RETROGRADE TGF-β SIGNALING AND ITS EFFECTS ON WD40 AT THE
DROSOPHILA NEUROMUSCULAR JUNCTION

BY

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Abstract

TGF-β signaling is particularly well studied for its role in early embryonic patterning and cell proliferation specific to cancer progression. At the larval neuromuscular junction (NMJ) of the model organism *Drosophila melanogaster*, several ligands from the TGF-β superfamily have been shown to promote synaptic growth and evoked neurotransmitter release. We focused on the retrograde bone morphogenic protein (BMP) signaling pathway and its potential role for the presynaptic expression of WD40, a potential cofactor of the E3 ubiquitin ligase Cullin 4 (Cul4) that is required for evoked neurotransmitter release. The goal of this project was to investigate whether this TGF-β signaling pathway regulates the expression of WD40. In conclusion, our data shows evidence that WD40 might a critical effector of retrograde BMP signaling at *Drosophila* NMJs.
**Introduction**

The *Drosophila* neuromuscular junction (NMJ) is a well-studied chemical synapse. The NMJ is a specialized synapse that mediates information transfer from a motor neuron to the muscle fiber it innervates. Synapses are specialized structures that allow rapid inter-cellular communication among neurons and between neurons and other cells, such as a muscle fiber in this case. As aforementioned, the best-studied synapse in *Drosophila melanogaster* is the larval NMJ in larval body walls muscles (Keshishian et al., 1996; Koh et al., 2000). This glutamatergic synapse is exhibits a critical form of synaptic plasticity termed synaptic homeostasis, which requires TGF-β signaling (Goold et al., 2007). Synaptic homeostasis ensures proper excitability of the muscle fiber by upregulating evoked neurotransmitter through a retrograde signal that is initiated by an impairment of postsynaptic glutamate receptors (Davis et al., 2013).

Transforming growth factor beta (TGF-β) signaling particularly well studied for its role in early embryonic patterning and cell proliferation including during cancer progression (Cohen, 2003; Derynck and Zhang, 2003; ten Dijke and Hill, 2004). TGF-β signaling is also critical for synaptic development and function in invertebrates and vertebrates (Poon et al., 2013; Sun et al., 2010). At the fly larval NMJ, several ligands of the TGF-β superfamily promote synaptic growth and/or neurotransmitter release (Poon et al., 2013). TGF-β ligands are broadly categorized into three subfamilies: the activin-Nodal group, the subfamily group and the bone morphogenetic protein (BMP) group, which typically act in separate pathways (Shi and Massague, 2003). Generally, TGF-β signaling pathways are made up of three components: 1) a secreted ligand, 2) a
receptor complex, consisting of two different receptors, each with serine/threonine kinase domains (the type I and II receptors), and 3) intracellular transducers, known as Smads. The pathway is activated by a ligand binding to a type II receptor, which activates its kinase activity allowing phosphorylation of the associated type I receptor in its guanine-serine rich region. The type I receptor then phosphorylates a receptor-regulated Smad (R-Smad) that binds to a common-mediator Smad (co-Smad) and/or inhibitory Smad (I-SMAD). SMADs form a trimer of two R-SMADs and one co-SMAD for translocation into the nucleus where they act as transcription factors regulating gene expression of their target genes (Derynck and Zhang, 2003).

At the Drosophila NMJ, two major TGF-β family members have been shown to act as positive regulators of synaptic growth (Poon et al., 2013). The canonical BMP ligand, Glass Bottom Boat (Gbb), acts in a retrograde manner to promote synaptic growth. Gbb is secreted from muscles, after which it binds to the presynaptic type II receptor wishful thinking (Wit) and the type I receptors thickveins (Tkv) and Saxophone (Sax) (Figure 1). Wit is the Drosophila ortholog of human BMPRII receptor and is necessary in motor neurons for proper synaptic growth and neurotransmitter release (Aberle et al., 2002; Marqués et al., 2002). Gbb is the critical ligand for Wit at the fly NMJ as shown by analysis of loss-of-function mutants and biochemical experiments (McCabe et al., 2003). Wit activates type I receptors Tkv and Sax to promote synapse growth at NMJ (Aberle et al., 2002; Marques et al., 2002; McCabe et al., 2003: Rawson et al., 2003). Gbb binding to Wit/Tkv/Sax ultimately results in phosphorylation of the R-
Smad mothers against decapentaplegic (Mad), which then associates with the co-
SMAD Medea to translocate to the nucleus and alter transcription.

