A FRONTOTEMPORAL DEMENTIA AND MOTOR NEURON DISEASE MOUSE MODEL OF TDP-43 PROTEINOPATHY

BY

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A THESIS

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DEDICATION

To my parents who supported me throughout all my schooling and to my grandfather who passed away during this program. Experience firsthand with my grandfather having Alzheimer's disease led me to study neuroscience.

ACKNOWLEGEMENTS

I would like to thank everyone in the Gitcho lab for all their help and encouragement: Dr. Michael Gitcho, Dr. Stephani Davis, Sheed Itaman, Sam Gan, and a special thanks to Deidre Carter for always telling me to breathe when I start to stress. Without their help, hard-work, and mentorship none of this would be possible. I also would like to thank Dr. Malcolm D'Souza for getting me started in research as an undergraduate and many thanks to Dr. Melissa Harrington and Dr. Hakeem Lawal for serving on my committee along with Dr. Michael Gitcho and Dr. Malcolm D'Souza.

ABSTRACT

Motor neuron disease (MND), of which amyotrophic lateral sclerosis (ALS) is one example is a neurological disorder that involves the progressive loss of motor neurons. The death of these motor neurons leads to a loss of voluntary muscle control that can affect speaking, walking, breathing, and swallowing leading eventually to death. There is currently no cure for ALS therefore understanding mechanisms associated with the progression of this disease is crucial to developing a treatment. Transactive response DNA binding protein of 43kda (TDP-43) functions as a heterogeneous nuclear ribonucleoprotein (hnRNP) and is the major pathological protein in frontotemporal dementia (FTD, 50%) and sporadic ALS (95%). Mutations in TDP-43 account for ~5% of familial ALS cases. In a subset of cases with TDP-43 proteinopathy, patients clinically display both frontotemporal temporal dementia and motor deficits (FTD-MND). We have characterized an age-dependent decrease in motor function associated with pathological changes in mice selectively driving TDP-43 expression in the spinal cord and brain using a neuronal-specific (Prion) driver. Expression of a nuclear localization defective (ΔNLS) TDP-43 mutant in spinal cord and brain showed severe motor deficit and significant changes in anxiety in 16-18 month old mice. Although there was a relatively late onset of symptoms compared to the human disease, that may be related to the low level of exogenous TDP-43 expression, these models may provide a better understanding of TDP-43 proteinopathies leading to the development of therapeutics to target these devastating diseases.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ALS	Amyotrophic lateral sclerosis
AMPk-α1	AMP-activated protein kinase
ANG	Angiogenin, Ribonuclease, RNase A Family 5
EM	Electron microscopy
FTLD	Frontotemporal Lobar dementia
FTLD-TDP	FTLD with TDP-43 pathology
FTLD-U	FTLD with ubiquitin inclusions
FUS	Fusion of sarcoma
H&E	Hematoxylin and eosin
IHC	Immunohistochemistry
MND	Motor Neuron Disease
NLS	Nuclear localization signal
OPTN	optineurin
pTDP-43	Phosphorylated TDP-43
SOD1	Cu/Zn superoxide dismutase
TDP-43	Trans-response DNA binding protein 43kDa
WT	Wild-type

CHAPTER 1

INTRODUCTION

Motor Neuron Disease (MND) is a progressive neurodegenerative disease causing muscle weakness and a loss of voluntary muscle movement such as walking and swallowing due to degeneration of motor neurons in the brain and spinal cord. This greatly impacts a patient's quality of life as the disease progresses resulting in death. Approximately 90-95% of MND cases are sporadic and 5-10% familial however the cases are clinically indistinguishable. MND also includes and immunological response that includes microglia activation especially in the motor cortex in humans, leukocytes migrating to the inflamed areas, blood stream immunological changes, and reactive astrocytes/astrogliosis.[1–3]In frontotemporal lobar degeneration (FTLD) there are language and behavioral changes[4]. In MND, TDP-43 pathology is found in ~95% of cases while familial mutations in TDP-43 account for ~5% of cases [5–7]. TAR DNA-binding protein 43 (TDP-43) is the major pathological proteins found in inclusions in sporadic and familial ALS and FTLD [8]. Histopathological studies in MND demonstrate the clearance of TDP-43 from the nucleus as an early event[9]. In 95% MND and ~50% FTLD cases tested, phosphorylated TDP-43 ubiquitinated neuronal cytoplasmic and intranuclear inclusions are present[10–12]. FTLD can also have two distinct subtypes, FTLD with MND and FTLD without MND, also called FTLD-U and FTLD-TDP. Each of which involve severe frontotemporal neuronal loss and gliosis[13].

In mice, overexpression of human TDP-43 is sufficient to cause neurodegeneration, motor dysfunction, paralysis, and death associated with MND [14]. Overexpression of human TDP-43 aggregates in the cytoplasm in mouse neurons and induces neuronal death [15]. Neuronal

overexpression of TDP-43 in mice has been shown to be toxic and TDP-43 knockout mice are embryotic lethal[14,16]. Defects in the nuclear trafficking of TDP-43 leads to disease like, MND and FTLD, redistribution of TDP-43 into insoluble cytoplasmic and nuclear aggregates made up of fragments of TDP-43 [17,18]. TDP-43 fragments, 25kDa TDP-43 fragment and the 35kDa, are possibly intermediate steps in degradation process of TDP-43[18].

Behavioral testing, rotarod and open field, will be conducted to determine motor impairment, stereotyped behavior, and anxiety of the mouse strains consistent with MND and FTLD. Muscle coordination and impairment was determined using an accelerating rotating rod that the mice are trained to walk on. The open field test examines exploratory behavior through recording a mouse in a novel enclosed environment. Exploratory behaviors occur when a mouse enters a novel environment because mice explore new environments through activity and rearing behaviors. Stereotyped behaviors are defined as any repetitive behavior with with no apparent purpose.

We are utilizing an inducible doxycycline expression system (Prion-tTA) to selectively drive mutant TDP-43 A315T and TDP-43 ΔNLS in the spinal cord and brain. This system of low spinal cord expression compared with other models of high toxic expression allows for age-dependent characterization in both motor function and anxiety similar to those with MND and FTLD-MND.[19,20]. This study characterizes motor function (rotorarod) and anxiety (open field) associated with pathological TDP-43 expression in the brain and spinal cord. These mice exhibit phosphorylated TDP-43 pathology in the spinal cord and brain and muscle atrophy similar to human cases of TDP-43 proteinopathy. In addition we examine the ultra-structure of the spinal cord.

CHAPTER 2

LITERATURE REVIEW

ALS/MND and FTLD

Amyotrophic lateral sclerosis (ALS)/motor neuron disease (MND), also called Lou Gehrig's disease, are fatal neurodegenerative disease where there is a loss of voluntary motor control through the progressive degeneration of motor neurons [21]. Amyotrophic refers to the muscle weakness, atrophy, and loss of motor control in the lower motor neurons[21]. Lateral sclerosis part of the disease refers to the gliosis that occurs after the degeneration of the corticospinal tract that causes the spinal tract to be hardened lateral columns [21]. The symptoms patients present with for diagnosis are mainly weakness. There are different variants of motor neuron disease, when the lower motor neurons are only affected the disease is primary spinal muscular atrophy and when the upper motor neurons are only affected the disease is primary lateral sclerosis [21]. ALS is characterized by both upper and lower motor neuron involvement.

Familial mutations account for 5-10% of ALS. Affected genes include SOD1(Cu/Zn superoxide dismutase), TDP-43, FUS (fusion of sarcoma), ANG (angiogenin, Ribonuclease, RNase A Family 5), and OPTN (optineurin) which have been used as mouse models for ALS [6,22,24–27] SOD1 mutations account for 20% of familiar ALS cases[27]. However ~90-95% of ALS is considered sporadic. Sporadic ALS is clinically indistinguishable from familial ALS[28–30]. The disease can manifest with dysregurlation of axonal transport before ALS

symptoms occur. Once the symptoms appear, pathology is abundant and degeneration has progressed [31]. There is an immunological reaction, which will be discussed more in the microglia section, in the brains and spinal cords of ALS patients[1].

ALS presents many problems when it comes to clinical trials and mouse models because many times the cause of ALS is unknown in a patient's case. Therefore many drugs that had positive preclinical results in rodents failed in human clinical trials. Therefore more knowledge is needed about the disease mechanisms of ALS, biomarkers, and early signs of the disease for early intervention[32].

