send the neurotransmitter across the synapse to the receptor sites on the postsynaptic partner cell in order to pass on information. Mammalian nervous system synapses use glutamate as the principal excitatory neurotransmitter; hence, the postsynaptic cells are full of glutamate receptors. Neuromuscular junctions (NMJs) are a type of synapse in which nerve meets muscle (Figure 1A, B). The NMJs of *Drosophila melanogaster* larvae are also glutamatergic, so they are analogous to synapses found in the mammalian brain. Drosophila NMJs are large, easy to study, and *Drosophila melanogaster* is a powerful genetic model organism, allowing the manipulation of gene expression. Furthermore, since Drosophila NMJs display developmental plasticity, which involves modifications to the synapse over developmental time while maintaining consistent patterns of connectivity between animals (making any abnormalities quantifiable), the *Drosophila* NMJ is a great model system for the study of glutamatergic synapses similar to the mammalian central nervous system (Figure 1B; Menon et al., 2013).

The NMJ, like all other synapses in the nervous system, relies on synaptic activity for proper function. The complex cellular processes and interactions that determine synapse formation and function are not well understood; however, the large degree of morphological variation among Drosophila species may provide some insight into the mechanisms regulating synaptic growth, the

relationship between the structure and function of a synapse, and even the evolution of nervous systems (Campbell and Ganetzky, 2013; Darabid et al., 2014).

Formation of the NMJ involves the outgrowth of an axonal growth cone, the long arm of a motor neuron, towards its target muscle that is to be innervated (Darabid et al., 2014). Precise nerve-muscle contact at this stage is regulated by many trans-synaptic signaling pathways, including Glass bottom boat, a Bone Morphogenetic Protein (BMP), which signals from the muscle to the motor neuron, and Wingless, a Wnt protein, which signals from the neuron to the muscle (Collins and DiAntonio, 2007). These points of contact between nerve and muscle develop into structures called synaptic boutons. Boutons contain many active zones, which release neurotransmitter and are located across the synapse from a cluster of glutamate receptors in the target muscle. Following NMJ maturation, a functional NMJ made up of a stereotypical number of boutons is present on each muscle fiber (Figure 1A). The number of boutons present at each synapse is indicative of synaptic stability and plasticity (Menon et al., 2013).

Boutons form by budding off of existing boutons at the synaptic terminal (Figure 1C). The proper growth of boutons is dependent on the stability of the microtubule (MT) cytoskeleton; thus, bouton number at the NMJ is linked to MTs. MTs are dynamic structures, characterized by repeated cycles of stabilization and destabilization, which is mediated by the binding of microtubule associated proteins (MAPs). End boutons have a MT loop in them, which is a mark of stable MTs (Nechipurenko and Broihier, 2012). Dynamic MTs are required for appropriate growth of the neuronal axon towards its target muscle as well as overall development of the NMJ (Goellner and Aberle, 2012). The relationship between MT stability and NMJ development is complex and can be thought of as a stop-and-go process. As the muscle grows during development, the synaptic terminal must keep up in order to function properly. As previously mentioned, boutons

form by budding off of already existing boutons and dynamic MTs are essential for the budding process. In order for this to occur, the MT loop inside of the existing bouton must be temporarily destabilized to permit budding of the new bouton (Figure 1C). The new bouton must then be stabilized in order to form a functional synapse, but then must be transiently destabilized to allow the budding of the next bouton. The newest bouton now needs to be stabilized until the budding of the next bouton and the process continues (Goellner and Aberle, 2012). Again, MT stability is mediated by MAPs, including Futsch and tau, which rapidly come on and off of the MTs (Janning et al., 2014). Therefore, MT stability is a transient process and is continually marked by rounds of stabilization followed by destabilization. Loss of Futsch at the NMJ leads to severely destabilized MTs and without any threshold stability, there is no NMJ development (Roos et al., 2000). So, severe destabilization of the MT cytoskeleton results in fewer boutons and smaller NMJs (Goellner and Aberle, 2012; Nechipurenko and Broihier, 2012). Tau also plays a role in NMJ development, but, unlike Futsch, it is not the master regulator of MTs (Barber et al., 2017). In summary, there cannot be too much stabilization or destabilization of the MTs, meaning there is an optimum level of stabilization. Dynamic MTs must be maintained in order to allow proper bouton formation and, therefore, appropriate synaptic growth and function.

