Quantitative Imaging of Net Axonal Transport in vivo: A Biomarker for Motor Neuron Health and Disease

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QUANTITATIVE IMAGING OF NET AXONAL TRANSPORT \textit{IN VIVO}: A BIOMARKER FOR MOTOR NEURON HEALTH AND DISEASE

A Dissertation Presented

By

Pin-Tsun Justin Lee

Submitted to the Faculty of the

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QUANTITATIVE IMAGING OF NET AXONAL TRANSPORT IN VIVO: A BIOMARKER FOR MOTOR NEURON HEALTH AND DISEASE

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This work was undertaken in the Morningside Graduate School of Biomedical Sciences Neuroscience Program

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a lethal, progressive neurodegenerative disorder that selectively affects both upper and lower motor neurons, leading to muscle weakness, paralysis and death. Despite recent advances in the identification of genes associated with ALS, the quest for a sensitive biomarker for rapid and accurate diagnosis, prognosis, and treatment response monitoring has not been fulfilled. In this thesis, I report a method of quantifying the integrity of motor neurons in vivo using imaging to record uptake and retrograde transport of intramuscularly injected tetanus toxin fragment C (TTC) into spinal motor neurons. This method tracks and profiles progression of disease (transgenic SOD1\textsuperscript{G93A} and PFN1 ALS mice) and detects subclinical perturbations in net transport, as analyzed in C9orf72 transgenic mice. It also defines a progressive reduction in net transport with aging. To address whether our technique enables drug development, I evaluated therapeutic benefits of (1) gene editing and (2) mutant gene silencing (with RNAi targeting \textit{SOD1}) in SOD1\textsuperscript{G93A} transgenic mice by characterizing their net axonal transport profiles. I constructed a computational model to evaluate key molecular processes affected in net axonal transport in ALS mouse model. The model allows prediction of key parameters affected in a \textit{C9ORF72} BAC transgenic mouse line. Prior immunization with tetanus toxoid does not preclude use of this assay, and it can be used repetitively in the same subject. This assay of net axonal transport offers broad clinical application as a diagnostic tool for motor neuron diseases and as a biomarker for rapid detection of benefit from therapies for transport dysfunction in a range of motor neuron diseases.
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PREFACE

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CHAPTER 1: INTRODUCTION

1.1 Axonal transport

1.1.1 General components, coordination and regulation

Intracellular trafficking of cargoes is essential for all cellular processes in eukaryotic cells. The precise and efficient delivery of cellular cargoes to discrete targets is tightly regulated and governs the function of any cellular machinery. This is especially evident in neurons due to their large size, high polarity, high energy demand, and most remarkably, their extended axon, which can reach up to one meter in length in humans. Thus, the active axonal transport of cargoes, spanning from membranous organelles to non-membranous proteins, ribosomes, and RNAs serves as a central mechanism to ensure normal neuron development, function, and survival.

Ever since the initial observation reported by Weiss and Hiscoe of “the axoplasmic flow,” a term coined to describe the continual yet slow movements of materials along the axon after nerve constriction experiments (Weiss and Hiscoe 1948), major progress has been made in our understanding of the molecular biology of axonal transport. Two distinctive cargo-specific rate components of axonal transport have been characterized through technological breakthroughs, from early ligation studies and pulse-chase labeling experiments to later fluorescently labeled protein tracers or molecular dyes visualized by high-resolution microscopy. Cargoes involved in the “fast component” are vesicular/ membranous cargoes that move rapidly either anterogradely—moving from cell body to synapse (e.g. Golgi-derived vesicles), retrogradely—moving from synapse to cell body (e.g. signaling endosomes,
autophagosomes), or bi-directionally (e.g. mitochondria) at speeds ranging from 0.8μm/s to 5μm/s (Grafstein and Forman 1980; Lorenz and Willard 1978). Cargoes involved in the “slow component” are mainly cytoskeletal proteins (e.g. tubulin, neurofilament proteins, actins) and a plethora of cytosolic proteins (e.g. metabolic enzymes, synaptic proteins, and molecular motor dynein) that move with an anterograde bias at speeds ranging from 0.01 to 0.09 μm/s (Lasek, Garner, and Brady 1984). One of the challenges in the field is to elucidate how targeted delivery of various cargoes is regulated by this essential cellular process. For example, what is involved to ensure that synaptic vesicles are targeted to axon terminal while sodium channels are targeted to nodes of Ranvier? In the past four decades, there have been major mechanistic insights pointing to multiple levels of regulation that contribute to functional and responsive transport networks, three of which have been extensively characterized. The first layer of regulation is track-specific regulation. Long-distance axonal transport depends on the molecular tracks, which are primarily microtubules in the axon. Several track-specific regulatory mechanisms have been identified that modulate microtubule stability. In addition, microtubules are subject to compartment-specific regulations along the axon (e.g. distal axon versus mid-axon). The second layer of regulation is cell type-specific and cargo-specific recruitment of specialized molecular motors that are composed of diverse subunit variants. The last layer of regulation is cell type-specific and cargo-specific kinase-based regulation for axonal transport modulation. Collectively, the interplay of all these regulatory mechanisms ensures robust axonal function and integrity.
Axon microtubules are made of αβ-tubulin polymers that assemble in a polarized array, with the faster-growing plus ends pointing toward the synapse, and the more stable minus ends pointing toward the cell body. Intriguingly, unlike the microtubules in non-neuronal cells which can be highly dynamic and become depolymerized after treatments including cold, Ca\(^{2+}\), and antimitotic drugs, axon microtubules are generally and intrinsically stable and long-lived even under those treatments. In extracting axon microtubules of the retinal ganglion cells from whole brains of guinea pigs and rats, Brady and colleagues identified a biochemically distinct tubulin fraction that was cold-stable from the conventional methods for microtubule preparations. Following a more extensive differential extraction protocol, the cold- and Ca\(^{2+}\)-insoluble fraction that was rich in axons displayed a basic shift in the isoelectric point compared to the cold-extractable or labile fraction (Brady, Tytell, and Lasek 1984). Recently, Song et al. discovered that a novel post-translational modification (PTM), in which polyamine is covalently added to tubulin through transglutaminase, causes axon microtubules to be positively-charged, and thus remain stable in the face of cold- or Ca\(^{2+}\)-induced depolymerization (Song et al. 2013). Such fine regulation of axon microtubule stability ensures stable structural framework for normal axon growth, functional axonal transport, and maintenance of axon morphology. In addition to polyamination, numerous PTMs including detyrosination, polyglutamylation, polyglycylation, acetylation, and other minor modifications to tubulin and microtubules have been well documented (Janke and Kneussel 2010; Janke 2014; Song and Brady 2015). These PTMs of microtubules are found in different degrees in different subcellular compartments in neurons. For
example, tubulin acetylation is associated with long-lived microtubules (Webster and Borisy, 1989), and acetylation of lysine 40 on α-tubulin has been reported to promote docking of the molecular motors kinesin-1 (Reed et al. 2006). However, a conflicting result by in vitro motility assays has demonstrated that tubulin acetylation alone does not affect kinesin-1 transport (Walter et al. 2012). Detyrosination of α-tubulin is also preferentially enriched on stable microtubules in the mid-axon (A. Brown et al. 1993), whereas tyrosinated-rich microtubules are mostly found in compartments of high dynamic nature such as the distal axon. Consistent with this finding, using in vitro reconstitution and live-imaging assays in dorsal root ganglion (DRG) neurons, Nirschl and colleagues observed a gradient of increased tyrosinated α-tubulin at the distal axons (Nirschl et al. 2016). Furthermore, these PTMs also change during neuronal differentiation. For example, polyamination of axon microtubules increases as neurons mature, which correlates with stabilized neuronal connectivity; however, the stability of axon microtubule in mature neurons may also confer their susceptibility to injury or neurodegeneration due to limiting microtubule dynamics (Song et al. 2013).

Microtubule-based molecular motors are the engines that power diverse cargoes along the axon and are primarily from the plus-end-directed kinesin superfamily that transport cargoes anterogradely and the minus-end-directed cytoplasmic dynein that drives cargoes retrogradely. It is worth noting that while the general role of conventional kinesin and cytoplasmic dynein have been studied and discussed extensively, the diversity of isoforms and subunit variants of each molecular motor, which confer their cell-specific and cargo-specific involvement of transport, are rarely addressed or even
examined. The oversimplification of molecular motor activities thus has the peril of overlooking the complex regulation of cargo-motor interaction. For example, conventional kinesin, a heterotetramer composed of two heavy chain (KIF5A-C, kinesin-1), and two light chain (KLC1-2) subunits (DeBoer et al. 2008), is responsible for the fast anterograde transport of most vesicles, organelles, and proteins and the slow axonal transport of cytoskeletal proteins. Kinesin-1 is encoded by three genes KIF5A, KIF5B, and KIF5C. Whereas KIF5A and KIF5C are differentially and primarily expressed in the neurons, KIF5B is expressed ubiquitously in mammals (Hirokawa et al. 2009; Kanai et al. 2000). DeBoer and colleagues have demonstrated through co-immunoprecipitation of mouse brain tissue that conventional kinesin holoenzyme are formed of kinesin-1 homodimers and KLC homodimers, and suggest six variant forms of conventional kinesin (DeBoer et al. 2008). These kinesin variants provide a basis of molecular regulation of motor-cargo specificity and targeted delivery of specific cargoes. Similar to kinesin, the pool of subunit variants in cytoplasmic dynein includes a single dynein heavy chain (DHC; encoded by DYNC1H1), two isoforms of intermediate chain (DIC; encoded by DYNC1IC1 and DYNC1IC2), two isoforms of intermediate chain (DLIC; encoded by DYNC1LI1 and DYNC1LI2), and three dynein light chain (DLC) including Roadblock, LC8, and Tctex (Reck-Peterson et al. 2018). In addition, a plethora of adaptor proteins and activator proteins of cytoplasmic dynein have been proposed to contribute in regulating cargo-specific transport (Olenick and Holzbaur 2019). Taken together, the diversity and heterogeneity of subunit variants of the molecular motors with associated adaptors bestow specific cargo-motor interaction and delivery.
There has been growing evidence in the past two decades that specific protein kinases regulate axonal transport through direct or indirect phosphorylation of specific motor (McIlvain et al. 1994; G. Morfini et al. 2002; 2004). For example, using vesicle motility assays in isolated squid axoplasm (Brady, Richards, and Leopold 1993), Morfini and colleagues demonstrated that glycogen synthase kinase-3 beta (GSK3β) phosphorylates KLC2, leading to its release from cargoes, without affecting the ATPase and microtubule-binding functionality of the motor, whereas c-Jun N-terminal kinase 3 (JNK3) inhibits anterograde transport by phosphorylating KIF5C, which affects its ability to bind to microtubules (G. Morfini et al. 2002; G. A. Morfini et al. 2009). Notably, kinases that regulate axonal transport can display high specificity, as illustrated by the isoform-specific effects of p38 MAPK. In squid axoplasm, p38 MAPKα selectively inhibited anterograde transport by phosphorylating kinesin-1, whereas p38 MAPKβ inhibited both anterograde and retrograde axonal transport (G. A. Morfini et al. 2013). Lastly, all these kinases are differentially expressed in a cell type-specific manner and their regulation is likely to affect specific isoforms of molecular motor and specific cargoes. In summary, the intricate and delicate cross-talk of multiple regulatory pathways in axonal trafficking is crucial to ensure precise delivery of cargoes at the proper time and space for neuronal development, metabolism, and function.

1.1.2 Axonal transport and neurodegenerative diseases

Since all neurons are post-mitotic and rely heavily on axonal transport for their function and survival, it is apparent that even minor dysregulation or dysfunction of the transport
machinery can result in deleterious neuropathological ramifications. Indeed, axonal transport deficits have been documented as one of the common and early hallmarks in various neurodegenerative diseases, including Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, Charcot-Marie-Tooth peripheral neuropathy, and motor neuron diseases (Sleigh et al. 2019; X.-A. Liu, Rizzo, and Puthanveettil 2012; De Vos et al. 2008; Millecamps and Julien 2013; Chevalier-Larsen and Holzbaur 2006). Research on the relationship between axonal transport defects and neurodegeneration gives rise to two central questions. First, do axonal transport deficits serve a causal, contributory, or consequential role in neurodegeneration? Second, how is axonal transport machinery differentially dysregulated in different neuronal cell types that are affected by different neurodegenerative diseases?

In addressing the first question, current evidence supporting axonal transport as a cause of neurodegeneration comes from studies of mutations in transport machinery genes. These genes encode key transport machinery components such as the microtubule network, the molecular motor complex, and the cargo-specific adaptors. These genes are ubiquitously expressed in most eukaryotic cells and are essential for developmental processes such as cell division, neuronal migration, and axon guidance. It is not surprising, then, that the majority of axonal transport gene mutations leads to death during embryogenesis or neurodevelopmental phenotypes, rather than neurodegenerative phenotypes. For example, mechanistic insight of dynein function first came from studies of three separate mouse lines with mutations in DYNC1H1: Legs at odd angles (Loa), Cramping 1 (Cra1), and Sprawling (Swl) mice. The two mouse
mutants with missense mutations of \textit{DYNC1H1}, heterozygous \textit{Loa} and \textit{Cra1}, display age-related progressive motor neuron loss in the spinal cord anterior horn, whereas heterozygous \textit{Swl} mice, with a nine base-pair deletion in \textit{DYNC1H1}, exhibit an early-onset proprioceptive sensory neuropathy without motor neuron pathology (Hafezparast et al. 2003; X.-J. Chen et al. 2007). Human mutations in \textit{DYNC1H1} was initially described to cause Charcot-Marie-Tooth disease, an inherited neurodegenerative condition that affects both peripheral motor and sensory nerves, leading to progressive loss of muscle tissue and touch sensation (Weedon et al. 2011). However, later clinical studies have shown that the same mutation leads to spinal muscular atrophy with lower extremity predominance, a neurodevelopmental condition characterized by non-progressive loss of motor neurons in infancy or early childhood (Tsurusaki et al. 2012; Hwang et al. 2016). Thus, retrograde transport machinery may have greater impact on neurons during the developmental stage when pro-survival signal and apoptotic signal depend heavily on functional transport. Nevertheless, a direct link between axonal transport defects and neurodegeneration has been suggested by the identification of mutated genes encoding motor protein subunits. One of these genes is \textit{DCTN1}.

\textit{DCTN1} encodes the dynactin subunit p150\textsubscript{Glued}, an adaptor for cytoplasmic dynein. Point mutations just a few amino acids apart at the CAP-Gly motif of this gene can lead to two distinctly different phenotypes. One phenotype is adult-onset distal human hereditary motor neuropathy type 7 (HMN7B), caused by G59S mutations, where selective loss of lower motor neurons occurs. The other is Perry’s syndrome, a progressive neurodegenerative disease caused by G71R/E/A, T72P, and Q74P
mutations and characterized by parkinsonism and psychiatric symptoms without any motor neuron degeneration. Mutant genes are expressed in all types. It is unclear how point mutations in the same CAP-Gly motif dictate selective vulnerability in neurons, and differentially in motor and nigral neurons. Whether these mutations result in defects in axonal transport is still under debate. In a Drosophila model of HMN7B, imaging of Rab7:green fluorescent protein in larval segmental nerves show normal axonal transport metrics as compared to the controls (Lloyd et al. 2012). In contrast, impairment of axonal transport is reported in primary sensory neurons overexpressing the HMN7B-specific mutation. Such impairment is absent in neurons with the Perry syndrome mutants (Moughamian and Holzbaur 2012). In summary, mutations in dynactin do not seem to affect retrograde axonal transport in a general way, but in a cargo-specific and cell type-specific way.

Disruption of axonal transport could be a consequence of other perturbed cellular processes upstream. Several aberrant cellular processes seen in neurodegenerative diseases have been suggested to play a causative role for axonal transport defects. These pathophysiological mechanisms include, but are not limited to, protein aggregation, mitochondrial dysfunction, and endoplasmic reticulum (ER) stress. For example, proper mitochondria homeostasis is critical to axonal maintenance and function. Some of the most common genetic causes of familial Parkinson’s disease are mutations in PINK1, which encodes PTEN-induced kinase 1 (PINK1), and PARKIN, which encodes Parkin RBR E3 ubiquitin-protein ligase (PARKIN). Both proteins are involved in mitophagy, the destruction pathway for damaged mitochondria (Valente et
al. 2004; Kitada et al. 1998). Under normal conditions, PINK1 is deactivated by proteasomal degradation, and mitochondrial transport is regulated by the outer mitochondrial membrane protein Miro, which attaches to the motor proteins through the adaptor Milton to facilitate its trafficking to various cellular locations. Upon mitochondrial damage, the level of PINK1 is stabilized, resulting in the recruitment of PARKIN, which directs Miro’s degradation. This causes mitochondria to be detached from the molecular motor and to undergo mitophagy. However, the dysfunctional quality control by mutant PINK1 and PARKIN in Parkinson’s causes accumulation of damaged mitochondria, and disrupts their axonal transport (S. Liu et al. 2012).

In summary, whether axonal transport deficit play a causative role or is secondary to other pathogenic defect is subject to debate. What is known is that it is a common target that seems vulnerable to perturbations and can be a converging point that leads to axonal degeneration.

How is dysregulated axonal transport, thought to be a common pathological hallmark in various neurodegenerative diseases, induced in selectively vulnerable neuron populations? As mentioned briefly in the previous section, protein kinase-based regulation of axonal transport modulates cargo-motor interactions, and has been involved in essential neuronal developmental processes such as the establishment of neuronal polarity (Hida et al. 2015) and synapse formation (Chua et al. 2012). Furthermore, kinases also modulate the transport of neurotrophic factors or stress signaling (Colin et al. 2008; Harrington and Ginty 2013; Perlson et al. 2010). It is worth noting that specific kinases are differentially expressed in a cell type-
subtype-specific manner, resulting in distinct transport characteristics. Such a fine regulatory network makes axonal transport susceptible to dysregulation when the former is altered by disease-causing mutations. Indeed, several alterations in kinase-based signaling pathways have been characterized in the past decades. In Huntington’s disease, for example, the pathogenic polyglutamine (polyQ)-huntingtin (HTT) inhibits anterograde axonal transport by selectively activating neuron-specific JNK3 to phosphorylate KIF5C. This phosphorylation releases kinesin-1 motor from binding to the microtubules (G. A. Morfini et al. 2009). As mentioned briefly in the previous section, aberrant p38 MAPK activation can inhibit anterograde fast axonal transport by phosphorylating kinesin-1. This aberrant activation of p38 MAPK is mediated by mutant and misfolded WT Cu-Zn superoxide dismutase 1 (SOD1) (Bosco et al. 2010; G. A. Morfini et al. 2013). Interestingly, a recent study demonstrates that ALS-linked Fused in Sarcoma (FUS) mutations also activate p38 MAPK to inhibit anterograde axonal transport (Sama et al. 2017), implicating the two ALS-linked genes may share the same pathological signaling pathway in impairing axonal transport.

1.2 Amyotrophic lateral sclerosis

1.2.1 Clinical manifestations, genetic features and pathogenesis

Heterogeneity of ALS

Amyotrophic lateral sclerosis (ALS or Lou Gehrig’s disease) is an adult-onset, fatal neurodegenerative disorder that targets specific motor neuron populations in the brain and the spinal cord, leading to progressive paralysis and death from respiratory failure,
typically within 5 years (Taylor, Brown, and Cleveland 2016). The hallmark of ALS is focal onset muscle weakness, muscle atrophy, fasciculations, muscle cramps and stiffness that spreads progressively to adjacent body regions in an organized way. Clinically, ALS is a heterogeneous disorder differing by which body regions are first affect, age of onset, disease progression, and life expectancy. There are also distinct motor and extra-motor manifestations (e.g. neurons from the frontotemporal lobes) leading to differential clinical presentations. ALS accounts for the majority of the motor neuron diseases, and its classic phenotype is the loss of both upper motor neurons (UMN), located in the cortex, and lower motor neurons (LMN), located in the brainstem or spinal cord. Two subtypes can be further categorized: spinal ALS, also known as the limb onset, and bulbar ALS, or the bulbar onset. The limb onset affects roughly two-thirds of the patients with initial symptoms appearing in the distal limb muscles, whereas the bulbar onset presents in the remaining 25% to 30% of cases with symptoms begins at the bulbar muscles that control mastication, swallowing, tongue movement, and articulation. In addition to these classic clinical presentations, additional rare and variant motor neuron diseases exist where a spectrum of differential involvement of UMN or LMN are characterized. At one end, primary lateral sclerosis (PLS) displays predominant lesions of UMN with signs such as progressive spasticity of the lower limbs and possibly of the bulbar region without signs of muscle atrophy or denervation (Pringle et al. 1992). Conversely, patients with progressive muscular atrophy present with predominantly LMN involvement, resulting in muscle weakness and atrophy, usually without UMN signs, though nearly a quarter of these patients develop UMN
symptoms within five years of diagnosis (W.-K. Kim et al. 2009). Importantly, various
ALS subtypes display predictable rates of disease progression and life expectancy. For
example, bulbar ALS is characterized by a worse prognosis and a shorter survival
compared to spinal ALS (Goldstein, Atkins, and Leigh 2002), and PLS shows a much
slower disease progression with higher survival rate (Tartaglia et al. 2007) compared to
ALS overall.

ALS has also been associated with extra-motor manifestations. Approximately
50% of ALS cases exhibit neuronal loss in the frontal and anterior temporal lobes,
resulting in varying degrees of behavioral changes and cognitive or language
impairments. Among those patients, 10% meet the criteria for a diagnosis of
frontotemporal dementia (FTD), the most common cause of pre-senile dementia. This
association between ALS and FTD has been recognized as a clinicopathological
spectrum (ALS-FTD) as they share overlapping symptoms. One of the shared
pathological hallmarks of ALS-FTD is the presence of trans-active response (TAR)
DNA-binding protein 43 (TDP-43) immunoreactive inclusions, which are present in most
of the ALS patients and half of the FTD patients, underscoring ALS as a multisystem
disorder for which motor neuron involvement is one aspect of a complex syndrome
(Neumann et al. 2006; Arai et al. 2006).

The heterogeneous nature of ALS is also seen in the identification of genetic
mutations in patients. Most ALS cases occur without family history of the disease and
are therefore designated as sporadic ALS (sALS). Up to 10% of ALS cases have a
positive family history with an autosomal dominant inheritance pattern and are thus
classified as familial ALS (fALS). Such classification, however, should not mistakenly define sALS as being strictly caused by environmental and stochastic factors without a genetic component. More and more genetic variants and mutations of existing ALS genes have been found in patients with sALS, demonstrating the importance of genetic elements in ALS susceptibility. Also noteworthy is that fALS and sALS share similar pathological features (e.g. the patterns of motor neuron vulnerability), suggesting that common molecular pathologies exist in both conditions. This brings hope for therapeutic development for targeting common affected pathways. To date, with the advances in genetic technological developments, more than 50 potential genes have been identified that are associated with ALS (Taylor, Brown, and Cleveland 2016). These genes demonstrate a complex and multifactorial pathology of three mutually compatible pathogenic cellular mechanisms: perturbations of protein homeostasis, alteration of RNA biology and trafficking, and disruption of cytoskeletal maintenance and dynamics (Peters, Ghasemi, and Brown 2015).

**Perturbation of protein homeostasis**

During normal cellular conditions, accumulation of misfolded, damaged, or aged proteins can induce cellular stress. To alleviate such perturbation and re-establish protein homeostasis, the cell employs a number of pathways to ensure protein quality control. To attenuate disturbances caused by misfolded or unfolded proteins in the endoplasmic reticulum (ER), a condition referred as ER stress, a cascade of complex signaling networks called the unfolded protein response (UPR) is activated to enlarge
ER folding capacity and upregulate the expression of molecular chaperones, leading to reduction of ER or cytoplasmic protein aggregation (Ron and Walter 2007). However, when this goal is not fulfilled, cells can target misfolded or damaged proteins in the cytoplasm for degradation by either the ubiquitin-proteasome pathways (UPS) or autophagy (Lilienbaum 2013). In ALS (and a number of neurodegenerative diseases), however, the protein quality control pathways are compromised, leading to the formation of cytoplasmic inclusions. Accordingly, ALS is marked pathologically by various small protein aggregates and multiple cytoplasmic inclusions with distinct morphologies, including Bunina bodies, ubiquitinated skein-like inclusions that are immunoreactive for TDP-43, and neurofilament-containing hyaline inclusions (Wada et al. 1999; Liscic et al. 2008; Wong, He, and Strong 2000). In fALS cases, the insoluble inclusions are typically composed of misfolded proteins encoded by the respective mutant genes; these include Cu-Zn superoxide dismutase 1 (SOD1), TAR DNA-binding protein (TARDBP), the fused in sarcoma (FUS), and dipeptide repeat (DPR) proteins from pathogenic hexanucleotide repeat expansion in the chromosome 9 open reading frame 72 (C9orf72). Although most of these misfolded proteins are associated with ubiquitin-positive inclusions, there are suggested distinct processes with which various inclusions are formed (Farrawell et al. 2015). For example, in sALS, it is rare to find colocalization of SOD1 with TDP-43 in the same inclusion, and the same holds true that SOD1 fALS cases display SOD1 inclusions without TDP-43 colocalization, highlighting fundamental difference in SOD1 and TDP-43 pathobiology (Maekawa et al. 2009).
Mutation of *SOD1* was the first identified genetic cause of ALS, which accounts for 20% of fALS and 2% of sALS cases (Rosen et al. 1993). A metalloenzyme that reduces free superoxide radials into molecular oxygen and hydrogen peroxide (McCord and Fridovich 1969), SOD1 is linked to over 180 disease-associated variations, and of which, the majority are autosomal dominant missense mutations (http://alsod.iop.kcl.ac.uk/). Although most mutant SOD1 proteins are associated with a decrease in dismutase activity, there seems to be no correlation between disease severity and disruption of dismutase function both in ALS patients and in a *SOD1*-knockout mouse model, suggesting a toxic gain of function that leads to motor neuron degeneration (Cleveland et al. 1995; Andersen et al. 1995; Reaume et al. 1996).