The activin pathway constitutes a second TGF-β signaling pathway at the fly
NMJ. The activin ligand Dawdle (Daw) acts through the postsynaptic activin type I
receptor Baboon (Babo) and the Smad2 transcription factor to promote postsynaptic
growth/differentiation at the NMJ (Ellis et al., 2010). In addition, Daw and Babo regulate
presynaptic differentiation by regulating Gbb expression (Ellis et al., 2010). A third TGF-
β signaling pathway is defined by the TGF-β ligand Maverick (Mav), which is secreted
from glial cells (Figure 2) and binds to the post-synaptic receptor Punt, which increases
Gbb signaling (Fuentes-Medel et al., 2012). Together, the BMP homolog Gbb and the
activin ligand Daw are strong activators of synapse growth at the NMJ. The Tkv
receptor and Mad transcription factor are also present in the muscle and may affect
postsynaptic development and function (Dudu et al., 2006).
**Figure 1:** TGF-β signaling at the fly NMJ. Glia-secreted Mav stimulates Mad phosphorylation in the muscle. Phosphorylated Mad induces expression of Glass bottom boat (gbb) which when bound to a TGF-β receptor wishful thinking (Wit), starts the retrograde signaling pathway in the neuron. (Kim et al., 2014)

**Figure 2:** The activin TGF-β pathway. The binding of Maverick released from the glia, binds to the activin receptor Punt. The binding of Maverick to Punt induces expression of Glass bottom boat (Gbb) through Mad (Poon et al., 2013)
Here, we focused on the BMP signal transducers Mad (R-Smad) and Medea (co-Smad). Our hypothesis has been that WD40 gene expression is a potential target of the canonical retrograde BMP signal. The goal of this project has been to test this hypothesis. Biochemical \textit{in vitro} data predicted that dWDR40A may associate with the E3 ubiquitin ligase cullin4 (CUL4)/DNA damage-binding protein (DDB1) complex as a cofactor (Lee and Zhou, 2007). However, the true role of WD40 is currently unknown.

Our unpublished research has shown that WD40 is found in motor neurons, both in the nucleus and cytoplasm. WD40 mutants exhibit a severe loss of neurotransmitter release at larval NMJs without any significant structural defect. Further analysis of WD40 mutants also showed that WD40 directly or indirectly controls TGF-β signaling since loss of functions mutant synaptic boutons exhibit a significant increase in phosphorylated Mad (p-Mad) levels at the NMJ, which indicates a hyper-activation of TGF-β signaling.

\textbf{Methods}

\textbf{Animal Model and Dissections}

The model organism used was \textit{Drosophila melanogaster}. Manipulating Mad and Medea in a compartment-specific manner required knocking down these components using RNAi. The RNAi transgenes were transcriptionally controlled by Gal4 (UAS-Gal4 system, (Brand and Perrimon, 1993). The C57-Gal4 driver expresses Gal4 specifically in the muscle, while D42-Gal4 driver expresses in motor motor and a few interneurons.
With two Gal4 drivers (C57 and D42) and two UAS-RNAi lines (Mad-RNAi and Med-RNAi), a total of four experimental heterozygous genotypes were created by appropriate genetic crosses:

\[
\begin{align*}
\text{C57-MadRNAi: +; +; C57Gal4/Tm6TbSb} & \times +; \text{UAS-MadRNAi}; + \\
\text{C57-MedRNAi: +; +; C57Gal4/Tm6TbSb} & \times +; \text{UAS-MedRNAi}; + \\
\text{D42/MadRNAi: +; +; D42Gal4/Tm6TbSb} & \times +; \text{UAS-MadRNAi}; + \\
\text{D42/MedRNAi: +; +; D42Gal4/Tm6TbSb} & \times +; \text{UAS-MedRNAi}; +
\end{align*}
\]

The first two genotypes knocked down either Mad or Medea in the muscle while the latter two knocked down either Mad or Medea in the neuron. All flies were raised at 23°C.

*Drosophila melanogaster* wandering 3rd instar larvae were selected for proper genotypes and dissected in a fillet preparation using a HL-3.2 solution (modified after Stewart 1994). Larvae were placed on Sylgard dishes equipped with metal pins. An experimental larva along with either one or two control larvae were dissected in each dish. The dissection consisted of first making an incision in the posterior end of the larva. The second incision is then made down the dorsal side, and the intestines, along with non-neural tissue were removed. Removing the organs reveals the larval central nervous system, allowing an unobstructed view of the neuromuscular junction. The pins were then used to fillet the larva.