FTLD (Frontotemporal lobar dementia) is a neurodegenerative disorder where there is progressive neuronal death in the frontotemporal lobar region of the brain. FTLD differs from Alzheimer's disease because a large portion of FTLD patients have abnormal behavior specifically with language, social interaction, and emotional blunting[33] . There are heterogeneous pathologies found in FTLD patients, FTLD and FTLD-TDP (ubiquitin inclusions) [34,35]. The ubiquitin inclusions largely have TDP-43 pathology, phosphorylated TDP-43, that is consistent with ALS/MND pathology[11,36]. Therefore FTLD is said to form a spectrum with ALS due to TDP-43 pathology and the term FTLD-TDP is being implemented to describe FTLD with TDP-43 pathology [37–39]. Also in FTLD it has been determined that TDP-43 mutations can cause FTLD without MND/ALS where previously it was believed that TDP-43 mutations will cause MND/ALS[40].

Astrocytes and Microglia

ALS and FTLD disease also involves astrocytes and microglia. Astrocytes are star shaped glial cells that are associated with synapses. Astrocytes can be come activated in response to

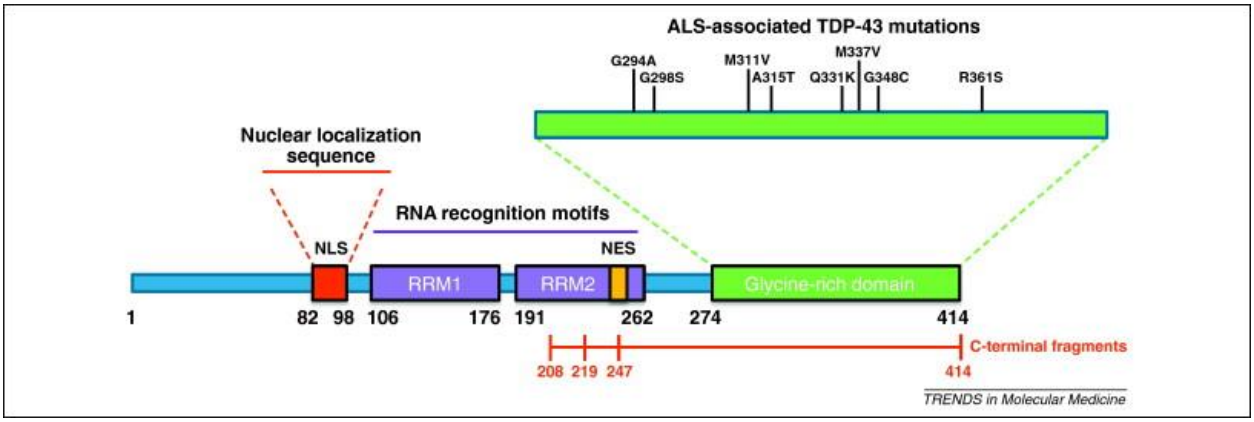
neurodegenerative diseases[41]. In FTLD it has been found that astrocyte apoptosis increases as disease severity increases[42]. Sporadic human ALS astrocytes secrete neurotoxic factors that are toxic to motor neurons [43,44]. It was found that the driving force behind astrocytes and the selective death of motor neurons is through necroptosis[26]. Recently it was also found that astrocytes from ALS patients reduce expression of major histocompatibility complex class 1 on motor neurons. This reduction causes the motor neurons to be more vulnerable to astrocyte induced death[45]. Reactive astrocytes from many different neurodegenerative disorders, including ALS and FTLD, can release lipocalin 2 which is selectively toxic to neurons thus promoting neuronal death[46]. Therefore a theory is that astrocytes, which normally protect motor neurons, are involved in the degeneration of motor neurons in the disease progression of sporadic ALS.

Microglia are activated during ALS as seen through the immunological reactions and microglia activation [1,2,47]. It has been found that TDP-43 is able to activate microglia through the NF-κB and NLRP3 inflammasome, which are cytoplasmic protein complexes that are responsible for the activation of inflammatory proteins[47]. The NF-κB pathway also when activated has also been shown in ALS to induce death of moto neurons through microglia[48]. Therefore microglia which is a part of neuroinflammation plays an important role in ALS disease progression. Microglia activation in neurodegenerative dementia, FTLD and Alzheimer's disease, has also been shown and can be used to predict disease severity and distribution[49]. Microglia are also activated in specific patterns in FTLD that are different then control[50].

TDP-43

TDP-43 (TAR DNA-binding protein 43kDa) is the major pathological protein found in ALS (95% of cases) and FTLD-TDP (50% of total FTLD cases) [10–12]. TDP-43 is a heterogeneous

nuclear ribonucleoproteins (hnRNPs) that is involved in RNA splicing, exon skipping, RNA stability, RNA transport, and other cellular functions[51,52]. TDP-43 is encoded by the gene TARDBP in humans.TDP-43 is composed of two RNA recognition motifs, nuclear localization signal (NLS), nuclear export signal, and a C-terminal glycine rich region [17,51–54]. TDP-43 is typically a nuclear protein. Pathological TDP-43 typically leaves the nucleus and form cytoplasmic insoluble ubiquitinated aggregates that include phosphorylated TDP-43[11,15,17,55–58].



TDP-43 Structure. **Reprinted from "TDP-43 functions and pathogenic mechanisms implicated in TDP-43 proteinopathies"** [59]

Increases in mislocalized cytoplasmic TDP-43, which is in the cytoplasm instead of the nucleaus, is toxic to neurons and this effect is enhanced by the familial A315T mutation[60]. It has been shown that chaperone protein HSB8 reduces truncated cytoplasmic TDP-43 and thus reduces TDP-43 toxicity [61]. Also that AMPk-α1 is activated for the mislocalization of TDP-43 in ALS patients[23]. TDP-43 also can have a prion like effect *in vitro* with the ability to once pathological seed cells and propagate phosphorylated pathological TDP-43 using lysates from ALS patients to seed cell cultures[62]. When ubiquitinated TDP-43 is degraded 25kDa and 35kDa fragments are produced that may reach a certain threshold before forming insoluble aggregates in the cytoplasm that also include full length TDP-43 [18].

Not all TDP-43 pathology is the same and recently it has been found that rare TDP-43 mutations can also cause abnormal RNA splicing and/or loss of function for other RNAs in ALS that may contribute to ALS without TDP-43 aggregates being produced unlike a majority of ALS cases [63] Also there in some evidence that TDP-43 is not the ubiquitinated target in TDP-43 inclusions instead other proteins that are apart of the inclusion are ubiquitinated but TDP-43 may not be is no ubiquitinated[64] . TDP-43 is essential and knockout of TDP-43 is embryonically lethal[16,65].

Mouse Models of ALS/MND and FTLD-TDP

There are many different approaches for creating a mouse model of ALS. Many use the discovered familial mutations to create a transgenic mouse with that mutation however only 5-10% of ALS cases are caused by familial mutations. There are limitations with these mouse models because the mutation for familial ALS in humans does not always show pathology in mice. For example an SOD1 mutation mouse line was created that has none of the TDP-43 abnormalities seen in ALS patients with the SOD1 mutations[24]. Other mouse lines developed overexpress TDP-43. One such line overexpressed human wild typeTDP-43 using a Thy-1, cell surface antigen protein that is brain specific, promoter which is expressed in all neurons in the central nervous system. These two TDP-43 lines expressed 2 fold and 1.2 fold. Both had impaired limb reflex and decreased stride with symptoms at 2 months of age. The life span of these lines were 24 days (2x expression of human wild-type TDP-43) and 6.7 months (1.2x expression of human wild-type TDP-43)[14]. An FTLD-TDP model overexpressed TDP-43 in the forebrain, consistent with where disease pathology is patients is found, found the elevated levels of TDP-43 caused motor dysfunction, impaired learning/memory, and hippocampal atrophy at 6

months. The increased TDP-43 level in the forebrain also produced TDP-43 ubiquitin positive aggregates [66].

Another mouse ALS and FTLD mouse line disrupted the nuclear localization signal of TDP-43. Under the CamK2α promoter the mice showed motor impairment after several months and neuronal loss in the hippocampus [15]. Different TDP-43ΔNLS mouse line, using a NEFH (Neruofilament heavy) promoter, was able to have a functional recovery through the clearance of TDP-43 from the cytoplasm. This mouse line had a survival time of an average of 10 weeks. They formed pTDP-43 inclusion, had motor impairment, reduced endogenous TDP-43, clearance of nuclear TDP-43, and muscle atrophy. This phenotype was able to be rescued with the reintroduction of dox shutting the TDP-43ΔNLS expression down[20]. These mouse lines for ALS and FTLD-TDP have severe symptoms beginning 6 months old and younger and generally a short life span, however, this is not consistent with the disease pathology in humans. Humans develop ALS and FTLD-TDP later in life and the disease progresses before death. This study is to determine if the mouse lines used have a similar pathology and disease progression as human ALS and FTLD-TDP.