Although the molecular basis for the toxicity of mutant SOD1 remains to be elucidated, disruptions of multiple cellular processes including excitotoxicity, mitochondrial dysfunction, oxidative stress, and neuroinflammation have been observed. In addition, aggregates and inclusions of mutant SOD1 have been linked to failed protein homeostasis by inducing chronic ER stress and impairing protein degradation. Notably, studies of protein structure alterations in 132 ALS-associated SOD1 mutants reveal a shared conformational change that induce chronic ER stress by binding to ER integral membrane protein derlin-1 (Fujisawa et al. 2012). Moreover, cytoplasmic mutant SOD1 can also induce ER stress by impairing ER-Golgi transport (Atkin et al. 2014). The chronic ER stress will trigger ER-associated degradation (ERAD) to mediate degradation by the proteasome. In the case of mutant SOD1, however, there is evidence showing dysfunction for both UPS and autophagy pathways.
(Bendotti et al. 2012; S. Chen et al. 2012). It is noteworthy that wild-type SOD1 undergone oxidation is also aggregation-prone and can confer toxic function as the mutant SOD1, such as inhibiting axonal transport. Additionally, using a conformation-specific antibody that recognize a misfolded SOD1—both the oxidized wild-type SOD1 and mutant SOD1—Bosco and colleagues were able to detect positive immunoreactivity on postmortem sALS human spinal cord with high prevalence, underscoring a common pathogenic pathway shared by misfolded SOD1 in all ALS cases (Bosco et al. 2010). Consistent with this finding, there is accumulating evidence suggesting that misfolded SOD1 proteins are toxic and can contribute to sALS pathogenesis (for review, see Rotunno and Bosco 2013). In addition to C4F6, two sets of antibodies generated specifically against denatured SOD1 detect small granular SOD1-positive inclusions in spinal motor neurons of all 29 sporadic cases examined and two of the 19 non-neurological controls (Forsberg et al. 2010). Interestingly, these sets of antibodies also detected intranuclear misfolded SOD1 in glial cells in all 43 spinal cord sections from sALS patients, but produced negligible staining in other neurodegenerative and non-neurological controls (Forsberg et al. 2011). Recently, the validity of these findings has been verified by extensive blinded histological and biochemical analyses of postmortem brain and spinal cord tissues from 19 sALS cases. Although conformation-specific antibodies targeting various domains and specific epitopes of misfolded SOD1 display different intensity and distinct immunostaining patterns in each patient, misfolded SOD1 is shown to be a common pathological feature in sALS cases (Paré et al. 2018). Collectively, these findings suggest that aggregation of wild-type misfolded SOD1 is a
pathological hallmark and may contribute to the pathogenesis of sALS. Furthermore, it is the propensity for aggregation of misfolded SOD1, rather than the loss of function by specific mutations in SOD1 that drives the toxicity to motor neurons.

TDP-43, a 43-kDa protein encoded by TARDBP gene, has been identified in both fALS and sALS patients and is the main component of ubiquitinated cytoplasmic inclusions in 97% of ALS cases, and 45% of frontotemporal lobar dementia (FTLD) cases (Tan et al. 2017). Belonging to the large heterogeneous nuclear ribonucleoprotein (hnRNP) family, TDP-43 localizes in the nucleus under normal condition and is responsible for regulating a series of RNA processing steps. However, in most cases of ALS and FTLD, TDP-43 redistributes from the nucleus to the cytoplasm where it undergoes hyperphosphorylation, ubiquitination, and proteolytic cleavage to form toxic cytoplasmic inclusions in the affected neurons and glia (more discussion below concerning TDP-43 and defects in RNA metabolism).

There is developing evidence implicating impairment and dysregulation of ALS-associated TDP-43 clearance. First, TDP-43 positive inclusion bodies in ALS and FTLD patients are found to be marked with sequestosome-1 (also known as p62), a marker for autophagy (King et al. 2011). Second, ALS-associated mutations in UBQLN2 (ubiquilin-2), and VCP (valosin-containing protein) induce TDP-43 inclusion formation, and impair autophagy and UPS pathways, respectively (Osaka, Ito, and Suzuki 2016; Gitcho et al. 2009). Lastly, TDP-43 has been suggested to regulate autophagy through its association with the mRNA of Atg7, whose gene product, autophagy related 7 (ATG7) is a major autophagy component. When TDP-43 level is depleted by gene
silencing, ATG7 reduction and impairment of autophagy ensue, implying the link between TDP-43-induced toxicity and protein clearance dysfunction (Bose, Huang, and Shen 2011).

**Defects in RNA metabolism and toxicity**

In the late 2000s, through the identifications of mutations in *TARDBP* and *FUS* as primary causes of ALS, there has been a shift in focus to deficits of RNA metabolism as a common pathogenic mechanism of ALS (Gitcho et al. 2008; Kabashi et al. 2008; Sreedharan et al. 2008; Kwiatkowski et al. 2009; Vance et al. 2009). As stated above, TDP-43 is critical to ensure proper RNA processing including splicing, transcription, mRNA transport, regulation of non-coding RNAs, and formation of stress granules in response to various cellular stress (Ratti and Buratti 2016). Although it is not certain whether TDP-43 mutants cause ALS through the loss of their physiological function or the gain of aberrant function, studies on the physiological roles of TDP-43 demonstrate a slim margin of error for regulating proper expression and localization of TDP-43 in healthy neurons; aberrant TDP-43 regulation will lead to ALS. First, for the loss-of-function hypothesis, TDP-43 is required for normal developmental processes as mice with deletion of TDP-43 at both the embryonic stage and postnatal stage are not viable (Chiang et al. 2010; Kraemer et al. 2010). Hemizygous *TARDBP* mutant mice, though viable, displayed motor phenotype, yet without signs of motor neuron degeneration (Kraemer et al. 2010). Second, for the gain-of-function hypothesis, mouse models overexpressing wild-type or mutant TDP-43 developed motor neuron degeneration
reminiscent of ALS (Wils et al. 2010; Stallings et al. 2010; Cannon et al. 2012). Together, these studies suggest a tight regulation for steady cellular concentration of TDP-43 is crucial for maintaining development and motor neuron health. In line with these observations, nuclear TDP-43 regulates its own expression through a negative feedback mechanism by which the protein can bind to the 3’ untranslated region (UTR) of its own pre-mRNA and direct the use of distal poly A sites to promote alternative splicing, resulting in a spliced variant that is sensitive to degradation through nonsense-mediated decay (Ayala et al. 2011; Avendaño-Vázquez et al. 2012; Koyama et al. 2016). In the case of TDP-43 pathology, depletion of nuclear TDP-43 leads to abnormal upregulation of TDP-43 synthesis, resulting in a vicious cycle of imbalance in TDP-43 expression (Koyama et al. 2016). The mutant-induced mislocalization of nuclear TDP-43 thus is proposed to confer a toxic function by forming cytoplasmic aggregates and concomitantly a loss of function by sequestering and depleting nuclear TDP-43 pool for RNA processing (Peters, Ghasemi, and Brown 2015). Notably, other RNA/DNA-binding proteins that are associated with ALS, including FUS, TAF15, hnRNPA2B1, and hnRNPA1 have been documented with cytoplasmic mislocalization that resembles mutant TDP-43 (Kwiatkowski et al. 2009; Couthouis et al. 2011; Kim et al. 2013).

Hexanucleotide (GGGGCC) repeat expansion mutations within the non-coding region of the C9ORF72 gene are the most common cause of ALS, FTD and ALS-FTD (DeJesus-Hernandez et al. 2011; Renton et al. 2011; Gijselinck et al. 2012). In normal individuals, there are less than 30 copies of the repeat expansion present, whereas in C9ORF72-associated ALS patients, there are hundreds of copies of the repeat
There are several proposed disease mechanisms through which mutations in \textit{C9ORF72} may contribute to ALS and FTD pathology. Three major pathomechanisms have arisen including 1) a reduction of physiological C9ORF72 protein expression and function (Waite et al. 2014), 2) a toxic gain of function by the intranuclear RNA foci, composed of RNA transcripts containing the repeat expansion that may sequester RNA-binding proteins (Donnelly et al. 2013; Sareen et al. 2013; Mohan, Goodwin, and Swanson 2014), and, 3) a toxic gain of function by dipeptide repeat (DPR) proteins produced from the repeat expansion transcripts and form cytoplasmic and intranuclear inclusions (Zu et al. 2013; Gendron et al. 2013).

Much evidence has been reported suggesting a loss of function of C9ORF72 contributing to disease. First, reduced levels of one or more of the \textit{C9orf72} transcript variants have been demonstrated in both induced pluripotent stem cell (iPSC)-derived neurons and CNS tissues of \textit{C9ORF72}-ALS/FTD patients (Gijselinck et al. 2012; Donnelly et al. 2013). Second, both C9ORF72 loss-of-function models in \textit{Caenorhabditis elegans} and zebrafish resulted in motor deficits (Therrien et al. 2013; Ciura et al. 2013). However, \textit{C9ORF72} knockout mice have no detectable neurodegeneration, suggesting that haploinsufficiency of C9ORF72 is not the primary mechanism for neurodegeneration (Atanasio et al. 2016; Burberry et al. 2016; Koppers et al. 2015; O'Rourke et al. 2016). Notably, ablation of \textit{C9ORF72} expression in several mouse models resulted in immune system dysregulation, including enlarged lymph nodes and spleen and autoimmune phenotype. This phenotype is likely caused, at least
partially, by haploinsufficient C9ORF72 regulating endosomal trafficking, lysosomal function, and autophagy (Levine et al. 2013; Farg et al. 2014; Sellier et al. 2016; Sullivan et al. 2016; Corbier and Sellier 2017; Tang et al. 2020). Thus, haploinsufficient C9ORF72 may lead to disturbed protein homeostasis.

RNA foci-mediated toxicity has also been suggested as a main pathogenic process for the disease. In vitro studies have demonstrated that GGGGCC repeat RNAs are prone to form highly stable secondary structures including G-quadruplexes and other heteroduplexes (Fratta et al. 2012; Haeusler et al. 2014). These stable secondary structures can mediate sequestration of RNAs, and RNA-binding proteins (RBPs), which leads to secondary pathological processes. For example, hexanucleotide repeat expansion RNA foci have been shown to bind to nucleolin (Haeusler et al. 2014), Pur-α (Sareen et al. 2013), and RanGAP (K. Zhang et al. 2015), whose depletion can activate nucleolar stress, dysregulation of stress granule formation, and disruption of nucleocytoplasmic transport, respectively.

There is accumulating evidence suggesting dipeptide repeat (DPR) as the major toxic molecules in C9ORF72-ALS/FTD. While the distribution of RNA foci does not correlate with the pattern of neurodegeneration in post-mortem tissues from C9ORF72 patients, sense-encoded poly-GR DPRs show colocalization with TDP-43 and correlate to neurodegeneration (DeJesus-Hernandez et al. 2017; Saberi et al. 2018). Like RNA foci, DPRs have shown to be involved with 1) nucleocytoplasmic transport defect by disrupting the nuclear pore complex, 2) sequestration of RBPs, including ribosomal proteins and components of the stress granules, 3) induction of nucleolar stress and

In addition to these three major pathomechanisms, other pathological processes have also been documented, including perturbation in neuronal excitability (Devlin et al. 2015). Taken together, although it has not been established which of these mechanisms is the primary contributor to disease progression, they are not mutually exclusive and all highlight the interplay of pathological protein homeostasis and RNA biology in ALS.

**Perturbations in cytoskeletal dynamics and axonal transport**

One of the earliest pathological alterations in ALS occurs in the axon and the distal synapse. In addition, there are growing evidence characterizing axonal transport deficit as one of the earliest axonal pathologies in ALS. See below for further discussion. (Section ALS and axonal transport).

**1.2.2 Diagnosis and treatment of ALS**

Current diagnosis of ALS remains grounded in medical history and clinical evaluation for abnormal mechanical properties of muscles (e.g. muscle weakness or fasciculations) with electrodiagnostic techniques, neuroimaging, and assays on blood or cerebrospinal fluid (CSF) to exclude other possible diagnoses. Due to the heterogeneous clinical features and broad spectrum of ALS and related subtypes of motor neuron diseases,
their accurate and timely diagnosis is challenging. Accordingly, the mean diagnostic delay is over one year, during which, a critical window for effective therapeutic potential may be missed (Paganoni et al. 2014).

For patients presenting with typical signs of both UMN and LMN dysfunction that progressively spreads from one body region to another, diagnosis can be made relatively simply; however, for patients with slow disease progression or at early stage with mild symptoms, it is difficult to discriminate ALS from other ALS-mimicking disorders. To this end, the differential diagnosis is important to exclude ALS from other neurological conditions, subtypes of motor neuron disorders, and clinical conditions that damage specific regions of the motor system.

Currently there are only two drugs approved by the U.S. Food and Drug Administration (FDA) and show modest efficacy in treating ALS. Riluzole, a sodium channel blocker that blocks glutamatergic neurotransmission can improve patient survival for an average of three months (Lacomblez et al. 1996). Edaravone, an antioxidant that reduces oxidative stress, shows reduced functional loss in patients after six months of treatment compared to patients in the placebo group (Writing Group and Edaravone (MCI-186) ALS 19 Study Group 2017).

The general delay in diagnosis and the modest drug efficacy point to the value of developing and validating a diagnostic and pharmacodynamic biomarker for ALS as this will allow personalized care-planning and maximize therapeutic benefit to patients at the early course of the disease.
1.2.3 Current biomarkers for ALS

A biomarker is defined by the National Institute of Health as a “characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathologic processes, or biological response to a therapeutic intervention” (Biomarkers Definitions Working Group. 2001). A more detailed categorization of biomarkers by the FDA include 1) diagnostic, 2) prognostic, 3) predictive, and 4) pharmacodynamic biomarkers (US Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER). Guidance for Industry and FDA Staff: Qualification Process for Drug Development Tools2014; Benatar et al. 2016). In the past two decades, there have been promising biomarker candidates, but none have been validated for measuring disease progression or for drug development. Current candidate biomarkers for ALS include protein-based, electrophysiological, and neuroimaging biomarkers (Bowser, Turner, and Shefner 2011).

Protein-based biomarkers

At disease onset and during disease progression, changes in protein or cytokine expression level can be detected and quantified in the blood, CSF, or urine by using antibody-based immunoassays. Current leading candidates by this method include neurofilament light chain (NfL), CSF phosphorylated neurofilament heavy chain (pNfH), CSF SOD1 levels, p75 neurotrophin receptor extracellular domain (p75NTR<sup>ECD</sup>), and DPR from patients with <i>C9orf72</i> repeat expansion. A recent discovery of inflammatory cytokines including monocyte chemoattractant protein-1 (MCP-1) and interleukin-18 (IL-
18) has demonstrated a promising potential as an indicator of neurodegeneration and disease progression in ALS (Huang et al. 2020). Some common hurdles and limitations when selecting these protein-based and biofluid-based biomarkers are the stability of the protein (i.e., whether the proteins can be quantified longitudinally), the specificity of the proteins to ALS (i.e., whether the proteins can differentiate ALS from other similar neurological conditions), and the availability of specific immunoreagents (e.g., the lack of reliable antibodies to detect individual DPR protein species) (Benatar et al. 2016).

**Electrophysiological biomarkers**

Current diagnosis of ALS employs needle electromyography (EMG) and nerve conduction studies to assess LMN functions as a measure of active denervation from their corresponding muscles. Although these diagnostic tests are able to distinguish motor from sensory nerve pathology, they are limited for timely and accurate diagnosis because the muscle weakness or fasciculations are not necessarily a prelude to the onset of ALS. Indeed, the sensitivity of EMG in diagnosing ALS is not high (60.7%) (Douglass et al. 2010). Over the years, many electrophysiological methods have been developed to track motor unit loss and to measure therapeutic effects of treatments. These include compound motor action potential (CMAP), motor unit number estimation (MUNE), and transcranial magnetic stimulation. These promising techniques also have a set of limitations, including challenges in test-retest repeatability (e.g., CMAP), the lack of longitudinal studies, the need of extensive training and expertise to operate (e.g., MUNE), and insufficient validation.
Neuroimaging biomarkers

Current candidates for ALS neuroimaging biomarker focus on 1) detecting gross structural changes and atrophy of the brain in the gray matter or white matter tracts, and 2) molecular imaging of markers reflecting pathological mechanisms, such as hypometabolism and neuroinflammation in the brain.

Developing and validating robust ALS biomarkers for early diagnosis, monitoring of disease progression, and drug evaluation are pressing needs in the field. Since ALS is heterogeneous, progressive, and involves multisystem dysfunctions, it is essential that a biomarker, or a combination of biomarkers can target a common pathology that reflects overall motor neuron health.

1.2.4 Motor neuron susceptibility in ALS

While ALS is recognized as the loss of both the upper and lower motor neurons in a broad sense, not all motor neurons are affected equally. In patients and animal models of ALS, specific motor neuron subpopulations are spared even at end stage disease, while other subtypes of motor neurons are targeted for progressive degeneration. Oculomotor neurons and Onuf’s nuclei motor neurons, for instance, are relatively preserved from neurodegeneration in ALS, allowing patients at progressive disease stage to still be able to have eye movements and pelvic sphincter control (Mannen et al. 1977; Gizzi et al. 1992; Kaminski et al. 2002). Importantly, even among vulnerable
spinal somatic motor neurons that innervate skeletal muscles, there is different selective susceptibility to degeneration in ALS (Frey et al. 2000; Pun et al. 2006).

In the spinal cord, a single motor neuron that innervates multiple muscle fibers within a given muscle form one motor unit, whereas multiple motor neurons innervating a single muscle form a motor pool. Each motor pool is composed of three types of somatic motor neurons—alpha (α), beta (β), and gamma (γ) motor neurons, according to the type of muscle fibers each innervates. Alpha motor neurons innervate extrafusal skeletal muscle fibers for muscle contraction; γ-motor neurons innervate intrafusal muscle spindles for increasing their sensitivity to stretch; and β-motor neurons, whose function are yet to characterized, innervate both extrafusal and intrafusal skeletal muscle fibers. Alpha-motor can be classified as fast- (F) and slow-twitch (S) fatigue-resistant types based on their contractile and metabolic properties. The F-motor units control the Type II muscle fibers, whereas the S-motor units control the Type I muscle fibers. Lastly, F-motor units can be further divided into fast-twitch fatigable (FF) type that innervates Type IIb muscle fibers or fast-twitch fatigue-resistant (FR) type which innervates Type IIa muscle fibers (Burke and Tsairis 1973; Kanning, Kaplan, and Henderson 2010).

Using the SOD1G93A mouse model, Caroni and colleagues reported the time course of motor unit loss and denervation pattern in the gastrocnemius muscle with a degenerating sequence from FF to FR to S motor units (Pun et al. 2006), illustrating a predictable temporal and spatial pattern of preferential vulnerability in specific motor neuron subtypes. A separate study also demonstrates the preferential degeneration of
α-motor neurons and the complete sparing of γ-motor neurons in SOD1\textsuperscript{G93A}, TDP-43\textsuperscript{A315T} and FUS\textsuperscript{P525L} mouse models (Lalancette-Hebert et al. 2016). These data concur with the observations that twitch force of F-motor units is affected first in sALS patients, suggesting this may be a common feature to all ALS patients (Dengler et al. 1990).

As to what may cause motor neurons to be selectively targeted for degeneration in ALS and why specific motor neuron subtypes are more vulnerable than others, few possible intrinsic and extrinsic factors of motor neuron subtypes have been investigated to speculate this selectivity. Comparing gene expression profiles of oculomotor nuclei, the hypoglossal nucleus, and the motor neurons in the spinal cord of both ALS patients and rat model reveal differential expression of 1) pro-survival or toxic factors (e.g. insulin-growth factor, semaphorin A3, EPH receptor A4 (EPHA4)), 2) isoforms of pump for neuronal excitability (e.g. α1 and α3 isoforms in Na\textsuperscript{+}/K\textsuperscript{+} ATPase); 3) AMPA receptors for calcium permeability (e.g. glutamate receptor 2 subunits); 4) protein chaperones (e.g. SIL1) to relieve ER stress among the motor neuron subtypes (Ragagnin et al. 2019). Other intrinsic differences from and among motor neurons include: 5) their large size with high demand for energy and 6) their vital need to maintain functional axonal trafficking (Kanning, Kaplan, and Henderson 2010; Nijssen, Comley, and Hedlund 2017; Ragagnin et al. 2019).

1.2.5 ALS and axonal transport

There are several lines of investigation implicating a strong contributing role of cytoskeleton disorganization and axonal transport deficits to ALS pathology. First,
histological-, biochemical-, radiolabeled-, fluorescence-based studies, including fluorescent reporter strains, have documented alterations in axonal trafficking of various cargoes for both anterograde and retrograde transport occurring months before other signs of neurodegeneration. These alterations exist across ALS patients and multiple transgenic ALS models, including several ALS-linked mutant TDP-43 and mutant SOD1, suggesting a global defect in axonal trafficking in ALS (Table I) (Breuer et al. 1987; Sasaki and Iwata 1996; B. Zhang et al. 1997; Warita, Itoyama, and Abe 1999; Williamson and Cleveland 1999; Ligon et al. 2005; De Vos et al. 2007; Bosco et al. 2010; Bilsland et al. 2010; Magrane et al. 2012; G. A. Morfini et al. 2013; Wang et al. 2013; Magrané et al. 2014; Alami et al. 2014).

Second, several ALS-linked mutations in genes encoding proteins with involvement in cytoskeletal network and transport machinery have been identified. These include neurofilament heavy chain (NFH), an intermediate filament; peripherin (PRPH), an intermediate filament in the periphery nervous system; dynactin-1, the dynein motor activator; profilin-1 (PFN1), an essential regulator of actin polymerization; tubulin α4A (TUBA4A), a constituent of microtubule; never in mitosis gene a (NIMA)-related kinase 1 (NEK1), a kinase essential for microtubule stability and dynamics; and annexin A11 (ANXA11), a tether for RNA granules and lysosome transport, highlighting a caustic link between dysfunctional axonal network and ALS (Figlewicz et al. 1994; Gros-Louis et al. 2004; Puls et al. 2003; Wu et al. 2012; Smith et al. 2014; SLAGEN Consortium et al. 2016; Smith et al. 2017; Liao et al. 2019).
Third, studies of differential expression in vulnerable spinal motor neurons and resistant oculomotor neurons demonstrate higher expressions of both peripherin and cytoplasmic dynein motor in spinal motor neurons, which may confer their susceptibility to axonal transport defects (Hedlund et al. 2010; Comley et al. 2015). Indeed, overexpression of peripherin results in axonal transport impairment (Millecamps et al. 2006). Additionally, reduction of dynein function in a SOD1<sup>G93A</sup> mouse model was able to rescue motor neuron disease progression and increase life span, possibly through inhibition of retrograde transport of stress signals induced by mutant SOD1, implying unnecessary dynein expression may not benefit SOD1-linked ALS pathology (Kieran et al. 2005; Perlson et al. 2009). Both these observations reflect the importance of axonal transport regulation and underscore what makes vulnerable motor neurons sensitive to perturbations.

Fourth, pharmacological, genetic-modified, and gene-editing treatments that stabilize microtubule dynamics have provided neuroprotective effects in several ALS models, suggesting a potential therapeutic strategy for ALS treatment. (Fanara et al. 2007; Taes et al. 2013; Dewil et al. 2007; W. Guo et al. 2017).

Lastly, recently identified modifier genes for ALS, such as EphA4 and kinesin-associated protein 3 (KIFAP3), are involved with axonal network and cytoskeletal dynamics (Van Hoecke et al. 2012; Landers et al. 2009). Reduced expression of both EphA4 and KIFAP3 increase survival of ALS patients, making them potential therapeutic targets for ALS.
Table I. List of axonal transport impairment in various ALS models. An increase in transport is marked with +, and a decrease in transport is marked with -.