Hence, mutations in MAPs, such as Futsch and tau, can lead to defects in NMJ development, which negatively affects the functioning of the nervous system (King and Herberlein, 2011). The activity of mammalian tau is highly regulated by the Par-1 kinase, a prominent cell polarity regulator also known as microtubule-affinity-regulating-kinase (MARK), which phosphorylates the MAP tau, deactivating tau (Timm et al., 2003). In turn, Par-1 is regulated by Tao-1 through phosphorylation. Tao-1, also known as microtubule-affinity-regulating-kinase-kinase (MARKK), is a member of the large Ste20 kinase family. The Ste20 family consists of

serine-threonine kinases that are active in various signaling pathways (Dan et al., 2001). In addition to phosphorylating Par-1, Tao-1 is a known member of the Hippo tumor suppressor pathway, which regulates growth of mitotic tissues (Boggiano et al., 2011, Halder and Johnson, 2011). In our lab, we have found that Tao-1 regulates NMJ development, independently of Hippo signaling. Tao-1 is important in regulating the MT cytoskeleton in cultured *Drosophila* cells, which may be more directly related to its role in NMJ development (Liu et al., 2010). Given that Tao-1 has been previously described in a signaling pathway involving Par-1 and tau in both mammals and flies (Timm et al., 2003, King et al., 2011), we wished to test whether the defects in NMJ formation we observed in loss of *Tao-1* function were mediated through a Par-1—tau—MT pathway.

RESULTS

Tao-1 negatively regulates bouton number at the NMJ

Since Tao-1 functions independently of Hippo signaling in the development of the Drosophila NMJ, the hypothesized Tao-1 pathway I tested included Par-1 and tau (Fig 1D). I examined the effects of each of these genes on NMJ development by driving either their overexpression or loss-of-function by RNAi, then quantified the number of boutons present at NMJ muscle 4 in segments A2-A4 of the larvae. We modified the gene expression levels by using the UAS-Gal4 system, which utilizes the yeast transcription factor Gal4 (Brand and Perrimon, 1993). Gal4 initiates transcription in a gene-specific manner after binding to an upstream activation sequence (UAS). Using transgenic lines that express the Gal4 protein only in certain tissues gave us the ability to control the expression of particular genes by mating the Gal4 flies to transgenic flies containing our genes of interest, such as *Tao-1*, which were designed to be under the transcriptional control of UAS. We expressed these genes in motor neurons only.

The number of boutons typically present at NMJ muscle 4 for a wild-type (WT) strain is between 18 and 21 boutons (James and Broihier, 2011; Viquez et al., 2006). As seen in Figure 2B, crossing a WT strain, w^{1118}, with the D42-Gal4 presynaptic driver, resulted in an average of 19.2 boutons. I used this as my control in each of my experiments.

According to my hypothesized pathway, when overexpressing *Tao-1*, there should be fewer boutons present than in WT because Tao-1 would be strongly inhibiting Par-1 (Figure 2A). When Par-1 is inhibited, tau is not phosphorylated so it remains on the MTs and overstabilizes them, leading to a decreased number of boutons at the NMJ. To test this hypothesis, I crossed two independent *Tao-1* overexpression lines, *Tao-1* line #8 and *Tao-1* line #61, with the D42-Gal4 motoneuron driver in order to drive overexpression of *Tao-1* presynaptically. Unexpectedly, both lines showed a WT number of boutons at NMJ4, as did a kinase-dead version of *Tao-1* (Figure 2B).

On the other hand, according to my hypothesized pathway *Tao-1* loss-of-function should result in more boutons at the NMJ because Par-1 would not be inhibited (Figure 2A). As a result, Par-1 would be phosphorylating and deactivating tau, causing tau to fall off of the MTs, thereby destabilizing them and allowing further bouton formation. This is what I observed when scoring the presynaptic knockdown of *Tao-1* in three independent RNAi lines: *Tao-1 RNAi HMS01126, Tao-1 RNAi GL00015*, and *Tao-1 RNAi x2* (Figure 2B, D). Each of these lines had a statistically significant increase in bouton number when compared to the WT w^{1118} strain (Figure 2B, C, D). This indicates that the Tao-1 protein is required presynaptically to restrict NMJ development.

The overexpression of *par-1* leads to more boutons at the NMJ

After concluding that Tao-1 negatively regulates bouton number, I moved on to the next candidate in my hypothesized pathway, Par-1. Interestingly, Par-1 has already been implicated in

vertebrate axon outgrowth and is thought to function with Tao-1 and tau in the context of MT stability during that process (Timm et al., 2003). I crossed *UAS-par-1* overexpression and *UAS-par-1 RNAi* lines to the D42-Gal4 motor neuron driver to observe Par-1's role in NMJ development. Based on my hypothesis, overexpression of *par-1* presynaptically should result in more boutons at the NMJ because it would be greatly phosphorylating and, therefore, deactivating tau (Figure 3A). Phosphorylated tau would fall off of the MTs, transiently destabilizing them, therefore allowing bouton formation (Figure 3A). Overexpression of *par-1* line 9.1 presynaptically led to 31 boutons per NMJ, which was a statistically significant increase in bouton number at the muscle 4 NMJ compared to WT (Figure 3B).

Similar to experiments with *Tao-1*, I overexpressed a kinase-dead version of *UAS-par-1* in order to study its kinase activity in the context of NMJ development. Interestingly, I saw no change in bouton number when compared to WT. This indicates that the kinase activity of Par-1 is required for the increase in bouton number observed in *par-1* line 9.1 overexpression (Figure 3B).