This all links together into a group of experiments that need to be done to classify a mouse model for ALS and FTLD. First there needs to be behavioral determinations such as motor impairment, anxiety, stereotyped behavior, and exploratory behaviors. Pathologically, there should be TDP-43 pathology in the form of pTDP-43 cytoplasmic inclusions since it is the main pathological protein in ALS and in approximately half the cases of FTLD. Also because it is an ALS model the impact on motor neurons needs to be determined, degeneration and demyelination. The length of time it takes before pathology and phenotype emerges in the mouse

lines will also be important to the relevance of this model as an ALS and FTLD-TDP mouse model because these diseases occur later in life. Most mouse models show a phenotype very early and the survival of these mouse lines is approximately 6 months of age.

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Animals

Prion-tTA (Prnp-tTA, tet-off, Jackson Lab 018124), . TetO-hTDP-43-NLS line 4 (Jackson Lab 014650), TetO-hTDP-43 A315T (developed by M. Gitcho and N. Cairns, Washington University School of Medicine, St. Louis, MO) mice were maintained on a C57BL/6J X FVB mixed background for this study. TetO-hTDP-43 A315T mice were developed by PCR cloning of human TDP-43 A315T cDNA (site-directed mutagenesis, sequence verified) into the pTRE-Tight response plasmid (Clontech Laboratories, 631059) containing a tetracycline-response element and linearized (XhoI excision) for pronuclear injections at the Washington University Transgenic core facility. Transgenic mice utilized the tetracycline-controlled transactivator protein (tTA, Tet-off).

Behavioral testing group size was 11-12 month age group WT n=8, A315T n=6, and NLS n=9. 14-15 month age group WT n=9 and NLS n=9 (There was only one A315T mouse in this age group which is why the group is eliminated from the behavioral testing analysis). 16-18 month age group WT n=14, A315T n=15, and NLS n=9. Animals were not assigned to age and genotype groups until after the testing was over to blind the study. Only the animal tag number was associtated with the animal during testing.

3.2 Rotarod test

Before testing the mice were placed on the rod rotating at 4 revolutions per minute for 5min. If the mouse fell off they were put back on the rod continually until the 5min were up.

Practice/training occurred 1x per week for 2 weeks. On testing day the mice were placed on the rod which was programmed to accelerate from 4rpm to 30rpm over the course of 10min. Data recorded by apparatus was latency of falling and velocity.

3.3 Open field

Open field testing was performed using Anymaze software and apparatus, a 40cm x 40 cm square with opaque walls fit into a grey base plate. All tested mice were individually placed into the apparatus for 10 min the day before testing to familiarize them with the new environment. On testing day the mice were recorded for 10min in the open field environment. The open field was cleaned between each animal tested.

3.4 Immunohistochemical staining

Brain and spinal cord were embedded into paraffin and sections were cut at 6µm. Slides were deparaffinized and rehydrated using a protocol of xylene (4 min) and then 20 dips in each 100% EtOH 2x, 95% EtOH 2x, 70% EtOH 2x, 50% EtOH, and then DH_2O. Slides were microwaved for 4min in citrate buffer (10mM sodium citrate, 0.05% Tween 20, pH 6.0 (HCL)). For florescence slides were blocked for 1hr in 5% Bovine serum albumin and 0.3% triton x in PBS. Primary antibodies; GFAP, cell signaling, 1:1000, Iba1, 1:1000, TDP-43, N-terminal,1:1000 dilution) incubation occurred overnight at 4°C. Secondary antibody (Alexa Fluro, Invitrogen; 488nm and 555nm 1:4000 dilution) incubation occurred at room temperature for 1hr. Mounting solution was DAPI Fluromount-G (Southern Bioscience). For alkaline phosphatase detection Vectastain ABC-AP Kit (Vector) was used with a modified protocol. The protocol was modified with 1hr blocking time, overnight primary antibody(pTDP, 1:5000, cosmobio and Iba1, 1:1000 dilution, Wako), 1hr biotinylated secondary antibody, and 1hr Vectastain ABC-AP reagent. It was followed by Vector Black following its protocol of being

developed for 20 minutes in the dark (Vector). Then hematoxylin counterstain (hematoxylin, 1% acid alcohol, followed by ammonia water). The slides were then dehydrated. Dehydrating is 20 dips in 50% EtOH 2x, 70% EtOH 2x, 95% EtOH 2x, 100% EtOH 2x and then xylene. The slides are then cover slipped (peramont).

3.5 Electron Microscopy (EM) imaging

Spinal cord was removed and post fixed in 4% Paraformaldehyde (PFA) in PBS for 6 hrs then exchanged into 2% glutaraldehyde. Electron microscopy was performed by Jean Ross, Delaware Biotechnology Institute, BioImaging Center, University of Delaware, Electron Microscope Facility.

3.6 Hematoxylin and Eosin staining

Brain and muscle were embedded into paraffin and sections were cut at 6µm. Slides were deparaffinized and rehydrated using the same protocol for deparaffinization and rehydrating as IHC. Slides were placed in hematoxylin (Thermo Shandon) for 8 minutes. It was followed by being placed in 1% acid alcohol (1 drip, 1% HCL in 70%EtOH) and then ammonia water (8 dips, 3 drops of ammonia hydroxide/100mL DH_2O) for bluing. Slides were then dipped in 95% alcohol and then placed in Eosin Y(Sigma) for 3 minutes . Then the slides were rinsed in DH_2O for 5 minutes. The slides were dehydrated to xylene and cover slipped (vectamount).

3.7 Luxol Fast Blue staining

Brain and spinal cord were embedded into paraffin and sections were cut at 6µm. Slides were deparaffinized in xylene (4 min) and run down to 95% alcohol (20 dips in 100% EtOH 2x, and 95% EtOH 2x). Slides were placed in luxol fast blue (1g luxol fast blue, 1L 95% reagent alcohol, and 10% acetic acid, acos organics) solution for 2hr at 60°C. Differentiated occurred

using 95% alcohol 2 times and 70% alcohol 2 times. Slides were then counterstained in hematoxylin followed by a 1% acid alcohol (1 dip) and ammonia water (8 dips) before dehydrating to xylene and cover slipped (Fisher Permount)

3.8 Nissl staining

Spinal cords were embedded into paraffin and sections at 6µm. Slides were deparaffinized and rehydrated to water. Slides were placed into the CEV solution (5g of Cresyl Etch violet acetate, acos organics) and 1L dH_2O) for 10 min and then rinsed in dH_2O. Slides were then dehydrated to xylene and cover slipped (Fisher Permount).

3.9 Statistics analysis

Statistical analysis was performed using Analysis of variance (ANOVA) followed by Bonferroni post-hoc anaylsis or T-test using GraphPad Prisim 6.

CHAPTER 4

RESULTS

4.1 Behavioral testing

The first experiment determined was if there were any impairments in the mice lines that are similar to MD and FTLD. It was noticed that the TDP-43ΔNLS have a decreased weight compared to WT (Figure 1). Motor impairments on the rotarod are apparent through a decreased latency to fall. TheTDP-43ΔNLS have shorter latency to fall, therefore spend less time on the accelerating rotarod then WT (Figure 2 a-c). From observation certain mice, which were later determined to beTDP-43ΔNLS mice, were unable to walk on the rotarod and had a latency to fall of 0 sec. This suggests motor impairment, possible partial paralysis, in these mice which is seen in MND. The mean speed (calculated by dividing total distance traveled by time mobile) of theTDP-43ΔNLS are greater coupled with the increase in rotations in the same age group (Figure 2 d-f,3 a-c). Anymaze software calculates rotations as when the animal completes a full 360° rotation using the body as a center of origin and the head as a vector. The rotation only accumulates as long as the direction is the same way from start to 360°. This suggests the rotations are connected to the speed of the mice and the increased rotations are increasing the speed recorded during the open field. Therefore the rotations that are being exhibited may have an influence on other motor markers that were recorded during open field testing. The mean speed was unchanged in the other two age groups which suggests the mice do not have motor impairment walking on a flat surface. Track plots of the 11-12 month age group mice during the open field at the 2 minute time point show the rotational pattern the TDP-43ΔNLS mice are exhibiting (figure 4c).