<table>
<thead>
<tr>
<th>Mutated genes</th>
<th>Model</th>
<th>Anterograde</th>
<th>Retrograde</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>N.D.</td>
<td>human ALS (nerve biopsies)</td>
<td>+</td>
<td>-</td>
<td>video-enhanced contrast microscopy</td>
<td>(Breuer et al. 1987)</td>
</tr>
<tr>
<td>N.D.</td>
<td>human ALS (postmortem autopsy)</td>
<td>-</td>
<td>-</td>
<td>ultrastructural change of proximal axon</td>
<td>(Sasaki and Iwata 1996)</td>
</tr>
<tr>
<td>SOD1^{G93A}</td>
<td>mouse (in vitro)</td>
<td>-</td>
<td>-</td>
<td>methionine pulse labeling</td>
<td>(B. Zhang et al. 1997)</td>
</tr>
<tr>
<td>SOD1^{G93A}</td>
<td>mouse (in vitro)</td>
<td>-</td>
<td>-</td>
<td>ligation</td>
<td>(Warita, Itoyama, and Abe 1999)</td>
</tr>
<tr>
<td>SOD1^{G37R}, SOD1^{G85R}</td>
<td>mouse (in vitro)</td>
<td>-</td>
<td>-</td>
<td>methionine pulse labeling</td>
<td>(Williamson and Cleveland 1999)</td>
</tr>
<tr>
<td>SOD1^{G93A}</td>
<td>mouse (in vitro)</td>
<td>-</td>
<td>-</td>
<td>fluorogold</td>
<td>(Ligon et al. 2005)</td>
</tr>
<tr>
<td>SOD1^{A4V}, SOD1^{G93A}, SOD1^{G85R}, SOD1^{G37R}</td>
<td>mouse (in vitro)</td>
<td>-</td>
<td>-</td>
<td>time-lapse microscopy</td>
<td>(De Vos et al. 2007)</td>
</tr>
<tr>
<td>SOD1^{H46R}</td>
<td>isolated squid axoplasm</td>
<td>-</td>
<td>-</td>
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<td>(Bosco et al. 2010; G. A. Morfini et al. 2013)</td>
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<td>SOD1^{G93A}</td>
<td>mouse (in vitro)</td>
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<td>fluoresceinly labeled TTC imaging</td>
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<td>SOD1^{G93A}</td>
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<td>-</td>
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<td>-</td>
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<td>kinetics of TDP43 granules</td>
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### 1.3 Aging

#### 1.3.1 Aging as a major risk factor for susceptibility to ALS

Aging is broadly defined as the time-dependent deterioration of physiological functions for survival and reproduction (Gilbert and Gilbert 2000). This deterioration is the major risk factor for most human diseases, including cardiovascular diseases, cancer, diabetes, and neurodegenerative diseases. Notably, the mean age of ALS at onset of symptoms is 55 to 65 years, implicating aging as a primary risk factor for ALS pathology. Interestingly, ALS and aging share some common pathological features, including axon loss, decrease in synaptic vesicles, decrease in muscle mass and strength (sarcopenia), and increase in oxidative stress (Marner et al. 2003; Ceballos et al. 1999; Jang and Van Remmen 2011; Park 2015). One of the best examples demonstrating a link between age- and ALS disease-related alterations is that they share the same susceptibility to the denervation process, based on the motor neuron subtypes; the motor neurons subject to selective vulnerability to neuromuscular junction (NMJ) denervation and axonal swelling in diseased mice are the same in older mice during normal aging. Similarly, motor neurons that are selectively resistant to degeneration in ALS are also relatively resistant to aging (Lexell 1995; Valdez et al. 2010; 2012).

<table>
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<tr>
<th>TDP43&lt;sup&gt;MM37V&lt;/sup&gt;, C9orf72)</th>
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<tr>
<td>TDP43&lt;sup&gt;MM37V&lt;/sup&gt; mouse (in vivo)</td>
<td>- fluoresently labeled TTC imaging</td>
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1.3.2 Aging and axonal transport

Early studies of spinal motor neurons show decline in axonal transport as a function of age (McMartin and O’Connor 1979; Frolkis et al. 1985; Frolkis, Tanin, and Gorban 1997; McQuarrie, Brady, and Lasek 1989; Viancour and Kreiter 1993; Fernandez and Hodges-Savola 1994). Recently, a meticulous investigation by Coleman and colleagues has documented the age-associated changes in axonal transport in both the peripheral nerve and central nervous system (CNS) in two fluorescence-reporter mouse models. They observed a common pattern of declining axonal transport (for both vesicular, and mitochondria transport) in both peripheral and central nervous tissues: an early drop of axonal transport rate in the “young adult” age (between 3 to 6 months), followed by a relatively stable “adult plateau” (around 18 months), and a second drop in old age (between 18 to 24 months) (Milde et al. 2015). A similar study by Schiavo and colleagues reported conflicting results. Using intravital imaging of fluorescently-labeled tetanus toxin fragment C (TTC), they did not observe alterations in axonal transport in mice aged from one to 13 months, although this study did not evaluate mice over 13 months old (Sleigh and Schiavo 2016). Further studies are required to validate these results and ascertain the impact of normal aging on axonal transport.
1.4 Neuroimaging (MRI and SPECT/CT)

1.4.1 Methods of neuroimaging

*Magnetic resonance imaging*

Magnetic resonance imaging (MRI) is a commonly used clinical technique to image structures of the brain including gray matter, white matter, vasculature and potential pathological tissue. Four steps occur in the MRI machine. First, MRI applies a strong magnetic field on the hydrogen protons abundant in the human body, as it is mostly made up of water and fat. Hydrogen protons in the body spin on randomly oriented axes, going in various directions, but when a strong magnetic field is applied to them, most of them align in the direction of the field and move in the same frequency as the magnetic field. Second, the machine then applies a radio frequency pulse toward the area of interest on the body. The radio frequency excites the protons and causes them to spin in phase, changing the direction of the net magnetization. Third, when the radio frequency is off, the excited protons return to alignment with the external field and emit a detectable signal that is captured by a computer. The rate of realignment with the magnetic field, also known as relaxivity, is different for each proton, depending on what tissues surround it and the presence of any contrast agents, such as gadolinium. Thus, the signal released by each proton is different. Fourth, the signal is converted into an MR image. Using the MRI system, injectable contrast reagents can be applied to alter the local magnetic field in the tissue being examined.
**SPECT/CT**

Single-photon emission computed tomography (SPECT)/ computed tomography (CT) is a molecular imaging technique that uses radioisotopes attached to a carrier protein as a radiotracer to visualize the region of interest. The radiotracer, once introduced into the subject, releases gamma rays that are captured by the SPECT camera to create a 3D image. Due to a low spatial resolution from the SPECT image, an accompanied CT scan is usually required.

**1.4.2 MRI and SPECT/CT involving ALS**

MRI is commonly used in the diagnosis of ALS, mainly to exclude alternative diseases such as spinal cord tumors and cervical spondylosis. Among different structural MRI techniques, diffusion tensor imaging has shown high sensitivity to detect axon pathology within white matter tracts. In recent years, functional MRI has shown to be capable of detecting subtle difference in brain activation.

SPECT or positron emission tomography (PET) imaging has been involved in the study of ALS. Specifically, glucose metabolism and cerebral blood flow are both reduced in patients with ALS. Currently, fludioxyglucose (FDG) has been used to study abnormal pattern of hypometabolism in ALS and FTD patients (Cistaro et al. 2014).
1.5 Tetanus toxin and tetanus toxin fragment C

1.5.1 Tetanus toxin and structure

Tetanus toxin produced by *Clostridium tetani* is a neurotoxin known for causing tetanus (also known as lockjaw), a condition characterized by painful, uncontrolled spasms with rigidity of the voluntary muscles. It is made of a single peptide of approximately 150 kDa which gets post-translationally cleaved to produce a heavy chain and a light chain, linked by a disulfide bond. The light chain contains a zinc metalloprotease that cleaves a member of the SNARE proteins, while the heavy chain comprises two functional domains: the N-terminal translocation domain and the C-terminal receptor-binding domains. While studying the protein structure of tetanus toxin in the late 1970s, Helting and colleagues carried out partial proteolysis of the toxin with papain and yielded two major protein fragments, B and C, respectively. Fragment B of the toxin contains the light chain and the N-terminal portion of the heavy chain, whereas fragment C is derived from the C-terminal portion of the heavy chain (Figure I) (T. Helting and Zwisler 1974; T. B. Helting and Zwisler 1977; T. B. Helting et al. 1978).
**Figure I-1. Schematic of a tetanus toxin molecule.** Tetanus toxin is composed of a light chain and a heavy chain. The light chain contains a zinc-dependent protease that cleaves synaptobrevin, whereas the heavy chain contains a N-terminal translocation domain and a C-terminal receptor-binding domain. Under papain treatment, the toxin yields two fragments—a 100 KDa fragment B and a 50 KDa fragment C as the receptor-binding domain. This diagram was adopted from Calvo et al (Calvo et al. 2012).

### 1.5.2 Mechanism of action

In the last few decades, the cellular mechanism of tetanus toxin action, or its “journey” from the peripheral neurons to the CNS, has been characterized in detail. This mechanism can be summarized in the following steps: 1) neurospecific binding, 2) internalization, 3) retrograde transport, 4) trans-synaptic transfer to connected interneurons, 5) membrane translocation and catalytic cleavage of the SNARE proteins.

Once tetanus toxin enters the body, perhaps through a wound that is contaminated with *Clostridium tetani* spores, it gets released and reaches the peripheral nerve terminals of the motor neurons, where it binds with high affinity to specific gangliosides, glycosphingolipids enriched on the presynaptic membrane. Several protein co-receptors have also been proposed for the toxin, including nidogens, synaptic vesicle proteins 2, and glycosylphosphatidylinositol-anchored protein (Bercsenyi et al. 2014; Yeh et al. 2010; Munro et al. 2001). Tetanus toxin fragment C (TTC) is what binds the receptor. Binding initiates the clathrin-mediated endocytosis (CME) pathway where tetanus toxin gets internalized and incorporated into endolytic vesicles that are transported toward the cell body via active, long-range, dynein-based fast retrograde axonal transport. Different from other proteins that move retrogradely, tetanus toxin does not accumulate at the cell soma but undergoes trans-synaptic transfer at the
intersynaptic space between the motor neuron and the connected inhibitory interneurons. The toxin is taken up by the synaptic vesicles at the pre-synaptic membrane of the interneurons through an undefined mechanism. After transcytosis into the inhibitory interneurons, tetanus toxin encounters acidification of the synaptic vesicle. This induces the translocation domain to form membrane-spanning pores followed by the release of the catalytic domain in the cytoplasm to cleave SNARE complex, thus preventing neurotransmitter release from the inhibitory interneurons. The result is the dis-inhibition of lower motor neurons, culminating with muscle rigidity and spasms.
**Figure I-2. Action of tetanus toxin.** *Clostridium tetani* spores can be introduced through a wound site where the bacteria germinate and produce tetanus toxin. The toxin is efficiently taken up by neurons, primarily motor neurons, in the neuromuscular junctions. Through clathrin-mediated endocytosis, TTC is subsequently transported retrogradely through BDNF-signaling endosome pathway to the cell body where it gets trans-synaptic transferred to the connected interneurons. The enzymatic domain of the toxin is released into the cytosol and cleaves the SNARE proteins, resulting in disinhibition of motor neuron firing and spastic paralysis. This diagram was adopted from Sykes (https://veteriankey.com/tetanus-and-botulism/).

### 1.5.3 Application of TTC as a neuronal tracer and a therapeutic carrier

With the advance of molecular cloning techniques and separation of toxicity assays, TTC has become a useful tool for investigating neuronal circuits, a tracer for normal neuronal physiological condition, and a delivery system for neuroprotective reagents. Due to its nontoxicity, neurospecificity, and ability to access the CNS through retrograde transport, TTC has been chemically or genetically conjugated to various reporter molecules including horse radish peroxidase, β-galactosidase, and green fluorescent protein to visualize specific neuronal structure or neural pathways (P. S. Fishman and Savitt 1989; Coen et al. 1997; 1999; Miana-Mena et al. 2003; 2004).

TTC used as a carrier is efficient and improves internalization of large proteins (e.g. human IgG) (P. S. Fishman, Savitt, and Farrand 1990). Several investigations have exploited TTC as a carrier for delivering neuroprotective reagents. Hybrid proteins made of TTC fused with survival motor neuron 1, SOD1, or neurotrophic or growth factors (including insulin-like growth factor 1, glial derived neurotrophic factor, and brain derived neurotrophic factor) have all been generated (Francis et al. 2004; Chian et al. 2009; Larsen et al. 2006; Li et al. 2009). In most of these studies, TTC and their passenger proteins both retain their biological activities; however, these treatments did
not see strong efficacy, and it is not clear if the passenger proteins can be released from the vesicular compartment to the cytosol.

Using TTC as a cargo tracer has also shed light on endosomal trafficking mechanisms in neurons. By fluorescently labeling TTC, Schiavo and colleagues have detailed signaling endosome trafficking that TTC “hitchhikes” to be retrogradely transported. Upon its internalization at the presynaptic membrane via CME, TTC is incorporated into early endosomes containing activated neurotrophin receptor p75NTR and undergoes a sorting and maturation process, which leads to its eventual active retrograde axonal transport. It has been proposed that small GTPase Rab5 and Rab7 are essential for the initial sorting and maturation process, respectively (Deinhardt et al. 2006).

Intravital imaging of TTC transport has also proven instrumental in studying the pathophysiology of motor neuron diseases. Schiavo and colleague have demonstrated axonal transport deficits as an early sign of pathology in a SOD1G93A mouse model months before the manifestation of any motor symptoms (Bilsland et al. 2010). Using the same technique, they also examine the status of axonal transport impairment in two separate mouse models of ALS, one harboring a mutant TDP-43, and the other a mutant FUS gene (Sleigh et al. 2020). Interestingly, a transport defect was detected in the mutant TDP-43 model, which does not show spinal motor neuron loss, even though it has a neuromuscular pathology. On the contrary, in the FUS model, a transport defect was observed only at late disease stage when 20% motor neuron loss has already occurred. This implicates that different ALS-linked genes do not disrupt retrograde
axonal transport of signaling endosomes in a general way, due to different pathogenic mechanisms.

Lastly, over the past decades, our lab has been interested in developing a reliable and minimally invasive biomarker that would allow timely and accurate diagnosis of ALS, a sensitive and steady measurement for disease progression, and a means for evaluating therapeutic benefits. Our team has conjugated TTC with an MRI-contrast enhancer, gadolinium (Gd), and administered this conjugate in an ALS mouse model for visualization of retrograde transport as a measure of motor neuron function. Unfortunately, due to limitations in the sensitivity of the MRI modality, variability in our measurements and the lack of proper controls, our results remain inconclusive (data not shown; Seth Townsend). However, this preliminary result does not negate the potential of employing TTC as a robust biomarker of axonal transport in motor neuron disorders.

1.6 Net axonal transport

One of the hallmarks in ALS is “dying back” axon degeneration. This suggests that the earliest pathological changes of ALS occur in motor neuron terminals. Fischer and colleagues examined this by characterizing the spatiotemporal disease progression in SOD1\(^{G93A}\) mice. The pathological sequence begins with denervation at the NMJ, followed by severe loss of motor axons from the ventral root. Then, in the symptomatic stage, there is loss of motor neuron cell bodies at the lumbar spinal cord (Fischer et al. 2004). Another early axon pathological event appears to be deficit of retrograde axonal transport, which has been shown to precede muscle denervation (Bilsland et al. 2010).
Therefore, it would be unequivocally important to have a diagnostic biomarker to detect these early motor neuropathies before the onset of clinical symptoms, such as muscle weakness. We use the term “net axonal transport” to refer collectively to these parameters of concern, including subtle deficit in axonal transport, denervation at the NMJ, degeneration of axons, and mild loss of motor neurons.

1.7 Research aims and hypotheses

In summary, axonal transport is vital for neuron survival and function. Deficits of axonal transport precede onset of symptoms in ALS. TTC can be used to measure axonal transport rate given TTC’s ability to hijack retrograde axonal transport of signaling endosomes. It is significant to mention, while the quantification of bulk TTC trafficking directly measure the net change in the amount of retrograde transport, it indirectly evaluates the status of the whole axonal connectivity, which is represented by a proper balance between anterograde and retrograde axonal transport. Therefore, our assay allows assessment of the net axonal transport, a quantitative readout of synaptic function, axonal connectivity, and motor unit number. To this end, the goal of this dissertation is to determine 1) whether intramuscularly injected radiolabeled TTC can be serially visualized and its uptake at the motor neuron cell bodies quantified, 2) whether the characterization of its uptake reflects motor neuropathy in different mouse models of motor neuron-related disease, including aging, and 3) whether TTC uptake is improved by effective treatment in the ALS mouse models.
Hence, we hypothesize that SPECT/CT imaging following intramuscular injection of $^{125}$I-TTC enables the quantification of the rate of its uptake. We also hypothesize that transgenic mouse models carrying ALS-linked mutations and aging wild-type animals will exhibit an altered net axonal transport of TTC in a disease-stage-dependent and age-dependent manner. To evaluate the potential of TTC as a pharmacodynamic biomarker, we hypothesize that ALS mouse models under effective treatment exhibit an improved net axonal transport when compared to non-treated transgenic controls.
PREFACE TO CHAPTER 2:

Parts of this chapter will appear in:


The work and analysis presented in this chapter was performed by Pin-Tsun Justin Lee with the following exceptions:

Synaptosomal studies was carried out together with Dr. Carolina Cefaliello. Some of the SPECT/CT imaging time points were scanned by Dr. Yuzhen Wang. Scanning of the double labeling sections with TTC and other cell markers was done by Dr. Christina Baer and the Sander Center for Optical Experimentation (University of Massachusetts Medical School). Quantitative analysis of the colocalization data was done by Dr. Zack Kennedy.
CHAPTER 2: DEVELOPMENT OF A TETANUS TOXIN FRAGMENT C-BASED BIOMARKER FOR IN VIVO VISUALIZATION

2.1 Introduction

Amyotrophic lateral sclerosis (ALS) is a heterogeneous neurodegenerative disorder characterized by progressive motor neuron loss in the primary motor cortex, the brainstem, and the anterior horn of the spinal cord, leading to paralysis and death within five years from initial symptoms. Most cases occur in mid-life with an unknown cause; only a small subset of cases are linked to genetic mutations (R. H. Brown and Al-Chalabi 2017). Unfortunately, there is no cure for ALS and the two FDA-approved treatments show very modest effect on disease course.

Two major hurdles to ALS clinical trials and the development of new treatments are the delay in diagnosis and the metric with which to measure treatment response. Because ALS is a diagnosis of exclusion, the average time from symptom onset to diagnosis is over a year. This narrows the window of opportunity to trial therapeutics. In evaluating new treatments, current practice relies primarily on clinical response, which can be time-consuming, error-prone, and inconsistent. Therefore, one of the most urgent clinical, scientific, and pharmaceutical demands in understanding the pathophysiology of motor neuron disorders including ALS is the development of one or more biomarkers that provide a metric of motor neuron health and function.

Microtubule-dependent axonal transport is a well-characterized cellular process that is essential to normal neuron viability and function. It is particularly important in motor neurons due to their large size, high metabolic need, and the extended axon
length, which can reach up to a meter in human. It is not surprising that axonal transport dysfunction has been established as one of the early hallmarks of ALS cytopathology, documented both in patients and in various ALS models.

The non-toxic receptor-binding domain of tetanus toxin, tetanus toxin fragment C (TTC), has been marked as one of the most efficient retrogradely transported molecules in motor neurons from the periphery to the central nervous system (CNS). Due to its robust function and motor neuron-specificity, we and many others have utilized TTC as a tracer for neuroanatomy and a delivery system for neuroprotective molecules. Importantly, the retrograde transport rate of TTC has been characterized in the recent years, and a proof-of-concept study has validated its potential to be used for tracking disease progression in a SOD1 ALS mouse model (Bilsland et al. 2010). This brings us to consider using TTC as an in vivo molecular imaging biomarker, which, to our knowledge, has never been done before. This in vivo biomarker will allow us to evaluate the motor unit function and viability through measuring the net axonal transport, or the net uptake of TTC in motor neurons and their connected neurons, after intramuscular injection.

To that end, we modified TTC with radiolabeled isotopes for in vivo quantitative visualization. In this chapter, we demonstrate that radiolabeled TTC retains its receptor binding functionality in vitro. Furthermore, we document that after radiolabeled TTC is injected into the tongue or the gastrocnemius muscle, it can be visualized in vivo respectively in the hypoglossal nuclei motor neurons and the L4-5 spinal motor neurons. Third, through colocalization study, we characterize that TTC is taken up by GAD67+
GABAergic interneurons through trans-synaptic transcytosis. Fourth, we evaluate the effect of aging, sex, and strain diversity on net axonal transport.

2.2 Results

To generate a radioactive TTC, we chose isotope iodine-125 (\(^{125}\)I) due to its long half-life (~60 days), since our goal is to trace TTC uptake longitudinally. Although TTC has been utilized extensively in studying neuronal circuitry and network, only a handful of studies focus on its transport properties after retrograde trafficking to the motor neuron cell body. We are interested in gaining a better overall understanding of the TTC transport kinetics.

**Iodination of TTC does not alter its binding affinity to TTC-binding gangliosides**

To verify that iodination does not alter the specificity of TTC binding to its receptor, we treated N18-RE-105 cells, which express ganglioside GT\(_{1b}\), a binding partner for TTC (Staub et al., 1986), with unlabeled TTC or iodine-127-TTC (\(^{127}\)I-TTC), a non-radioactive isoform. In this assay, we observed comparable binding between TTC and \(^{127}\)I-TTC to the N18-RE-105 cells. On the contrary, both HeLa and COS7 cells, which do not express the ganglioside receptor for TTC (specifically GT\(_{1b}\)) (Staub et al. 1986), showed no specific binding to TTC or \(^{127}\)I-TTC (Fig. II-1A).

To further characterize binding affinity of \(^{125}\)I-TTC to neural receptors, we prepared and treated synaptosomes from mouse brain membrane with TTC and \(^{125}\)I-TTC. The dissociation constant of \(^{125}\)I-TTC (\(K_d\) approximately 100 nM) was similar to
that of TTC as previously reported (MacKenzie et al., 1997), suggesting, again, that iodination does not impair TTC’s neurospecific binding ability (Fig. II-1B). Furthermore, TTC and I-TTC bind specifically and with equivalent affinity to cells and tissue expressing the tetanus receptors.
Figure II-1. Iodination of TTC does not impair receptor binding. (A) Staining of TTC after incubating TTC or cold iodinated TTC ($^{127}$I) with HeLa, COS7, and N18-RE-105 (N18) cells show that (1) binding is selective for cells that express the TTC binding ganglioside GT1b (N18 but not HeLa or COS7), and (2) binding of iodinated TTC to the cell surface was not eliminated by iodination. Bar = 100 μm. (B) $^{125}$I-TTC binds with high affinity to its binding sites on mouse brain synaptosomes. The concentration of unlabeled TTC, labeled TTC and synaptosomal preparations were done in quintuples. Error bars represent standard error mean (S.E.M.).

$^{125}$I-TTC retains retrograde transport activity

Next, we examined the feasibility of detecting retrograde transport with $^{125}$I-TTC in vivo. Following tongue injections in 60-day-old Sprague Dawley rats, we imaged $^{125}$I-TTC uptake longitudinally at various time points. Our initial experiment design was to trace TTC signal for the first two days. We scanned the animals at 30 minutes-, 1, 6, 20, and 45 hours post-injection. Since we did not pre-treat the animals with cold iodine, we observed strong signals of radioactivity in both the tongue and the thyroid gland (due to accumulation of free radioactive iodine), with no trace of signal in the hypoglossal nuclei even up at 45 hours post-injection (Fig. II-2A). To ensure that we do not miss uptake of TTC at a later time point due to longer than expected transport kinetics, we imaged the animals a week after the injection (216 hours post-injection). Surprisingly, aside from seeing the residual signals from the tongue and the thyroid gland, we observed the emergence of a distinct signal at the anatomical position of the hypoglossal nuclei. Repeated imaging in the following days, up to 336 hours post-injection, has validated this persistent signal at the same position. The specificity of this signal was further confirmed after the animal was sacrificed and the whole brain was isolated and imaged.
ex vivo at 14 days post-injection (Fig. II-2B). We verified that this represented uptake of TTC into these nuclei with immunostaining for TTC in the hypoglossal nuclei in the brainstem (Fig. II-2C).