I predicted that loss of *par-1* function would result in fewer boutons at NMJ4, that is, show the opposite phenotype as did overexpression of *par-1*. However, knocking down *par-1* presynaptically with RNAi resulted in WT bouton numbers rather than seeing my hypothesized decrease in bouton number (Figure 3B).

Loss of tau results in more boutons at the NMJ

Next, I tested my hypothesized signaling pathway on tau. Because *tau* null mutations result in embryonic lethality (Barber et al., 2017), I could only test partial *tau* knockdown via RNAi, which, according to my hypothesis, should lead to more boutons present at the NMJ as tau would fall off and temporarily destabilize the MTs, allowing further bouton formation (Figure 4A). I crossed four independent *tau RNAi* lines to the D42-Gal4 driver and examined their NMJs. All of

them had an increase in bouton number, although only three, including *tau RNAi VDRC lines 25023* and *25024* and *BL line 28891*, were statistically significant when compared to WT (Figure 4B). Taken together, my results suggest that my proposed signaling pathway could be functioning downstream of Tao-1.

DISCUSSION

Although our lab has discovered that the Hippo pathway was not functioning in NMJ development, we were able to conclude that one of the pathway's members, *Tao-1*, was. We were unsure of what proteins Tao-1 was functioning with at the NMJ, so I tested a candidate pathway involving Tao-1, Par-1, and tau, all predicted to affect the MT cytoskeleton. A pathway involving Tao-1, Par-1, and tau has been previously implicated in the development of the *Drosophila* central nervous system during axon outgrowth (King et al., 2011); however, I am interested in the development of the peripheral nervous system. After studying the effects of each of these genes on bouton number at the NMJ, I can only conclude that Tao-1 could be working with Par-1 and tau via MTs to direct NMJ development and bouton formation, but my results are not definitive.

Based on my data and data from others in the lab, we can conclude that *Tao-1* restricts NMJ growth. *Tao-1* has already been implicated in *Drosophila* neural development functioning with *par-1* and *tau* in the central nervous system, but not in the context of MT stability. That work indicated that Tao-1 phosphorylates and inhibits Par-1, leading to increased tau activity in the adult fly CNS (King et al., 2011). This is similar to my results in the PNS using larvae. I tested two *Tao-1* overexpression lines and a *Tao-1* kinase-dead line, which all appeared wild-type for bouton number, although I predicted that overexpression of *Tao-1* would lead to fewer boutons. Because

having a lot of Tao-1 around led to a WT phenotype, this means that Tao-1 is not a rate-limiting step in the pathway.

However, when I reduced presynaptic *Tao-1* function using three independent RNAi lines, each led to a significant increase in bouton number, suggesting that taking *Tao-1* away had a major effect on NMJ development. I hypothesized that this could be because of decreased MT stability. According to my model, loss of Tao-1 leads to increased Par-1 activity, which inhibits tau and moderately destabilizes MTs (Figure 1D). Moderately destabilized MTs result in inappropriate growth and visibly dense, disorganized NMJs due to an increased number of boutons, consistent with what I observed (Nechipurenko and Broihier, 2012). These findings suggest that Tao-1 might be needed for proper MT dynamics in order to control NMJ growth.

The Par-1 kinase has already been found to function in NMJ development on the postsynaptic (muscle) side of the NMJ (Zhang et al., 2007). However, I am studying its effects on MT dynamics in presynaptic (neuronal) tissue. In mammals, Tao-1 activates Par-1, which is known to deactivate tau via phosphorylation. This destabilizes the MT cytoskeleton and increases MT dynamics for neurite outgrowth (Timm et al., 2003; Timm et al., 2006). However, in flies, Par-1 is deactivated by Tao-1 via phosphorylation and, as a result, phosphorylated tau levels are lower (King et al., 2011). I overexpressed wild-type *par-1*, which lead to a significant increase in bouton number. I also overexpressed a kinase-dead version of *par-1* that resulted in a WT phenotype. This suggests that, unlike Tao-1, the amount of Par-1 in the (presynaptic) neuron is important and that Par-1's kinase activity is required for its effect, as loss of its kinase activity resulted in normal NMJs. So, it is possible that Par-1 is phosphorylating a MAP, maybe tau, which destabilizes MTs and leads to improper NMJ growth and bouton formation.

To test the necessity of presynaptic Par-1, I performed *par-1 RNAi* and observed a WT number of boutons, which was inconsistent with my hypothesis. However, the line that I used is known to be a "weak" RNAi line, so it is possible that the amount of Par-1 protein was not actually reduced much, or at all. Quantification of Par-1 levels from RNAi-treated and control animals would be necessary to confirm this. I tried another *par-1 RNAi* line, but it was lethal prior to our assay age. Because overexpression of *par-1* had a significant effect at the NMJ, but loss of *par-1* function did not lead to the opposite phenotype, *par-1*'s role remains unclear. It is possible that Par-1 does not have a presynaptic function as we had thought. In order to draw a conclusion about Par-1 function, it is necessary to do further experiments, including using *par-1* transheterozygous genotypes, whose larvae do live long enough that this phenotype can be scored. I have rebalanced two *par-1* mutant alleles, so that these experiments can be performed.

