Brandeis University

Blazeman Foundation for ALS Research

Research Progress Report 5/1/2017 – 5/30/2018

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1. Introduction and summary of previous results (Years 1-4):

Motor neuron survival and growth depends on cues from the surrounding tissues, including other neurons, muscles, or support cells. This communication between neurons and other cells involves a number of growth factors that bind to receptors on the neuron and are transported into the neurons through a network of internal membrane compartments, leading to activation of growth- and survival-promoting signals. There have been many clues that membrane traffic of growth signals is defective in ALS, since mutations or changes in traffic have been seen in patients and in animal models of disease. However, we do not yet understand enough about how these processes go wrong in ALS to design effective treatments.

Our approach has been to model ALS in animal systems (including fruit flies and mammalian neurons in culture) to examine what has gone wrong with membrane traffic in neurons. Our goal is to use these animal models to discover the underlying biology that is defective when TDP-43 is misexpressed, with the goal of identifying relevant biological processes and molecules that would benefit from therapeutic intervention. We chose to model ALS by manipulating the expression of TDP-43, a gene linked to familial ALS. Aggregates containing TDP-43 have been reported in most cases of ALS, both sporadic and familial, indicating that TDP-43 has a common underlying role in the biology of the disease.

In 2016, we published a research article (Deshpande et al., 2016) describing our results on the effect of TDP-43 on growth signaling at the fruit fly neuromuscular junction (NMJ). Our main conclusions from this work were that changes in TDP-43 levels result in very strong **local** defects in the growth signaling at the NMJ, but that **long-range signaling** at the cell body was not affected. This was true when we expressed either more or less TDP-43 protein in neurons, suggesting that tight control over TDP-43 levels is important for its normal function, which is consistent with other findings in the field (Robberecht and Philips, 2013). Remarkably, we were able to bypass this local

signaling defect by rerouting membrane traffic of the signaling proteins, suggesting a new way to rescue NMJ defects in ALS (Deshpande et al., 2016). What remained a mystery is why and how TDP-43 specifically affected local signaling in the first place. In year 5, we focused on identifying mechanisms by which this occurs.

We have also been investigating the mechanisms underlying the defects in growth and branching of mammalian neurons caused by TDP-43 misregulation, in collaboration with Dr. Suzanne Paradis and Josiah Herzog (a graduate student the Paradis Lab). We found that altering levels of TDP-43 led to a strong defect in branching and growth of neurons from the cortical region of the brain, an area that is important in ALS. Reminiscent of our results from the fruit fly NMJs, these data suggest that both up and downregulation of TDP-43 protein levels can lead to similar phenotypes. Changes in neuron branching and growth could lead to problems with connectivity and neuronal firing. TDP-43 normally works by binding to many different RNAs, which encode or control hundreds of proteins in the cell. Using different versions of TDP-43 protein where its RNA binding domain is either modified or truncated, we established that the neuronal branching defect depends on the ability of TDP-43 to bind its RNA targets. We also used live imaging to show that the defect is likely to occur at the step of neurite elongation, rather than neurite shrinking or retraction. These results set up a system for studying how growth signaling is altered by TDP-43 in mammalian cells, and were published in November 2017 (Herzog et al., 2017).

2. Progress and Results from year 5

2A. Mechanism for local growth factor signaling defects in the fruit fly ALS model

To understand why TDP-43 specifically caused local (at the synapse) but not long-range (at the cell body) defects in growth signaling in fruit fly neurons, we investigated two possible mechanisms. First, excessive transport of signals away from the synapse could be contributing to synapse-specific depletion. Second, recent work has suggested that in addition to canonical signaling, neurotransmitter receptors on the muscle could help to regulate the local synaptic growth signals, and therefore reduction of post-synaptic glutamate receptors could lead to a decrease in pre-synaptic signaling.

We tested the first hypothesis by disrupting transport away from the synapse in flies over-expressing TDP-43, by testing if two different mutations in the motor proteins that carry materials away from the synapse could trap the signals at the synapse and restore function. We found that