Figure II-2. $^{125}$I-TTC uptake at the hypoglossal nuclei was visualized following tongue injection. (A) Sagittal SPECT/CT imaging of the head of a Sprague Dawley rat following $^{125}$I-TTC injection to the tongue (asterisk) shows accumulated $^{125}$I-TTC (arrows) at the region of hypoglossal nucleus in the brainstem. The signal in the tongue
clears over time, while the hypoglossal signal lasts. (B) Ex vivo SPECT/CT imaging of the brain of the same Sprague Dawley rat from panel (A) shows specific signal at the hypoglossal nuclei at different orientations in the brainstem at 339 post-injection. (C) Staining of TTC on coronal section of the brainstem from the same Sprague Dawley rat 339 hours after TTC administration to the tongue shows labeling of hypoglossal nuclei. Bar = 100 μm.

To precisely characterize the kinetics of ¹²⁵I-TTC uptake, we evaluated the time to peak of ¹²⁵I signal in the hypoglossal nuclei with repeated imaging taken at an interval of every 3 to 4 hours up to 66 hours post-injection. This time the Sprague Dawley rats were pre-treated with potassium iodine in the water to block thyroid uptake of free ¹²⁵I. By analyzing the volume-of-interest of the SPECT acquisitions, we were able to quantitatively measure the radioactivity of ¹²⁵I signal intensity from each time point. Our results revealed the emergence of ¹²⁵I signal at the hypoglossal nuclei approximately eight hours post-injection. This highly focal signal increased over time, reaching a peak at approximately 20 hours post-injection. This was followed by a plateau and then a slow decline in signal, during which period all other background radioactivity was gradually eliminated through normal clearance mechanisms (Fig II-3). Over three weeks, this plateau signal typically declined by about 40-50%. By plotting the quantified radioactivity of ¹²⁵I over time, we constructed the net axonal transport profile of TTC in the hypoglossal motor system.

We next evaluated the net axonal transport profile of ¹²⁵I-TTC in mice following injection into limb muscles innervated by lumbosacral motor neurons. In our initial studies, we injected ¹²⁵I-TTC into the gastrocnemius muscle of healthy 12-week-old C57BL6 mice. We monitored uptake into the L4-5 motor neurons ipsilateral to the
injection by longitudinally imaging in each animal at approximately 6 hours for the first 48 hours, then every 24 hours up to two weeks (336 hours). For the first several hours, $^{125}$I-TTC was detected diffusely with strong accumulations in the abdomen, the subcutaneous tissue, and the bladder, reflecting distribution of TTC by the circulation. This diffuse signal was largely cleared, however, within the first 48 hours, reducing the whole body’s $^{125}$I radioactivity to approximately 50 times less than the initial injection amount. In the ipsilateral L4-5 region, we observed an emergent signal as early as 14 hours post-injection. This signal peaked at 48 hours post-injection and then slowly declined in a plateau analogous to that defined in the hypoglossal motor neuron (Fig. II-4A). This signal was persistent, and detectable even after 21 days post-injection. To verify that this signal was dependent on the integrity of retrograde transport of TTC within the sciatic nerve, we repeated this study in animals in which a fragment of sciatic nerve had been transected three days prior to the ipsilateral $^{125}$I-TTC gastrocnemius muscle injection. No uptake of radioactivity was detected in the L4-5 lumbar regions in the sciatic nerve-transected mice (Fig. II-4). Again, these studies documented that TTC can be labeled to permit retrograde transport from muscles to motor neurons in the same animal over many points in time. This process reflects the summation of both uptake into the motor neurons from the muscles and then the actual retrograde transport; for this reason, we designate this process the net axonal transport. Taken together, these data demonstrate that $^{125}$I-TTC retains its biological activity and is retrogradely transported from muscle to its corresponding motor neuron, making it a reliable reagent for visualizing net axonal transport in vivo.
Figure II-3. $^{125}$I-TTC signal at hypoglossal nucleus permits characterization of net axonal transport in Sprague Dawley rats. Upper panels: sequential sagittal images of the head show that $^{125}$I-TTC injected into the tongue muscles of 8-week-old Sprague Dawley rats ($n = 4$) is taken up and transported to the hypoglossal nucleus (pointed arrows) in the brainstem. The thyroid gland (pointed arrow heads) is labeled due to accumulation of free radioactive iodine. Lower panel: the quantification representation of net axonal transport to hypoglossal nucleus. Error bars represent S.E.M.
Figure II-4. $^{125}$I-TTC uptake at L4-5 lumbar segment requires retrograde axonal transport. Upper panels: sequential sagittal SPECT/CT images of the thoracolumbar region of 85-day-old C57BL6 mice with (top row) or without (bottom row) transection of the sciatic nerve are compared. The arrowhead points to the accumulated TTC at L4-5 lumbar segments. Bottom panel: the graph plots the quantitative uptake of TTC in the L4-5 region over time. Uptake is expressed as radioactivity in μCi and represents net axonal transport ($n = 4$ in the sciatic nerve-intact group, and $n = 6$ in the sciatic nerve (SN)-transected group). Data are presented as least-square mean absolute radioactivity from timepoints 24-hr to 336-hr (14 days) based on the mixed model of repeat measure (MMRM) analysis, along with individual mouse datapoint. Least-square mean differences ± standard errors (S.E.) (95% confident interval (C.I.)) were $0.072 \pm 0.01$ (0.05, 0.09) with a $P < 0.0001$. 
**TTC undergoes trans-synaptic transcytosis of into GAD67+ GABAergic interneurons**

It has been demonstrated that once TTC reaches motor neuron dendrites, it can pass out of motor neurons into the surrounding neuropil, presumably to afferent presynaptic terminal, although the specific cell types or post-synaptic structure require further validation (P. S. Fishman and Carrigan 1987). Given our ability to see a persistent signal of transported TTC in the spinal cord even up to three weeks post-injection due to a slow turnover rate, we next evaluated the localization of TTC in the anterior horn at 12-, 24-, and 96 hours post-injection. In addition, to investigate the specific cell types that associate with TTC after its trans-synaptic transfer, we examined the colocalization of TTC with various cell types in the spinal cord by co-labeling the spinal cord sections with TTC and either ALDH1L1 (a marker for astrocytes), IBA1 (a marker for microglia), or GAD67 (a marker for GABAergic interneurons synaptic boutons in the spinal cord).

At 12 hours post-injection, staining of TTC has a punctate appearance and is exclusively within the motor neuron cell bodies at the ipsilateral side. There is no colocalization with any of the other cell types (Fig. II-5). At 24 hours, the accumulated signal of TTC within the motor neurons is enhanced, with sparse puncta labeled outside the motor neurons. Interestingly, colocalization of TTC and GAD67 is evident while no significant increase in colocalization was observed between TTC and other cell markers. At 96 hours, staining of TTC dispersed more broadly throughout the extracellular space of the motor neurons, and appeared within reticulated structures.
outside of the motor neuron cell bodies. Importantly, co-labeling of TTC and GAD67 exhibited a robust increase in colocalization, which was not observed between TTC and other cell markers (Fig. II-5C, bottom row). This observation is validated by measurement of fold change of the ratio of colocalized pixels to total cell signal pixels after normalizing to non-TTC containing tissue (Fig. II-5D). Taken together, our data validate previous findings that TTC undergoes trans-synaptic transcytosis, although to our knowledge, this colocalization with GAD67+ GABAergic interneurons has never been documented (P. S. Fishman and Carrigan 1987; Perreault et al. 2006).
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<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**A**

**ALDH1L1**

**B**

**IBA1**

**DAPI TIC ALDH1L1**
Figure II-5. TTC colocalizes with GAD67+ interneurons following retrograde transport and transcytosis from spinal motor neurons. Immunohistochemistry in transverse section of L4-5 spinal cord shows no pronounced colocalizations of TTC and
(A) ALDH1L1 (astrocytes), or (B) IBA1 (microglia) at any of the timepoints. Bar = 20 μm. Immunohistochemistry in transverse section of L4-5 spinal cord shows gradually increased colocalization of TTC and (C) GAD67 (GABAergic interneurons) over the course of net axonal transport. TTC emerges from the motor neurons as early as 24 hours post-injection (indicated by the arrowhead). Bars = 20 μm. (D) Quantification of colocalization shows strong association between TTC and GAD67+ interneurons over time.

Aging animals exhibit mild delay in net axonal transport

Nearly all neurodegenerative disorders are age-dependent, although why age confers susceptibility to these diseases is not clear. Many age-dependent physiological changes have been delineated, many of which may compromise viability of neurons. The impact of aging on axonal transport has not been evaluated in detail until recently. As discussed in the introduction (Section 1.3.2), there are a handful of studies examining motor nerves ex vivo and one study examining intravital imaging. However, the effect of aging on net axonal transport has never been described in vivo. We have therefore sought to quantify net axonal transport in cohorts of wild-type mice at six different ages, including 50-day-, 100-day- (young), 300-day-, 500-day- (middle-age), 700-day- (old) and 900-day-old (very old) C57BL6 mice. Following injection of 125I-TTC into the gastrocnemius muscle, we characterized the net axonal transport profile of each age cohort (Fig. II-6A). The peak uptake is the highest in the group of young animals, which is followed by a significant reduction (~15%) between the 100-day-old mice and the 300-day-old mice. Between 300-day-old to 700-day-old mice, we see a stable plateau, which is followed by another significant reduction between 700-day-old and the 900-day-old mice (~42%) (Fig. II-6C). On the other hand, the delay in time to peak
demonstrated a biphasic profile (Fig. II-6B), with an initial slowing phase as a function of age between the 50-day-, 100-day, and 300-day-old animals then a stable phase in the older animals (between 300-day-old to 900-day-old mice). Taken together, these results document a change of net axonal transport by age, implicating a decreased uptake and transport efficiency. Notably, our observations correspond well with the recent findings by Coleman and colleagues, that axonal transport declines with aging in two distinct phases separated by a plateau period (Milde et al. 2015).
Figure II-6. Net axonal transport declines with normal aging. (A) Net axonal transport of C57BL6 mice is illustrated at six different ages: 50 (n = 9), 100 (n = 11), 300 (n = 13), 500 (n = 14), 700 (n = 13), and 900 days (n = 5). Data are presented as least-squares means relative to the Day-50 (50d) group from timepoints 24-hour to 336-hour based on the MMRM. The overall least-squares means of net axonal transport of C57BL6 mice from timepoints 24-hour to 336-hour were compared between the young 50d group versus the other age groups. The least-squares mean differences (95% C.I.) were 0.3 ± 0.08 (-0.13, 0.19) between the 50d and the 100d groups, 0.17 ± 0.08 (0.02, 0.33) between the 50d and the 300d groups, 0.13 ± 0.08 (0.02, 0.28) between the 50d
and the 500d groups, 0.16 ± 0.08 (0.004, 0.32) between the 50d and the 700d groups, and 0.41 ± 0.1 (0.21, 0.61) between the 50d and the 900d groups. Net axonal transport is significantly smaller in very old group than the young and healthy groups, with \( P \) value of 0.0001. (B) The time to peak is delayed with age, and appears to have a biphasic profile with an initial slowing (time 0 to 300 days, blue line; \( R^2 = 0.9879 \)) followed by an apparent plateau (300 to 900 days, magenta line; \( R^2 = 0.5949 \)). (C) The peak amplitude of uptake relative to the 50d mice shows a trend toward decline as the animals age. Error bars represent S.E.M.

**Net axonal transport can be impacted by strain-specific effects but not gender effect**

Other than age, there are a few risk factors associated with ALS, including sex, race, and ethnicity. ALS is more common among men than women, with a male predominance of approximately 1.5:1 (Salameh, Brown, and Berry 2015). Several studies also suggest that ALS rates are higher among non-Hispanic Caucasians (whites) compared with those of African, Asian, and Hispanic descent (Cronin, Hardiman, and Traynor 2007; Rechtman et al. 2015). As these are important parameters as possible confounders for our analysis, we sought to examine whether there is any specific effect of sex or genetic background on net axonal transport.

To examine strain-specific effects on net axonal transport, we quantified and compared the transport profiles in males of three inbred strains: 10-week-old FVB/NJ, B6SJLF1/J, and C57BL/6J mice, as these are common inbred strains used as controls of genetic mouse models. We chose the C57BL6 as the “wild-type” controls for normalizing the net axonal transport profiles of other strains. Using the same technique, \(^{125}\text{I}-\text{TTC} \) was injected into the gastrocnemius muscle, followed by quantification of net
axonal transport. Among the three strains, B6SJLF1/J mice display a modest but statistically significant change of net axonal transport profile with a reduced peak uptake (~15% reduction compared to C57BL6) when compared with the age-matched FVB/NJ and C57BL6 cohorts. However, the time to peak profile is unchanged. There was no difference detected between the FVB/NJ and C57BL6 groups (Fig. II-7).

To evaluate the impact of gender on net axonal transport, we characterized net axonal transport profiles between 10-week-old males and females of the C57BL6 strains. We observed statistically comparable profiles between the males and females (Fig. II-7). This result concurs with a recent study that reports no distinction in mitochondrial and vesicular axonal transport between male and female (Milde et al. 2015). Taken together, net axonal transport of TTC is a sensitive measure to quantitatively and non-invasively examine motor unit integrity and function in the wild-type animals.
Figure II-7. Net axonal transport is impacted by strain-specific effects but not gender effect. Upper panels: time course of sagittal SPECT/CT images of 70-day-old FVB/NJ male (n = 4), B6SJLF1 male (n = 4), C57BL6 male (n = 4), and C57BL6 female
mice (n = 4) shows accumulated radioactivity in the L4-5 lumbar segments. Lower panel: The graph plots the quantification of uptake as a function of time. The B6SJLF1 male mice display a modest but statistically significant change (~15% difference) of net axonal transport profile with a reduced amplitude when compared with the age-matched FVB/NJ and C57BL6 cohorts. No difference is found between the C57BL6 male and female cohorts. Data are presented as least-squares means relative to C57BL6 male group from timepoints 24-hour to 336-hour based on the MMRM analysis, along with individual mouse datapoint. The overall least-squares means of net axonal transport from timepoint 24-hour to 336-hour were compared between the C57BL6 male versus other groups. Least-squares mean differences ± S.E. (95% C.I.) were 0.27 ± 0.094 (0.046, 0.49) with a P < 0.025 between the C57BL6 male and the B6SJLF1 male groups, 0.072 ± 0.088 (-0.13, 0.28) with a P < 0.44 between the FVB/NJ male and the C57BL6 male groups, and 0.15 ± 0.07 (-0.016, 0.323) with a P < 0.07 between the C57BL6 male and female groups (Table II).
2.3 Methods

Recombinant expression, purification, and radiolabeling of TTC

Recombinant TTC (residues 865-1315 of tetanus toxin) with an N-terminal poly-histidine tag was overexpressed in *Escherichia coli* host BL21 (DE3) using plasmid pET28a (Novagen). The TTC expression vector, pET28a:TTC, was constructed from plasmid pMALTetC to pET28a by employing a codon-substituted cDNA clone of TTC (Makoff et al. 1989; Figueiredo et al. 1995). After induction with 1 mM IPTG for 5 hours at 37°C, the recombinant protein was purified from soluble bacterial extracts by immobilized metal affinity chromatography (IMAC) followed by ion exchange chromatography. For IMAC, histidine-tagged TTC was bound to nickel NTA agarose (Qiagen) and eluted by increasing 200 mM of imidazole. For SP Sepharose cation exchange (GE Healthcare), IMAC-purified samples were first dialedyzed against 20 mM of 2-[N-morpholino]ethanesulfonic acid (MES) buffer, pH 5.8, and were chromatographed with SP Sepharose resin. The bound material was eluted with 800 mM of NaCl. For Q Sepharose anion exchange chromatography (Amersham Biosciences), the SP-column-purified samples were dialyzed against 20 mM of BisTris buffer, pH 6.5, and were chromatographed with Q Sepharose resin. The flowthrough was first dialyzed against 20 mM PBS, pH 7.5, and was concentrated using a Centricon Plus-20 centrifugal filter unit (Millipore). The recombinant TTC was purified using a Sephacryl S-300 column (GE Healthcare) on a FPLC platform, and concentrated with a Centricon unit. All fractions of flowthrough are analyzed by Coomassie gel to confirm band size.
For iodination of TTC, two iodogen tubes were prewashed with Tris buffer. Into each iodination vial was added Na$^{125}$I for a combined activity of 10 mCi. The tubes were incubated at room temperature for 6 minutes with gentle swirling every 30 seconds. The contents of the iodogen tubes were mixed with 0.3 mg of TTC. After 10 minutes of incubation, tyrosine was added to quench the reaction. The crude product showed a labeling efficiency of 96% by ITLC (Whatman paper, saline). The material was isolated by Bio-Rad P6 spin column with DPBS. After purification, a radiochemical purity of 99% was reached by ITLC.

**Binding of TTC in N18-RE-105 cells**

N18-RE-105 neuroblastoma cells were kindly provided by Dr. Paul Fishman (University of Maryland) and were cultured in DMEM (Gibco) and supplemented with HT supplement (Gibco) (100 µM sodium hypoxanthine, 16 µM thymidine), 400 µM aminopterin (Sigma Aldrich), 10% fetal bovine serum (Gibco), and 1% penicillin/streptomycin (Gibco). HeLa and COS7 cells were cultured in DMEM (Gibco) and supplemented with 10% fetal bovine serum (Gibco), and 1% penicillin/streptomycin (Gibco). All cells were grown in an incubator at 95% air and 5% carbon dioxide at 37°C and passaged with EDTA-trypsin when confluent. To evaluate binding of TTC or TTC labeled with $^{125}$I ($^{125}$I-TTC), cells were plated on glass coverslips in 24-well plate pre-coated with poly-L-lysine (Sigma Aldrich) and were incubated with TTC or I-TTC (final concentration 2.5 µg/mL) for 1 hour at 37°C. The cells were then fixed with 4% paraformaldehyde (PFA) in PBS for 10 min, blocked with 4% donkey serum in PBS for 1
hour, stained with polyclonal antibody against TTC (Rockland) for 1 hour, and then stained with FITC-conjugated fluorescent anti-rabbit secondary antibody (Abcam) for 1 hour. Cells were mounted in ProLong Diamond antifade mountant with DAPI (Life Technology).

**Evaluation of TTC affinity to its binding sites on mammalian membrane**

To determine binding affinity of radiolabeled TTC to its receptors on mammalian membrane, synaptosomal preparation was carried out as previously described by Rogers and Snyder (Rogers and Snyder 1981). Briefly, neocortices from 10-week-old C57BL6/J mice were isolated and homogenized using glass Dounce tissue grinders (Kimble Kontes) in 10 volumes of cold 0.32 M sucrose. The homogenate was first centrifuged at 2,000 \( x \ g \) for 10 minutes and the supernatant was centrifuged at 13,000 \( x \ g \) for 20 minutes. The resulting synaptosomal pellet was resuspended in distilled water and ready to be used for the binding assay. For the \(^{125}\text{I}-\text{TTC}\) binding affinity assay, 0.5 nM of \(^{125}\text{I}-\text{TTC}\) (20,000 cpm) was incubated with the various amounts of synaptosomal preparation in the incubation buffer made of 50 mM Tris-acetate and pH 6.0, 0.5% bovine serum albumin, with a final volume of 200 \( \mu l \). After 3 hours of incubation at 0\( ^\circ \text{C} \), the suspensions were centrifuged at 12,000 \( x \ g \) for 15 minutes. The resulting pellet was washed and resuspended with incubation buffer followed by another centrifugation at 12,000 \( x \ g \) for 15 minutes. After a total of three washing steps, the pellet was resuspended in 100 \( \mu l \) of incubation buffer and radioactivity was measured on a Searle gamma counter.
To determine the affinity of $^{125}$I-TTC for its binding sites, a displacement assay of TTC was performed by incubating synaptosomal preparations with both radiolabeled TTC and excess unlabeled TTC. Each of the incubation samples were done in triplicate, and the read of radioactivity in each sample was normalized by subtracting to the radioactivity of the blank sample (no synaptosomal preparations added).

**Animals**

All animal studies reported in our studies were compliant with the protocols approved by the IACUC committee at the University of Massachusetts Medical School. Sprague Dawley rats were obtained from Taconic and C57BL/6J, FVB/NJ, and B6SJL/F1/J mice at various ages were obtained from Jackson Laboratories.

**In vivo injections of TTC**

One week prior to the start of an imaging study, potassium iodine was added to the drinking water of the animal to block thyroid uptake of free $^{125}$I. For the intramuscular tongue injection, animals were anesthetized with isoflurane and briefly removed from the nose cone. Radiolabeled TTC (~100 µg) was injected at the tongue muscles using a Hamilton syringe. For intramuscular gastrocnemius muscle injection, animals were anesthetized. The area for injection was shaved and swabbed with 70% ethanol, and radiolabeled TTC (~100 µg) was injected at the caudal gastrocnemius muscle. As a positive control for reduction of axonal transport, a cohort of mice underwent sciatic
nerve lesioning. Under anesthesia, an incision exposed the sciatic nerve, from which a fragment (3-4 mm in length) was excised, followed by suturing of the incision. After three days of recovery, radiolabeled TTC was injected ipsilaterally to the lateral gastrocnemius muscle. At the end point of the experiments, animals were sacrificed by intracardial perfusion with 4% PFA in PBS. The isolated spinal cord or brain tissues were fixed in 4% PFA for 24 hours and then incubated in 20% sucrose for 24 hours. The tissues were then frozen in Tissue-Tek O.C.T. medium (Sakura) at -80°C until cryostat sectioning.

**Immunohistochemistry**

For immunohistochemistry, all brain and spinal cord tissues were sectioned using a cryostat, blocked with 0.4% Triton X-100, 4% donkey serum, 4% goat serum and 4% bovine serum albumin (Sigma Aldrich) in PBS for two hours. For sections treated with primary antibodies that were raised in mice, the M.O.M. (mouse on mouse) blocking reagent (Vector Laboratories) was added for an extra hour. After three hours of blocking, the tissue sections were incubated with polyclonal antibody against TTC (Rockland) and IBA1 (Novus Biologicals), or monoclonal antibodies against ALDH1L1 (Millipore) and GAD67 (Abcam). Thereafter, sections were incubated with Alexa Fluor 488 anti-rabbit secondary antibody (Abcam) and Alexa Fluor 594 anti-mouse secondary antibody (Invitrogen). The sections were mounted in ProLong Diamond Antifade Mountant with DAPI (Life Technologies). Images were acquired using TissueFAXS iQ
tissue cytometer (Tissuegnostics), captured with an Orca Fusion BT camera (Hamamatsu), and analyzed with the TissueFAXS Viewer software.

**SPECT/CT Imaging**

All animals were imaged on a NanoSPECT/CT™ small animal imaging camera (Bioscan Inc.) at various time points following radiolabeled TTC injection. The CT scanning was performed first at standard resolution, using a 45 kVp voltage and 500 milliseconds exposure time. The SPECT image parameters were 1.0 mm/pixel, 256 x 256 frame size and 60 seconds per projection for a total of 24 projections. Acquisition time was approximately 30 minutes. During imaging, the animals were anesthetized with 1-2% isoflurane in 1.5 l/min oxygen. The completed CT and SPECT images were reconstructed and the volume-of-interest (VOI) analysis of the SPECT acquisitions were carried out by VivoQuant 1.23 software (InvivoCRO). At each time point, we subtracted from uptake in the designated VOI the background uptake of $^{125}\text{I}$-TTC in an equivalent but remote VOI; this yielded the uptake specific to the region of interest in either the hypoglossal nucleus or the anterior gray matter of the spinal cord. The radioactivity of VOI in each animal was normalized to that of its wild-type and age-matched controls; in the aging study, VOI radioactivity was normalized to that of young (50-day-old) mice.

**Statistical analysis**

Comparison of different experimental groups of transport profiles was analyzed based on a mixed model for repeated measures (MMRM) by Dr. Yimeng Lu at VIR.
Biotechnology. The model included all measurements (excluding the first two time points—time at 0 hour and time at 30 minutes—due to poor signal-to-noise ratio and the non-specific localization of free $^{125}$I) as the dependent variable; experiment group, visit, and treatment-by-visit interaction as the fixed effects; and individual mice as a random effect. A compound symmetry covariance structure was used to model the within-subject errors. With a mixed-effects model based on restricted maximum likelihood estimation used for the analysis, no imputation of missing data was performed, assuming data were missing at random, conditional on the fixed and random effects. The primary result obtained from the model was the least-squares mean differences between the experimental groups from 24-hour time points through the last time points. The least-squares mean differences with the corresponding 95% confidence intervals and the 2-sided $P$ values were provided. The least-squares means for each treatment group, as well as the corresponding 95% confidence intervals, were provided as well (Table II).