The final test of my hypothetical Tao-1 signaling pathway involved tau, a MAP that when bound to MTs, stabilizes them and thus restricts NMJ growth. Evidence suggests that Tao-1 can regulate Par-1 and tau in the development of the Drosophila central nervous system because when examining the coexpression of *Tao-1 RNAi* and *par-1* overexpression, there was an increase in the phosphorylation of tau by Par-1 compared to *par-1* overexpression alone (King et al., 2011). I performed RNAi against *tau* with four independent lines, which all led to an increase in bouton number as I had hypothesized. Although these results are consistent with my pathway, we are unconvinced of its biological significance. Despite the fact that three of the *tau RNAi* lines lead to a statistically significant increase in bouton number, the increase was subtle. The average number of boutons per NMJ for each line was under 25 boutons, which is much lower than the increase we observed with *Tao-1 RNAi* and *par-1* overexpression. The *tau* data would be more convincing if it led to a larger increase in bouton number. However, RNAi frequently results in only a partial

loss-of-function phenotype, and since *tau* null mutants die as embryos, that suggests these RNAi lines may only reduce *tau* mRNA levels partially. If that is the case, then perhaps my *tau RNAi* data are biologically relevant after all.

These data suggest that tau may be playing a more minor role in a pathway affecting MT stability and bouton formation in NMJ development. As previously mentioned, dynamic MTs play a critical role in proper NMJ development, but it is a complicated relationship. MT stability is mediated through the binding of MAPs, which are constantly coming on and off of the MTs (Janning et al., 2014). Evidence suggests that Futsch may be the primary MAP involved in regulating MT stability in neurons because loss of *futsch* results in severely destabilized MTs, which leads to fewer boutons and smaller NMJs (Roos et al., 2000; Nechipurenko and Broihier, 2012). Tau also affects MT stability and NMJ development, but it may not be as critical to the process as Futsch (Barber et al., 2017). Loss of *tau* is similar to taking away the on/off switch of NMJ growth because, without tau, the MTs are mildly destabilized (not as severely as they are without Futsch). This permits inappropriate growth of the NMJ and results in more boutons and a disorganized NMJ. On the other hand, if tau is unable to fall off of the MTs, the MTs are too stable, which inhibits bouton formation as transient destabilization of MTs is required for the budding of new boutons (Goellner and Aberle, 2012; Nechipurenko and Broihier, 2012). Therefore, MTs must be dynamic enough in order to allow transient rounds of stabilization and destabilization. So, it is possible that tau may be playing a role in this pathway, but it is probably not the only protein affecting bouton formation downstream of Tao-1 and Par-1.

Future directions include staining for Futsch as a marker of MT stability in all of my experimental conditions that changed bouton number (presynaptic *Tao-1 RNAi*, *par-1* overexpression, and *tau RNAi*). In addition, I will also look at the amount of phosphorylated tau

from larvae of these genotypes. This can be done by performing immunoblots from intact tissue and observing phosphorylated tau levels vs. total tau. Finally, I would like to generate flies that express *Tao-1 RNAi* and lack *par-1* function in the same animal, to see if Tao-1 requires Par-1 to exert its effect on NMJ development. Showing a dependence on Par-1 would be strong evidence for my hypothetical pathway model for *Tao-1* function and would be the first indication that *par-1* plays both a presynaptic and postsynaptic role during NMJ development.

EXPERIMENTAL PROCEDURES

Crosses

Adult male flies of varying genotypes and female virgin *UAS-dcr2; D42-Gal4* flies were crossed at 25ºC in individual vials to create desired genotypes, such as overexpression or loss-of-function by RNAi of a particular gene. *D42-Gal4* expresses the Gal4 transcription factor (which binds to UAS sequences) in motor neurons only, and *UAS-dcr2* enhances the RNAi effect by overexpressing the rate-limiting enzyme in the RNAi process.

Larvae Dissection and Labeling by Immunofluorescence

Third instar wandering larvae were dissected, or filleted, and then direct immunofluorescence was performed using a rabbit anti-horseradish peroxidase (HRP) primary antibody conjugated to a Cy3 fluorophore at a 1:500 dilution (Jackson Immunoresearch). When an additional mouse anti-Dlg primary antibody was used at a 1:500 dilution (DSHB), an anti-mouse secondary antibody conjugated to a 488nm fluorophore was utilized at a 1:1000 dilution (Jackson Immunoresearch). All dissected and stained larvae were mounted in ProLong Anti-Fade media (Invitrogen).

Bouton Scoring

Boutons of NMJ 4 in fillet segments A2-A4 were counted using an Olympus BX60 epifluorescence microscope.

Imaging

Fillet dissections were imaged using a Leica TCS SP8 scanning confocal microscope. Images were then compiled and analyzed using ImageJ software.

