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14. ABSTRACT

In this Therapeutic Idea Award proposal application, we will conduct proof-of-concept studies to evaluate the therapeutic potential of a dual PPAR-delta/gamma nuclear receptor agonist, T3D-959, to ameliorate ALS neurotoxicity. This will be accomplished by pursuit of the following two Specific Aims. In Aim #1, we will test the hypothesis that T3D-959 can prevent ALS disease-relevant phenotypes in primary cortical neuron models. We will employ two different ALS neurotoxicity systems, which recapitulate key features of ALS disease pathology in sporadic ALS and in the most common form of familial ALS. In Aim #2, we will test the hypothesis that T3D-959 can rescue ALS disease phenotypes in two different mouse models, the PrP-TDP-43:Q331K transgenic mouse and the (G4C2)-149 repeat C9orf72 mouse model. We will perform two preclinical trials, after establishing a safe, effective dose and route of delivery for T3D-959. Preclinical trial read-outs will be to evaluate general health of T3D-959-treated ALS mice in comparison to vehicle-treated ALS mice; neuromuscular and cognitive function; histopathology; and disease-specific molecular pathology read-outs.

15. SUBJECT TERMS

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1. INTRODUCTION:

In this project, we tested if the PPAR-delta/gamma agonist drug T3D-959 could prevent ALS disease phenotypes in cultured cortical neurons expressing ALS disease proteins. We also performed preclinical trials of T3D-959 in two different ALS mouse models, one featuring the expression of a disease protein (TDP-43) implicated in ALS, and the other expressing a gene (C9orf72) known to cause the most common form of familial ALS.

2. KEYWORDS:

PPAR-delta; PPAR-gamma; agonist; nuclear receptor; transcription factor; TDP-43; C9orf72 gene; dipeptide; cortical neuron; preclinical trial; mouse model; cognitive function; histopathology; molecular pathology; outcome measures

3. ACCOMPLISHMENTS:

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

This was a two-year project.

Goals for end of Year 1 were as follows:

Major task 1 = determine if T3D-959 can rescue disease phenotypes in cortical neurons expressing TDP-43 (100% completed)

Major task 2 = determine if T3D-959 can rescue disease phenotypes in cortical neurons expressing dipeptide repeat constructs (100% completed)

Major task 3 = perform a pharmacodynamics study to identify the optimal dose of T3D-959 to be used in the preclinical trials (100% completed)

Goals for the end of Year 2:

Major task 4 = Preclinical trial of T3D-959 in TDP-43 transgenic mice (100% completed)

Major task 5 = Preclinical trial of T3D-959 in G4C2-149R C9orf72 transgenic mice (100% completed)

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

1) Major activities

We spent the first year of this project performing Major Tasks 1 and 2, which comprised Aim #1 of this project, which was to determine if **PPAR δ/γ activation can prevent ALS neurotoxicity phenotypes in primary neuron models of ALS.** During the first year of the project, we also completed Major Task 3, the first step of Aim #2, the goal of which was to complete a **pharmacodynamics study to establish the maximal safe dose and the delivery regimen of T3D-959 to achieve engagement with target in the CNS of treated mice** as a prelude to initiating two preclinical trials of T3D-959 in two different ALS mouse models. At the end of the first year of the project, we initiated Major Tasks 4 and 5, which comprise the remainder of Aim #2, which was to perform two preclinical trials of T3D-959 in two different ALS mouse models (the PrP-TDP-43:Q331K transgenic line and the (G4C2)-149R model). We recently completed the two preclinical trials, and thereby fulfilled all tasks and milestones proposed for this project.

2) Specific Objectives

MAJOR TASKS 1 + 2:

We tested the hypothesis that T3D-959 can prevent ALS disease-relevant phenotypes in primary cortical neuron models. **We employed two different ALS neurotoxicity systems, which recapitulate key features of ALS disease pathology in SALS and in the most common form of FALS.** As TDP-43 histopathology is a defining feature of SALS and most forms of FALS, the first model system was based upon expression of full-length TDP-43 or C-terminal truncated TDP-43. As C9orf72 ALS due to expansion of a G4C2 repeat is the most common form of FALS, the second model system was based upon expression of synthetic dipeptide repeat constructs [(GR)30 and (GA)30] to model C9orf72 gain-of-function neurotoxicity due to RAN translation.

MAJOR TASKS 3, 4 & 5:

We sought to test the hypothesis that **PPAR δ/γ activation can prevent ALS neurotoxicity phenotypes in transgenic mice**, and so initiated the preclinical trial work by performing a pharmacodynamics study to establish the maximal safe dose and the delivery regimen of T3D-959 to achieve engagement with target in the CNS of treated mice. We then established the requisite cohorts for the two different preclinical trials and successfully initiated dosing of mice with either drug or vehicle. The two preclinical trials were carried out in parallel and required that we follow each set of cohorts to one year of age, obtaining read-outs and outcome measures at 2 month to 3 month intervals.

3) Significant results / key outcomes

Major task 1: We were able to confirm that treating primary cortical neurons with T3D-959 increases the expression of key PPAR-delta target genes at doses of 100 nM, 1 uM, and 10 uM, but has no effect at 10 nM (**Figure 1**). Based upon these data, experiments were performed using T3D-959 at the three effective doses.

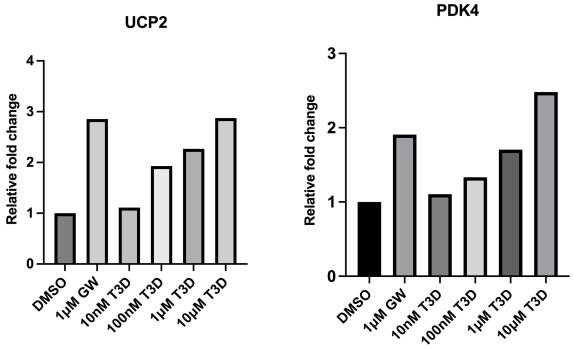


Figure 1. qRT-PCR analysis of PPAR-delta target genes in primary cortical neurons treated with T3D-959

Primary cortical neurons (PCNs) were cultured from postnatal day 1 mice and treated at DIV9 with the indicated dose of T3D-959 or the PPAR-delta agonist GW501516 (positive control). Neurons were cultured until DIV16 and RNA was isolated for downstream analyses.