TheTDP-43ΔNLS mice displayed characteristics consistent with anxiety and anti-exploratory behavior. Time freezing is the measurement of how long the mouse during the open field spent completely stationary. The amount of time freezing was significantly increased in the 14-15 month and 16-18 month age groups (Figure 3). Rearing time active is the total measurement of the mouse when its body/head rises above the laser beam set for the level of adult mice in a non-rearing position during the open field test. TheTDP-43ΔNLS mice show decreased rearing

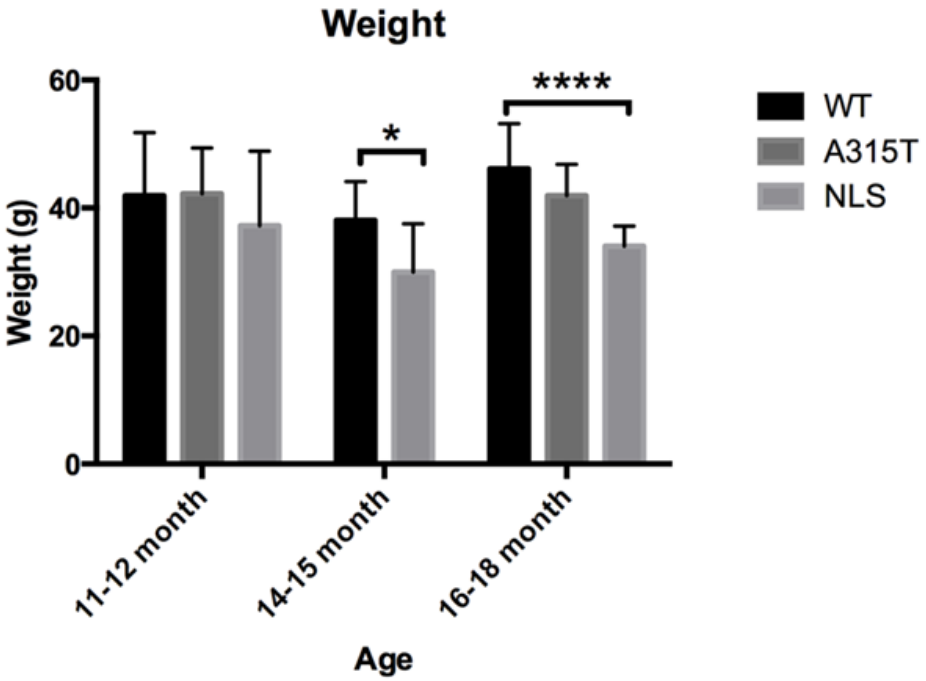


Figure 1 Weight change in TDP-43ΔNLS. The TDP-43ΔNLS mice have a reduced weight in the 14-15 month and 16-18 month age groups. Unpaired T-test (14-15 month) and One way ANOVA Bonferroni post hoc (16-18month).*= p <0.05 **** = p value < 0.0001

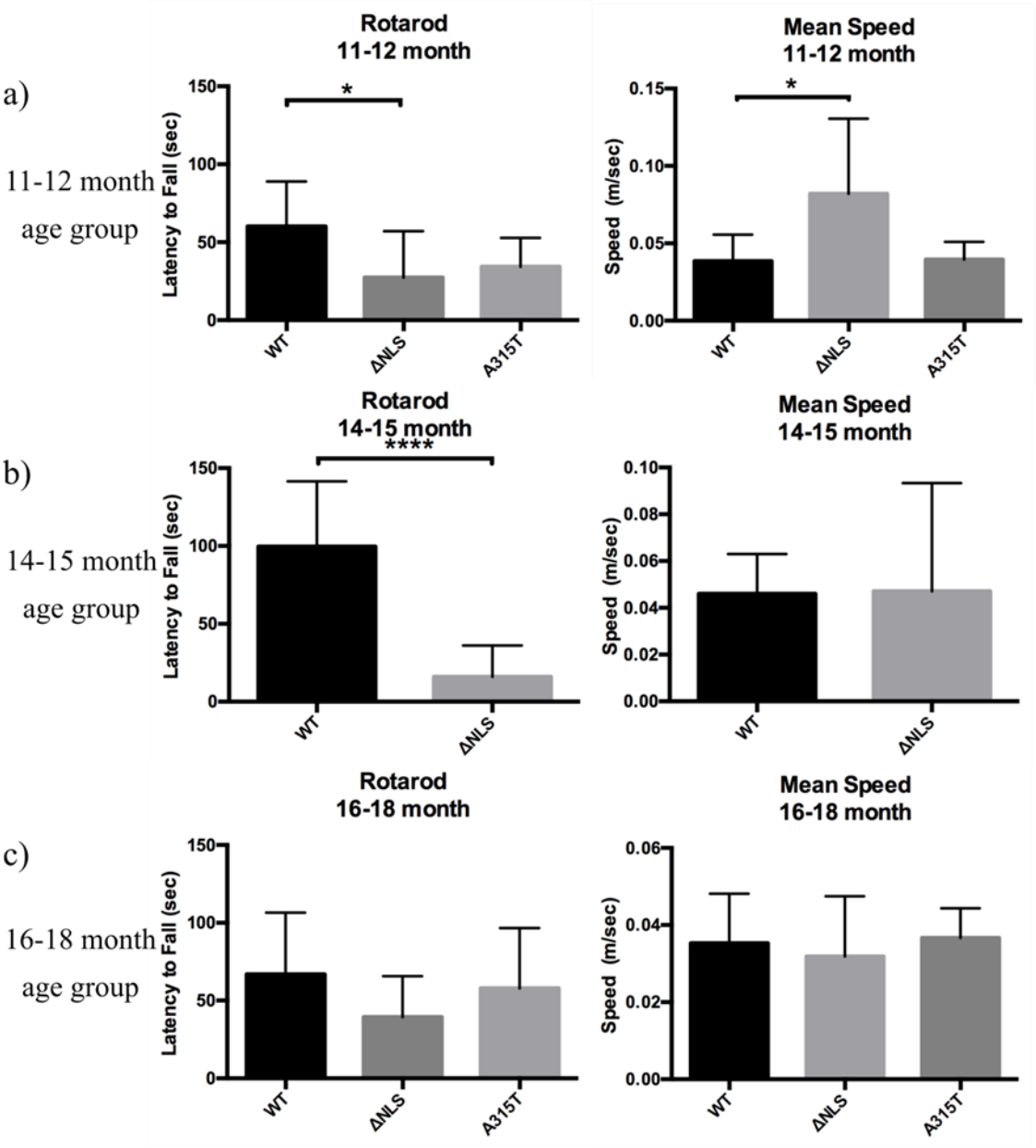


Figure 2 Impaired motor skills in TDP-43ΔNLS a) Reduced latency to fall on accelerating rotarod for TDP-43ΔNLS and increased mean speed in open field in 11-12 month age group one way ANOVA Bonferroni post-hoc b) Reduced latency to fall on accelerating rotarod in the TDP-43ΔNLS 14-15 month age group. Unpaired t test. c) 16-18 month latency to fall on accelerating rotarod one way ANOVA Bonferroni post hoc. *= p <0.05 ** = p value < 0.005 **** = p value < 0.0001