ACKNOWLEDGEMENTS

I wish to thank the Colleran-Weaver fund for supporting this research, as well as Jeff Johansen for the use of his epifluorescence microscope. Also, thanks to Stephen Politano, Ryan Salemme, and Toren Bakula for sharing *Tao-1* and w^{1118} data. Finally, I wish to thank the John Carroll University Honors Program for encouraging the completion of this manuscript.

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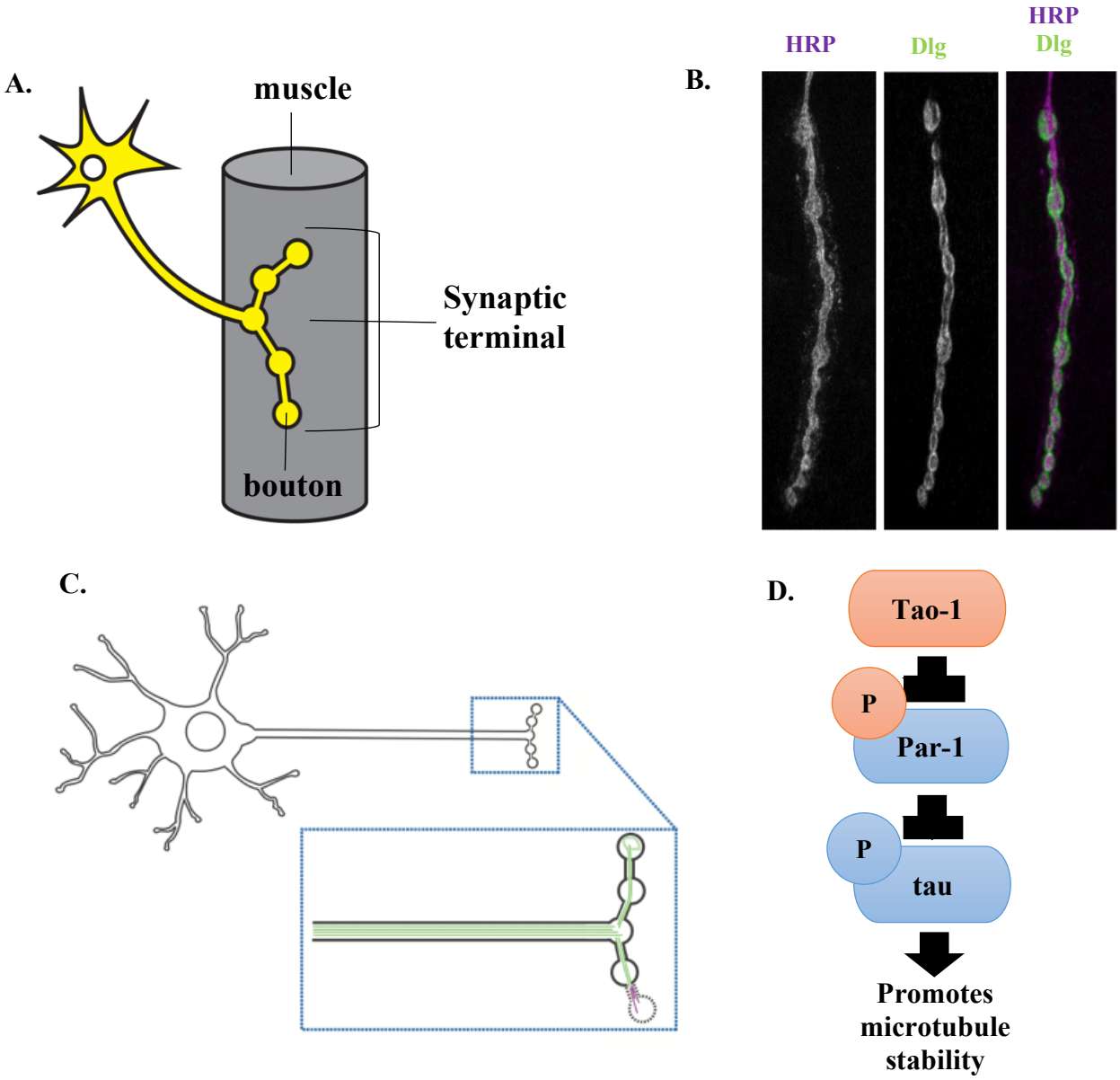
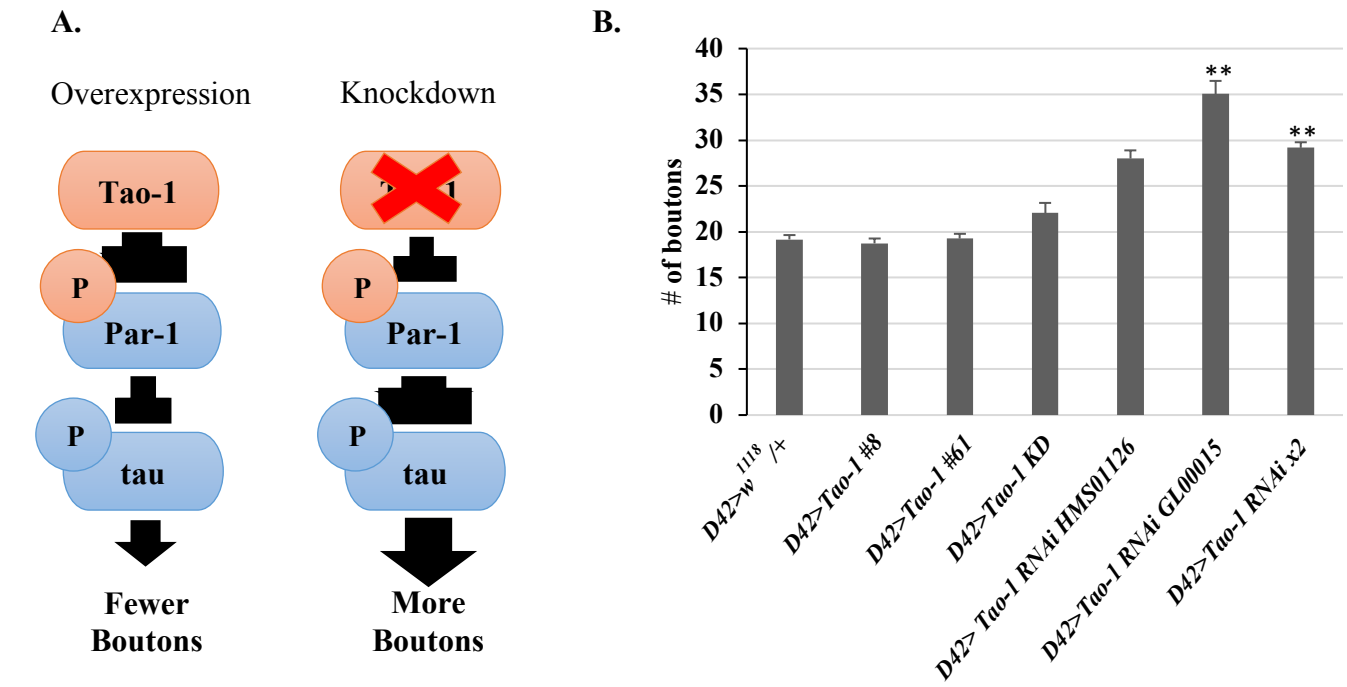