**Immunostainings**

The dissected larval preparation was fixed with 4% paraformaldehyde solution (PFA, pH = 7.4) for 20 minutes at room temperature. The preparation was then washed
three times with 0.4% PBS-T (100 mM phosphate buffer solution supplemented with 20% Triton, pH = 7.4) for ten minutes. Lastly, primary antibodies were added to the dish; the primary antibodies used were 1:10,000 dilution of guinea pig anti-WD40 and 1:250 dilution of Cy3-conjugated goat anti-HRP in 1mL of 0.4% PBS-T. The preparations were incubated in a dark 4°C cold room overnight.

After overnight incubation, the preparations were washed with 0.4% PBS-T three times in 10 minute increments. This was followed by a one hour incubation with secondary antibody (AF488-conjugated anti-guinea pig) in a 1:500 dilution at room temperature. Next, the preparations went through a third set of three ten-minute PBS-T washes. Finally, the preparations were washed in PBS (100 mM phosphate buffered saline, pH 7.3) for ten minutes. The washes were performed twice and afterwards were set aside for imaging.

**Neuromuscular Junction Imaging**

The fixed larvae were imaged using an Olympus FV300 laser confocal microscope. The lasers used were Argon488 and HeNe543. The Argon488 laser was used to visualize the AlexaFluor488 (which was used to view WD40 expression) and the HeNe543 laser was used to visualize Cy3. The dissected larvae were placed under an Olympus LUMPanF1 60x water lens. The neuromuscular junction (NMJ) imaged was located on the third abdominal segment of the larvae between muscles 6 and 7.

**Analysis of Boutons and Distal Axons**
Analysis of WD40 expression was done using ImageJ software. The fluorescence of WD40 in boutons and distal axons was analyzed for each control and experimental genotype. This was done by first circling the region of interest, either the distal axon or the bouton, and then normalizing the intensity of the fluorescence by subtracting the background signal. The background signal was determined by selecting a region surrounding the axon or synapse that was the most representative background fluorescence.

**Results**

*Postsynaptic knock-down of Mad reduces synaptic but not axonal WD40 levels.*

The postsynaptic C57-driven Mad-RNAi knockdown in muscles had the most consistent results, showing a clear trend towards a reduction of WD40 protein levels in both distal axons and boutons. There was a significant difference in WD40 levels at synaptic boutons between the C57 heterozygous control and the C57-driven MadRNAi knockdown (Figure 3; \( p = 0.0011 \)). However, there was no significance between the “silent” Mad-RNAi control and the C57-driven Mad0RNAi knockdown (\( p = 0.0594 \)).

The two controls (C57/+ and MadRNAi/+) showed a significant difference in HRP immunostaining (\( p<0.0001 \), not shown). This could be indicative of leaky expression and could therefore make it difficult to achieve significance between the undriven MadRNAi and the knockdown. There was no significance between the mutant and the control neuromuscular junctions in HRP or WD40 fluorescence in the distal axons. However, in the boutons, there was a visible increase in HRP fluorescence in the C57 heterozygous controls.
Figure 3: Expression of WD40 (top row), HRP (middle row) and an overlay of both HRP and WD40 expression (bottom row) in the NMJ at the 3rd abdominal segment between muscle 6/7. These animals were treated with the experimental protocol as listed above. The C57/+ and MadRNAi/+ animals showed more fluorescence in WD40 levels in the synapse but not the axons. HRP expression showed differences in only synaptic fluorescence as well.
Graph 1: Expression of WD40 in distal axons (left) and boutons (right). There was no indicated significance in the WD40 axonal fluorescence. The WD40 synaptic fluorescence showed significance between the C57/+ and C57/MadRNAi animals (p=0.0011).

Graph 2: Expression of HRP in distal axons (left) and boutons (right). There was no indicated significance in the HRP axonal fluorescence. However, HRP synaptic fluorescence showed significance between the C57/+ and C57/MadRNAi animals (p < 0.0001).

**Postsynaptic knock-down of Medea reduces synaptic but not axonal WD40 levels.**

The C57 mediated Medea knockdown caused no significant differences between any of the phenotypes in either HRP and WD40 fluorescence in distal axons (Graph 3
and 4). However, it can be clearly seen that the WD40 and HRP levels in the C57/MedRNAi mutants are decreased as shown in Figure 4. Yet, there is no significance between the driver alone and the knockdown. With WD40 expression, the only significance seen in the boutons was seen between the MedRNAi/+ and C57/MedRNAi mutants (Graph 3, p= 0.0079. There was no significance between the driver alone and the mutant. With HRP expression, there was a significant difference between the two controls C57/+ and MedRNAi/+ with a p-value of 0.0002, and between MedRNAi/+ and C57/MedRNAi with a p-value of 0.0186 (Graph 4).