However, we were unable to transfect primary cortical neurons with the GFP-only and TDP-43 expression constructs at a high enough level of efficiency. Hence, we decided that we needed to switch to a system of transduction via infection with lentivirus. We therefore cloned each expression construct into a lentivirus vector, and provided the lentivirus vectors to a core service for production of high titer lentivirus for these experiments. We received the lentivirus vectors, and we were able to efficiently transduce primary cortical neurons with GFP-only and GFP-TDP-43 expression constructs. **However, when we assayed for cell death, we did not detect a significant increase in cell death in neurons expressing TDP-43 or C-terminal TDP-43**. We tested various stressors, but we did not detect any significant differences between TDP-43 expressing neurons and mock transfected neurons. We evaluated nucleo-cytoplasmic transport and did not observe any significant defects in TDP-43 expressing neurons, and when we compared overall mitochondria content and assayed mitochondrial morphology, we did not observe any alterations in TDP-43 expressing neurons. Treatment of primary neurons expressing TDP-43 with the PPAR-delta agonist T3D-959 yielded modest, but not significant improvements in mitochondrial content and morphology, and in neuron survival; however, these improvements were also noted in control neurons.

Major task 2: We were able to efficiently transduce primary cortical neurons with GFP, (GA)30 [ggacga], and (GR)30 [ggaaga] vectors. We performed three biological replicates to assess nucleolar morphology by quantifying the mislocalization of NPM1 from the nucleolus to the nucleoplasm. Treatment with T3D-959 did not consistently alter nucleolar morphology in any of the conditions tested (**Figure 2**). Primary cortical neurons were also transduced with an NLS-NES-tdTomato construct along with GFP, (GA)30, or (GR)30, and fluorescence recovery after photobleaching (FRAP) was performed of the nucleus to assess nucleo-cytoplasmic transport. While transduction with GR(30) had little effect on fluorescent recovery, **(GA)30 expressing neurons did exhibit impaired nuclear transport** (**Figure 3**). Treatment with T3D-959, however, did not significantly alter fluorescent recovery in either condition (**Figure 3**).

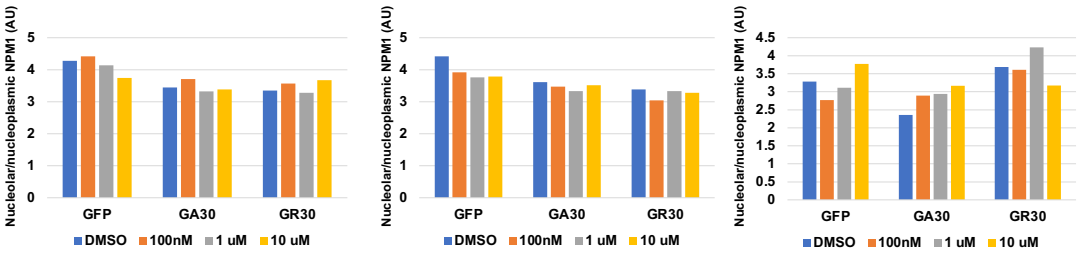


Figure 2. T3D-959 does not alter NPM1 nuclear localization in dipeptide repeat expressing cortical neurons

Primary cortical neurons (PCNs) were cultured from postnatal day 1 mice and treated with the indicated dose of T3D-959 or DMSO (negative control). Neurons were cultured for 24 hrs and then assayed for immunostaining intensity in the nucleolus or in the non-nucleolar portions of the nucleus (nucleoplasm). We did not detect any significant differences in the subnuclear localization of NPM1 in negative control GFP-only expressing neurons in comparison to GA(30) or GR(30)-expressing neurons. Nor did we observe any significant effect of T3D-959 treatment on subnuclear localization of NPM1 in GFP-only, GA(30), or GR(30)-expressing neurons.

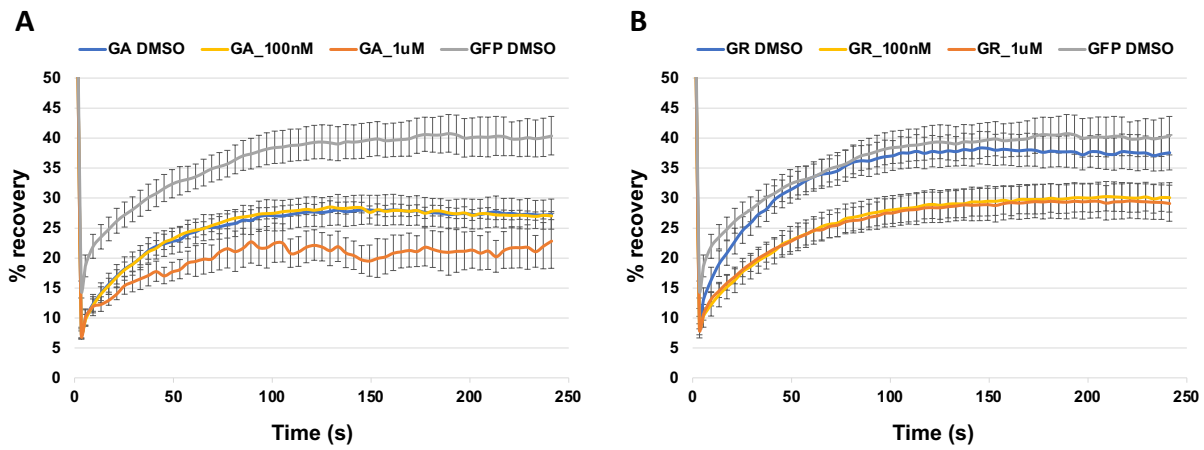


Figure 3. T3D-959 does not improve nucleocytoplasmic transport in dipeptide repeat expressing cortical neurons

Primary cortical neurons (PCNs) were cultured from postnatal day 1 mice and were transduced with lentivirus vectors expressing either GA(30) or GR(30), or empty vector GFP-only (negative control). We performed fluorescence recovery after photobleaching (FRAP) using a NLS-NES-eGFP reporter and measured the % recovery of GFP signal in the nucleus after photobleaching, here shown as a function of time in seconds. While there were no significant differences in % recovery rate for GR(30)-expressing neurons vs. control GFP-expressing neurons [RIGHT], we did detect significant differences between untreated GA(30)-expressing neurons and GFP-only-expressing neurons [LEFT]. However, T3D-959 treatment did not affect fluorescence recovery in GA(30)-expressing neurons, and did not improve fluorescence recovery in GR(30)-expressing neurons, but rather there was a trend toward worsened fluorescence recovery in GR(30)-expressing neurons.