Figure 1. Schematic of NMJ and Tao-1's predicted pathway

A) Schematic of the neuromuscular junction: The axon extending from the cell body of the motor neuron reaches its target muscle cell and forms the synaptic terminal, establishing precise neuron-muscle contact. The synaptic terminal is made up of rounded structures called boutons, which house multiple active zones for the release of the neurotransmitter glutamate. Clusters of glutamate receptors are located on the muscle cell opposing the active zones in the neurons. The synapse is the gap between them. **B)** *D42>w*^{1118}/+ 3rd instar larvae muscle 4 NMJs stained with anti-HRP (marks the presynaptic neuron membrane), anti-Dlg (marks the subsynaptic reticulum of the postsynaptic muscle), and merge of HRP (purple) and Dlg (green). Each rounded structure highlighted in both purple and green represents one bouton, and the total number of boutons at NMJ4 was the phenotype I quantified. **C)** Microtubule stability and bouton formation: Purple microtubules are destabilized, allowing for bouton formation, which is signified by the dotted lines. The green microtubules are stable, which are characterized by the loops inside of the bouton, and no NMJ growth is occurring. **D)** Tao-1's hypothesized signaling pathway: Tao-1 phosphorylates and inhibits Par-1, preventing the phosphorylation and deactivation of the MAP tau. This means that tau remains on the microtubules and stabilizes them.