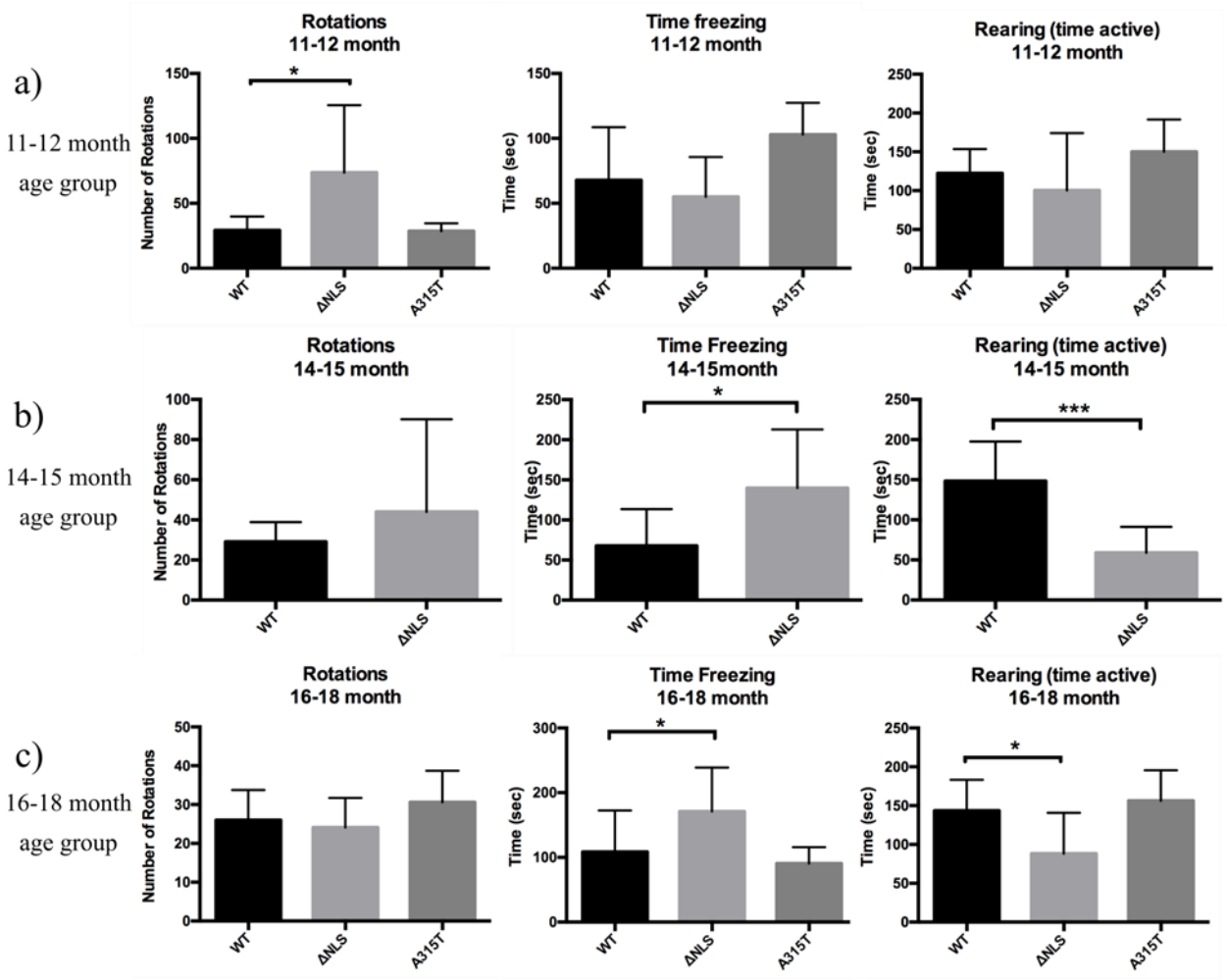


Figure 3 Anxiety and anti-exploratory behavior in TDP-43ΔNLS during open field a) Increased rotations (circling) inTDP-43ΔNLS 11-12 month. One way ANOVA Bonferroni post-hoc b) Increased time spent freezing in the TDP-43ΔNLS 14-15 month age group and decreased rearing time during open field in the TDPΔNLS 14-15 month age group. Unpaired t-test c) Increased time spent freezing in the TDP-43ΔNLS 16-18 month age group and decreased rearing during open field in TDP-43ΔNLS 16-18 month age group. *p <0.05 ***p value < 0.0005

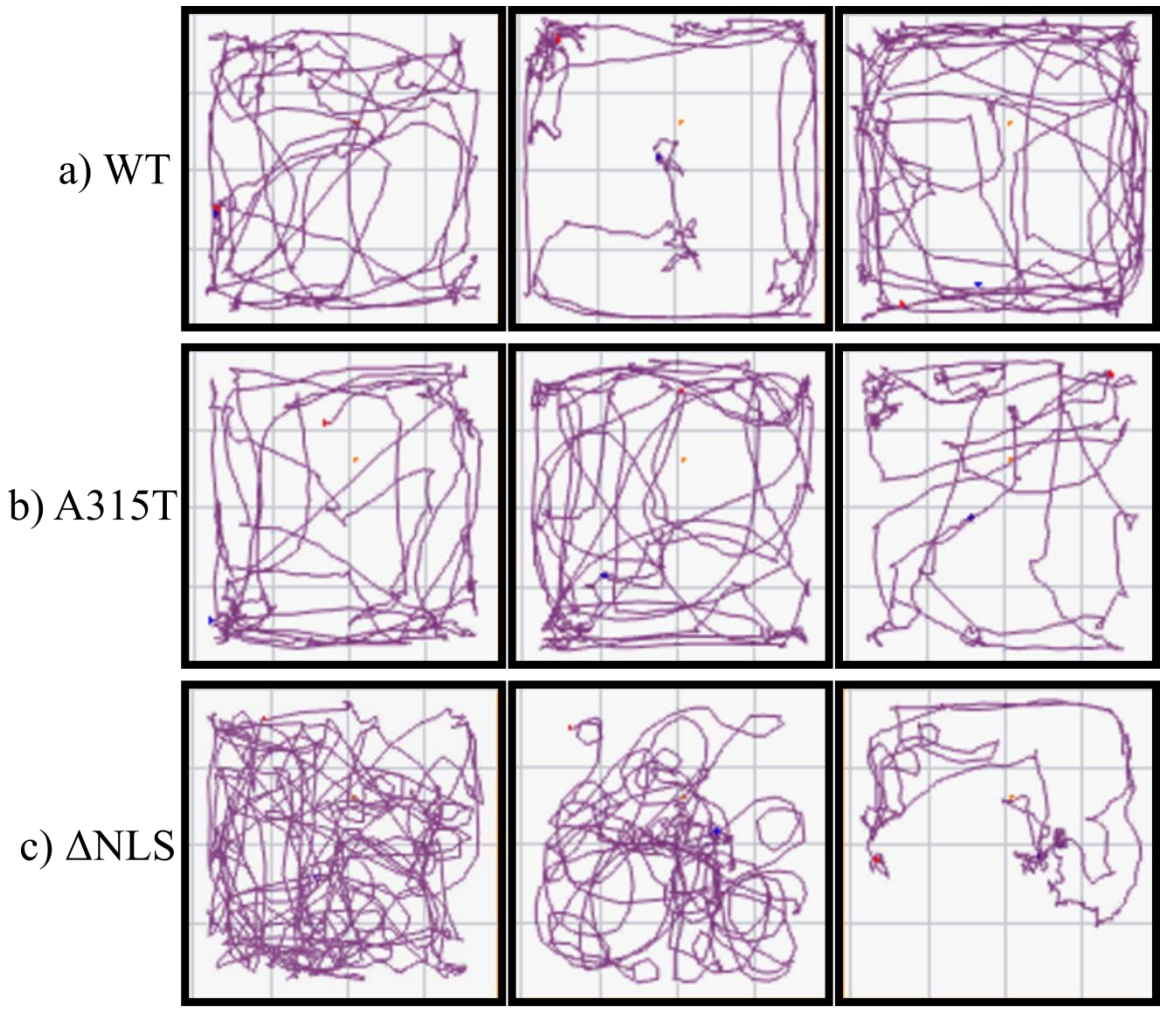


Figure 4. Track plot of 11-12 month age group showing increased rotations in TDP-43ΔNLS mice. a)WT b)A315T c)TDP-43ΔNLS Track plots during the open field at 2 min time point during testing**.**

4.2 Change in muscle morphology inTDP-43ΔNLS mice

The muscle cross sections stained with H&E show skeletal muscle hypertrophy in theTDP-43ΔNLS mice in both age groups 14-15 month and 16-18 month (Figure 5c,f). The myofibers increased in diameter and the spaces between the myofibers is reduced changing the

morphology of the myofibers in theTDP-43ΔNLS mice compared to its control littermates. A315T muscle cross sections have centralized nuclei. Muscle atrophy or grouping was not seen in the TDP-43 ΔNLS.