Finally, we compared overall mitochondria content and morphology between neurons transduced with GFP, (GA)30, (GR)30 by measuring fluorescence intensity of the mitochondrial protein TOM20. We observed a significant increase in TOM20 intensity (mitochondrial content) upon transduction of primary neurons with GA(30) and GR(30); however, treatment with T3D-959 did not affect this phenotype in a consistent manner. We did not observe any significant differences in mitochondrial morphology or effects of T3D-959 upon this read-out.

Major task 3: We did intraperitoneal injections in wild-type C57BL/6J control mice with different doses of T3D-959 dissolved in saline (30 mg/kg, 50 mg/kg, 70 mg/kg and 90 mg/kg) for one week to optimize the final dose for the preclinical trial. We monitored the body weight of these WT mice every day and assessed the general health of the T3D-959-treated WT mice in comparison to vehicle-treated WT mice. The expression levels of three PPAR-delta targets (ucp2, pdk4 and angptl4) in both cortex and spinal cord were measured by qRT-PCR. Based on the data of the induction of all three genes, along with absence of signs of disease, stress, or toxicity, we decided that 50 mg/kg would be the best option for the dosage to be used in the preclinical trial and because of toxicity noted at higher doses, and we modified the dosing regimen to a Mon-Wed-Fri frequency. We repeated the pharmacodynamics experiment in WT mice using this dose and frequency, and we observed significant expression increases in the three PPAR-delta targets after a two-week dosing interval.

Major task 4: We bred PrP-TDP-43:Q331K mice for the preclinical trial of T3D-959, and the baseline behavioral tests were finished at the age of 5 weeks. Based on the performance of the baseline of motor function, we balanced the genders and assigned these mice into T3D-959-treatment group and vehicle-treatment group. Due to concerns over toxicity noted in Major task 3, we initiated Mon-Wed-Fri intraperitoneal injections of 50 mg/kg/day T3D-959 at 6 weeks of age.

The first outcome measure for the preclinical trial was the General Health cluster, which consisted of body weight and a composite neurological screening exam.

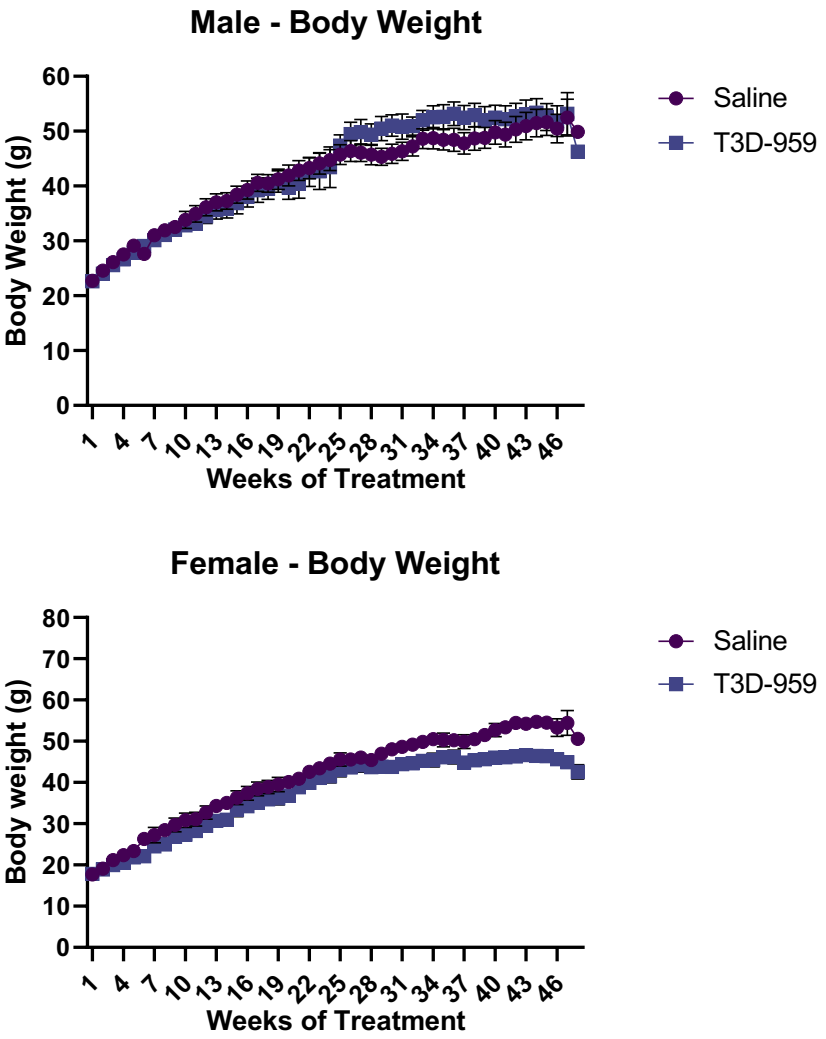


Figure 4. T3D-959 does not alter body weight in males, but causes a decrease in body weight in females.

We obtained weekly body weights on TDP-43 transgenic mice beginning at the start of treatment (6 weeks of age). Male TDP-43 transgenic mice from the treatment group and vehicle (saline) group displayed virtually identical body weights over the course of the study. Female TDP-43 transgenic mice treated with T3D-959 showed a significant drop in body weight compared to saline-treated TDP-43 mice, but actually weighed the same as non-transgenic WT control mice ($P < .05$ for TDP-43 treated vs. TDP -43 saline; ANOVA with post-hoc Tukey test). All cohorts consisted of $n \geq 6$ mice / group. As the absence of a marked increase in body weight in T3D-959-treated TDP-43 female transgenic mice matches with the weight chart for normal WT non-transgenic female littermates, it may represent a retention of normal physiological metabolism in the disease state.

We also performed a composite neurological phenotyping examination, consisting of evaluations for clasping, balance, kyphosis, and gait. Each metric is scored from 0 – 3, with 0 = normal, and 3 = most severe. This testing was performed every two months, and while we documented worsening neurological function in TDP_43 transgenic mice as they aged, we did not observe any improvement in neurological function in TDP-43 transgenic mice treated with T3D-959 (**Figure 5**).

