BEYOND BIOMARKER DISCOVERY: RETINOID SIGNALING IN MOTOR NEURONS AND AMYOTROPHIC LATERAL SCLEROSIS

by

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Christi L. Kolarcik, PhD

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Amyotrophic lateral sclerosis (ALS) is the most common form of adult onset motor neuron disease and is characterized by the progressive degeneration and death of motor neurons. The pathologic mechanisms underlying ALS are poorly understood although our laboratory identified decreased levels of transthyretin (TTR), a protein that impacts the retinoid signaling pathway, in the cerebrospinal fluid of ALS patients. Differential expression of retinoid signaling components has been reported in ALS patients and transgenic animal models of familial ALS. We sought to further characterize TTR and retinoid signaling proteins in ALS and to evaluate the role of retinoid signaling in motor neuron cell death.

Mass spectrometry and immunoblotting were used to investigate TTR. Immunohistochemistry using lumbar spinal cord tissue from ALS patients and non-neurologic disease controls was used to characterize retinoid signaling pathway proteins. Spinal cord tissue homogenates were used for co-immunoprecipitation studies and electrophoretic mobility shift assays. Motor neuron-enriched cultures established from embryonic day 14 rats were utilized for *in vitro* studies. RAR-mediated signaling was modulated with pan-agonists and isotype-specific agents and hydrogen peroxide used to model oxidative stress/injury.

Altered post-translational modifications and high molecular weight species of the TTR protein were observed in ALS. Cellular retinoic acid binding protein-II (CRABP-II) and retinoic acid receptor beta (RARβ) exhibited increased nuclear localization in motor neurons of sporadic

ALS patients. Protein-protein interactions (between CRABP-II and RARα or RARβ) did not differ although retinoic acid response element binding was increased in ALS as compared to controls. Treatment with a pan-RAR or RARβ-specific agonist significantly decreased oxidative stress-induced motor neuron cell death *in vitro* and genes downstream of RARβ were increased with treatment.

Our results indicate that TTR genetic polymorphisms do not represent a novel susceptibility factor for ALS, although protein modification and aggregation appear to be altered in ALS. Localization of proteins of the retinoid signaling pathway is altered in ALS patients and these changes translate to the transcriptional level. Our *in vitro* work indicates that stimulating the RARs (particularly RARβ) is neuroprotective and that pharmacologic agents that target this nuclear receptor may be of value in slowing the progression of ALS.

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PREFACE

When I decided to apply to graduate school, I knew that the journey would be filled with challenges and the battle an uphill one. Fortunately, decisions I made early in the process enabled my personal and scientific development although I am not sure that I fully appreciated the gravity of them at the time. Joining the laboratory of Robert Bowser was certainly an example of this type of decision. Dr. Bowser has encouraged and enabled my scientific development and been an inspiration in terms of community involvement and scientific integrity. In addition, my colleagues in the Bowser laboratory particularly Sam Darko, Henrik Ryberg and Tina Kovalik helped make coming to work each day an enjoyable experience and have become friends instead of just colleagues. My mentor relationship with David Lacomis was another invaluable aspect of my graduate training. The opportunity to attend Multidisciplinary Clinics with Dr. Lacomis and to see patients with ALS first-hand provided perspective and fueled my passion to make a difference in the lives of these individuals. I greatly admire Dr. Lacomis and the relationship he has established with his patients along with his humor and genuine interest in my training.

In addition to these relationships and as a graduate student in the Cellular and Molecular Pathology (CMP) Program, there were a number of other people that helped me along this journey and made its completion possible. Wendy Mars, our program director, was a critical counselor, instructor and advocate. Shari Tipton kept me organized and was constantly available

for whatever forms or information I needed. Although almost each and every member of the Department of Pathology has also provided some form of advice, encouragement or assistance, I think the majority of the thanks for this should be attributed to George Michalopoulos for recruiting these excellent faculty members and creating the collaborative and scientifically-rich working environment in which I was fortunate enough to train. The administrative staff of the School of Medicine in general was also of great assistance including Cindy Duffy, Veronica Cardamone, Carol Williams, Claire Gauss and Jennifer Walker.

My four committee members (Charleen Chu, Satdarshan Monga, Kacey Marra and Michael Zigmond) were another critical component of my training. Their guidance, support and commitment to my development as an independent scientist were beyond what I could have asked. In addition, these individuals served as collaborators throughout my training. Dr. Monga kindly offered liver extracts for use as positive controls. Dr. Marra and Han Li provided hypoxia chambers for studies investigating non-physiologic oxygenation. Dr. Chu and Sam Cherra assisted with morphological assessments of neuronal processes.