Statistical significance inferences of immunohistochemistry of TTC co-labeling in the anterior horn of the spinal cord were reported as fold exchange of pixel colocalization over time. ImageJ’s JACoP script was used to compute Mander’s Overlap Coefficient, which is a ratio of the number of pixels containing both TTC and cell marker signal to the total number of pixels containing cell marker signal. Fold change was normalized to pixel colocalization of negative control images to correct noise (i.e. images of the contralateral anterior horn which contains no TTC signal). Statistical significance of
changes in colocalization over time was determined using a mixed effects model corrected with Tukey’s multiple comparison test. GraphPad Prism 8 was used to compute statistical analysis.
### Table III. Statistical significance of changes in net axonal transport efficacy

<table>
<thead>
<tr>
<th>Cohort (strain; sex)</th>
<th>Age (d)</th>
<th>Sample size (N)</th>
<th>Cohort Overall LSMMeans ± SE normalized to the control group (95% CI)</th>
<th>Difference between experimental vs control group Overall LSMMeans ± SE (95% CI)</th>
<th>P value for differences between experimental and control groups</th>
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<tr>
<td><strong>Sciatic nerve-dependent transport</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(C57BL6; males)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Intact</td>
<td>85</td>
<td>4</td>
<td>0.075 ± 0.006 (0.06, 0.089)*</td>
<td>Control group</td>
<td>-</td>
</tr>
<tr>
<td>Sciatic nerve-transacted</td>
<td>85</td>
<td>6</td>
<td>0.002 ± 0.008 (-0.015, 0.02)*</td>
<td>0.072 ± 0.01 (0.05, 0.09)*</td>
<td>0.0001</td>
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<td><strong>SOD1G3A (SJL/B6; males)</strong></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>nTg</td>
<td>45-125</td>
<td>30</td>
<td>0.76 ± 0.03 (0.69, 0.83)</td>
<td>Control group</td>
<td>-</td>
</tr>
<tr>
<td>SOD1G3A</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Pre-symptomatic</td>
<td>45</td>
<td>5</td>
<td>0.87 ± 0.08 (0.7, 1.03)</td>
<td>-0.11 ± 0.09 (0.29, 0.07)</td>
<td>0.24</td>
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<td>Pre-symptomatic</td>
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<td>5</td>
<td>0.42 ± 0.08 (0.26, 0.59)</td>
<td>0.34 ± 0.09 (0.16, 0.52)</td>
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<td>Symptomatic</td>
<td>85</td>
<td>6</td>
<td>0.35 ± 0.08 (0.2, 0.51)</td>
<td>0.4 ± 0.08 (0.24, 0.57)</td>
<td>&lt;0.0001</td>
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<td>Late symptomatic</td>
<td>110</td>
<td>9</td>
<td>0.17 ± 0.06 (0.04, 0.29)</td>
<td>0.59 ± 0.07 (0.45, 0.74)</td>
<td>&lt;0.0001</td>
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<tr>
<td>End stage</td>
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<td>N.E.</td>
<td>N.E.</td>
<td>N.E.</td>
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<td><strong>SgSOD1 treatment (SJL/B6; females)</strong></td>
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<tr>
<td>nTg</td>
<td>45-125</td>
<td>30</td>
<td>0.75 ± 0.06 (0.61, 0.88)</td>
<td>Control group</td>
<td>-</td>
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<tr>
<td>SOD1G3A</td>
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<tr>
<td>SgSOD1-treated</td>
<td>85</td>
<td>7</td>
<td>0.8 ± 0.06 (0.67, 0.93)</td>
<td>0.05 ± 0.09 (-0.13, 0.24)</td>
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<td>SgControl-treated</td>
<td>8</td>
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<td>0.29 ± 0.06 (0.18, 0.41)</td>
<td>0.45 ± 0.09 (0.27, 0.63)</td>
<td>0.0001</td>
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<td><strong>C57BAC (SJL/B6; males/females)</strong></td>
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<tr>
<td>nTg</td>
<td>700</td>
<td>6</td>
<td>0.79 ± 0.05 (0.68, 0.9)</td>
<td>Control group</td>
<td>-</td>
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<tr>
<td>C9BAC</td>
<td>700</td>
<td>10</td>
<td>0.58 ± 0.04 (0.5, 0.67)</td>
<td>0.21 ± 0.07 (0.07, 0.35)</td>
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<td><strong>Aging; strain (C57BL6; males)</strong></td>
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<td>50d</td>
<td>50</td>
<td>9</td>
<td>0.75 ± 0.06 (0.63, 0.87)</td>
<td>Control group</td>
<td>-</td>
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<td>100d</td>
<td>100</td>
<td>11</td>
<td>0.72 ± 0.05 (0.62, 0.83)</td>
<td>0.03 ± 0.08 (-0.13, 0.19)</td>
<td>0.74</td>
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<tr>
<td>300d</td>
<td>300</td>
<td>13</td>
<td>0.58 ± 0.05 (0.48, 0.67)</td>
<td>0.17 ± 0.08 (0.02, 0.33)</td>
<td>0.026</td>
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<tr>
<td>500d</td>
<td>500</td>
<td>14</td>
<td>0.62 ± 0.05 (0.53, 0.71)</td>
<td>0.13 ± 0.08 (0.02, 0.28)</td>
<td>0.087</td>
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<tr>
<td>700d</td>
<td>700</td>
<td>12</td>
<td>0.59 ± 0.05 (0.49, 0.69)</td>
<td>0.16 ± 0.08 (0.004, 0.32)</td>
<td>0.045</td>
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<tr>
<td>900d</td>
<td>900</td>
<td>5</td>
<td>0.34 ± 0.08 (0.18, 0.5)</td>
<td>0.41 ± 0.1 (0.21, 0.61)</td>
<td>0.0001</td>
</tr>
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<td><strong>Immunization (C57BL6; males)</strong></td>
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<tr>
<td>Non-immunized</td>
<td>13</td>
<td></td>
<td>0.81 ± 0.06 (0.69, 0.92)</td>
<td>Control group</td>
<td>-</td>
</tr>
<tr>
<td>Immunized plus 1 booster</td>
<td>10</td>
<td></td>
<td>0.35 ± 0.06 (0.22, 0.47)</td>
<td>0.46 ± 0.08 (0.28, 0.63)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Immunized plus 2 boosters</td>
<td>8</td>
<td></td>
<td>0.35 ± 0.07 (0.2, 0.5)</td>
<td>0.46 ± 0.09 (0.27, 0.65)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Immunized 1 vs 2 boosters</td>
<td>10, 8</td>
<td></td>
<td>-</td>
<td>0.004 ± 0.096 (-0.2, 0.19)</td>
<td>0.97</td>
</tr>
</tbody>
</table>

LSMeans = least-squares means; SE = standard Error; CI = confidence interval; N.E. = not estimable.

*Based on absolute radioactivity (μCi) without being normalized to the control groups.

Analyses were based on Mixed Model for Repeated Measures (MMRM). For each experiment, the model included all measurements (excluding the first two timepoints—time at 0 hour and time at 30 minutes post-injection) as the dependent variable; cohort, visit, and cohort-by-visit interaction as the fixed effects; and individual animal as a random effect. Due to the relatively small sample sizes, a compound symmetry covariance structure was used to model the within-individual mouse errors.
PREFACE TO CHAPTER 3:

Parts of this chapter will appear in:


The work and analysis presented in this chapter was performed by Pin-Tsun Justin Lee with the following exception:

The statistical analyses based on Mixed model for repeated measures (MMRM) was carried out by Dr. Yimeng Lu.
CHAPTER 3: CHARACTERIZATION OF NET AXONAL TRANSPORT OF TTC AND DETECTION OF EARLY THERAPEUTIC BENEFITS IN ALS MOUSE MODELS

3.1 Introduction

The advance of genome sequencing in the recent years has led to the identification of new genes that are either causing or contributing to amyotrophic lateral sclerosis (ALS). Equally important to our understanding of the pathophysiological roles by these genetic mutations is the development of experimental models, the most valuable of which are animal models. Indeed, the first model of ALS, the SOD1$^{G93A}$ transgenic mouse that harbors a human mutant SOD1 protein containing the G93A mutation (Gurney et al. 1994), has been instrumental in revealing dysfunctional cellular mechanisms in ALS pathogenesis, including glutamate toxicity, non-cell autonomous involvement by the glia cells, protein misfolding, mitochondrial abnormalities, and impairment of axonal transport (Philips and Rothstein 2015; Ilieva, Polymenidou, and Cleveland 2009). In addition, transgenic SOD1 mutant mice and rats have been the workhorse for preclinical efficacy studies, including the FDA approved treatment, Edaravone (Ito et al. 2008). Moreover, several genetic mouse models have been developed recently with a wide range of distinct characteristics and phenotypes, ranging from subtle perturbations of specific cellular processes to gross and progressive pathological features of motor neuron degeneration. These differences may underscore mutant-specific, strain-specific, and overexpression-specific effects. In the context of developing a robust physiological biomarker for motor neuron diseases, with particular focus on ALS, we hypothesize that 1) defects in net axonal transport is a common pathological
denominator; 2) various ALS mouse models representing distinct motor neuron alterations display distinct net axonal transport profiles; 3) improved motor unit function and viability through therapeutic interventions can be reflected by the amelioration of net axonal transport deficit.

Our pilot studies documented in the previous chapter persuaded us to investigate several directions in this chapter. First, we characterized net axonal transport in several genetic ALS mouse models, including SOD1, PFN1, TDP43, and C9ORF72. Second, we demonstrated that net axonal transport can be used as a metric to measure therapeutic benefits and dose response for drug screening. Lastly, we also examined tetanus toxoid’s potential as a net axonal transport tracer, and the effect of active tetanus (tetanus toxoid) immunization on net axonal transport of TTC.

3.2 Results

Net axonal transport is deficient in pre-symptomatic stage and impaired with disease progression

One of the best-characterized ALS rodent models is the SOD1^{G93A} mouse, which displays deficiencies in retrograde axonal transport as early as one month prior to the onset of muscle denervation (Bilsland et al. 2010). To test its use as a biomarker for this denervating process, we recorded uptake of ^{125}I-TTC from the gastrocnemius muscle of SOD1^{G93A} mice at different disease stages: pre-symptomatic (45- and 65-day-old), minimally symptomatic (85-day-old), late symptomatic (110-day-old) and terminal stages (125-day or older). Following intramuscular injection, the animals were imaged at
24- or 48- hour intervals in a two-week span, and the radioactivity at corresponding L4-5 lumbar regions was quantified (Fig. III-1A and B). Age-matched non-transgenic littermates served as controls. At 45 days, radioactivity at the L4-5 lumbar regions of SOD1\textsuperscript{G93A} and age-matched control mice were comparable (Fig. III-1B), though the SOD1\textsuperscript{G93A} mice displayed a (non-significant) modest increase in peak radioactivity. However, at 65 days (presymptomatic stage), the SOD1\textsuperscript{G93A} mice revealed a 43% reduction in peak radioactivity at the L4-5 regions (Fig. 2C, \( P < 0.0005 \), Table II) without any change in the time to the peak uptake (Fig. III-1D). As the disease progressed with age, the abnormality of net axonal transport worsened. By 85 days (pre- or minimally symptomatic), there was a 58% reduction in amplitude of relative peak radioactivity at the L4-5 regions, as well as a delay of approximately 20 hours in the time to the peak. Further progression was evident at late symptomatic and end stages, at which respectively there were reductions in peak radioactivity of 64% and 70%, associated with 48% and 76% delays in the time to peak (Table II). These studies demonstrate that in this mouse model this assay defines a quantitative, progressive defect that correlates with progressive worsening of the motor neuron disease.
Figure III-1. Net axonal transport is progressively impaired in mice harboring transgenes with mutant SOD1 gene. (A) The time course of net axonal transport at L4-5 lumbar segments (arrowheads) was determined by quantifying $^{125}$I signals from SPECT/CT images as illustrated by sagittal thoracolumbar images of mice. SOD1$^{G93A}$ mice at various ages (middle row: 85 days; bottom row: 110 days), as compared to their 85-day-old non-transgenic littermates (top row). (B) The net axonal transport becomes progressively impaired as the disease stage progresses through four stages: pre-symptomatic (45 days, $n = 6$; 65 days, $n = 5$), pre-symptomatic or minimally symptomatic (85 days, $n = 6$), late-symptomatic (110 days, $n = 9$) and terminal stage (> 125 days, $n = 6$). Data are presented as least-squares means relative to nTg group from timepoints 24-hour to 336-hour based on the MMRM. The overall least-squares means of net axonal transport from timepoint 24-hour to 336-hour were compared between the non-transgenic controls versus the other groups. The least-squares mean differences (95% C.I.) were $-0.11 \pm 0.09 (-0.29, 0.07)$ between the D45 and the nTg groups, $0.34 \pm 0.09 (0.16, 0.52)$ between the D65 and the nTg groups, $0.40 \pm 0.08 (0.24, 0.57)$ between the D85 and the wild type groups, $0.59 \pm 0.07 (0.45, 0.74)$ between the D110 and the nTg groups. Net axonal transport is significantly smaller in all the groups except D45 than the nTg group. See Table II for the full MMRM analysis for $P$ values. $P = 0.671$ for the pre-symptomatic animals, and $P < 0.0001$ for the early-symptomatic, symptomatic, late-symptomatic, and terminal stage animals. (C) The time to peak becomes prolonged with age in SOD1$^{G93A}$ mice (red with magenta line, $R^2 = 0.98$) but not non-transgenic controls (black with blue line, $R^2 = 0.432$). (D) The relative amplitude of peak radioactivity declines ($R^2 = 0.783$) as the disease progresses in SOD1$^{G93A}$ mice. Error bars represent S.E.

Unlike TTC, tetanus toxoid is not an effective tracer for retrograde transport

Vaccinations against tetanus are essential and effective means to provide immunity against this infection. The World Health Organization recommends worldwide tetanus vaccination every 10 years throughout life. Current tetanus vaccines are based on chemical inactivation of the toxin with formaldehyde to produce the toxoid antigen. We were intrigued to examine whether tetanus toxoid (TT) has similar properties as TTC, since TT is already safely used in human.
As the toxoid antigen has been heavily modified and cross-linked, its native conformation and functionality can be greatly altered. To evaluate net axonal transport of tetanus toxoid, we labeled clinical grade TT produced at the Mass Biologics Laboratory, with $^{125}\text{I}$ and injected it into the tongue muscles of 60-day-old Sprague Dawley rats. We then characterized the net axonal transport of TT to the hypoglossal nuclei. To our surprise, we were able to detect the emergence of $^{125}\text{I}$-TT signal at the hypoglossal nuclei despite harsh detoxifying treatment. However, in contrast to $^{125}\text{I}$-TTC, the time to peak for $^{125}\text{I}$-TT is at 48 hours post-injection, a day later than $^{125}\text{I}$-TTC. Moreover, the peak amplitude of uptake of $^{125}\text{I}$-TT has a fourfold decrease. Our greatest concern is the inconsistent quantified radioactivity at the hypoglossal nuclei, which fluctuated at different imaging time points. This may imply a higher turnover rate in $^{125}\text{I}$-TT compared to TTC (Fig. III-2A).

To investigate whether $^{125}\text{I}$-TT was able to be traced reliably as a quantifiable biomarker for net axonal transport in the healthy and diseased mouse models, we administered it into the gastrocnemius muscle of 85-day-old SOD1$^{G93A}$ transgenic mice and their non-transgenic littermates, followed by imaging its uptake every 24 hours in a span of 10 days. Unlike the hypoglossal motor system which was secluded from organs that are highly responsible for metabolic clearance, the L4-5 lumbar region is juxtaposed to the kidneys. As $^{125}\text{I}$-TT has been heavily modified, it displayed a long retention rate in the animals, especially in the kidneys. The strong signal of free $^{125}\text{I}$ in the kidneys greatly masked the signal at the L4-5 lumbar region, even after the first few days. Furthermore, as described earlier, $^{125}\text{I}$-TT signal was not persistent and strong,
making it harder to be differentiated from non-specific signals in the neighboring areas (Fig. III-2B).

To avoid the masking effect due to the high retention of free $^{125}\text{I}$ in the kidneys, we chose to observe the $^{125}\text{I}$-TT uptake at the C6-T1 cervicothoracic region following forelimb triceps muscle injection. To include a positive control, we injected radiolabeled TTC alongside the radiolabeled TT into 105-day-old SOD1$^{G93A}$ mice with their age-matched non-transgenic controls. At 24 hours post-injection, our first imaging time point following injection, we observed an uptake of $^{125}\text{I}$-TTC in the C6-T1 cervicothoracic region in both nTg and SOD1$^{G93A}$ animals, while no $^{125}\text{I}$-TT signal was detected in either animal (Fig. III-2C). Like the previous experiment, strong signal of free $^{125}\text{I}$ was seen in the arm, kidneys, stomach, and bladder and has a much slower clearance rate in the $^{125}\text{I}$-TT-treated animals than the $^{125}\text{I}$-TTC-treated animals, implicating a major attenuation of the neurospecific trafficking by TT.
Figure III-2. Net axonal transport of tetanus toxoid is attenuated. (A) Upper panels: sequential sagittal (top row), horizontal (mid row), and coronal (bottom row) images of the head show that 125I-TT injected into the tongue muscles of 8-week-old Sprague Dawley rats ($n = 2$) is taken up and transported modestly to the hypoglossal nucleus in the brainstem. Lower panel: quantification representation of net axonal transport to hypoglossal nucleus.
Figure III-2. Continued. (B) Sequential sagittal thoracolumbar images show that the uptake of $^{125}$I-TT at the L4-5 lumbar segments following gastrocnemius muscle injection was faint and hazy in 85-day-old non-transgenic and SOD1$^{G93A}$ mice ($n = 4$ for each group).
Figure III-2. Continued. (C) Sequential sagittal cervicothoracic images show clear uptake of $^{125}$I-TTC, but not $^{125}$I-TT, at the C6-T1 spinal segments following triceps muscle injection in 105-day-old non-transgenic and SOD1$^{G93A}$ mice (nTg with $^{125}$I-TTC: top row, $n = 2$; nTg with $^{125}$I-TT, second row, $n = 2$; SOD1$^{G93A}$ with $^{125}$I-TTC: third row, $n = 2$; SOD1$^{G93A}$ with $^{125}$I-TT: bottom row, $n = 5$).
Impairment of net axonal transport is seen in transgenic mice expressing mutant profilin-1

Our group and others have reported that mutations in profilin-1 (PFN-1) cause ALS (Wu et al. 2012; Daoud et al. 2013; Yang et al. 2016). PFN1 is an important regulator of formin-based actin polymerization, maintenance of cytoskeletal integrity and other cellular functions. Recently, we and Dr. Zuoshang Xu have generated a transgenic mouse model expressing the ALS-associated mutation (C71G) of PFN1 (Yang et al. 2016). These mice display late onset motor neuron loss and muscle atrophy, resulting in paralysis and death. On the cellular level, mutant PFN1 aggregate formation, alteration in cytoskeleton, and accumulation of ubiquitin and p62 have been seen in motor neurons of this transgenic line. To ascertain whether our method can detect aberrant net axonal transport in this model, we administrated $^{125}$I-TTC into the gastrocnemius muscle of Thy1.2-PFN1$^{C71G/C71G}$ mice at the age of 230 days, a symptomatic stage in which motor function in these mice is noticeably abnormal with obvious foot dragging yet without full paralysis. At this stage, we observed a $\sim$73% reduction of peak radioactivity compared to the controls; the time to the peak uptake was comparable in the Thy1.2 PFN1$^{C71G/C71G}$ and control mice (Figure III-3). The data from this Thy1.2-PFN1$^{C71G/C71G}$ model are analogous to those in the SOD1$^{G93A}$ mice, demonstrating that this functional biomarker may be broadly useful in diverse categories of motor neuron disorders.
Figure III-3. Net axonal transport is diminished in transgenic mice harboring mutant PFN1 genes. Upper panels: time course of radioactivity as it accumulates in the L4-5 lumbar segments were shown in these sagittal images of PFN1^{C71G/C71G} transgenic mice at the symptomatic stage (230-day-old). Bottom panel: Quantification of net axonal transport in two symptomatic PFN1^{C71G/C71G} transgenic mice (230 d, n = 2, solid and dotted red lines) and age-matched, nTg controls (n = 4, black lines) were illustrated. Net axonal transport was greatly altered in the PFN1^{C71G/C71G} mice.
No net axonal transport alteration is seen in a TDP-43<sup>Q331K/Q331K</sup> knock-in mouse model

We recently generated a TDP-43 knock-in mouse model with the human-equivalent Q331K mutation in the mouse TDP-43 gene (White et al. 2018). These mice do not display motor neuron loss or denervation but have FTD-like cognitive dysfunction. To examine whether this mutant TDP-43 caused perturbation of net axonal transport, we quantified ¹²⁵I-TTC uptake in 300-day-old TDP-43<sup>Q331K/Q331K</sup> female mice and age-matched, gender-matched wild-type mice. With a small sample size (n = 3 for each group), we observed comparable net axonal transport between these groups, demonstrating the absence of a motor phenotype in this mouse model (Fig. III-4).
Figure III-4. Net axonal transport is not altered in a TDP-43Q331K/Q331K knock-in mouse model. Upper panels: time course of radioactivity as it accumulates in the L4-5 lumbar segments were shown in these sagittal images of 300-day-old TDP-43Q331K/Q331K knock-in mice (bottom row, n = 3) and their age-matched controls (top row, n = 3). Bottom panel: quantification of net axonal transport shows no alteration between the wild-type and the TDP-43Q331K/Q331K knock-in mice.

Impairment of net axonal transport is seen in C9ORF72 transgenic mice

A useful attribute of a biomarker for the integrity of motor neurons and the motor unit is the ability to detect perturbations in function that are biologically important but too subtle to be reflected in clinical manifestations. We have therefore used the uptake of $^{125}$I-TTC to L4-5 motor neurons to study a line of ALS mice in which there is a very clear
molecular phenotype but without weakness. We recently reported a novel line of transgenic mice that harbor a bacterial artificial chromosome (BAC) containing the human transgene with the ALS-associated C9ORF72 gene with ~600 copies of the disease-causing intronic hexanucleotide GGGGCC intronic repeat (Peters et al. 2015). Although these animals do not recapitulate the motor phenotypes of ALS, they have clear disease-specific histopathological features, including deposits of intranuclear RNA foci and an abundance of poly(glycine-proline) dipeptides. We asked whether quantitative analysis of $^{125}$I-TTC uptake can be used as a marker of sub-clinical motor unit pathology in this model \textit{in vivo}. We administered $^{125}$I-TTC into the gastrocnemius muscle of 24-month-old C9BAC transgenic mice and age-matched non-transgenic controls and followed uptake at L4-5 by SPECT/CT imaging at 24-hour or 48-hour intervals for two weeks. While the time profile of uptake, including time to peak uptake, was equivalent post-injection in both cohorts, the peak uptake of $^{125}$I-TTC was significantly lower (~22% reduction) in the C9BAC mice (Fig. III-5A). Additionally, we obtained from collaborators an independent line of younger (15-month-old) transgenic mouse carrying the same C9BAC construct. In these mice, the profile of net $^{125}$I-TTC transport was identical to that in our mice (Fig. III-5B). These data further support net axonal transport of TTC as a promising biomarker for the detection of subclinical motor neuron pathology.
Figure III-5A. Net axonal transport is reduced in aged C9BAC transgenic mice. (A) Upper panels: sequential sagittal SPECT/CT images of the thoracolumbar region of 700-day-old C9BAC transgenic mice (from Brown lab; bottom row) and the non-transgenic littermates (top row). Net axonal transport in 24-month-old C9BAC transgenic mice (n = 10, red lines) displayed subtle alteration compared to the corresponding non-transgenic littermates (n = 10, black lines). Data are presented as least-squares means relative to nTg group from timepoints 24-hr to 336-hr based on the MMRM. The overall least-squares means of net axonal transport from timepoint 24-hr to 336-hr were compared between the nTg and the C9BAC groups. The least-squares mean differences (95% C.I.) were 0.21 (0.07, 0.35) between the two groups. Net axonal transport is significantly smaller in the C9BAC group than the nTg group, with P value of 0.0071 (Table II).
**Figure III-5B. Continued.** Upper panels: sequential sagittal SPECT/CT images of the thoracolumbar region of 450-day-old C9BAC transgenic mice (from Lagier-Tourenne lab; bottom row) and the non-transgenic littermates (top row). Net axonal transport in 450-day-old C9BAC transgenic mice ($n = 6$, red lines) displayed subtle alteration compared to the corresponding non-transgenic littermates ($n = 7$, black lines). Data are presented as least-squares means relative to nTg group from timepoints 24-hr to 336-hr based on the MMRM. The overall least-squares means of net axonal transport from timepoint 24-hr to 336-hr were compared between the nTg and the C9BAC groups. The least-squares mean differences (95% C.I.) were 0.17 ± 0.064 (0.034, 0.323) between the two groups. Net axonal transport is significantly smaller in the C9BAC group than the nTg group, with $P$ value of 0.0212.
**CRISPR/Cas9 gene targeting ameliorates net axonal transport deficiency in**

**SOD1\textsuperscript{G93A} mice**

We next asked if the measurement of net axonal transport might be a sensitive, early indicator of benefit from a therapeutic intervention in ALS. To that end, we characterized the effect of CRISPR-Cas9-mediated gene targeting of SOD1 on the net axonal transport in a SOD1\textsuperscript{G93A} ALS mouse model. The treated animals showed reduced level of SOD1 protein in both the cortex and spinal motor neurons (~50% reduction) as illustrated by Dr. Zackary Kennedy.