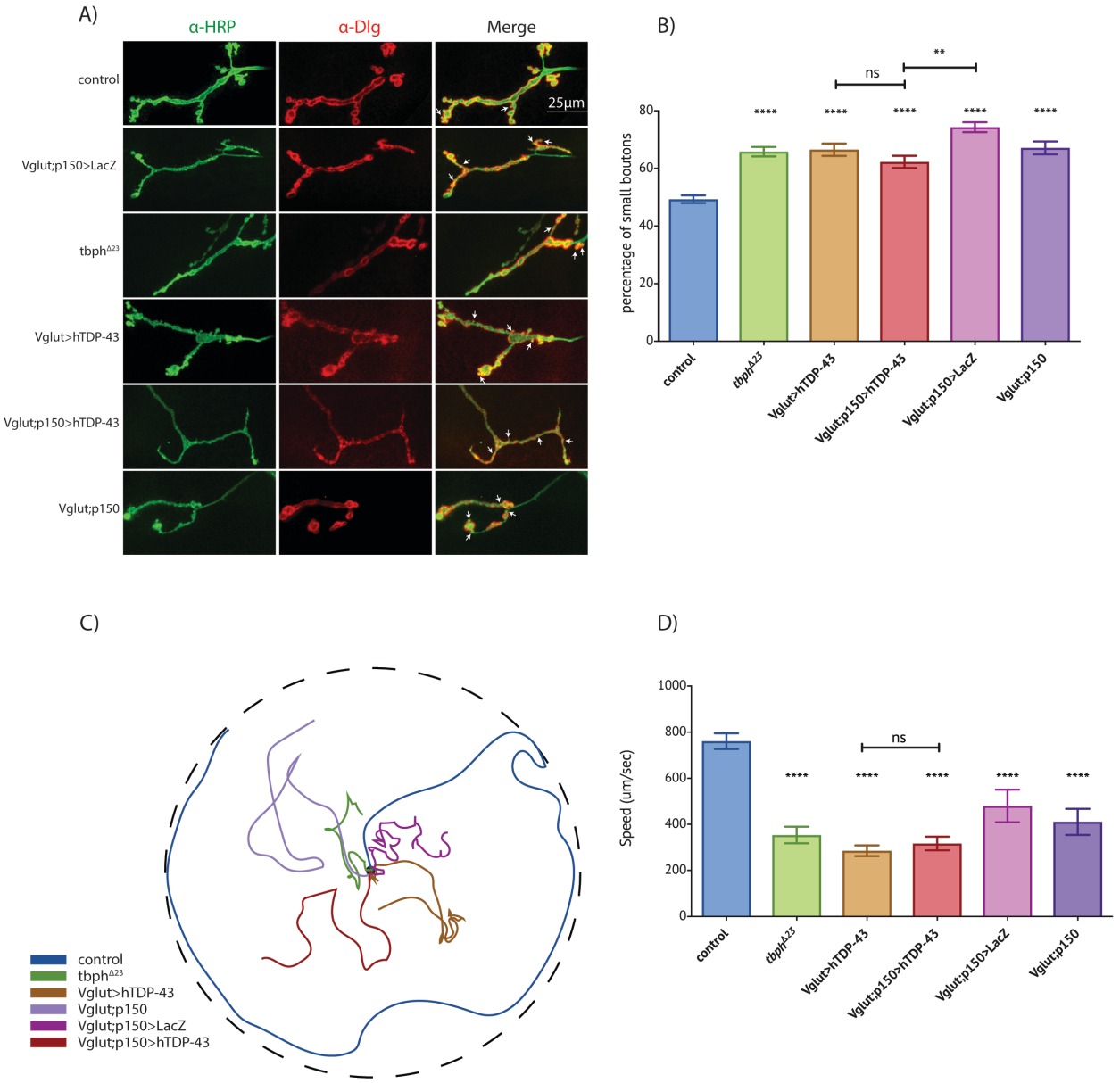

Figure 1. Overexpression of p150 (a component of a motor protein that transports materials away from synapses) does not rescue TDP-43 overexpression defects. A) Maximum intensity representative images depicting NMJ morphology at muscle 4. α-HRP staining the neuron is shown in green and α-Dlg staining the post-synaptic density is shown in red, merge is shown in yellow. White arrows on the merged images represent small boutons (<2 μm in diameter). Only boutons on the main branch were quantified. B) Quantification of the percentage of small boutons compared with the total number of Type Ib boutons at the NMJ ± SEM. Statistical significance was calculated with One-Way ANOVA and Tukey's comparison. C) Representative traces of larval crawling showing total distance moved during a 3-minute period. Each genotype is marked by a separate color. The edge of the agar plate is marked with the black dashed circle. D) Quantification of larval crawling speed ± SEM. Statistical significance was calculated with One-Way ANOVA and Tukey's comparison.

this did not rescue the synapse growth or motility defects in fly larvae (see **Figure 1** for one of the mutations), suggesting that excess retrograde transport is not likely to be the problem.