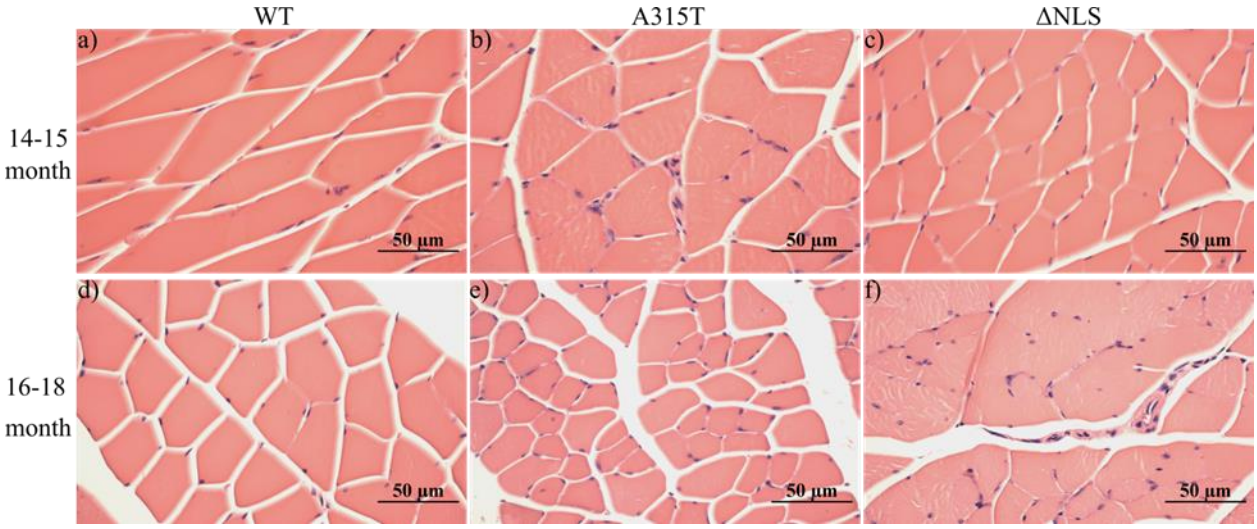


Figure 5.Skeletal muscle hypertrophy in TDP-43ΔNLS leg muscle cross sections H&E staining a) 14-15 month WT (control) b) 14-15 month A315T c) 14-15 month TDP-43ΔNLS d) 16-18 month WT (control) H&E e) 16-18 month A315T H&E with central nuclei f) 16-18 month TDP-43ΔNLS H&E Muslce sections are from the gastronemous muscle and n=1 for each genotype in each age group.4.3 Loss and inclusions in the dentate gyrus

4.3 Loss and Inclusions in the dentate gyrus

The dentate gyrus stained with luxol fast blue (myelin stain) has a loss of myelin in the TDP-43ΔNLS line 16-18 month (figure 6c,f). The morphology of the dentate gyrus appears to be changed in the A315T andTDP-43ΔNLS. This could be due to loss of neurons, demyelination of neurons, or a characteristic of the transgenic mouse line. Loss of neurons can be seen in the pTDP-43 and hematoxylin (nuclear) staining at low magnification along with pTDP-43 inclusions at higher magnification. The pTDP-43 is clearly in the cytoplasm of cells in the dentate gyrus and may be a contributing factor to the loss of cells. There is also an increase in microglia in the dentate gyrus (figure 7c) in theTDP-43ΔNLS mice then in control and A315T. TheTDP-43ΔNLS 16-18 month age group showed cytoplasmic inclusions in the dentate gyrus.

These cytoplasmic inclusions are pathologically similar to MND and FTLD with TDP-43 inclusions. The disease pathology includes hyper-phosphorylated TDP-43 aggregated in cytoplasmic and nuclear inclusions.

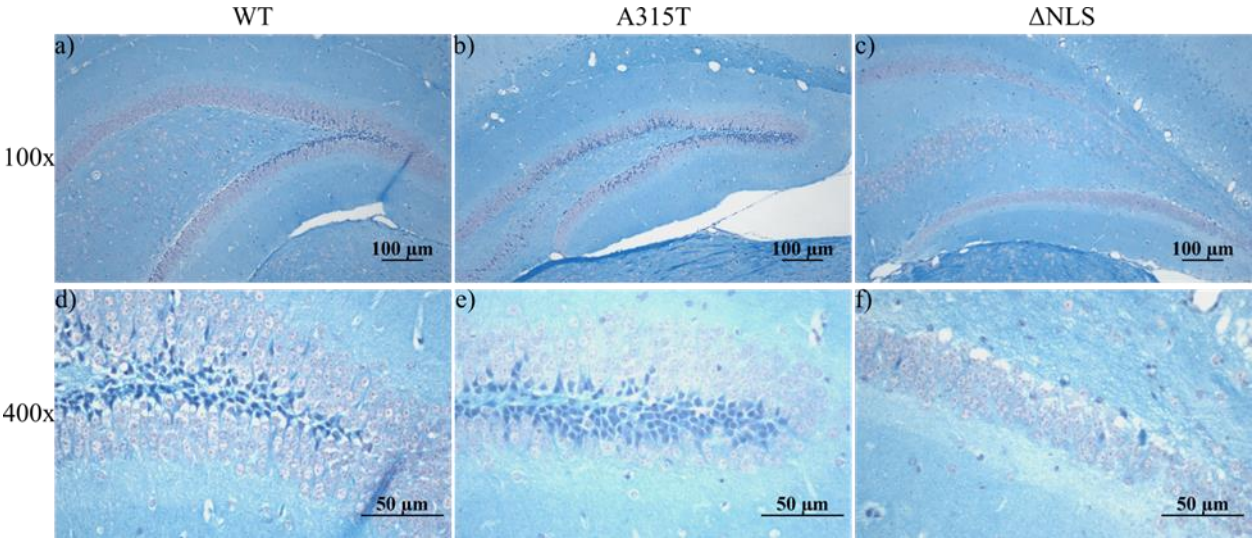


Figure 6 Myelin degeneration and neuronal loss in dentate gyrus of 16-18 month age group a-c) 100x WT, A315T, and TDP-43ΔNLS respectively. d-f) 400x WT, A315T, and TDP-43ΔNLS respectively n=1 for each genotype in each age group.

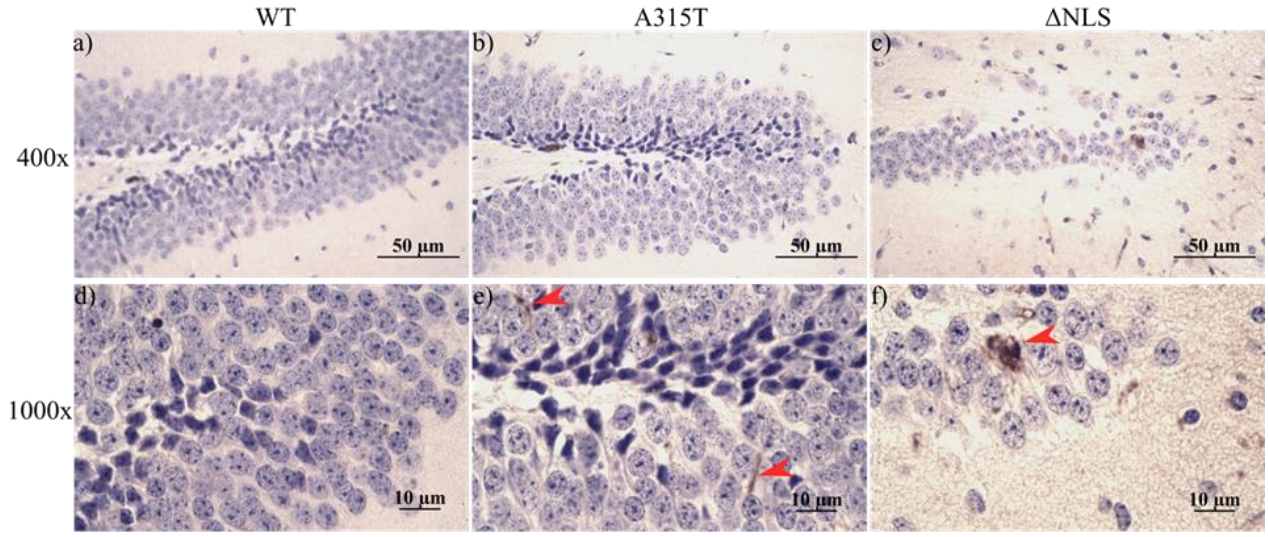


Figure 7 pTDP-43 inclusions in the dentate gyrus a-c)40x dentate gyrus with pTDP-43 (brown) and hematoxylin (purple, nuclear). d-f) 100x dentate gyrus A315T has small pTDP-43 cytoplasmic inclusions and TDP-43ΔNLS has large pTDP-43 cytoplasmic inclusion. Arrows point to pTDP-43 cytoplasmic inclusions n=1 for each genotype in each age group..