![Figure 4: Expression of WD40 (top row), HRP (middle row) and an overlay of both HRP and WD40 expression (bottom row) in the NMJ at the 3rd abdominal segment between muscle 6/7. These animals were treated with the experimental protocol as listed above. The C57/+ and MedRNAi/+ animals showed more fluorescence in WD40 levels in the synapse but not the axons. HRP expression showed differences in only synaptic fluorescence as well.](image)
**Graph 3:** Expression of WD40 in distal axons (left) and boutons (right). There was no indicated significance in the WD40 axonal fluorescence. The WD40 synaptic fluorescence showed significance between the MedRNAi/+ and C57/MedRNAi animals (p=0.0079).

**Graph 4:** Expression of HRP in distal axons (left) and boutons (right). There was no indicated significance in the HRP axonal fluorescence. The HRP synaptic fluorescence showed significance between the MedRNAi/+ and C57/MedRNAi animals (p=0.00186) and between the C57/+ and MedRNAi/+ genotypes as well (p=0.0002).
**Presynaptic knock-down of Mad reduces synaptic but not axonal WD40 levels.**

The D42-driven mediated Mad-RNAi knockdown in the presynaptic motor neuron showed no significant difference in WD40 expression levels in distal axons across all genotypes (Graph 5, Figure 5). However, for HRP expression in distal axons, there was significant difference between the two controls (Graph 6; p=0.0386). There was no significant difference between the mutant genotype with either controls. In boutons, there was a significant difference in WD40 levels between all genotypes (Graph 5. C57/+ and MadRNAi/+ showed a significance with WD40 expression with a p value of 0.0028, the MadRNAi/+ and D42/MadRNAi showed significance with a p<0.0001 and D42/+ showed significance with D42/MadRNAi with a p-value of 0.0012. HRP expression in the boutons showed significance between the two controls (p < 0.001) and between the Mad control and the mutant genotype (p < 0.001) (Graph 6).
Figure 5: Expression of WD40 (top row), HRP (middle row) and an overlay of both HRP and WD40 expression (bottom row) in the NMJ at the 3rd abdominal segment between muscle 6/7. These animals were treated with the experimental protocol as listed above. The D42/+ and MadRNAi/+ animals showed more fluorescence in WD40 levels in the synapse but not the axons. WD40 expression showed significance across all genotypes in the synapse. HRP expression showed differences in axonal and synaptic fluorescence.
**Graph 5:** Expression of WD40 in distal axons (left) and boutons (right). There was no indicated significance in the WD40 axonal fluorescence. C57/+ and MadRNAi/+ showed a significance with WD40 expression with a p value of 0.0028, the MadRNAi/+ and D42/MadRNAi showed significance with a p<0.0001 and D42/+ showed significance with D42/MadRNAi with a p-value of 0.0012.

**Graph 6:** Expression of HRP in distal axons (left) and boutons (right). HRP expression was significant in the axon between the two controls (p=0.0386). In the synapse, the significance between the two controls and between the MadRNAi/+ and D42/MadRNAi both had p-values of less than 0.0001.

**Presynaptic knock-down of Medea reduces synaptic but not axonal WD40 levels.**

Lastly, D42-driven Med-RNAi showed no significant difference in WD40 expression at the distal axon (Figure 6). However with HRP, there was a significant
difference between the silent control and the D42/MedRNAi (p=0.0405). The silent control was not significant when compared to the mutant. In boutons, WD40 expression was significantly different between the heterozygous silent Med-RNAi and the D42/MedRNAi (Graph 7 p= 0.0193) The silent control was not significant to the mutant.

For HRP, all the genotypes were significantly different to each other. The silent control was significantly different from the D42/MedRNAi with a p-value of 0.0238, the Med-RNAi heterozygous was significantly different from the D42/MedRNAi with a p-value less than 0.0001 and the two controls were significantly different from each other with a p-value equal to 0.0075 (Graph 8).
Figure 6: Expression of WD40 (top row), HRP (middle row) and an overlay of both HRP and WD40 expression (bottom row) in the NMJ at the 3rd abdominal segment between muscle 6/7. These animals were treated with the experimental protocol as listed above. The D42/+ and MedRNAi/+ animals showed significant differences in fluorescence of WD40 levels in the synapse but not the axons. HRP expression showed differences in axonal fluorescence between the D42/+ and D42/MedRNAi animals. HRP synaptic fluorescence showed significance between all three genotypes.
**Graph 7:** Expression of WD40 in distal axons (left) and boutons (right). WD40 expression was insignificant across all genotypes in the axon. In the synapse, WD40 expression showed significance between the MedRNAi/+ and D42/MedRNAi animals (p=0.0193).