We then tested our second hypothesis, that neurotransmitter receptor levels in the postsynaptic cell could be influencing presynaptic signaling. We found that levels of the neurotransmitter receptor GluRIIA were strongly reduced (**Figure 2**), suggesting that this pathway could be playing a role in disease pathology. Indeed, very recent work showed that levels of the glutamic acid decarboxylase enzyme (Gad1) are reduced in TDP-43 null animals (Romano et al., 2018). Gad1 plays an important role in limiting accumulation of the neurotransmitter glutamate in the extracellular space, which in turn plays a role in the organization of post-synaptic neurotransmitter receptor. In this study, it was found that TDP-43 null animals have toxic extracellular levels of glutamate, which lead to reduced accumulation of Glutamate receptors, mirroring our observations. Overall, our findings provide some important insight into what factors TDP-43 misregulation could be leading to defective local growth signaling.

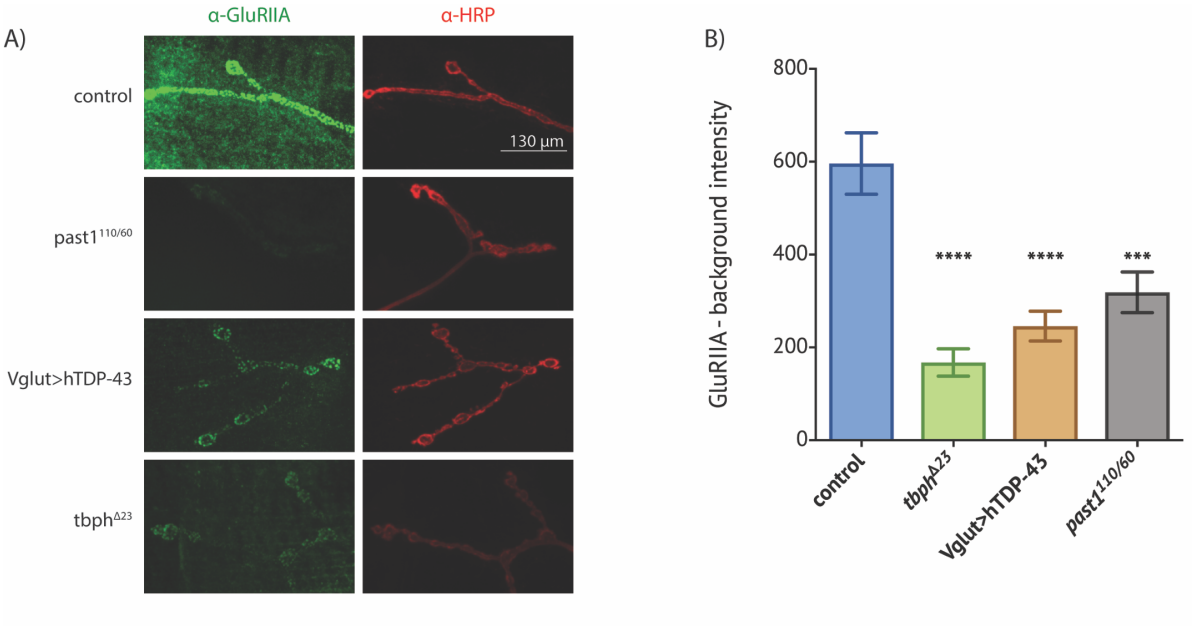

Figure 2. GluRIIA levels are altered in animals with altered TDP-43. A) Maximum intensity representative images showing GluRIIA staining at the NMJ at muscle 4. α-HRP staining the neuron is shown in red and α-GluRIIA is shown in green. Past1 is used as a control for GluRIIA depletion (Koles et al., 2015). B) GluRIIA background staining was variable and so quantification shows background-subtracted GluRIIA intensity ± SEM. Statistical significance was calculated with One-Way ANOVA and Tukey's comparison.