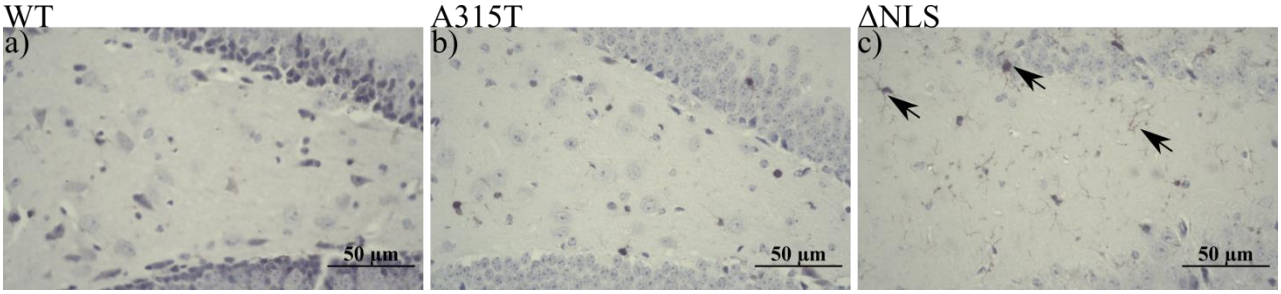


Figure 8 Iba1 is increased in TDP-43ΔNLS 16-18 month age group a) WT b) A315T c)TDP-43ΔNLS Iba1 (dark brown) and hematoxylin(purple, nuclear) arrows point to Iba1 (micorglia). 400x magnification n=1 for each genotype in each age group..

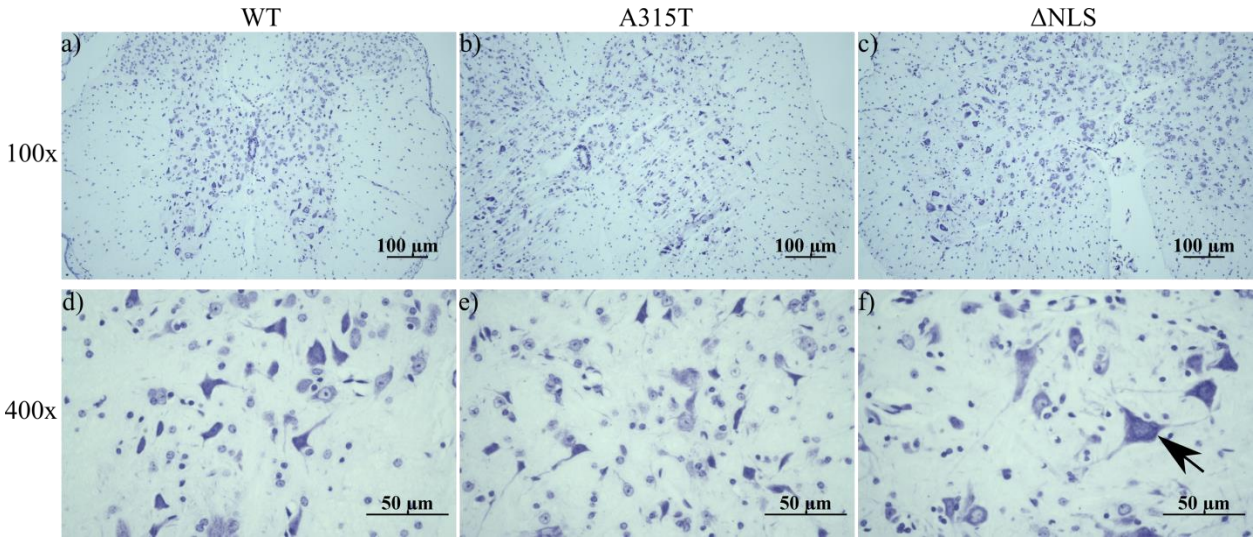


Figure 9 Nissl staining shows spinal cord motor neuron changes of the 16-18 month age group. a,d) WT b,e) A315T c,f) TDP-43ΔNLS Neruons appear Dark purple and nuclei appear light purple. Arrow points to a motor neuron. n=1 for each genotype in each age group..

4.4 GFAP andTDP-43 immunofluorescence

Immunofluorescence of dentate gyrus of 16-18 month age group for GFAP (Glial fibrillary acidic protein), TDP-43, and Dapi (nuclear) show inTDP-43ΔNLS mice the TDP-43 is cleared out of the nucleus and is cytoplasmic (figure 10c). This is similar to one of the early stages of ALS where nuclear TDP-43 leaves the nucleus and becomes aggregated in the cytoplasm. Astrocyte activation inTDP-43ΔNLS is occurring in the dentate gyrus and throughout the entire brain. TheTDP-43ΔNLS mice brains have reactive astrocytes in response to the TDP-43 pathology (figure 10c).

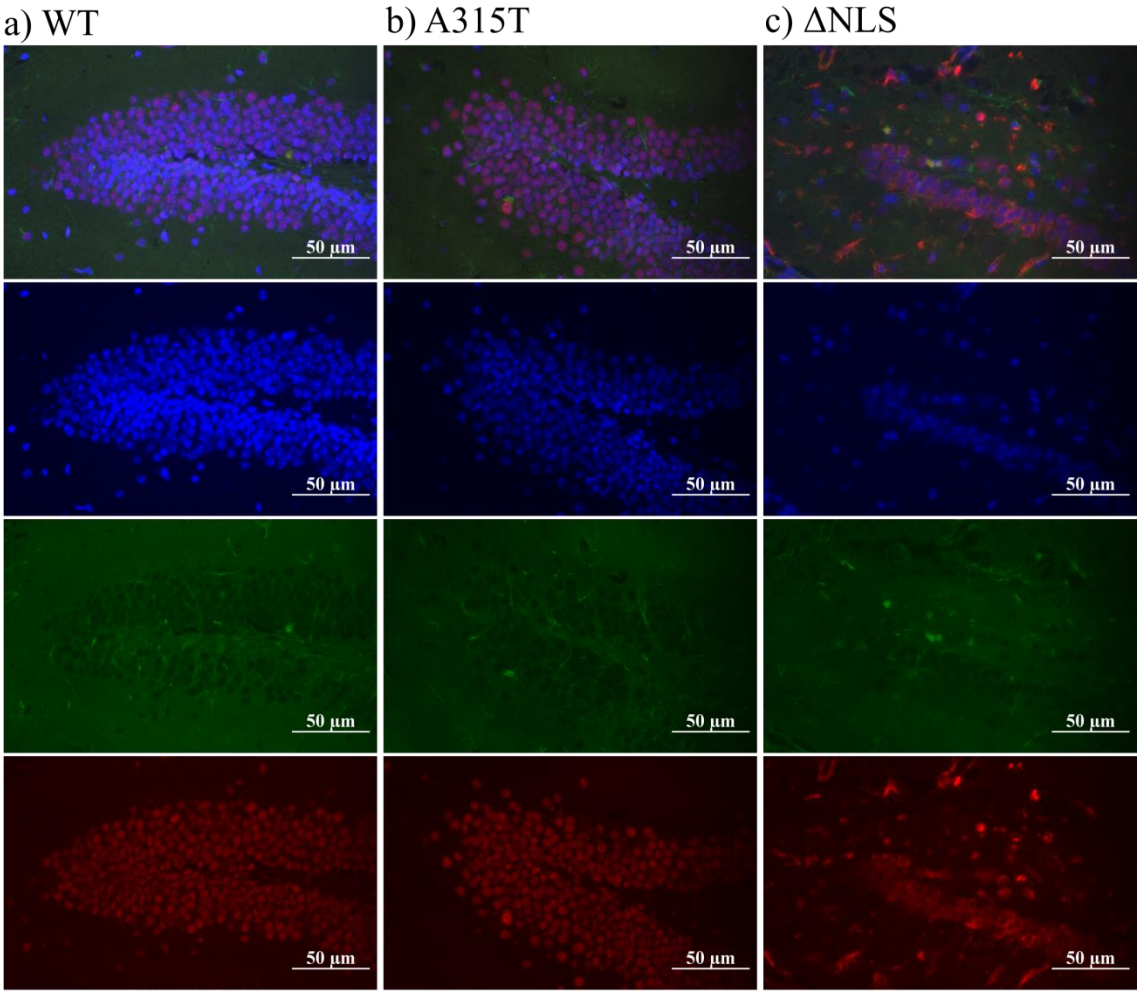


Figure 10. GFAP and TDP-43 in the dentate gyrus. a) WT b) A315T c) TDP-43ΔNLS in which activated astrocytes are seen. 400x stained with DAPI (blue, 358nm), GFAP (green, 488nm) and TDP-43 (TDPA, red, 555nm) n=1 for each genotype in each age group.