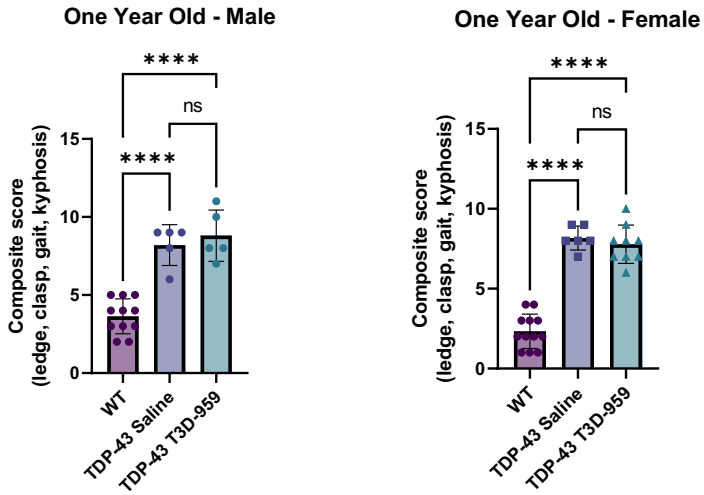


Figure 5. T3D-959 does not improve neurological function on a composite screening exam.

Here we see results from the terminal time point of 12 months, when the disease is most severe. Despite receiving 10.5 months of T3D-959 treatment, the treated group shows composite scores virtually identical to saline treated TDP-43 mice. ****$P < .0001$, ANOVA with post-hoc Tukey test; n ≥ 6 mice / group.

The second outcome measure was neuromuscular and cognitive function.

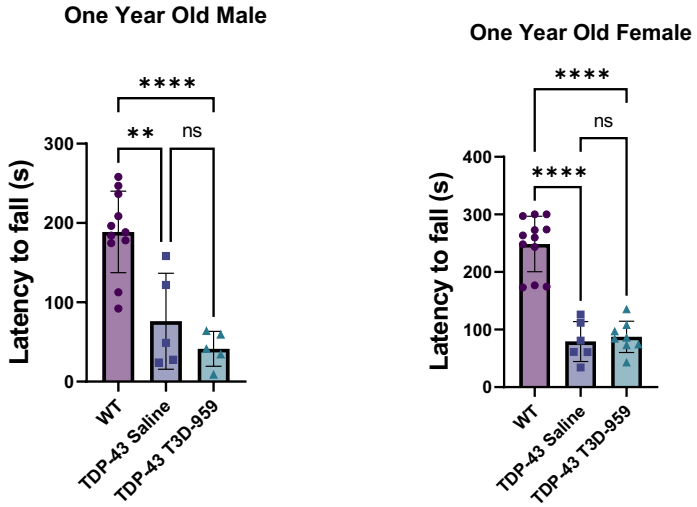


Figure 6. T3D-959 does not improve performance on the rotarod.

Here we see results from the terminal time point of 12 months, when the disease is most severe. Despite receiving 10.5 months of T3D-959 treatment, the treated group shows composite scores comparable to saline treated TDP-43 mice. **$P < .01$, ****$P < .0001$, ANOVA with post-hoc Tukey test; n ≥ 6 mice / group.

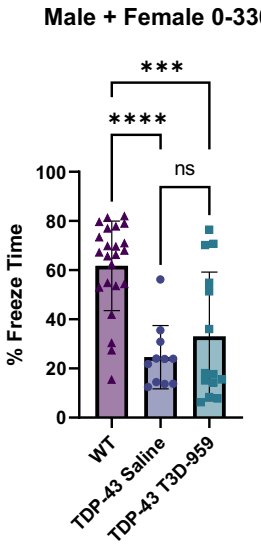


Figure 7. T3D-959 treatment yields a modest increase in contextual fear performance in T3D-959 treated TDP-43 mice.

Here we see results from the terminal time point of 12 months, when the disease is most severe. After receiving 10.5 months of T3D-959 treatment, the treated group's % freezing time was almost 10% higher than the saline-treated group's % freezing time. ***$P < .001$, ****$P < .0001$, ANOVA with post-hoc Tukey test; n ≥ 10 mice / group.

Similarly, we did not observe any improvements on the open field test and two-paw and four-paw grip strength tests. We performed cognitive testing using a contextual fear conditioning paradigm, which revealed a minor improvement in the freezing time in T3D-959 treated mice, as reflected by a less significant difference in performance vs. controls (**Figure 7**). However, the freezing times when compared between T3D-959 treated mice and saline-treated mice, were similar.

The third outcome measure was histopathology analysis:

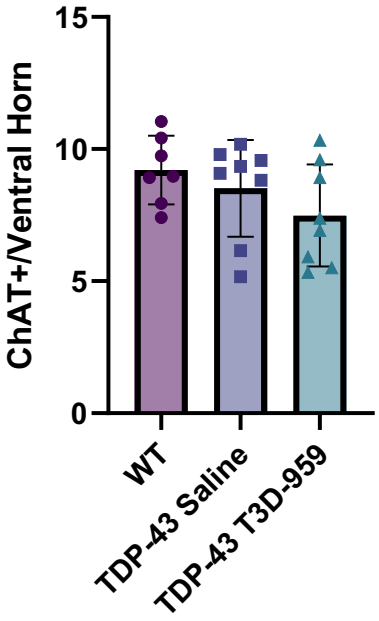


Figure 8. Motor neuron quantification at one year of age

- ChAT positive ventral horn motor neurons were counted from at least 14 horns per animal.
- Euthanized mice were transcardially perfused with PBS then 4% paraformaldehyde in PBS, cryoprotected in 30% sucrose in PBS and embedded in TissueTek OCT.
- 30 µm sections from the lumbar cord were taken via cryosectioning.
- The sections were washed three times in PBS before being incubated with 4% BSA and .1% Triton X-100 at room temperature for 1 hour.
- Sections were then incubated with ChAT (1:100) overnight.
- The following day sections were washed three times in PBS and incubated with secondary in PBS (1:1000) at room temperature for 1 hour.
- Following secondary the sections were washed 3 times in PBS and stained with DAPI (1:1000) and Neurotrace (1:1000)
- The sections were washed a final 3 times and cover-slipped with ProLong Gold antifade mounting media

We did not observe any significant differences in motor neuron numbers between the three cohorts (n tor neuron numbers between the three cohorts (n ≥ 7 mice / group). One-way ANOVA.