2B. Mechanism for branching defects in the mammalian cultured neuron ALS model

As our next step to understand why cortical neurons have less branching when TDP-43 is altered, we began exploring the involvement of calcium channels and neuronal activity, which are

known to affect branching. Our initial observations showed that neurons that overexpress TDP-43 fail to expand their dendritic arbors in response to neuronal firing. We also observed that calcium entry into the neuron following activation is impaired in neurons that overexpress or underexpress TDP-43 (**Figure 3A**). CREB is a transcription factor that is a well-established master regulator of neuronal branching in response to activity (**Figure 3B**). Our recent data indicates that in neurons that have excess of TDP-43 protein, CREB is not properly activated (**Figure 3C**), and that this depends on the ability of TDP-43 to bind RNA. Further, loss of TDP-43 causes a similar effect. This is similar to our branching results, suggesting that the ability of CREB to regulate expression

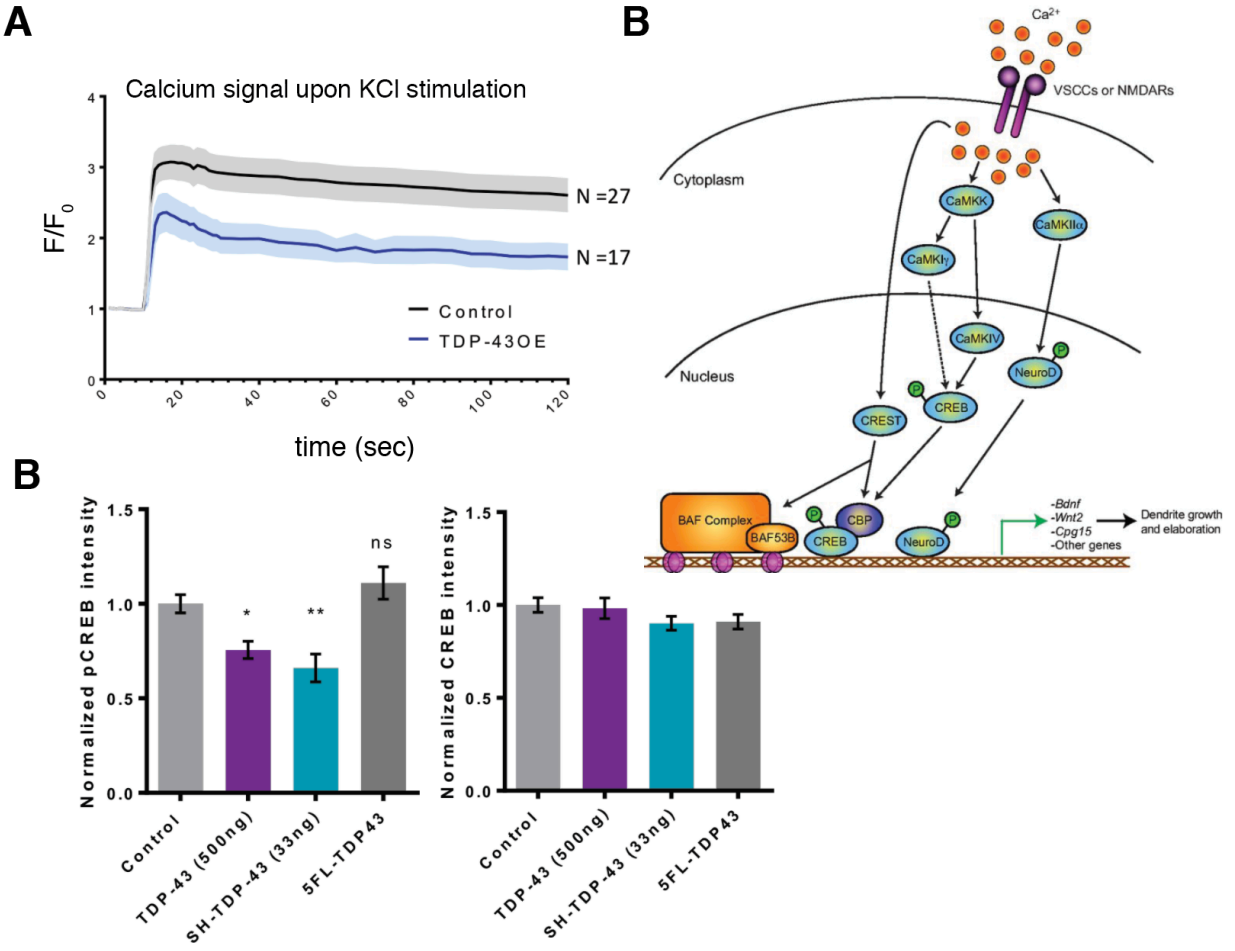

Figure 3. Calcium signaling defects in TDP-43 mis-expressing rodent cortical neurons. A) Depolarization induced calcium influx following exposure to 55mM KCl is significantly lowered by TDP-43 overexpression. Calcium is measured as a ratio of Fura-2 intensities (Excitation 340nm and 387 nm; emission 515nm) Two-way ANOVA (repeated measures) p= 0.013. B) Model for calcium-dependent Creb signaling to promote dendrite growth and elaboration (from (Puram and Bonni, 2013)) C) TDP-43 misexpression inhibits CREB activation, and this depends on RNA binding. 7 DIV cortical neurons transfected at 2 DIV. Neurons were immunostained with an antibody that recognizes phosphorylated CREB at serine 133 (pCREB) or total CREB. Graph shows Fluorescence intensity of pCREB or CREB immunostaining Data presented as mean ± S.E.M.