At day 85, net axonal transport was fully preserved in the CRISPR-treated SOD1\textsuperscript{G93A} mice and distinctly better than in the untreated mice (Fig. III-6A; Table II). At that timepoint, the mice were all asymptomatic or minimally symptomatic (some 85-day mice showed leg tremors). At day 110 (symptomatic to late symptomatic stage), although we observed a 45% reduction in peak uptake and a similar time to peak uptake compared to the non-transgenic animals, these metrics were significantly better than the mock-treated controls (Fig. III-6B). This TTC-based documentation of the benefit of SOD1 gene editing was evident well before standard behavioral tests distinguished treated from untreated mice (e.g. by grip strength at ~100 days and rotarod at ~120 days; demonstrated by Dr. Kennedy). These data show that net axonal transport demonstrates early and distinct benefit from a therapeutic intervention in clinically normal ALS mice, well in advance of behavioral or survival parameters.
Figure III-6. Net axonal transport detects early improvement after CRISPR-mediated knockdown of SOD1. Improvement in net axonal transport of (A) 85-day-old and (B) 110-day-old SOD1^{G93A} mice after gene editing. For net axonal transport profile of 85-day-old mice: Black: net axonal transport in wild-type nTg mice (n = 6). Grey: untreated SOD1^{G93A} mice (Cas9 + sgControl, n = 8). Blue: treated SOD1^{G93A} mice (Cas9 + sgSOD1, n = 7). Data are presented as least-squares means relative to nTg group from timepoints 24-hr to 336-hr based on the MMRM. The overall least-squares means of net axonal transport from timepoint 24-hr to 336-hr were compared between the nTg group versus the other group. The least-squares mean differences (95% C.I.) were 0.05 ± 0.09 (-0.13, 0.24) with P value of 0.57 between the nTg control and the treated SOD1^{G93A} groups, 0.45 ± 0.09 (0.27, 0.63) between the nTg group and the untreated SOD1^{G93A} group. Net axonal transport is significantly lower in the untreated mice than the treated mice, with P value of < 0.0001 using the MMRM model (Table II).
Figure III-6. Continued. Improvement in net axonal transport of (A) 85-day-old and (B) 110-day-old SOD1<sup>G93A</sup> mice after gene editing. For net axonal transport profile of 110-day-old mice: Black: net axonal transport in wild-type nTg mice (n = 4). Grey: untreated SOD1<sup>G93A</sup> mice (Cas9 + sgControl, n = 5). Blue: treated SOD1G93A mice (Cas9 + sgSOD1, n = 4).

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**Dose-dependent benefit of AAV-microRNA suppression of SOD1-silencing on net axonal transport in SOD1<sup>G93A</sup> transgenic mice**

A quality and sensitive biomarker should be able to optimize therapeutic efficacy by assessing the dose-response relationship. Through collaboration with Dr. Mueller, who
has employed adeno-associated viral delivery of a synthetic microRNA to suppress human mutant SOD1 expression (Borel et al. 2016), we were able to demonstrate a dose-responsive improvement in net axonal transport assay with doses of the AAV-miR virus. We characterized the net axonal transport of six SOD1\textsuperscript{G93A} mouse treatment groups, with each group receiving a different dose of the therapy at birth. At 85 days, both the mock-treated and the least-dosage-treated SOD1\textsuperscript{G93A} mice showed no improvement of defective net axonal transport. The mice that received all other doses of treatment displayed similar improvement in net axonal transport; we did not observe a gradient or dose-response (Fig. III-7). These results also corresponded well with other metrics of therapeutic benefits. Together, our results suggested that net axonal transport is a reliable biomarker for assessing treatment efficacy for motor neuron disease.
Figure III-7. Dose-response effect of RNAi therapy is reflected by improvement of net axonal transport in SOD1<sup>G93A</sup> transgenic mice. Sequential sagittal SPECT/CT.
images of the thoracolumbar region of 85-day-old SOD1<sup>G93A</sup> transgenic mice after SOD1-silencing treatment with dosage range from untreated (red line, \( n = 3 \)), \( 2E^9 \) (purple line, \( n = 3 \)), \( 7E^9 \) (yellow line, \( n = 3 \)), \( 2E^{10} \) (blue line, \( n = 3 \)), \( 7E^{10} \) (green line, \( n = 3 \)) and the non-transgenic littermates (black line, \( n = 3 \)) were illustrated. Net axonal transport in the treated mice displayed dose-dependent response.

**Immunization reduces but does not eliminate net axonal transport of \(^{125}\text{I}-\text{TTC}\)**

This technique of visualizing net axonal transport has broad application in human motor neuron disorders. An important consideration is that most individuals in North America and Europe have been immunized with tetanus toxoid (TT). We therefore sought to investigate the impact of immunization of TT on the analysis of TTC uptake. We administered two consecutive immunizations of TT or PBS to wild-type mice at eight and 14 weeks of age. We then assayed the net axonal transport of \(^{125}\text{I}-\text{TTC}\) at 20-weeks of age (12 weeks from the initial immunization; red profile in Fig. III-8B). After immunization with TT, the mice developed antibody titers against TT, as expected. Mice immunized with TT demonstrated \(^{125}\text{I}-\text{TTC}\) uptake with a time profile identical to that of non-immunized controls, but with a reduced total amplitude of uptake (approximately 45% reduction), as compared to non-immunized controls (Fig. III-8B). One possible application of our transport assay is to determine how transport is affected by a trial compound, comparing net transport at baseline with net transport after starting a trial therapy. We therefore asked if repeat administrations of TTC would further attenuate the peak amplitude of net axonal transport. To test this, we conducted a second net axonal transport assay on these immunized mice ten weeks after the initial assay (22 weeks after the initial immunization, green profile in Fig. III-8B, Table II). This second transport assay was not associated with a further reduction of the net axonal transport.
This indicates that immunization of TT partially blocks the net axonal transport of TTC but that it remains subsequently feasible to use TTC transport as a biomarker for therapy efficacy in disorders like ALS.

Figure III-8A. Immunization with tetanus toxoid and TTC reduces but does not eliminate net axonal transport. (A) The titer of anti-tetanus toxoid antibodies is illustrated for the key time points in the experiment. Two cohorts of age-matched C57BL6 mice (n = 12 for each group) received two consecutive injections of either tetanus toxoid (TT) or PBS, first at 8 weeks of age and subsequently at 14 weeks. Twelve weeks after the primary vaccination (at age 20 weeks), both groups received \(^{125}\text{I}\)-TTC to characterize net axonal transport. Ten weeks later (at age 30 weeks), a repeat net axonal transport assay was performed on the previously immunized animals along with a new control set of naïve non-immunized controls (n = 4). The antibody titers against TT in mice that received a primary and booster shots of TT were significantly elevated (P < 0.0001; repeated measures two-way ANOVA after Bonferroni’s correction as compared to the mice with PBS treatments).
Figure III-8B. Continued. (B) Top panels: The time course of radioactivity as it accumulates in the L4-5 lumbar segments were shown in these sagittal images of mice that are immunologically naïve to TT (top row, \( n = 13 \), black line in the profile), mice that received two toxoid injections (middle row, \( n = 10 \), red line), and mice immunized twice with TT then TTC, whose TTC transport was re-assayed at age 30 weeks (bottom row, \( n = 8 \), green line). Bottom panel: The resulting net axonal transport profile is illustrated. For mice receiving two TT immunizations and either one (red) or two TTC injections (green), the reduction in transport as compared to wildtype naïve mice (black) is significant \((P < 0.0001, \text{Table II}; \text{the overall least-squares mean differences ± S.E. (95\% CI)}\) was 0.46 ± 0.08 (0.28, 0.63). However, those two groups had essentially the same net axonal transport profile as compared to the control group \((P = 0.968)\) with the least-square mean differences ± S.E. (95\% C.I.) being 0.004 ± 0.096 (-0.19, 0.2). In panel A, the group that received two PBS treatments followed by the \(^{125}\text{I-}\text{TTC}\) assay is designated as “PBS x 2 + TTC” (black). The group that received two TT treatments followed by the \(^{125}\text{I-}\text{TTC}\) assay is designated as “TT x 2 + TTC” (red). Panel B also
depicts the third group that underwent a follow-up $^{125}$I-TTC transport assay; this group is designated as “(TT x 2 + TTC) + TTC” (green).

For most of the longitudinal data, we requested a professional statistician, Dr. Yimeng Lu, who used a mix model with repeated measures (MMRM). The MMRM model included all measurements (except the first two timepoints—0 hour, and 30 minutes—due to poor signal-to-noise ratio from free $^{125}$I at the injection time) as the dependent variable. Experiment group, visit, and treatment-by-visit interaction as the fixed effects, and subject as a random effect. For the full analyses of least-squares means, and least-squares mean difference between the experimental group along with the control group, and the 2-sided $P$ values, please see Table II.
3.3 Methods

We used the same method for SPECT/CT as described in the Method section of Chapter 2.

Animals

As mentioned in the previous chapter, all animal studies were compliant with the protocols approved by the IACUC committee at the University of Massachusetts Medical School. Transgenic mice carrying a human SOD1 mutant (B6SJL-Tg[SOD1-G93A]1Gur/J) and C57BL/6J mice at various ages were obtained from Jackson Laboratories. The Thy1.2-PFN1\textsuperscript{C71G/C71G} transgenic mice were obtained from Dr. Zuoshang Xu (Yang et al., 2016), and their non-transgenic (nTg) FVB/NJ controls were obtained from Jackson Laboratories. The C9ORF72 transgenic (C9BAC), and TDP-43\textsuperscript{Q331K} knock-in mice were produced previously in our lab and by collaborators (Peters et al., 2015; White et al., 2018). The second strain of C9ORF72 transgenic (C9BAC) mice were obtained by Dr. Lagier-Tourenne at the Massachusetts General Hospital (Jiang et al., 2016). The SOD1\textsuperscript{G93A} mice treated with AAV9.Cas9 was provided by Dr. Zachary Kennedy, and the SOD1\textsuperscript{G93A} mice treated with rAAVrh10-miRNA to silence SOD1 expression was provided by Dr. Chris Mueller at University of Massachusetts Medical School.

In vivo injections of TT
For the full protocol of intramuscular injection, please refer to Method section in Chapter 2. We applied the same method of $^{125}$I-TT injection as with $^{125}$I-TTC.

**Tetanus toxoid vaccination**

For immunization against tetanus, C57BL6 male mice received tetanus toxoid (TT) in the gastrocnemius muscle (MassBiologics; 90 Lf (flocculation units): 10 µl) at 8 weeks of age and a follow-up booster 6 weeks later in the same muscle. For the control/naïve mice, PBS was injected instead of TT. Six weeks after the booster shot, $^{125}$I-TTC was administered to the same muscle for both the immunized and naïve mice and the net axonal transport was quantified for two weeks. To test the effect of immunization against TTC, a second dose of $^{125}$I-TTC was administered ten weeks after the initial net axonal transport assay to a new set of naïve mice, the mice immune to TTC, and the mice immune to both TT and TTC. The radioactivity of VOI in each animal was normalized to that of naïve mice. To compare levels of anti-tetanus toxoid IgG, mouse sera were collected prior to four key timepoints: the primary tetanus toxoid injection, the booster injection, and the first and the second TTC uptake assay. Mouse IgG antibody against tetanus toxoid was quantified by ELISA (XpressBio).
PREFACE TO CHAPTER 4:

The work and analysis presented in this chapter was performed by Pin-Tsun Justin Lee except the following:

The conceptualization of the mathematical model was initiated by Dr. Robert H. Brown.
CHAPTER 4: A KINETIC MODEL OF NET AXONAL TRANSPORT PREDICTING KEY RATE DETERMINANTS IN ALS MICE HARBORING C9ORF72 MUTATIONS

4.1 Introduction

In the previous two chapters, we have demonstrated that radiolabeled TTC is a sensitive physiological tracer that allows quantitative measurement of net axonal transport. The altered radioactivity profiles, representing net axonal transport in various mouse models, reflect different motor neuron abnormalities. These are reflected in two parameters that describe the uptake profile: the time-to-peak, and the peak amplitude value. For example, in the SOD1G93A mice, we see a trend of constant decline with respect to the time to peak uptake, and a drastic drop (from 45- to 65-days; Fig III-1C and D) followed by a gradual decline in the peak radioactivity as disease progresses. This pattern is distinguishable from the aging models, where the time to peak uptake has an initial decline between the young and the middle age animals, then plateaus all the way even into very old age. On the other hand, the peak uptake exhibits two periods of decline (100 days and 300 days, and between 700 days and 900 days) sandwiching a stable plateau phase (Fig II-6B and C). Equally intriguing is the net axonal transport profile of C9BAC mice, which shows comparable time-to-peak uptake but significant lower peak uptake of 125I-TTC than the controls. Since our assay evaluates the summation of the integrity of neuromuscular junctions, the function of axonal transport, and the number of viable motor units, the differential alterations in the SPECT profiles represent some compound defects with complex dynamics. To delineate the different parameters that affect the overall kinetics shaping the curves of the net axonal
transport, we sought to create a kinetic model of net axonal transport. To our knowledge, a mathematical model of in vivo net axonal transport has not been explored, either in general or in the context of motor neuron disorders. Our goal for creating a mathematical model of net axonal transport is to address two questions: 1) can a model reproduce the complex phenomena that underlie the temporal pattern of TTC uptake into spinal cord from muscles, and 2) will this model generate new hypotheses about the pathophysiology of changes in axonal transport observed in ALS?

In this chapter, we designed a kinetic model of net axonal transport by mathematical modeling using a software named Berkeley Madonna. We selected our parameters and made assumptions to reproduce the net axonal transport profile of the experimentally-measured data. Furthermore, using local sensitivity analyses, we defined key parameters that determine the net axonal transport profile. Based on our model, we were able to compare parameters that can be potentially affected by C9orf72 mutation. We concluded that our model is valuable for generating hypotheses for experimental investigation.

4.2 Results

Designing model components: selecting input parameter values and assumptions

Mathematical modeling of net axonal transport assay simulates a system where there is supply of TTC from the gastrocnemius muscle and removal of TTC at the intersynaptic space at rates that depend on the rates of several cellular processes. Moreover, the output radioactivity at the L4-5 lumbar region consists of readouts from many motor
neurons that innervate the gastrocnemius muscle, thus reflecting the integrity of motor units and motor pool. Pico Caroni and colleagues elegantly characterized a topographic map of FF, FR and S motor units that innervate the lateral gastrocnemius muscle in mouse (Pun et al. 2006). With this information, we constructed a model as follows:

In this model, TTC transport is simulated within a simplified motor unit consisting of one FF, one FR, and one S motor neurons. The trafficking zones in each motor neuron consists of, in order, the muscle fibers (M_FF, M_FR, and M_S), the presynaptic membrane/NMJ (J_FF, J_FR, J_S), the distal axon terminal (T_FF, T_FR, and T_S), the axon (AX_FF, AX_FR, and AX_S), and the cell body/interneuron (IN). The rate of TTC trafficking in and out of the system is determined by the forward and reverse rate constants (\( k_f \) and \( k_r \)) of five distinct cellular processes or “reactions”. These cellular processes are summarized as: 1) TTC binding to its specific receptor at the NMJ; 2) clathrin-mediated endocytosis at the NMJ/presynaptic membrane; 3) TTC-containing endosome maturation characterized by slow short-range movements in the distal axon terminal (Deinhardt et al., 2006); 4) retrograde transport of TTC-containing endosomes through the axon; 5) somatic sorting and release of TTC through exocytosis from the motor neuron to be either taken up by connected GABAergic interneurons or cleared from the intersynaptic space. The three motor neurons, with their parallel trafficking zones, all converge at the cell body. It is important to note that since SPECT/CT imaging does not provide the spatial resolution for differentiating motor neuron cell body and interneuron presynaptic neuropil as we quantified the uptake of radioactivity, our experimentally-measured data of the peak radioactivity is the summation of these two structures. For
this reason, the trafficking mechanism of somatic sorting to transcytosis to interneurons by TTC is not explicitly tracked in this model (Fig IV-1).

**Figure IV-1. Model of net axonal transport of TTC in motor neurons.** This kinetic model incorporates five rate-determining steps in the process of net axonal uptake of TTC from muscles to the gray matter in the spinal cord. These steps are: (1) (M to J) receptor binding of TTC at the NMJ, (2) (J to T) clathrin-mediated endocytosis of TTC at the NMJ/presynaptic membrane, (3) (T to AX) endosome maturation involved with small Rab5 and Rab7 GTPase exchange at the axon terminal, (4) (AX to IN) processive retrograde axonal transport of TTC-containing endosomes toward the motor neuron cell body, and (5) clearance of TTC at the upstream connecting network. Because the main injection site of TTC is the lateral gastrocnemius muscle, our model assumes three parallel transport pathways from the three types of muscle fiber within the muscle. These are designated M_FF (type IIb), M_FR (IIa), and M_S (type I) muscle fibers. These muscle fibers are innervated respectively by fast-fatigable (to M_FF), fast fatigue-resistant (to M_FR), and slow (to M_S) motor neurons. Forward reaction rate constants are
designated by $f$, and the reverse reaction rate constants are designated by $r$. Subscripts for forward reactions are labeled according to the reaction source; reverse reaction subscripts are labeled according to the target. The five steps and corresponding rate constants for the three reactions are labeled ff, fr or s. M: muscle; J: NMJ; T: axon terminal; AX: axon; IN: neuron cell body/interneuron.

To select input parameters for our model, we did an extensive search in the published literature for the rate constants of the aforementioned reactions. We grouped our search results into three categories: 1) published values obtained using the same system as ours; 2) published values from experimental conditions or designs different than ours (e.g. in vitro vs in vivo; rats vs mouse; botulinum toxin vs TTC, and etc.), and 3) values that are not well characterized in the literature. Among these parameters, the initial concentration for the muscle fibers and the retrograde transport forward rate of TTC have been characterized in the same system as ours (Pun et al. 2006; Bilsland et al. 2010). On the contrary, the forward rate constants for the receptor binding and endocytosis have been characterized in different experimental conditions than ours. Finally, both the forward and reverse rate constants of endosome maturation, the reverse rate constants of receptor binding, clathrin-mediated endocytosis, and retrograde axonal transport were not well defined in the literature (Table IV-1).
Table IV-1. Summary of probable input parameter values from literature.

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Finding</th>
<th>Assumed values</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle fiber type contents</td>
<td>Motor unit (MU) numbers and muscle fiber subtype compositions of the gastrocnemius and soleus muscles were measured at different stages in SOD1&lt;sup&gt;G93A&lt;/sup&gt; mice</td>
<td>SOD1&lt;sup&gt;G93A&lt;/sup&gt; at 70d – IIb: lla: I = 3.37: 29.85: 11.58</td>
<td>(Pun et al. 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SOD1&lt;sup&gt;G93A&lt;/sup&gt; at 120d – IIb: lla: I = 0: 6.88: 11.58</td>
<td></td>
</tr>
<tr>
<td>Axon numbers in aging</td>
<td>Axon numbers as a function of age were measured in the gastrocnemius muscles of different aged rats.</td>
<td>MU numbers – 300-day: ~700-day:~900-day = 100%: 87%: 30% or 63.35: 50.35: 33.35</td>
<td>(Hashizume, Kanda, and Burke 1988)</td>
</tr>
<tr>
<td>Rate of bulk solution to receptor</td>
<td>The rate of botulinum toxin, another member of the clostridial neurotoxin, to the NMJ was measured.</td>
<td>50</td>
<td>(Simpson 1980)</td>
</tr>
<tr>
<td>Rate of endocytosis</td>
<td>Clathrin-coated vesicles take ~70s to mature and get endocytosed.</td>
<td>514.29</td>
<td>(Loerke et al. 2009)</td>
</tr>
<tr>
<td>Vesicle numbers and distribution</td>
<td>Synaptic vesicles have different distribution between motor neuron subtypes with twice the amount of vesicles docked in NMJs at type IIb fibers than that at type IIa/I fibers.</td>
<td>Rate of endocytosis for IIb: lla: I = 1028.58 : 514.29 : 514.29</td>
<td>(Rowley et al. 2007)</td>
</tr>
<tr>
<td>Rate of retrograde axonal transport</td>
<td>Retrograde axonal transport of TTC was measured in WT, SOD1&lt;sup&gt;G93A&lt;/sup&gt; transgenic mice at various symptomatic stages.</td>
<td>We chose 1.4 µm/sec as the average transport rate in WT controls. This is converted to 1.26, assuming the axon has a length of ~4 cm.</td>
<td>(Bilsland et al. 2010)</td>
</tr>
<tr>
<td>Rate of postsynaptic release</td>
<td>The time for neurotrophin to be released from the neuronal somata is ~120s</td>
<td>298.8</td>
<td>(Kolarow, Brigadski, and Lessmann 2007)</td>
</tr>
</tbody>
</table>

We also made the following assumptions:

1) The rate constants of a specific reaction or cellular process is uniform for FF, FR, and S motor neurons.
2) It has been reported that upon receptor binding, TTC uptake by synaptic vesicles can be mediated either through clathrin-mediated endocytosis, or synaptic vesicle recycling (Blum et al. 2014). We assumed that all TTC are taken through the clathrin-mediated endocytosis in this model.

3) The first exception to assumption 1) is the forward and reverse rate constants for endocytosis. Due to differences in motor unit size (Burke and Tsairis 1973) and vesicle density per synapse (Rowley et al. 2007) among the three different α-motor neuron subtypes, we factor in the clathrin-mediated endocytosis rate constants of FF, FR and S motor neurons with a ratio of 18:5:6.

4) The second exception to assumption 1) is the initial concentration of TTC in the gastrocnemius muscle. The composition of FF, FR, and S motor neurons that innervate the lateral gastrocnemius muscle has a ratio of 58:31:11 (Pun et al. 2006). In addition, the transport efficiency of TTC from the gastrocnemius muscle to L4-5 lumbar region is 150-to-1 (based on our empirical experience). We assumed the initial concentration value of TTC in the muscle zones (M_{FF}, M_{FR}, and M_S) to be 88:46:16.