4.5 Spinal Cord changes

EM imaging of the spinal cord of the 16-18 month age group of mice shows distinct differences between WT and TDP-43ΔNLS genotypes (figure 11). TheTDP-43ΔNLS have enlarged mitochondria. The TDP-43ΔNLS also appear to have disorganized and/or degenerated myelin sheaths. The nucleus of the TDP-43ΔNLS is also less distinct than WT nucleus. The A315T EM imaging also shows possible myelin degeneration however these mice have no

noticeable phenotype. Nissl staining of the spinal cord shows possible changes to motor neuron distribution in the TDP-43ΔNLS (figure 9).

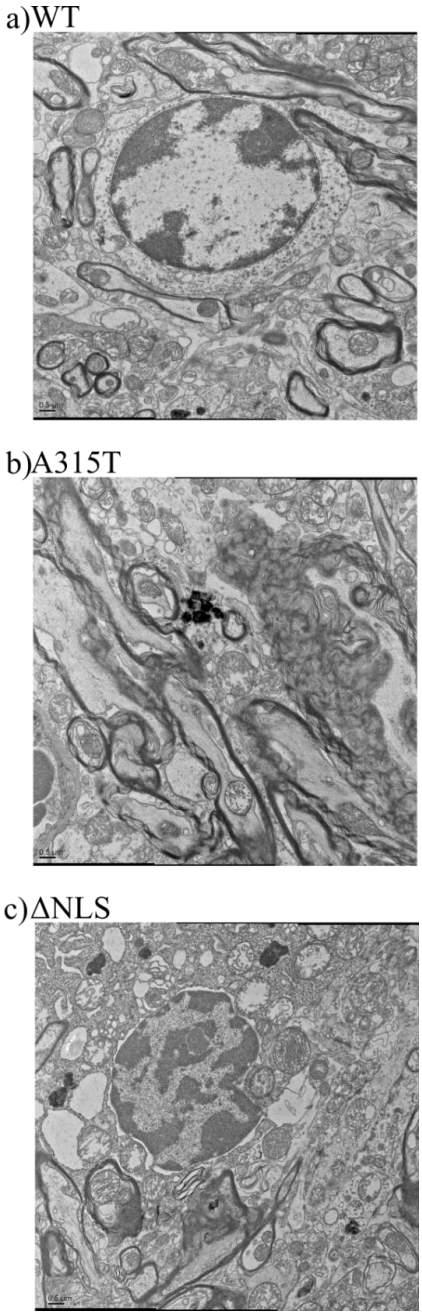


Figure 11 EM imaging of the spinal cord of 16-18 month age group. a) WT b) A315T c) TDP-43Δ NLS Myelin sheaths, nuclei, and mitochondria can be seen changed in TDP-43ΔNLS compared to WT. Images are from the ventral horn of the spinal cord n=1 for each genotype in each age group.

CHAPTER 5

DISCUSSION

The purpose of this study is to determine if the TDP-43 A315T and TDP-43ΔNLS mouse line using a hamster prion promoter can be used as an ALS and FTLD mouse model based on behavior and pathology. TDP-43 A315T is a familial ALS mutation in humans and TDP-43ΔNLS has a defect in the nuclear localization signal casusing TDP-43 to accumulate in the cytoplasm and thus TDP-43 proteinopathy. TDP-43 proteinopathy is found in ~95% of ALS cases and in ~50% of FTLD cases [10–12]. Therefore it is important to evaluate the behavior and pathology of these mice at different ages to determine the progression of the disease and its similarities and differences to ALS and FTLD. Using the prion driver for TDP-43ΔNLS and A315T it was found that the TDP-43ΔNLS has distinct TDP-43 proteinopathy, behavioral changes (open field), and motor impairments (rotarod) similar to ALS and FTLD. The A315T mouse line have a lower expression of the mutated TDP-43 under the prion driver and therefore do not show motor impairment or behavioral changes. In order to evaluate the lines for proteinopathy H&E, luxol fast blue (myelin), pTDP-43, microglia, astrocyte, and motor neuron staining. Also EM imaging was performed on the ventral horn of the spinal cord.

TDP-43ΔNLS mice have motor impairment on the rotarod. Rotarod tests motor coordination and/or motor impairment. The data suggests that the motor impairment is from motor coordination because the mean speed of the TDP-43ΔNLS mice is not reduced which suggests normal walking ability on a flat surface. For an MND/ALS disease mouse model it is important for there to be motor dysfunction. H&E staining suggests possible skeletal muscle hypertrophy. The skeletal muscle hypertrophy may be a compensatory method for the loss of

surrounding muscle or atrophy. However no atrophy or grouping of myofibers was seen. The EM images of the spinal cord of 16-18 month age group of the TDP-43ΔNLS line shows a disorganized/degenerated myelin sheath which may be a factor in the motor impairment. The nuclei are also less distinct and the mitochondria are enlarged compared to WT which suggest pathology in the spinal cord which also may play a role in the motor impairment found in the TDP-43ΔNLS line.

Mice naturally explore novel environments through activity and other behaviors such as rearing. The dentate gyrus has been implicated in novel spatial environment recognition [67]. Therefore neuronal loss in the dentate gyrus could be why during behavioral testing theTDP-43ΔNLS mice displayed anti-exploratory behavior in a reduced rearing and increased time spent freezing (figure 3). The dentate gyrus also has decreased myelination that is most likely due to neuronal loss as shown with the hematoxylin stain (nuclear stain) that the nuclei in the dentate gyrus are gone in the TDP-43ΔNLS. pTDP-43 cytoplasmic inclusions are also found in the dentate gyrus of TDP-43ΔNLS and smaller pTDP-43 cytoplasmic inclusions are also shown in the A315T mice. This pathology is very consistent with ALS and FTLD-TDP disease pathology where there are phosphorylated, ubiquitinated TDP-43 inclusions [11,15,17,55–58].

Also increased anxiety in mice has been linked to anti-exploratory behavior[68]. The loss of the dentate gyrus neurons could have the TDP-43ΔNLS mice perceive the novel environment as not novel and therefore not an environment to explore or theTDP-43ΔNLS mice have increased anxiety, consistent with FTLD, which decreases their exploratory behavior. It is not possible to determine the exact cause of the anti-exploratory behavior because this behavior has many possible causes. In future research other behavioral tests could be used to determine anxiety levels of the mice such as the light/dark box which measures the time it takes for a mouse to

leave the dark area and enter the lighted area. Another test could be to track the mouse in another novel environoment that is very different then their home cage and compare their reaction to that novel environment with WT.

The 11-12 month TDP-43ΔNLS mice also experience circling behavior (increased number of rotation (figure 3a), a stereotypic behavior which is repetitive and unvarying with no known goal or reason, which can be due to a vestibular, auditory, or disruption in the nigro-striatal region reducing dopamine levels all of have an established relationship with circling behavior[69–72]. Circling behavior is especially seen in Parkinson disease mice models. The circling behavior as seen by a significant increase of rotations done in the open field also appears to be correlated with the increase of mean speed for the 11-12 month age group.

Another characteristic of ALS and FTLD is astrocyte and microglia activation[2,43–45,73]. The 16-18 month age group was evaluated for reactive astrocytes and microgliosis using IHC and immunofluorescence. TDP-43ΔNLS have an increase in activated astrocytes compared to control as seen using immunofluorescence (figure 9c). Also there appears to be an increase of microglia in the dentate gyrus (figure 7c).This is similar to disease progression of ALS and FTLD.

The spinal cord of the 16-18 month age group of the TDP-43ΔNLS mouse line has differences when compared to WT. The butterfly pattern of gray matter is less distinct and more spread out (figure 8c). This could be suggestive of motor neuron degeneration. The motor neuron cell bodies appear larger in the TDP-43ΔNLS however the spinal cord slice could be at a slightly different level causing the cell bodies to appear larger then WT and A315T.

These two mouse models TDP-43ΔNLS and TDP-43 A315T have a longer life span than previous models ALS and FTLD[20,25,66,74]. Both mouse lines in this study survive until 18 months. The motor deficits are seen at 11-12 months while the anti-exploratory behavior is seen at 14-15 month and 16-18 month age group. This is consistent with a disease progression that occurs later in life as in ALS and FTLD. This makes these mouse lines, specifically TDP-43ΔNLS, a more relevant mouse model to investigate disease progression. There are very few pathological characteristics in the TDP-43 A315T mousseline and no behavioral phenotype. This could be due in part to the extremely low expression level of the A315T mouse line (data not shown). The prion driver also has a lower expression level then other mouse models used because there is a slower progression to the pathology and the survival rate is much longer than other ALS and FTLD mouse models.

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