of genes required for branching and growth is impaired when TDP-43 is not functioning properly. We are currently directly testing the idea that changes in specific calcium channels lead to these reduced levels of calcium in neurons overexpressing TDP-43.

2C. Testing signaling and branching defects in ALS patient induced pluripotent stem cells (iPSC)

The important next question to address is how these pathways relate to the progression of cellular pathology in human patients, whether they are perturbed similarly and result in equivalent cellular defects. A powerful cutting-edge technique that allows this investigation is reprograming stem cells (induced pluripotent stem cells or iPSC) derived from ALS patients (harboring mutations in TDP-43) to generate neuronal cell lines. We obtained a small grant ($19,000) from Brandeis to supplement our Blazeman Foundation-supported work and to initiate a collaboration with the Human Neuron Differentiation Core (HNDC) at Boston Children's hospital, a facility that specializes in working with such patient-derived neuronal cell lines and provides access to state-of-the-art facilities for imaging and recording neuronal activity. This enables us to compare the ALS patient neurons to control cell lines and probe for signs of neuronal dysfunction.

We obtained previously published iPSC lines harboring the TDP-43 A90V mutation and patient control (Zhang et al., 2013b). We differentiated the iPSCs into cortical-like neurons using a Neurogenin-2 (NGN2) rapid neural induction protocol (Zhang et al., 2013a). The advantage of this approach is that functional synapses are observed in these cultures after only 2 weeks of plating. Using this protocol, we did not observe changes in growth or CREB activation in TDP-43 expressing neurons. There are several possible explanations for why the iPSC results differ from our results in primary rodent neurons. First, these differentiated neurons may not recapitulate the behavior of true neurons; we could instead try a more elaborate differentiation protocol to create motor neuron-like or other neuronal types. Second, the TDP-43 A90V mutation may only begin to exert its effects on aged neurons (indeed, ALS only shows up later in life), compared to the more drastic effects of over or underexpressing TDP-43, and therefore does not show an effect in short-term culture of iPSCs. However, we did note one surprising and interesting change – we observed a reduction of synapse formation in iPSCs after 2.5 weeks in culture. This may be important since synapse loss is an early hallmark of ALS, and the iPSC system could be a powerful model for testing the basis of this defect and finding routes to reverse it. We request to use the remaining Blazeman Foundation funds to pursue this effect in iPSCs and in primary neurons.

3. Next Steps:

We are planning to focus our next steps on wrapping up our calcium signaling paper, and exploring the molecular mechanisms underlying the defects in synapse formation caused by TDP-43 misregulation in iPSC models. For the next 6 months, we would like to focus on the following goals.

1. In order to investigate how TDP-43 misregulation is affecting calcium signaling in neurons, we will test if levels of calcium channels are changed in neurons overexpressing TDP-43.
2. We will reinstate the activation of CREB pathway in the neurons overexpressing TDP-43, by mechanisms that bypass the requirement for calcium entry, to test if this can alleviate the defects in growth and branching.
3. We will test if motor neurons derived from TDP-43 mutant iPSC, which have previously been shown to have growth defects like our cultured neurons, also have defects in CREB signaling.
4. We will follow up our observation on defects in synapse formation in patient-derived neurons, by testing the density of synapses in cultured rodent neurons when the levels of TDP-43 expression are altered (overexpression and knock-down). This will provide us a powerful system in which potential involvement of TDP-43 targets, such as calcium channels, can be tested directly.

3. Budget

Please see attached budget summary. There were no funds spent other than Dr. Deshpande's salary and benefits. A balance of $ 9,986.66 remains. We request to dedicate these remaining funds to wrap up our CREB signaling paper, or to push forward the iPSC work into new areas.

4. Publicity and dissemination

a. An article describing the patient iPSC work was published in Brandeis Now. http://www.brandeis.edu/now/2017/june/ALS-TDP43-rodal.html

b. Recent findings from the mammalian ALS model studies were published in a research article in *Scientific Reports*. (https://www.nature.com/articles/s41598-017-15914-4).

5. References

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