The mathematical model on net axonal transport is able to reproduce the transport profile

With this model, the concentration of TTC in each trafficking zone is calculated with an ordinary differential equation. Please see Appendix I for the complete list of differential equations. For the simulation, we were able to specify the initial TTC concentrations and
the rate constants for all reactions with a number of rough approximations. We imported our empirical data of the net axonal transport obtained for wild-type, SOD1\textsuperscript{G93A} ALS mice, and aging mice, and we requested the software to simulate based on our model the output parameters that would yield a good best-fitting curve to the empirical dataset. We reproduced the net axonal transport profile (Fig IV-2) with sets of output parameters (Tables IV-2, and IV-3). We recognize the uncertainty of a number of our input parameters may lead to the problem of overfitting our model. This concern was also evident in a number of negative rate constant values in the output parameters, which makes the result difficult for interpretation. Lastly, we also recognized the challenges of using our model to simulate deficits of net axonal transport in SOD1\textsuperscript{G93A} and aging animals, due to the complexity and potentially confounding elements of both conditions. For this reason, we devised and performed a series of sensitivity analyses that enable us to optimize our base parameter values and to determine the most important input parameters for net axonal transport.
Figure IV-2. Empirical validation of model of net axonal transport. Net axonal transport profiles obtained through empirical measurements in (A) the SOD1G93A transgenic mice, and (B) the aged mice can be reproduced with our kinetic model.
Table IV-2. Comparison of the probable input parameter values (mostly obtained from the literature) and the simulated values of the model of net axonal transport in SOD1<sup>G93A</sup> transgenic mice.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Non-transgenic</th>
<th>SOD1&lt;sup&gt;G93A&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>85d</td>
<td>65d</td>
</tr>
<tr>
<td>M&lt;sub&gt;FF&lt;/sub&gt;</td>
<td>63.35</td>
<td>3.37</td>
</tr>
<tr>
<td>M&lt;sub&gt;FR&lt;/sub&gt;</td>
<td>33.09</td>
<td>33.09</td>
</tr>
<tr>
<td>M&lt;sub&gt;S&lt;/sub&gt;</td>
<td>11.58</td>
<td>11.58</td>
</tr>
<tr>
<td>k&lt;sub&gt;fFF&lt;/sub&gt;</td>
<td>10.91</td>
<td>10.91</td>
</tr>
<tr>
<td>k&lt;sub&gt;fFR&lt;/sub&gt;</td>
<td>10.91</td>
<td>10.91</td>
</tr>
<tr>
<td>k&lt;sub&gt;fS&lt;/sub&gt;</td>
<td>10.91</td>
<td>10.91</td>
</tr>
<tr>
<td>k&lt;sub&gt;fJFF&lt;/sub&gt;</td>
<td>130</td>
<td>130</td>
</tr>
<tr>
<td>k&lt;sub&gt;fJFR&lt;/sub&gt;</td>
<td>130</td>
<td>130</td>
</tr>
<tr>
<td>k&lt;sub&gt;fJS&lt;/sub&gt;</td>
<td>130</td>
<td>130</td>
</tr>
<tr>
<td>K&lt;sub&gt;fAXFF&lt;/sub&gt;</td>
<td>597.6</td>
<td>597.6</td>
</tr>
<tr>
<td>K&lt;sub&gt;fAXFR&lt;/sub&gt;</td>
<td>298.8</td>
<td>298.8</td>
</tr>
<tr>
<td>K&lt;sub&gt;fAXS&lt;/sub&gt;</td>
<td>298.8</td>
<td>298.8</td>
</tr>
<tr>
<td>K&lt;sub&gt;IN&lt;/sub&gt;</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Root mean square</td>
<td>0.00989</td>
<td>0.01056</td>
</tr>
</tbody>
</table>

Note: The number of each rate signifies the rate of a single TTC molecule per hour.
### Table IV-3. Comparison of the probable input parameter values (mostly obtained from the literature) and the simulated values of the model of net axonal transport in aging mice.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>50d Input</th>
<th>50d Simulated</th>
<th>100d Input</th>
<th>100d Simulated</th>
<th>700d Input</th>
<th>700d Simulated</th>
<th>900d Input</th>
<th>900d Simulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFF</td>
<td>63.35</td>
<td>63.35</td>
<td>62.25</td>
<td>62.25</td>
<td>50.35</td>
<td>50.35</td>
<td>33.35</td>
<td>33.35</td>
</tr>
<tr>
<td>MFR</td>
<td>33.09</td>
<td>33.09</td>
<td>33.09</td>
<td>33.09</td>
<td>33.09</td>
<td>33.09</td>
<td>33.09</td>
<td>33.09</td>
</tr>
<tr>
<td>kF_MF</td>
<td>10.91</td>
<td>6.229</td>
<td>10.91</td>
<td>7.362</td>
<td>10.91</td>
<td>3.305</td>
<td>10.91</td>
<td>5.347</td>
</tr>
<tr>
<td>kF_MR</td>
<td>10.91</td>
<td>0.349</td>
<td>10.91</td>
<td>1.089</td>
<td>10.91</td>
<td>11.557</td>
<td>10.91</td>
<td>5.86</td>
</tr>
<tr>
<td>kF_MS</td>
<td>130</td>
<td>71.817</td>
<td>130</td>
<td>117.278</td>
<td>130</td>
<td>157.335</td>
<td>130</td>
<td>57.534</td>
</tr>
<tr>
<td>kF_Ms</td>
<td>130</td>
<td>57.512</td>
<td>130</td>
<td>91.09</td>
<td>130</td>
<td>130.331</td>
<td>130</td>
<td>114.447</td>
</tr>
<tr>
<td>kF_JF</td>
<td>1028.58</td>
<td>81.14</td>
<td>1028.5</td>
<td>523.766</td>
<td>1028.5</td>
<td>1105.91</td>
<td>1028.5</td>
<td>275.83</td>
</tr>
<tr>
<td>kF_Jr</td>
<td>514.29</td>
<td>140.3</td>
<td>514.29</td>
<td>282.137</td>
<td>514.29</td>
<td>-70.934</td>
<td>514.29</td>
<td>132.604</td>
</tr>
<tr>
<td>kF_Js</td>
<td>514.29</td>
<td>-81.388</td>
<td>514.29</td>
<td>95.9</td>
<td>514.29</td>
<td>181.946</td>
<td>514.29</td>
<td>85.159</td>
</tr>
<tr>
<td>kF_TR</td>
<td>2400</td>
<td>1404.38</td>
<td>2400</td>
<td>1942.78</td>
<td>2400</td>
<td>2502.35</td>
<td>2400</td>
<td>1642.5</td>
</tr>
<tr>
<td>kF_TS</td>
<td>1200</td>
<td>657.402</td>
<td>1200</td>
<td>1084.31</td>
<td>1200</td>
<td>1650.44</td>
<td>1200</td>
<td>1041.66</td>
</tr>
<tr>
<td>kF_Ts</td>
<td>1200</td>
<td>418.46</td>
<td>1200</td>
<td>970.839</td>
<td>1200</td>
<td>1041.37</td>
<td>1200</td>
<td>496.305</td>
</tr>
<tr>
<td>kF_TF</td>
<td>1.2</td>
<td>1.2</td>
<td>1</td>
<td>1</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>kF_Tr</td>
<td>1.2</td>
<td>1.2</td>
<td>1</td>
<td>1</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>kF Ts</td>
<td>1.2</td>
<td>1.2</td>
<td>1</td>
<td>1</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>KfAX_FF</td>
<td>597.6</td>
<td>50.698</td>
<td>597.6</td>
<td>177.517</td>
<td>597.6</td>
<td>14.774</td>
<td>597.6</td>
<td>130.989</td>
</tr>
<tr>
<td>KfAX_FR</td>
<td>298.8</td>
<td>60.857</td>
<td>298.8</td>
<td>77.453</td>
<td>298.8</td>
<td>19.831</td>
<td>298.8</td>
<td>80.708</td>
</tr>
<tr>
<td>KfAX_S</td>
<td>298.8</td>
<td>104.47</td>
<td>298.8</td>
<td>150.811</td>
<td>298.8</td>
<td>12.83</td>
<td>298.8</td>
<td>76.393</td>
</tr>
<tr>
<td>KfAX_TR</td>
<td>1200</td>
<td>41.375</td>
<td>1200</td>
<td>298.662</td>
<td>1200</td>
<td>306.094</td>
<td>1200</td>
<td>168.246</td>
</tr>
<tr>
<td>KfAX Ts</td>
<td>600</td>
<td>617.414</td>
<td>600</td>
<td>830.134</td>
<td>600</td>
<td>172.838</td>
<td>600</td>
<td>576.438</td>
</tr>
<tr>
<td>KfAX JS</td>
<td>600</td>
<td>96.711</td>
<td>600</td>
<td>541.808</td>
<td>600</td>
<td>336.624</td>
<td>600</td>
<td>161.389</td>
</tr>
<tr>
<td>KfIN</td>
<td>0.001</td>
<td>0.00358</td>
<td>0.001</td>
<td>0.00045</td>
<td>0.001</td>
<td>0.00076</td>
<td>0.001</td>
<td>0.000311</td>
</tr>
<tr>
<td>Root mean square</td>
<td>0.01033</td>
<td>0.01312</td>
<td>0.01558</td>
<td>0.01012</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

**Optimizing uncertain input parameters**

Since we have a number of uncertain input parameters, we optimized each of them through evaluating the effect of changing one set of input parameters on the overall output parameters. We used the following steps:
1) Based on our model, we grouped rate constants in the same direction of the same cellular process together (e.g. a group was made for axonal transport forward rate constants—$k_{f\text{AXff}}$, $k_{f\text{AXfr}}$, $k_{f\text{AXs}}$).

2) We fixed one set of input parameters while allowing all other input parameters to be simulated.

3) Once output parameters were obtained, we evaluated i) the number of negative output parameter values produced from the simulation and ii) how well the simulating result fit based on the root-mean-square (RMS) values provided from the best-fitting curve function.

4) We then made a small change in either direction (i.e. increase or decrease the fixed input parameter values of $k_{f\text{AXff}}$, $k_{f\text{AXfr}}$, $k_{f\text{AXs}}$ by a fraction of 1% or 5% and so forth), and repeated steps 2) and 3).

5) We plotted the finite changes of the input parameter set of interest against the RMS to approximate the range of optimized input parameters (Fig IV-3). Thus, we approximate and optimize one input parameter set of interest by choosing input parameters that gives us i) minimized RMS of empirical vs simulated curves across the local domain of parameters and ii) minimal amount of negative rate constants from the simulated output parameters.

6) We then moved on to a different set of input parameters and repeated steps 2) to 5) until we optimized them.
Figure IV-3. Sensitivity analysis screens for optimal parameter values. Variations in $kf_{AX}$ have a large impact on RMS. There is a large local domain of broad RMS minimization for $1.15 < kf_{AX} < 1.2$.

Mathematical model of TTC uptake kinetics depict dominant cellular processes of net axonal transport

Once we optimized all the input parameters, we sought to prioritize which input parameters had the greatest impact on the overall output of the net axonal transport system. To that end, we used local sensitivity analysis by observing the degree of change of the net axonal transport profile using 0.5x, 1x, 2x, and 10x for each individual input parameter (not set of parameters). To our surprise, our model predicts that the most sensitive parameters are the forward and reverse rate constants of receptor binding, endocytosis, endosome maturation, and the forward rate constant of axonal transport from the FF motor neuron. In addition, the reverse rate constants of axonal transport of the FR and S motor neurons have a moderate impact on the overall model.
output. Explicitly, the transport rate constants in the FF motor neuron have the most impact on the overall uptake of TTC; doubling or halving these parameters causes a great change in the net axonal transport profile (Fig. IV-4A, and 4C). In addition, the anterograde transport rate in the FR and S motor neurons also impacts the uptake of TTC, especially with respect to the time-to-peak uptake (Fig. IV-4C).
**Figure IV-4. Sensitivity analysis of the net axonal transport model predicts parameters that would impact the relative peak uptake and the time to peak uptake of TTC.** Shown in the colored bars are the effect of the dominant parameters on the mathematical model for relative peak uptake (A-B) and time to peak uptake (C-D), as predicted and ranked by local sensitivity analysis of the model. Based on the prediction, C9ORF72 transgenic mice (B and D) and their nTg littermates (A and C) share the same dominant input parameters, mostly from the FF motor neuron pathway.

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**Model of net axonal transport in C9BAC mice predicts alteration of trafficking during endocytosis**

One interesting function of mathematical modeling of a physiological system is to allow prediction on factors that may cause perturbation in the pathophysiological setting. Since SOD1G93A has a multifactorial and progressive motor neuron phenotype, we decided to focus on the C9BAC ALS model that is rather subtly affected without, for example, any substantial denervation. Using the same steps for input parameter optimization and sensitivity analysis, we observed the same dominant parameters for the C9BAC mice (Fig. IV-4B and D). By comparing the output parameter from the simulated result, we observed, for the most part, comparable parameter value between the C9BAC and the non-transgenic, age-matched controls, except, strikingly, a 580x, and a 540x fold increases in the forward and reverse rate constants of endosome maturation process, respectively. This aspect of the model implicates an altered endosomal trafficking as an important distinguishing feature of the C9ORF72 model (Table IV-4). Whether this change is a key mechanism in the deficit of TTC peak uptake will depend on further experimental investigation. However, we are struck by this prediction of the model, since dysfunction in endosomal trafficking has been reported in
C9ORF72-deficient mice (O’Rourke et al. 2016). (Please see full discussion below in Chapter 5). These observations demonstrated that our model is able to generate hypotheses about the pathophysiology of changes in axonal transport observed in ALS.
Table IV-4. The simulated output parameters of nTg and C9BAC study. Parameter values in bold represent dominant parameters from the sensitivity analysis. Comparison between the two cohorts illustrate a significant higher $k_f$ and $k_r$ in C9BAC animals comparing to that of the nTg.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>nTg</th>
<th>C9BAC</th>
</tr>
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4.4 Methods

Data sources and search strategies

We searched PubMed and BioNumbers databased (Milo et al. 2010) to obtain research papers evaluating rate constants of specific cellular mechanisms with the key words, “rate constants of” endocytosis, CME, tetanus toxin receptor binding, clostridium toxin receptor binding, endosome maturation, retrograde transport initiation, retrograde axonal transport, synaptic retrieval, synaptic vesicle release, and number of type II muscle fibers with age, number of NMJ with age, number of synaptic vesicles with alpha motor neurons, number of synaptic vesicles with fast-fatigable alpha motor neurons, number of synaptic vesicles with fast-fatigue-resistant alpha motor neurons, and number of synaptic vesicles with slow-fatigue-resistant alpha motor neurons.

Software

We used Berkeley Madonna 10.2.8. to run our simulation, calculate the root mean square (RMS) for the best-fit curve function, and run sensitivity analyses.
CHAPTER 5: GENERAL DISCUSSION AND CONCLUSIONS

5.1 Summary of findings and overall significances

The current work demonstrates an *in vivo*, quantifiable method to evaluate the efficiency of motor unit uptake of an intramuscular injected retrograde tracer protein from the muscle to the cell body in the spinal cord. The characterization of the kinetics of this uptake depicts the integrity of the neuromuscular units that encompasses components including total number of viable motor neurons, the integrity of the neuromuscular junction, and the robustness of retrograde axonal transport. Although past studies have utilized the same tracer modified for fluorescence-based imaging and tracked TTC trafficking on the sciatic nerve of wild-type and ALS mouse models (Bilsland et al. 2010; Sleigh et al. 2020; Hafezparast et al. 2003), there has been no establishment of using TTC as a biomarker for tracking disease progression in the same mouse through repeated imaging, much less as a therapeutic biomarker.

We recognized that the net axonal transport assay measures a compound defect, which poses a challenge in distinguishing perturbations of specific biological processes when net axonal transport is abnormal, as measured by this TTC assay. Quantitative analysis of net axonal transport in its present form is nonetheless useful and informative, for the following reasons. First, using the current method, we can detect, on a macroscopic level, defects and loss of motor unit integrity in a wide range of neuromuscular pathologies. Notably, our comprehensive and detailed characterization of net axonal transport in five different ALS mouse models displaying various degrees of motor phenotypes evidently validates that net axonal transport...
disturbances, not exclusively axonal transport per se, is a broad and most common pathology in ALS. In addition, there have been reported mouse models where motor neuron loss and axonal transport deficits developed separately through independent mechanisms (Malik et al. 2011; Marinkovic et al. 2012; Sleigh et al. 2020). We believe that our system would still be well able to detect alterations of the various trafficking pathologies in these cases. In fact, it would be interesting to characterize the net axonal transport in the mouse model expressing FUS$^{Δ14/+}$ and the spinal and bulbar muscular atrophy (SBMA) mouse model containing 100 poly-Q AR repeats mutation generated by Schiavo and colleagues (Malik et al. 2011; Sleigh et al. 2020). These mice did not display axonal deficits but motor neuron degeneration, and they would be valuable for examining the impact of motor neuron loss alone on the transport profile.

Second, even though the etiology of ALS appears to be heterogeneous with varying phenotypic severity, pathological mechanisms—including axonal transport impairment, cytoskeletal disturbances, axonal withdrawal, synaptic dysfunction, and motor neuron loss—are common converging points from various upstream pathogenic events. This makes net axonal transport more meaningful as a metric for evaluating therapeutic benefits as compared to mutant-specific features acquired by a single model caused from strain-specific or overexpression effects. As documented in gene therapy studies in the SOD$^1^{G93A}$ ALS mice, net axonal transport is sensitive to reflect therapeutic benefit, and even displays a dose-response effect for treatment.

There are a number of findings and significances in this study.
The stability and the low clearance rate of TTC enables serial quantification for net axonal transport

Ever since Bizzini and colleagues pioneered the use of TTC as a therapeutic carrier to bypass the blood-brain barrier into the CNS (Bizzini, Stoeckel, and Schwab 1977; Bizzini, Grob, and Akert 1981), more than 200 investigations, our group included, have exploited TTC’s “motor neuron-philic” properties for characterizing neuroanatomical, cell physiological, and therapeutic endeavors (Toivonen, Oliván, and Osta 2010). In addition, Schiavo and colleagues have extensively delineated the biology of TTC uptake in the past few decades. It is based upon these discoveries and insights that we are convinced to apply TTC as a neuroimaging biomarker for motor neuron disorders. One of the areas that have not been explored over the years is a detailed uptake kinetics and clearance of TTC at the system level of a whole organism. Early investigations on using TTC to map neuronal network, for example, tracked its uptake up to less than a week without quantitative measure (Coen et al. 1997; P. S. Fishman and Carrigan 1987; Miana-Mena et al. 2003; Rind, Butowt, and von Bartheld 2005; Perreault et al. 2006). Furthermore, although transcytosis of TTC has been described (P. S. Fishman and Carrigan 1987; Perreault et al. 2006), the specificity of interneurons uptake of TTC has not been fully examined.

Since our goal is to visualize and quantify TTC longitudinally in the same subject, we were pleasantly surprised that its trans-synaptic release and subsequent uptake by interneurons has a slow turn-over cycle and is only minimally cleared from the anterior horn. In the preliminary study of $^{125}$I-TTC intramuscular injection in the tongue, we were
able to observe TTC signal even up to three weeks post-injection. This property permits longitudinal stability for quantification of TTC accumulation in the ventral horn of the spinal cord. Notably, part of this slow clearance rate may be attributed to its uptake by the GAD67+ GABAergic interneurons. This process can already be detected by 24 hours post-injection, a time that is before the measured peak uptake of our transport profile, suggesting that the stable, persistent radioactive signal in the SPECT/CT imaging comes predominantly from the accumulation of TTC in the interneurons. A more precise temporal uptake by immunochemistry analysis will help characterize this.

*Net axonal transport profile reflects complex and heterogeneous motor neuron and axon pathologies in various mouse models*

The net axonal transport profile of $^{125}$I-TTC allows us to extract two parameters for further analysis: the amplitude of peak uptake (normalized to controls) and the time-to-peak uptake (**Fig. III-1C and D**). Understanding exactly what these parameters represent biologically will require further dissection. We do know that they are extremely sensitive and subject to subtle perturbations and various forms of axon and motor neuron pathologies. For example, in SOD1$^{G93A}$ transgenic animals that are pre-symptomatic at 45-days old, a slight yet statistically insignificant increase of peak uptake is observed when compared to the control cohorts. This modest increase interestingly corresponds to an earlier observation where enhanced synaptic transmission occurs at such early symptomatic stage (Rocha et al. 2013). Between 45- and 65-days old, we observed a 47% decrease of peak uptake. Such drastic reduction
in relative peak amplitude is likely a reflection of gross FF motor unit denervation of the gastrocnemius muscle at 55 days (Pun et al. 2006). Between “late” pre-symptomatic animals (65-day-old), and the minimally symptomatic animals (85-day-old), we observed a slight decrease of the relative peak uptake, with a 17% delay of the time to peak uptake. The slow peak uptake may correspond to a combined effect of synaptic vesicle stalling in the FR motor unit axons and a minor motor neuron loss at day 65 (Pun et al. 2006; Martin et al. 2007), the sprouting re-innervation of the FF NMJs by the FR/S motor units at 70 days (Pun et al. 2006), and a significant deficit in retrograde axonal transport at 74 days (Bilsland et al. 2010). In the late symptomatic and end stage animals (110-day- and >125-day-old, respectively), a further ~72% decrease in the relative peak uptake and ~40-50% delay in time to peak uptake may reflect the progressive pathology of significant motor neuron loss and 70% FR/FF motor unit loss at 100 days, and 60% of remaining motor neurons being TUNEL-positive at 120 days (Martin et al. 2007; Hegedus, Putman, and Gordon 2007).

PFN1 is a major regulator of actin dynamics that involved in growth cone maintenance, neurite outgrowth, sprouting, and synaptic vesicles in neurons. Although we and our collaborators have demonstrated that mutations of PFN1 causes ALS, how mutant PFN1 leads to motor neuron death is still to be elucidated. One recent key finding by our collaborator is that mutations in PFN1 are destabilizing, and cause accelerated turnover of the PFN1 protein in cells (Boopathy et al. 2015). Additionally, another recent study reported that PFN1 has a direct effect on promoting microtubule growth and mutations in PFN1 may disrupt microtubule dynamics, leading to motor
neuron degeneration (Henty-Ridilla, Juanes, and Goode 2017). These findings, along with the progressive pathology recapitulated in our transgenic PFN1\textsuperscript{C71G} mice (Yang et al. 2016) fully support the pronounced reduction of relative peak amplitude in the net axonal transport profile (Fig. III-3). For further studies, it would be interesting to investigate the timing of onset of the net axonal transport deficit, and whether such deficit is apparent even at the pre-symptomatic stage.

TDP-43 is the major pathological protein found in the cytoplasmic inclusions in almost all ALS cases, and half of FTD cases (Neumann et al. 2006). One of the challenges with using genetic mouse models of specific disease is to differentiate overexpression-specific effect from the effect of the mutant gene. We and our collaborator have developed a TDP-43 knock-in mouse model that carries the human Q331K mutation. This mouse line does not exhibit motor neuron dysfunction but does have cognitive decline due to mutant gene disturbance of TDP-43 autoregulation, leading to reduction of parvalbumin interneurons in the frontal cortex (White et al. 2018). Interestingly, the lumbar motor neurons in these animals upregulate the expression of argin (Agrn), a protein secreted by neurons involved in promoting NMJ function, and the expression of aldehyde oxidase 1 (Aox1), an enzyme implicated in neuronal maintenance following axon injury (Burden, Yumoto, and Zhang 2013; Kolarcik and Bowser 2012). It is likely that the regulations of these proteins promote NMJ integrity and maintain a functional axonal transport, as documented by the absence of change of the net axonal transport profile between the TDP-43\textsuperscript{Q311K/Q311K} animals and their wild-type littermates (Fig. III-4).
A hexanucleotide repeat expansion within the C9orf72 gene is the most common genetic cause of ALS and FTD. Our transgenic mice carrying more than 550 copies of repeats display signature cytopathological hallmarks of RNA foci and dipeptide protein formation yet without apparent motor neuron pathologies, including motor neuron loss and NMJ denervation. Surprisingly, we detected a modest yet significant change of net axonal transport profile in which time to peak uptake was comparable, yet relative peak uptake was reduced in the 700-day-old C9BAC female mice compared to their age-matched non-transgenic littermates (Fig. III-5A). Strikingly, investigating on a separate line that has an identical BAC from the Lagier-Tourenne/Cleveland groups, we observed the same trend of deficit in the 450-day-old cohorts (Fig. III-5B). Although the pathogenesis of ALS by C9ORF72 mutation appears to be multifactorial, the loss of the physiological function of C9ORF72 protein has been suggested to contribute to ALS. To characterize protein encoded by C9orf72, Levine and colleagues conducted sensitive homology searches that link C9ORF72 to Differentially Expressed in Normal and Neoplasia (DENN), a GDP/GTP exchange factor (GEF) that activates Rab-GTPase (Levine et al. 2013). In addition, C9ORF72 has been suggested to be involved in endosomal trafficking and autophagy. Atkin and colleagues demonstrated that in control human spinal cord sections, C9ORF72 was colocalized with Rab5 and Rab7 proteins in the spinal cord motor neurons; in C9ORF72 ALS patients, however, an enhanced colocalization between C9ORF72 and Rab7 and Rab11 were observed compared with controls, suggesting that repeat expansion within C9ORF72 may lead to dysregulation of cellular trafficking (Farg et al. 2014). More recently, using human induced motor
neurons, Ichida and colleagues illustrated that normal C9ORF72 is required for vesicle trafficking and lysosomal biogenesis in motor neurons, whereas repeat expansion within C9ORF72 reduces its own expression, causing deficit in trafficking—leading to glutamate receptor accumulation and defect in protein degradation—leading to impaired clearing of the toxic dipeptide proteins. When a constitutively active Rab5 mutant was expressed in these cells, it rescues neurodegeneration in these mutant cells (Shi et al. 2018). Taken together, these results may suggest a link of a subtle defect in net axonal transport with the repeat expansion of our C9BAC mice. We know that it is neither denervation nor loss of motor neurons that may cause the subtle defects, suggesting instead that the amplitude loss reflects defects in actual endosomal trafficking, or in some other property of the C9ORF72 transgenic motor unit. While further study should unravel the underlying molecular mechanisms, we demonstrated the sensitivity of our method, which detected transport deficiencies even in absence of an overt clinical phenotype.