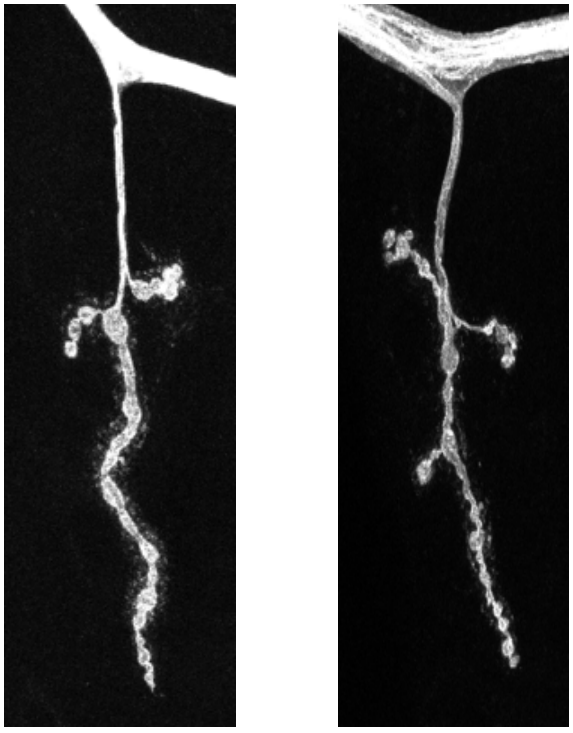


Figure 2. *Tao-1* negatively regulates bouton number at the NMJ

A) Predictions of total bouton number at muscle 4 NMJ for both *Tao-1* overexpression and knockdown by RNAi based on my model. *Tao-1* overexpression is hypothesized to lead to fewer boutons and *Tao-1* knockdown is hypothesized to lead to more boutons. **B)** Presynaptic overexpression and knockdown of *Tao-1*. Quantification of the total number of boutons present at muscle 4 NMJ synapses in segments A2-A4 in the larvae of various genotypes crossed with the D42-Gal4 presynaptic driver. All *Tao-1* overexpression had no effect on bouton number while all *Tao-1 RNAi* lines led to a statistically significant increase in bouton number. n=73, 91, 49, 18, 40, 23, 104 NMJs scored, respectively. Data were analyzed by a two-tailed *t*-test: ** = $p<0.0001$. Error bars represent the standard error of the mean (SEM). **C-D)** Representative confocal images of 3rd instar larval NMJs at muscle 4 for **C)** wild-type versus **D)** presynaptic *Tao-1* loss-of-function.

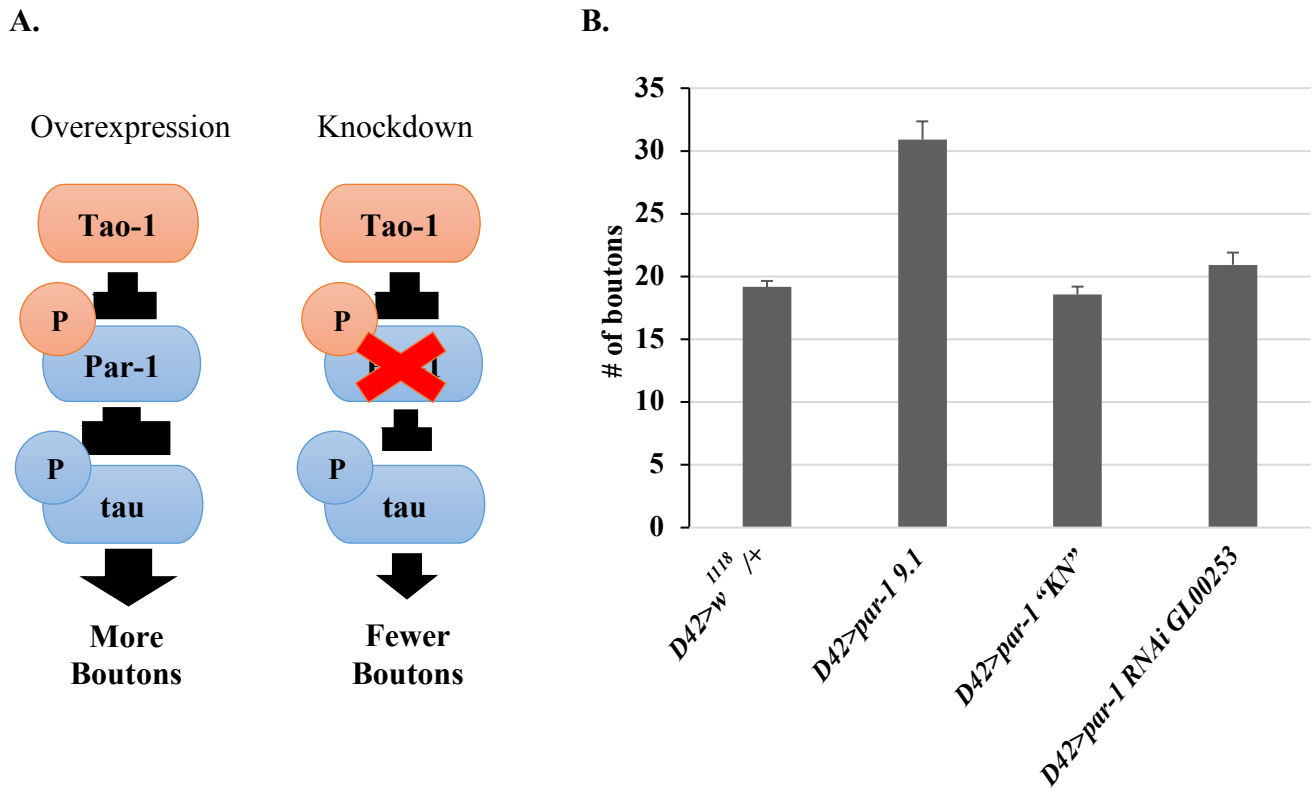


Figure 3. There are more boutons when *par-1* is overexpressed

A) Predictions of total bouton number at muscle 4 NMJ for both *par-1* overexpression and knockdown by RNAi, based on my model. *par-1* overexpression is hypothesized to lead to more boutons and *par-1* knockdown is hypothesized to lead to fewer boutons. **B)** Presynaptic overexpression/knockdown of *par-1*. Quantification of the total number of boutons present at muscle 4 NMJs in segments A2-A4 in the larvae of various genotypes crossed with the D42-Gal4 presynaptic driver. Overexpression of wild-type *par-1*, but not kinase-dead *par-1*, led to a statistically significant increase in bouton number. n=73, 35, 19, 29 NMJs scored, respectively. Data were analyzed by a two-tailed *t*-test: ** = $p<0.0001$. Error bars represent the SEM.