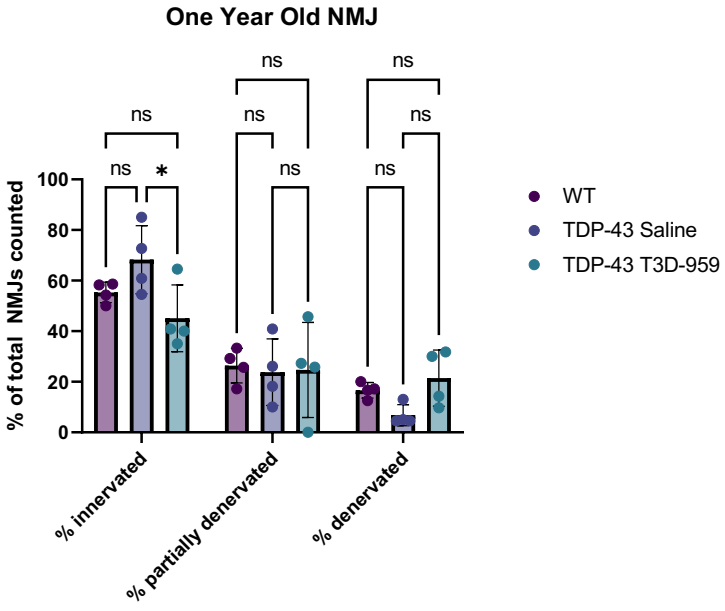
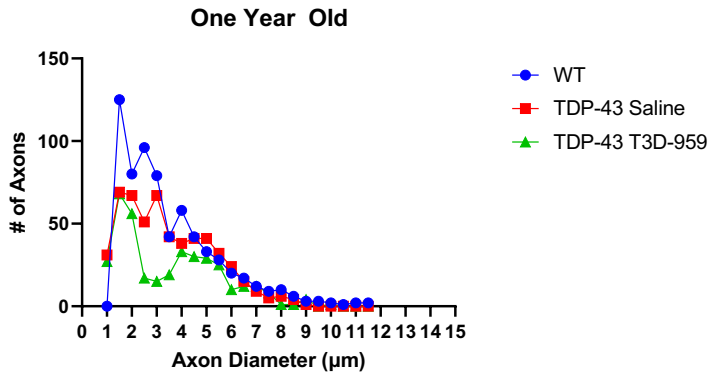


Figure 9. Neuromuscular junction (NMJ) quantification at one year of age

T3D-959 treated mice showed no change in percent of innervated, partially denervated or fully denervated NMJs after 10 month of treatment (12 months old). n = 4 per group; p > 0.05 using two-way ANOVA (mixed model) with Tukey's post hoc test. Error bars = SD

≥ 20 NMJs from the quadricep were counted from at least 6 sections per animal. Euthanized mice were transcardially perfused with PBS then 4% paraformaldehyde in PBS. The quadriceps were collected and cryoprotected in 20% sucrose in PBS and embedded in TissueTek OCT. 30 µm longitudinal sections were prepared. Sections were then incubated with Synaptophysin (1:250) overnight at 4C. The following day sections were incubated with secondary in PBS (1:1000), α-bungarotoxin (1:1000) and neurofilament-L (1:1000).



Genotype	# of large caliber axons (>7µm)	Percent of total axons
Wildtype	44	6.56 %
TDP-43 Saline	64	3.15 %
TDP-43 T3D-959	51	2.50 %

Figure 10. T3D-959 does not rescue loss of large caliber motor axons.

To quantify motor axons at the L5 lumbar root, mice were transcardially perfused with PBS, followed by 4% paraformaldehyde in PBS for fixation. L5 roots were collected and thin sections (1 µm) were cut and stained with Toludine blue. Entire roots were imaged and the cross-sectional diameter and area was measured with Nikon NIS-elements software. Both T3D-959 treated and saline-treated TDP-43 mice showed a reduction in the % of large caliber axons, consistent with motor neuron degeneration in the ALS model mice. However, the reduction in % of large caliber axons was similar between T3D-959-treated TDP-43 transgenic mice and saline-treated TDP-43 transgenic mice.

We did not observe any improvement in histopathology phenotypes in T3D-959 treated TDP-43 transgenic mice compared to saline-treated TDP-43 transgenic mice, including motor neuron numbers (**Figure 8**), NMJ numbers (**Figure 9**), number of large caliber axons at the L5 lumbar root (**Figure 10**), or numbers of neurons in the frontal cortex. In addition to these milestone read-outs, we also developed an assay to measure the levels of neurofilament light chain (NF-L) in blood plasma from mice, as this is emerging as a valuable biomarker in ALS and related diseases. We were able to detect marked increases in NF-L levels in TDP-43 transgenic mice at one year of age, but we did not observe any difference between NF-L levels in T3D-959 treated mice vs. saline-treated TDP-43 mice (**Figure 11**). NF-L in plasma correlates with the severity of the disease process.

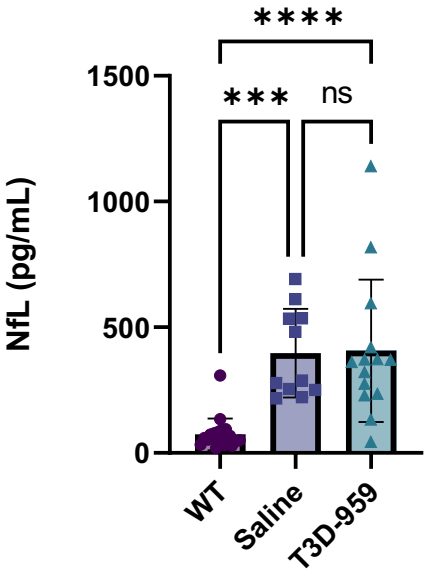


Figure 11. Neurofilament light chain (NF-L) levels in plasma are equivalently elevated in T3D-959-treated and saline-treated TDP-43 transgenic mice.