**Tetanus toxoid has attenuated binding and retrograde transport functionality**

Having demonstrated the robustness of $^{125}\text{I}$-TTC transport into the motor neurons, we applied the same method on an FDA-approved alternative of TTC, the tetanus toxoid (TT), and made two remarkable observations. First, the formaldehyde-treated tetanus toxoid with random cross-linking bridges was greatly attenuated in its specific cellular binding and retrograde transport. Unlike $^{125}\text{I}$-TTC, $^{125}\text{I}$-TT accumulation at the hypoglossal nuclei appears to be less intense and fluctuates over the duration of the
SPECT/CT imaging (Fig. III-2A). This is probably due to inefficient uptake of various sizes of TT agglomerate. In addition, the slow clearance or high retention of $^{125}$I-TT may also cause a strong immune response that triggers its clearance or block its entry into the motor neurons. Second, in our control groups, we documented the robust uptake of TTC at the C6-T1 spinal region following forelimb triceps muscle injection. Interestingly, the late symptomatic SOD1$^{G93A}$ ALS mice (105-day-old) displayed only a mild altered net axonal transport in motor units innervating the triceps muscle when compared to their age-matched non-transgenic controls, suggesting a less progressive motor neuron denervation and degeneration at the late symptomatic stage in this model (Fig. III-2C). This corresponds well with the findings by Capitanio and colleagues who characterized and compared muscle compositions of the forelimb triceps and the hindlimb gastrocnemius in ~100-day-old SOD1$^{G93A}$ and control mice. While there was a drastic change in muscle weight and muscle fiber composition with significant reduction of type IIb muscle fibers in the gastrocnemius muscle, a comparable muscle fiber composition with a slight decrease in muscle weight in the triceps muscle in SOD1$^{G93A}$ animals was observed compared to the wild-type (Capitanio et al. 2012). This observation reaffirms the reliability of TTC as a biomarker to gauge motor unit integrity. It also suggests that not all FF motor units are equally affected or are affected at the same rate. Further studies are required to investigate the underlying mechanisms.

Lastly, it is important to point out that most of our experimental controls for the various ALS mouse models (except the TDP-43Q331K/Q331K knock-in animals) are non-transgenic mice that shared the same strain as their transgenic cohorts. Although these
mice are valid controls in examining mutant-specific effect on net axonal transport, they may not be ideal to exclude the overexpression-specific effect of the mutant genes. For example, instead of using age-matched non-transgenic wild-type FVB/NJ mice as the control cohorts for PFN1\textsuperscript{C71G/C71G} transgenic mice, it would make our analysis more comprehensive and our interpretation more unbiased by also including transgenic mice that express exogenous PFN1\textsuperscript{WT} driven by the same promoter.

**Age-related net axonal transport alterations are seen in phases**

It is well recognized that aging is the major risk for neurodegenerative diseases including ALS. Even though “normal” aging and ALS may be caused by separate mechanisms, age-related changes can exacerbate disease process and limit the efficacy of the compensatory mechanisms (Adalbert and Coleman 2013). As mentioned earlier, links connecting aging and ALS includes the same selective vulnerability of spinal motor neuron subtypes to denervation, axon loss in the tibial nerves, and axonal transport deficits (Pun et al. 2006; Valdez et al. 2010; Gilley et al. 2012; Bilsland et al. 2010). One question that requires elucidation is whether age-related axon pathology occurs in a linear decline throughout the course of adult life or in an accelerated fashion at old age when such pathology exceeds the threshold for compensatory responses. We set forth, in this work, to delineate the effect of aging on net axonal transport in a cross-sectional analysis of six age groups of C57BL6 mice. We were able to detect gradual deficits of net axonal transport with age. The striking feature of the relative peak uptake is characterized by the initial decrease between 100- and 300-day-old mice,
followed by a relatively stable plateau phase from 300- to 700-day-old mice, and ended with a second decline between 700- to 900-day-old animals (Fig. II-6C). For the time to peak uptake, it is characterized by a biphasic profile with an initial slowing phase between 50- to 300-day-old animals and a plateau phase between 300- to 900-day-old animals (Fig. II-6B). While we recognize the challenge in interpreting the biological meaning of these changes due to the scarcity of longitudinal studies characterizing axon pathology with age in the current literature, these alterations certainly reflect the combined effect of age-related axon pathology, including changes in motor unit number, axonal transport, NMJ denervation and reinnervation, and motor neuron loss. To dissect this combined effect further, we evaluated Kajri and colleagues’ work that documented motor unit numbers by MUNE method on 300-, 390-, 450-, 510-, 600-, 660-, 720-day-old C57BL6 mice. They reported a significant reduction only at 600-, and 720-day-old animals, suggesting active denervation and reinnervation processes occur as the animal ages (Sheth et al. 2018). This study was done on the triceps muscle, which may reflect different motor unit composition than the gastrocnemius muscle. In addition, Sanes and colleagues compared young (3 to 6 months old) and old (24 to 28 months old) C57BL6 mice and reported only a ~10.7% denervation of the gastrocnemius muscle in the old animals compared to their younger cohorts (Valdez et al. 2012). In the context of these studies, the plateau phase in our relative peak uptake may represent the active denervation and reinnervation process, where partially denervated fast-twitch muscle fiber is reinnervated by slow resistant motor neurons, between the period of 300 to 700 days of age, until the slow motor neuron may reach a size that is beyond its
capacity to maintain, which may issue in further denervation. Coleman and colleagues characterized the decline of axonal transport with age. There is a consistent transport rate between young (30- and 90-day-old) mice, a decrease of transport rate from young to middle age (90- to 180-day-old) mice, a plateau between middle age to old (180- to 540-day-old) mice, and a second decrease between old (540- to 720-day-old) animals (Milde et al. 2015). Overall, the characterization from these studies and our findings demonstrate gradual and complex age-related changes of axonal structures and functions, which appears to be fundamentally distinct from the pathology seen in the ALS models with progressive paralysis (SOD1,PFN1).

**Net axonal transport is affected by mouse strain but not gender**

It is a common concern, when using animal models, to consider potential confounders such as gender and strain. A good example of strain-specific, and gender-specific effect on studying ALS mouse models is the development of the first TDP-43 transgenic mouse model that overexpresses the mutant human TDP-43\(^{A315T}\) under the mouse prion promoter. The original strain was generated on a mixed C57BL6xCBA background, and this transgenic mouse line displays progressive neurodegeneration reminiscent of both ALS and FTLD (Wegorzewska et al. 2009). However, in subsequent studies when this strain was made congenic on a C57BL6 background, instead of seeing a motor phenotype, a gastrointestinal dysfunction caused by neurodegeneration of colonic myenteric plexes was recorded, and this phenotype was more aggressive in the male mice (Y. Guo et al. 2012; Esmaeili et al. 2013). This disease phenotype,
however, can be restored by high fat diet treatment, allowing increase of life-span in the animals, and unmasking the motor neuron phenotype (Coughlan et al. 2016). To that end, we examine the gender- and strain-specific effect on net axonal transport. We observed a modest difference in net axonal transport between B6SJLF1 and the two strains FVB/NJ and C57BL6, indicating the importance of proper controls when evaluating net axonal transport. Between male and female C57BL6 mice, we did not see a significant difference of the net axonal transport. However, the female shows a slightly higher (but not significant) peak uptake (Fig. II-7). One possible explanation to this trend could be the fact that both male and female received the same dose volume of $^{125}$I-TTC instead of a dose volume by body weight.

*Net axonal transport detects early therapeutic benefits and informs dose-response relationship*

One of the most important roles of an effective biomarker is the ability to monitor drug effects in the preclinical model system and inform the dose-response effect of the drug. We documented a robust improvement of net axonal transport, comparable to the level of a healthy control, at the minimal symptomatic stage of SOD1$^{G93A}$ mice (85-day-old) under CRISPR-Cas9 treatment (Fig. III-6A). This is sharply different from the age-matched untreated SOD1$^{G93A}$ animals, which display a 60% reduction in the relative peak uptake. By the late symptomatic stage (110 days), the treated mice still exhibit a 39% reduction of peak uptake, as compared to a 64% reduction in the untreated animals (Fig. III-6B). These data demonstrate a reliable means to detect the efficacy of
SOD1 editing before any current standard behavioral assays, such as weight analysis, grip strength, and rotarod. Furthermore, using net axonal transport assay, we were able to optimize dosage for treatment. In the AAVrh10-miRNA-treated SOD1\textsuperscript{G93A} animals, we differentiated the least effective dose, the lowest dose, from the effective doses, the higher doses. Interestingly, we did not see a gradient of improved net axonal transport profiles, but saw more of an all-or-none response (Fig. III-7). Further studies with narrower dosage range may fine-tune the dose-response relationship. Importantly, net axonal transport is sensitive to detect early therapeutic benefits and repeat visualization in the same subject allows for longitudinal monitoring of drug effect. This makes the process of clinical trial more efficient and easing the need for large sample size.

**Prior immunization to tetanus toxoid attenuates net axonal transport of TTC but does not preclude TTC use for repeat assay**

Molecular imaging of TTC uptake provides an *in vivo*, non- or minimally invasive, quantitative method to assess motor unit integrity and function in a longitudinal way. In order to apply it translationally into the clinical setting, a potential hurdle to consider is the neutralizing anti-tetanus antiserum as a result of vaccination for tetanus. Current vaccination schedules in the United States and other developed countries start at early childhood with booster doses given every 10 years thereafter. To elucidate whether prior immunization dampens retrograde transport of TTC, Fishman and colleagues compared uptake of rhodamine-labeled TTC in the hypoglossal nucleus between vaccinated and immunologically naïve mice. They showed that the quantified uptake of
TTC was not attenuated in the vaccinated mice (Paul S. Fishman et al. 2006). This is different from what we observed with our immunized animals, whose net axonal transport showed a 50% reduction in the relative peak uptake compared to the immunologically naïve mice (Fig. III-8B). There are potential explanations to this discrepancy in the effect of immunization on TTC uptake. First, we injected a dose that is twice the amount used in Fishman’s group, which could cause a stronger immune response against TTC. Second, instead of performing intraperitoneal injections, we administered intramuscularly, which required a more concentrated injected reagent in a much smaller volume. From our prior tetanus toxoid experiment, we know TT remains at the injected muscles up to a week before its clearance. The slow clearance rate of TT in the muscle may also trigger a stronger immune response to block TTC entry. Fortunately, we did not observe a further attenuation of TTC uptake when we repeat the net axonal transport assay 10 weeks later. An indirect reason for this phenomenon is the low retention rate of TTC in the circulation. When comparing $^{125}$I-TT and $^{125}$I-TTC, we noticed a much quicker clearance of $^{125}$I-TTC from the system (i.e. within first 24-48 hours, as opposed to more than 100 hours as seen in $^{125}$I-TT). It is possible that such a quick clearance does not activate a strong immune response. Most importantly, this result validates the clinical potential of our assay; prior tetanus vaccinations will not mask the sensitivity of the net axonal transport profile to detect early evidence of benefit of a therapy. This property allows repeated measurement net axonal transport profiling in the clinical setting both to trace disease progression and measure beneficial therapeutic effects.
Mathematical model of net axonal transport in C9BAC mice predicts alterations in endocytic trafficking

Our net axonal transport profiles reflect the complexity of motor neuronal and neuromuscular circuitry. To construct a model that mimics the cellular environment of a simple neuromuscular circuitry, we employed three parallel processes represented by the three α-motor unit subtypes. There are two reasons for this selection. First, in most of the profile curves, we observed an exponential uptake at the earlier time points until it approaches the peak, followed by a gradual decline of uptake. We realized that to reproduce such a curve by ordinary differential equations, a single process would not be sufficient. Second, since the axon pathology in ALS displays a spatially and temporally highly predictable sequence of affected motor units, we decided to recapitulate this property by having three parallel processes in our system. Then, in view of the net axonal transport assay of TTC, the general cellular processes that determine its overall uptake kinetics include 1) receptor binding, 2) internalization through endocytosis, 3) transition from early endosome to late endosome by acquiring Rab5 and Rab7, respectively, 4) retrograde axonal transport, and 5) trafficking from motor neuron cell bodies to the connected interneurons (Fig. IV-1).

Once we set up our model and identified all the independent variables, we carried out two steps: 1) searching through publications that detail specific rate constants, and 2) importing our empirical data. Once these were accomplished, we asked the Berkeley Madonna software to simulate our model to provide us the best set
of output parameters for the best fitting curve to our empirical transport profile. This process was repeated literally hundreds of times through a long series of computational runs to select the most probable (best fit) parameters. As we sequentially examined all the parameters, the simulation provided coherent input parameters with least root mean square values as a measure of fit with the experimental observations. Through local sensitivity analysis by changing and examining one input parameter’s impact on the overall output parameter, we identified the cellular processes from receptor binding up to retrograde transport in the FF motor neuron that are dominant parameters determining the net axonal transport profile (Fig. IV-4). Most importantly, our model predicted that C9BAC animals have an alteration in endocytosis in the FF motor neuron, with forward and reverse rate constants 580x, and 520x, respectively, greater than that of the nTg littermates. This is the predicted cause accounting for the ~22% reduction in the relative peak uptake of the C9BAC mice (Fig. III-5A and B).

This prediction generates a very interesting link. On the one hand, as mentioned earlier, several investigations have suggested that C9ORF72 protein plays a role in regulating endosomal trafficking, autophagy, and lysosomal biogenesis (Farg et al. 2014; Shi et al. 2018; Liang et al. 2019). Specifically, normal C9ORF72 protein colocalizes with small Rab proteins including Rab1, Rab5, Rab7, and Rab11. In addition, normal C9ORF72 was found in both the extracellular space and cytoplasmic vesicles, regulates internalization of TrkB receptor, and is responsible for transporting Shiga toxin. In C9ORF72 motor neurons, however, there is an aberrant increased localization between C9ORF72 and Rab7 (Farg et al. 2014). In a separate study,
human induced motor neuron of \textit{C9ORF72} exhibiting neurodegeneration can be rescued by introducing a constitutively active Rab5 mutant (Shi et al. 2018). In summary, C9ORF72 regulates endosomal trafficking, including TrkB receptor internalization, and requires proper Rab5, and Rab7 expression. Detailed characterization of TTC uptake by Schiavo and colleagues has proposed that TTC sorting through the endocytic pathway requires Rab5 and Rab7, and is shared by the neurotrophin receptors p75\textsuperscript{NTR} and TrkB (Deinhardt et al. 2006). Collectively, mutation in \textit{C9ORF72} may reduce normal C9ORF72 function, leading to trafficking defects in endosome, with which TTC is transported. Furthermore, while whole tetanus toxin enters the neurons primarily via clathrin-mediated endocytosis, Blum and colleagues demonstrated that TTC endocytosis utilizes both synaptic-vesicle (SV) cycling, and clathrin-mediated endocytosis, with the former uptake occurring at high concentration of TTC (Blum et al. 2014). Based on these findings and our simulated result, a potential working model of mutation in \textit{C9ORF72} on net axonal transport of TTC is as follow: The lack of functional C9ORF72 dysregulates endosomal trafficking, leading to a defect in TTC uptake via clathrin-mediated endocytosis. The bulk TTC triggers SV cycling, as reflected by the increase rate constant of endocytosis. The overall uptake of TTC via retrograde transport, however, is attenuated slightly, resulting in a 22% reduction of peak uptake (\textbf{Fig. V}). A major contribution of this model is its capacity to generate hypotheses for further experimental investigation.

It is important to mention that while our working model looks at the effect of haploinsufficient function of C9ORF72 in regulating endosomal trafficking, the C9BAC
mouse lines examined by net axonal transport assay do have endogenous, wild-type C9ORF72 proteins. However, whether the expression level of the endogenous C9ORF72 is affected by the mutant C9ORF72 requires further analysis. A potential pathomechanism for a reduced protein level of C9ORF72 is that mutant C9ORF72 alters the RNA metabolism of endogenous C9ORF72, either through RNA foci- or DPR-mediated perturbations. Therefore, protein quantification of C9ORF72 in the spinal motor neurons of our C9BAC mouse should be evaluated to validate this hypothesis.

Alternatively, if the endogenous C9ORF72 expression is unaltered by the mutant C9ORF72 transgenes, two potential gain-of-toxic pathomechanisms can be proposed on why our mathematical model predicts an alteration in endocytosis in C9BAC mice. The first potential mechanism is through local mitochondrial dysfunction in the presynaptic terminal of motor neurons affected by DPRs. Mitochondria are essential to provide ATP and regulate $\text{Ca}^{2+}$ signals in order to modulate healthy and functional synapses (Devine and Kittler 2018). As mentioned earlier, poly-GR DPR has been shown to preferentially bind to mitochondrial ribosomal proteins and compromise mitochondrial function (Lopez-Gonzalez et al. 2016). A second potential mechanism is the altering of neuronal excitability by C9ORF72 repeat expansion. Devlin and colleagues, demonstrated a progressive loss of action potential output and synaptic activity in iPSC-derived motor neurons from C9ORF72 patients (Devlin et al. 2015). Since TTC bulk uptake can be stimulated by membrane depolarization, the dysregulated neuronal excitability may alter its uptake, resulting in change of endocytosis process (Blum et al. 2014).
Figure V. A model predicting the impact of C9ORF72 on net axonal transport of TTC. (A) Under normal condition, TTC is taken up by binding to gangliosides/receptors at the neuromuscular junction and internalized through clathrin-mediated endocytosis (CME) to the retrograde transport route. TTC and neurotrophin receptor (TrkB) gets sorted in a Rab5-positive early endosome, where it is subsequently progresses to a Rab7-positive late endosome to initiate a cytoplasmic dynein-dependent retrograde transport process. C9 has been implicated in regulating endosomal trafficking and associating with Rab5 and Rab7 proteins to promote this process. (B) In the case of C9BAC transgenic mouse, we predicted a perturbed net axonal transport at the endocytic steps in which insufficient C9ORF72 proteins attenuate TTC uptake through the CME route, and cause more TTC to enter the synaptic vesicles (SV) during retrieval of the SV proteins.

5.2 Challenges and limitations

The employment of neurospecific transport of TTC demonstrates a promising way to detect perturbations of motor neuron integrity and function, to trace disease progression, and to measure treatment responses. However, there are areas of limitations with this method as listed below:

1) This technique quantifies a compound defect of motor neuron and axon pathology. To understand the underlying pathobiological mechanisms by which retrograde transport is impaired require specific independent analyses to delineate various perturbations.

2) This technique employs the retrograde axonal transport properties of TTC to gauge integrity of neuromuscular circuitry. However, this result cannot be generalized for all other axonal cargoes. In the long term, it may be informative to study the same process with other cargoes such as rabies virus glycoprotein.

3) One area that remains unaddressed and is likely to affect the uptake of TTC is alteration by mutant-specific gene effect on dendrites, motor neuron cell bodies,
and interneurons. Measured radioactivity of accumulated TTC represents both the efficiency of its uptake and clearance. Gene mutations that affect dendritic structure and spine density, reduce cell body size, and affect interneuron health and function will all display alterations in the net axonal transport profiles. To distinguish between these defects, it would be necessary to analyze them independently. Moreover, a detailed immunohistochemical characterization of TTC trafficking over time provide the kinetic profiles of TTC transport in the cell bodies of the motor neurons from its transcytosis into the interneurons. Such characterization is useful for 1) understanding the biology of TTC transport, 2) understanding the defects of such processes by various ALS-linked mutants, and 3) optimizing our mathematical model.

4) Due to the route of injection, our technique is limited to quantifying retrograde transport, specifically in lower motor neurons that innervate muscle. For that reason, our method can be applied to a wide variety of disorders that affect lower motor neurons, including peripheral neuropathies, traumatic nerve injuries, ALS and a number of ALS-like motor neuron disorders. However, this strength is also a limitation: our approach does not detect or in any way quantify upper motor neuron involvement. Further clinical characterization combined with other biomarkers must be employed to detect and quantify upper motor neuron pathology.

5) To apply our technique to the clinical setting, we will need isotopes (e.g. $^{123}$Iodine, or $^{111}$Indium) that are available for use in clinical imaging. To avoid
radiation exposure, it would be ideal to apply other imaging modalities. Also, a current Good Manufacturing Practices (cGMP)-grade TTC is needed for clinical research. As well, we have used TTC produced from bacteria for these studies; we anticipate that clinical application of this method may require that TTC be derived from mammalian cells, such as Chinese Hamster Ovary (CHO) cells or other mammalian cell types.

6) Our preliminary data suggest repeated administrations of TTC do not attenuate TTC uptake further. However, we have not examined the net axonal transport profiles beyond more than two TTC injections, which is a potential scenario in clinical use. Further studies are required to evaluate the effect of immunity on more than two repeated administrations of TTC. It is also worth quantifying and characterizing the titer of anti-TTC antibodies following along repeated injections.

7) For the mathematical model, we have simplified the motor neuronal and neuromuscular system. For example, we did not account for the uptake of TTC into the sensory neurons. We also did not consider non-neuronal involvement in our model. Lastly, we need expertise and more powerful sensitivity analysis to strengthen the validity of our model.

### 5.3 Future directions

There are some interesting and unanswered questions for future investigations. These include but are not limited to: How does TTC get cleared by GABAergic interneurons? Does TTC transport beyond interneurons? Can we visualize and measure TTC
trafficking in individual compartments alone, so we can detail how various ALS mutations affect specific transport process? Then on the technical side, could we apply TTC with other radio-isotopes that are broadly used in the clinical utility? Is there a way we can modify TTC to be imaged with other imaging modalities such as MRI or ultrasound? We believe that these questions will help us gain further insight into TTC biology, ALS axon pathology, and TTC’s potential clinical use.

Here we demonstrated an in-vivo, minimally invasive visualization and quantification of net axonal transport as a measure for motor unit integrity. We can detect alterations in various ALS mouse models and in aging animals, where the kinetics of TTC transport show distinct characteristics for different genetic mutations. For this, we have developed a mathematical model to simulate and predict potential disturbance of transport caused by specific mutation. Lastly, we are also able to discern early therapeutic benefits and measure dose-dependent effects in a mutant SOD1 mouse model before other conventional clinical measures. We are hopeful that our method will be useful to serve as a prognostic and even a pharmacodynamic tool for screening effective therapeutic intervention for motor neuron diseases, such as ALS.
Appendix I

List of Ordinary Differential Equations used in the mathematical model

\[
\frac{d[M_{FF}]}{dt} = -k_f M_{ff} \times M_{FF} + kr_{Mff} \times J_{FF}
\]

\[
\frac{d[M_{FR}]}{dt} = -k_f M_{fr} \times M_{FR} + kr_{Mfr} \times J_{FR}
\]

\[
\frac{d[M_S]}{dt} = -k_f M_s \times M_S + kr_{Ms} \times J_S
\]

\[
\frac{d[J_{FF}]}{dt} = k_f M_{ff} \times M_{FF} - k_f J_{ff} \times J_{FF} - kr_{Mff} \times J_{FF} + kr_{Jff} \times T_{FF}
\]

\[
\frac{d[J_{FR}]}{dt} = k_f M_{fr} \times M_{FR} - k_f J_{fr} \times J_{FR} - kr_{Mfr} \times J_{FR} + kr_{Jfr} \times T_{FR}
\]

\[
\frac{d[J_S]}{dt} = k_f M_s \times M_S - k_f J_s \times J_S - kr_{Ms} \times J_S + kr_{Js} \times T_S
\]

\[
\frac{d[T_{FF}]}{dt} = k_f J_{ff} \times J_{FF} - k_f T_{ff} \times T_{FF} - kr_{Jff} \times T_{FF} + kr_{Tff} \times AX_{FF}
\]

\[
\frac{d[T_{FR}]}{dt} = k_f J_{fr} \times J_{FR} - k_f T_{fr} \times T_{FR} - kr_{Jfr} \times T_{FR} + kr_{Tfr} \times AX_{FR}
\]

\[
\frac{d[T_S]}{dt} = k_f J_s \times J_S - k_f T_s \times T_S - kr_{Js} \times T_S + kr_{Ts} \times AX_S
\]

\[
\frac{d[AX_{FF}]}{dt} = k_f T_{ff} \times T_{FF} - k_f AX_{ff} \times AX_{FF} - kr_{Tff} \times AX_{FF} + kr_{AXff} \times IN
\]

\[
\frac{d[AX_{FR}]}{dt} = k_f T_{fr} \times T_{FR} - k_f AX_{fr} \times AX_{FR} - kr_{Tfr} \times AX_{FR} + kr_{AXfr} \times IN
\]

\[
\frac{d[AX_{S}]}{dt} = k_f T_{s} \times T_{S} - k_f AX_{s} \times AX_{S} - kr_{Ts} \times AX_{S} + kr_{AXs} \times IN
\]

\[
\frac{d[IN]}{dt} = k_f AX_{ff} \times AX_{FF} + k_f AX_{fr} \times AX_{FR} + k_f AX_{s} \times AX_{S} - k_f IN \times IN - kr_{AXff} \times IN
\]

\[- kr_{AXfr} \times IN - kr_{AXs} \times IN\]


Bendotti, Caterina, Marianna Marino, Cristina Cheroni, Elena Fontana, Valeria Crippa, Angelo Poletti, and Silvia De Biasi. 2012. “Dysfunction of Constitutive and Inducible Ubiquitin-


Kwon, Ilmin, Siheng Xiang, Masato Kato, Leeju Wu, Pano Theodoropoulos, Tao Wang, Jiwoong Kim, Jonghyun Yun, Yang Xie, and Steven L. McKnight. 2014. “Poly-Dipeptides Encoded


Waite, Adrian J., Dirk Bäumer, Simon East, James Neal, Huw R. Morris, Olaf Ansorge, and Derek J. Blake. 2014. “Reduced C9orf72 Protein Levels in Frontal Cortex of Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration Brain with the C9ORF72 Hexanucleotide..."