**Graph 8:** Expression of HRP in distal axons (left) and boutons (right). HRP expression was insignificant across all genotypes in the axon. In the synapse, WD40 expression showed significance between the MedRNAi/+ and D42/MedRNAi animals (p=0.0193).
Discussion

Post-synaptic Mad knockdown models

The C57-driven knockdown of Mad was the most consistent out of the four collections of data presented. Although no significant differences in WD40 fluorescence were found in distal axons, a clear trend was found towards a decrease in both WD40 and HRP expression (Graphs 1 and 2). The C57 driven MadRNAi had the lowest WD40 expression and was significant with the C57/+ control (p=0.0011). With more NMJs, the lack of significance from the silent transgene (2nd control) is likely to change.

The driven control C57-MadRNAi showed significance in HRP expression in the boutons. The significance between the two control (C57/+ and MadRNAi/+ genotypes can be contributed to genetic background with the MadRNAi/+ genotype.

Post-synaptic Med knockdown models

The muscle-driven knockdown of Medea did not show importance in either WD40 or HRP expression in the distal axons. However, C57-driven knock down of Med decreased WD40 and HRP expression as seen in Graphs 3 and 4. Since there is a very visible decrease in fluorescence of the C57/MedRNAi animals, with more NMJs, this trend can be solidified towards significance or not.

The postsynaptic Medea knockdowns show significance in the synapses between the two controls. Once again, due to the very blatant decease in WD40, more NMJs due to this trend may solidify the data towards or away from significance.

Regarding HRP expression, there is significance between the two controls and between undriven control and knockdown animals. One explanation for this observation could be
due to the MedRNAi line not being a strong of a knockdown as wanted. In both WD40 and HRP expression in the boutons, the undriven control possessed the highest fluorescence; this observation also carries on to the presynaptic Medea knockdown data set where the fluorescence of the MedRNAi heterozygous control was the highest as well. The RNAi line used for Medea knockdowns has been quite inconsistent throughout the whole year. Breeding the flies was difficult as food would dry out frequently and would not provide a hospital environment for the larvae. Due to this conundrum, it was difficult to make crosses in a timely manner and to collect data.

**Pre-synaptic Mad knockdown models**

No significance was shown in WD40 expression of distal axons for the pre-synaptic Mad knockdown. However, there was significance shown between the two controls. Due to the variance of the D42/+ data as shown in Graph 5, the significance can be called into question. This data set also had the misfortune of some images that were overly fluorescent in the green channel. The aberrantly bright images may have considerably skewed the data.

**Pre-synaptic Med knockdown models**

Lastly, the pre-synaptic med knockdowns were perhaps the most variable data set collected. There was no significance in WD40 expression across the genotypes in the distal axon. However, the presented data points are not condensed and rather smeared. Regarding HRP expression, there was significance between driven control and Pre-synaptic Med knockdowns. In the boutons, there is significance between
MedRNAi heterozygous control and the pre-synaptic Med knockdowns. HRP expression is significant across all three genotypes when compared to each other.

Several reasons may attribute to my confusing and non-straightforward data set. The first could be the potential of affecting the growth pathway. Secondly, genetic background could also play a role. The MedRNAi/+ has been consistently bright for both the C57/MedRNAi set and the D42/MedRNAi set, so the problem could lie in the line itself. Lastly, the D42/MedRNAi set suffered the heaviest blow from the aberrantly bright images that were acquired towards the end of this data collection since half of the data came from those images.

**Conclusion**

Our primary goal has been to study effects of retrograde TGF-β signaling on synaptic and axonal WD40 expression levels. The most difficult obstacle to overcome was the viability of the Med-RNAi line, as it did not thrive as well as we wanted to. Towards the end of the experiments, the abnormally bright images obtained in the last several weeks were deeply concerning and efforts to rid of the brightness have been implemented by re-making the WD40 antibody making fresh stocks of solutions. In most of the experiments, there is a decrease of expression of WD40 in the mutant genotypes, which can provide us a lead to further studying the interaction between TGF-β’s effects on WD40 expression levels at the neuromuscular junction.
References Cited


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