Here we see results from the terminal time point of 12 months, when the disease is most severe. T3D-959-treated TDP-43 mice have markedly elevated (abnormal) NF-L in plasma at levels comparable to saline-treated TDP-43 mice. ***$P < .001$, ****$P < .0001$, ANOVA with post-hoc Tukey test; n $\geq$ 11 mice / group.

The fourth outcome measure was molecular pathology analysis:

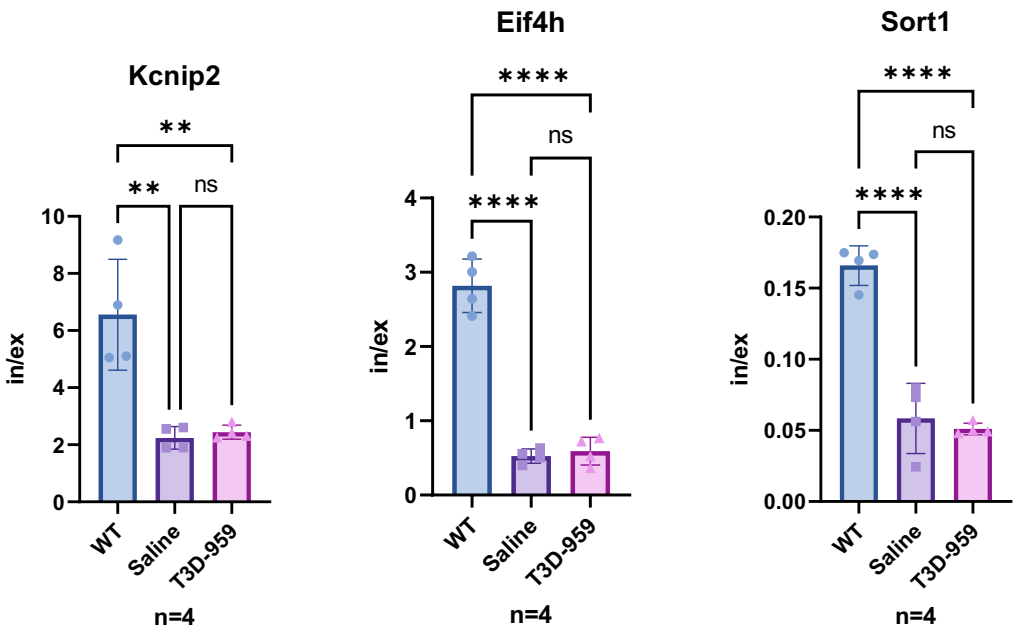


Figure 12. T3D-959-treated TDP-43 mice show no reduction of alternative splicing in cortex.

We isolated RNA from the cortex of TDP-43 transgenic mice either treated with T3D-959 or receiving vehicle (saline control) treatment, as well as from the cortex of WT control non-transgenic littermates. We performed RT-PCR on genes known to undergo aberrant alternative splicing in diseased TDP-43 mice and quantified the relative splicing of a normally included (in) exon vs. exclusion (ex) of this exon. AS shown here, aberrant alternative splicing is occurring in TDP-43 transgenic mice at the pre-terminal disease stage (one year), but this molecular pathology is not improved with T3D-959 treatment. $^{**}P < .01$, $^{****}P < .0001$, ANOVA with post-hoc Tukey test.

In TDP-43 mutant ALS mice, a hallmark molecular abnormality is altered splicing of certain genes in the cortex and spinal cord. We performed RT-PCR analysis of three genes known to undergo altered splicing in the CNS of ALS TDP-43 transgenic mice, and while we detected prominent altered splicing in the cortex (**Figure 12**) and the spinal cord (similar results; not shown) of one year-old TDP-43 transgenic mice, we did not observe any improvement in this anomalous splicing in T3D-959-treated mice compared to saline-treated mice.

Major task 5: We obtained the (G4C2)-149R transgenic mice from our collaborator, Dr. Leonard Petrucelli, and these mice arrived at our animal facility when they were 8 weeks of age to insure their survival. Due to the two-week quarantine requirement, the baseline of rotarod and grip strength of these mice were performed at 10 weeks of age and required 2 weeks to complete including comprehensive analysis of the data. Based on the baseline motor function performance, we balanced genders and randomly assigned these mice into the T3D-959-treatment group and vehicle-treatment group. We initiated Mon-Wed-Fri intraperitoneal injections of 50 mg/kg/day T3D-959 at 13 weeks of age. *As (G4C2)-149R transgenic mice do not develop appreciable disease phenotypes until 6 months of age, initiating drug dosing at 13 weeks of age still makes this study a preclinical prevention trial as proposed, because drug treatment was initiated 3 months before disease onset*.

The first outcome measure for the preclinical trial was the General Health cluster, which consisted of body weight and a composite neurological screening exam.

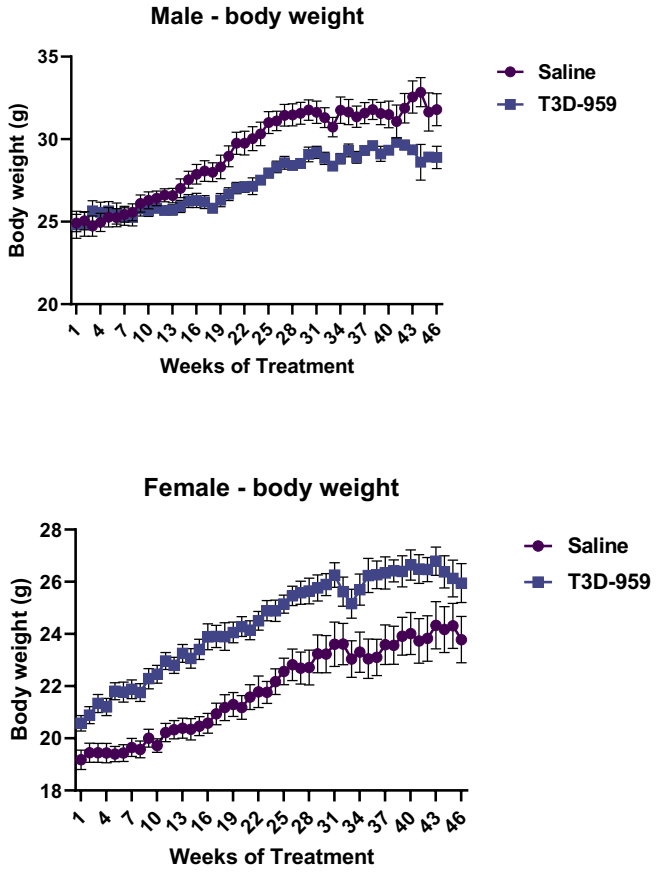


Figure 13. T3D-959 yields divergent effects on body weight in G4C2-149R C9orf72 mice

We obtained weekly body weights on G4C2-149R transgenic mice beginning at the start of treatment. Male G4C2-149R transgenic mice displayed a significant drop in body weight compared to saline-treated TDP-43 mice. Female G4C2-149R transgenic mice treated with T3D-959 experienced significant weight gain compared to saline-treated TDP-43 mice, All cohorts consisted of $n \geq 7$ mice / group. In each case, saline-treated weighed about the same as control mice, which for this experiment were G4C2-2R transgenic mice (that is, mice which express a normal length G4C2 repeat).