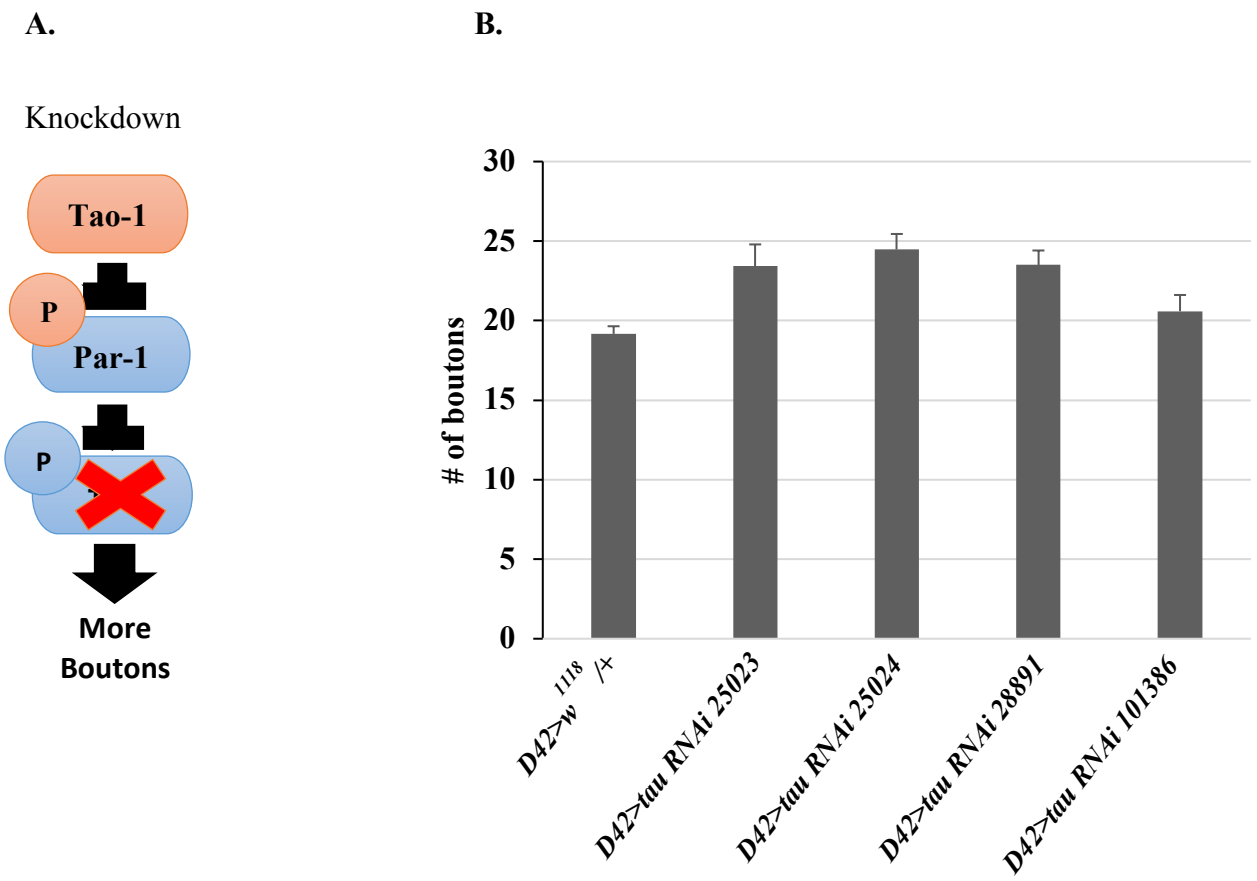


Figure 4. Loss of *tau* results in more boutons

A) Predictions of total bouton number at muscle 4 NMJ for knockdown of *tau* only, based on my model. The predicted increased in boutons is because loss of *tau* destabilizes the microtubules, which should result in more boutons. **B)** Presynaptic knockdown of *tau*. Quantification of the total number of boutons present at muscle 4 NMJs in segments A2-A4 in the larvae of various genotypes crossed with the same D42-Gal4 presynaptic driver used previously. Three out of four *tau RNAi* lines caused a statistically significant increase in the number of boutons at NMJ4. n=73, 14, 19, 24, 39 NMJs scored, respectively. Data were analyzed by a two-tailed *t*-test: * = $p<0.001$, ** = $p<0.0001$. Error bars represent the SEM.