We also performed a composite neurological phenotyping examination, consisting of evaluations for clasping, balance, kyphosis, and gait. We documented worsening neurological function in G4C2-194R transgenic mice compared to the G4C2-2R mice as they aged, and while we did not observe any improvement in neurological function in female G4C2-149R mice treated with T3D-959, we did observe improved neurological function in G4C2-149R male mice treated with T3D-959 after disease onset (**Figure 14**).

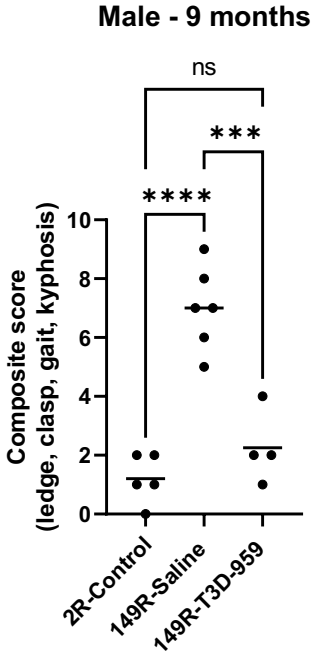


Figure 14. T3D-959 treatment ameliorates neurological dysfunction in male G4C2-149R C9orf72 ALS mice

We performed the ledge test, hindlimb clasping analysis, gait test, and kyphosis evaluation, as have previously previously. These exams are scored from 0 (absence of relevant phenotype) to 3 (severe phenotype). As shown here, T3D-959 treated male mice at 9 months of age were much improved compared to saline-treated G4C2-149R mice, with neurological function scores comparable to control G4C2-2R mice. ***$P < .001$, ****$P < .0001$, ANOVA with post-hoc Tukey test; n $\geq$ 4 mice / group.

The second outcome measure was neuromuscular and cognitive function. We did not observe any effect of T3D-959 treatment on rotarod performance, the open field test, or the two-paw and four-paw grip strength tests in both male and female G4C2-149R mice in comparison to saline-treated G4C2-149R mice. To evaluate cognitive function, we performed the contextual fear test on T3D-959 and saline-treated G4C2-149R mice, and we observed a modest improvement in the freezing time performance of aged T3D-959-treated male G4C2-149R mice (**Figure 15**), but not in T3D-959-treated female mice in comparison to saline-treated G4C2-149R mice.

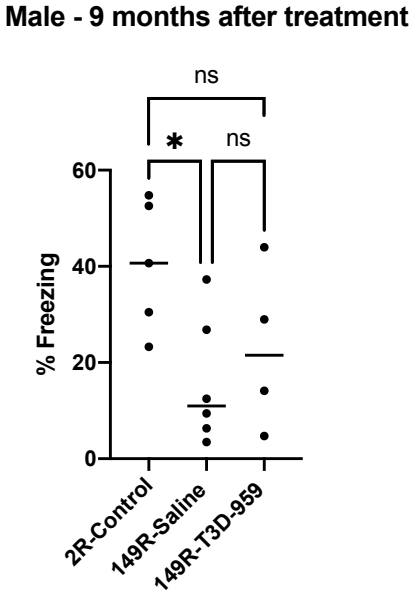


Figure 15. T3D-959 treatment mildly improves cognitive function in male G4C2-149R C9orf72 ALS mice

We performed the contextual fear conditioning test of 9 month-old male G4C2-149R mice and control G4C2-2R mice, monitoring % freezing. While saline-treated G4C2-149R mice performed worse than the 2R controls, T3D-959-treated G4C2-149R mice performed comparably to the 2R controls. However, there was no statistical difference between T3D-959- and saline-treated G4C2-149R mice, indicating that this is a very modest effect at best. *$P < .05$, ANOVA with post-hoc Tukey test; n $\geq$ 4 mice / group.

The third and fourth outcome measures were histopathology and molecular pathology analysis:

We did not observe any improvements in either motor neuron survival or NMJ numbers in T3D-959 treated G4C2-149R transgenic mice compared to saline-treated G4C2-149R transgenic mice. As G4C2-149R mice display accumulation of sense and antisense RNA foci and develop phospho-TDP-43 accumulation akin to the pathological situation in human C9orf72 ALS patients, we had also proposed to evaluate these outcome measures as part of the preclinical trial. Surprisingly, we actually observed increased RNA expression of the 149R G4C2 repeat sequence in the cortex of T3D-959-treated mice and also detected increased levels of the poly(GP) dipeptide in the cortex of T3D-959-treated mice (**Figure 16**). This was an entirely unexpected result and somewhat concerning, as accumulation of mutant RNA and dipeptide protein typically correlates with worse disease.

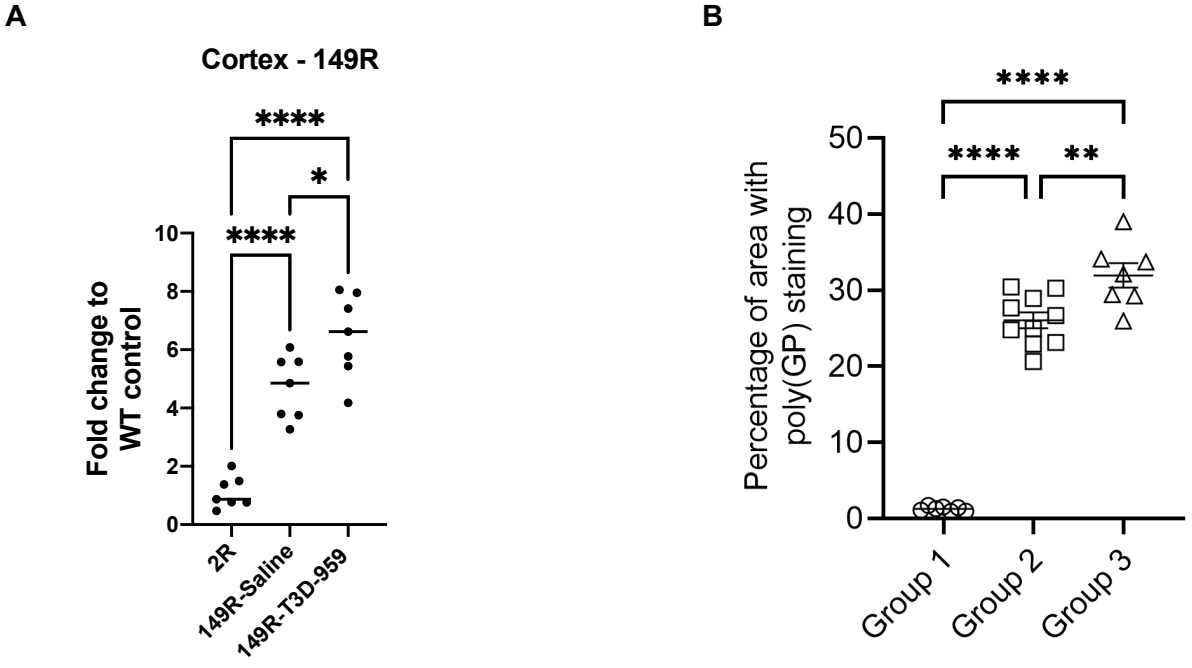


Figure 16. T3D-959 treatment of G4C2-149R transgenic mice yields increased G4C2-149R repeat expression and correspondingly increased poly(GP) dipeptide in the cortex

A) We measured the mRNA expression levels of exogenous AAV-derived G4C2-containing transcripts in the cortex of 9 month-old G4C2-149R mice by performing qRT-PCR on isolated RNAs, and noted markedly increased levels of the expanded repeat transcript in T3D-959-traeted mice compared to saline-treated mice. B) We performed immunostaining with an anti-poly(GP) antibody and determined the % area of sections immune-reactive for the antibody staining for the 2R control (Group 1), 149R saline-treated mice (Group 2), and 149R T3D-959-treated mice (Group 3) at 9 months of age. Poly(GP) accumulation in T3D-959-treated G4C2-149R mice was dramatically increased. *$P < .05$, **$P < .01$, ****$P < .0001$, ANOVA with post-hoc Tukey test. $n \geq 7$ mice / group. Error bars = s.e.m.

Our final outcome measure was quantification of phospho-TDP-43 (pTDP-43) accumulation in the cortex of G4C2-149R mice treated with T3D-959 or with saline. We observed a substantial accumulation of pTDP-43 in G4C2-149R compared to control mice (for whom pTDP-43 was undetectable); however, T3D-959-treated G4C2-149R mice and saline-treated G4C2-149R displayed similar amounts of pTDP-43 accumulation in the cortex at 9 months of age.

4) Other achievements

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to report

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

The PI has presented the preclinical trial work to different ALS patient groups, including 'I AM ALS' and the leadership team at the Johns Hopkins Packard Center for ALS Research.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

This study evaluated the potential utility of a novel drug therapy as a treatment for ALS. The results of the project do not support further research on PPAR-delta agonists as likely treatments for ALS, although many caveats remain, due to the uncertainty surrounding the predictive value of the two mouse models used in this project.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

One important development which can be used by others in the field is the blood plasma assay to measure Neurofilament light chain (NF-L), which is being evaluated as a potential biomarker in human ALS patients. The fact that the blood plasma NF-L assay worked in mice means that it could be used in future preclinical trials.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report

5. CHANGES/PROBLEMS: *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Nothing to report

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

No journal publications, conference papers or presentations, websites, novel technologies, patents, licenses, etc. were generated from this project at the time of preparing this report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

1. Albert La Spada, M.D., Ph.D. Role: PI
   Person months worked: 5.5

   Dr. La Spada supervised the project, directing the work of one research faculty member, one postdoctoral fellows, and two research technicians

2. Chenchen Niu, M.D., Ph.D. Role: Assistant Project Scientist
   Person months worked: 12

   Dr. Niu was responsible for the evaluation of T3D-959 neuroprotection in primary cortical neuron models of TDP-43 toxicity, and she coordinated all the preclinical trial work, beginning with the pharmacodynamics testing of T3D-959 dosage, continuing with the establishment of TDP-43 transgenic mice and (G4C2)-149 repeat transgenic mice cohorts, and proceeding through the performance of general health, behavioral testing, histopathology, and molecular pathology analysis cluster read-outs.

3. Frederic Arnold, Ph.D. Role: Postdoctoral Fellow
   Person months worked: 3.6

   Dr. Arnold performed all the studies of T3D-959 neuroprotection for the C9orf72 dipeptide repeat studies in primary cortical neurons, and he assisted Dr. Niu with setting up the cohorts for both preclinical trials.

4. Byeongga Cha, B.S. Role: Senior Research Assistant
   Person months worked: 12

   Mr. Cha was responsible for establishing all the primary cortical neuron cultures and assisted with all the neurotoxicity and functional assays performed on primary neurons. He also oversaw the management of the TDP-43 transgenic lines and the (G4C2)-149 repeat transgenic lines.

5. David Luong, B.A. Role: Laboratory Assistant
   Person months worked: 12

   Mr. Luong performed the intraperitoneal injections of drug or vehicle into mice during the preclinical trial phase of the project and worked alongside Dr. Niu in performing all general health, behavioral testing, histopathology, and molecular pathology analysis cluster read-outs.

In addition to the paid personnel in the La Spada lab, we received in-kind support from Dr. Leonard Petrucelli, whose lab provided the G4C2-2R and G4C2-149R transgenic mice for this project. We also received in-kind support from Dr. John Didsbury, CEO of T3D Therapeutics, who supplied us with quality control tested T3D-959 drug compound for the project.

8. SPECIAL REPORTING REQUIREMENTS

Not applicable to this project

9. APPENDICES

Nothing additional to report