I have also been extremely fortunate to collaborate with a number of exceptional scientists (in addition to the individuals mentioned above). The laboratory of Brent Harris including David Graber and Christopher Cogbill at Dartmouth University opened their laboratory doors to me and spent an intense day and a half teaching me how to isolate and culture primary motor neurons. At the University of Pittsburgh, Jonette Werley offered invaluable assistance and advice concerning immunohistochemistry. Don DeFranco and Marcia Lewis allowed me to practice spinal cord isolations while we waited for approval of our IACUC protocol. William Walker and Michelle Wood assisted with gel shift assays and the troubleshooting that followed. Aaron Bell and John Stoops were critical for primer design and

Q-PCR. The laboratory of Johnny Huard, specifically Jonathan Proto, Lauren Drowley, Joe Feduska and Mitra Lavasani gave me the opportunity to perform live cell imaging experiments, and I am immensely grateful for this assistance as these experiments added a great deal to our *in vitro* work.

Obviously, research cannot be conducted without funding, and I am grateful for the support I received from the Cellular Approaches to Tissue Engineering and Regeneration (CATER; NIH T32 EB001026) and Clinical and Translational Science Institute (CTSI; NIH T32 05 TL1 RR024155-02) Training Grants. I would especially like to thank Alan Russell and Marla Harris for their assistance during my time as a CATER fellow. Nicole Fowler, Benjamin Huffman, Galen Switzer, Mark Roberts and Wishwa Kapoor deserve my thanks for their help during my time as a member of the CTSI program.

Along my climb, I had the opportunity to reflect upon teachers from my past that aided in getting me to this point. Two of these previous mentors deserve my most sincere and heart-felt thanks. William Valilik, my high school biology professor, somehow knew that I would end up in a Ph.D. program before I knew they existed and encouraged me to apply for training programs as a high school student. My undergraduate mentor, John Simpson, gave me my first experience conducting research. His mentorship and friendship prepared me for what I would experience in graduate school, and I regret that he could not be here at this point in my career as having his approval always meant so much to me.

At times along this climb, I felt as if I would not be able to hold on much longer, that there was really no way for me to progress or that even if I moved forward, the obstacles in front of me were too great to move. At these critical times, my involvement with the Western Pennsylvania/West Virginia Chapter of the ALS Association served as a source of inspiration.

In particular, Michael Bernarding, Jenni Franz and the people and families with ALS motivated me to return to the bench even when my experiments did not want to cooperate. The Weekly Q and Wednesdays With Chad were two other sources of much-needed inspiration that I could not be more grateful to Brian McMahon and my brother Chad for sending to me each and every week.

The graduate students of the CMP, CATER and CTSI programs also deserve my most sincere thanks and appreciation. I am extremely fortunate that many of these colleagues have become friends and that some of these friends are now extensions of my family with whom I could not imagine life without. Although I will certainly miss names in this list, I am eternally grateful to the following people for their love, support, inspiration, willingness to listen, and genuine friendship throughout this journey: Cecelia Yates, Carolyn Kitchens, Miranda Sarachine, Lauren Drowley, Arlee Fafalios, and Paulina Liang. I would not hesitate for a second to do all of this all over again if for no other reason than to have these people become part of my life. Other members of the Royal Blue PSL Softball team (Bart Phillips, Austin Dulak, Brian Janelsins, Mike Ferenczy), graduate students from my incoming class (Jessica Chu, Gina Coudriet, Neil Bhola) and members of the BGSA Council (Hikmat Daghestani, Meghan Delmastro, Michelle Manni) have all added to my graduate school experience as well. In addition, all of my wonderful and amazing "non-science" friends were instrumental to my success, and I could not be more thankful for each and every one of them.

And last but most certainly not least, the people that have been there from the very beginning and experienced the trials and tribulations of this journey with me are my family. I could not ask for a better, more supportive group of cheerleaders and thank them for believing in me and my abilities when I was convinced that I just could not keep going. My Mom has always

been a role model and one of my biggest advocates; she has taught me how to persevere and reminds me of what is truly important. My sister, Jacquie, is an unbelievably supportive and loving friend that is always willing to listen, help or sacrifice to make things easier for others (especially me); a better sister does not exist and her humor and attention to detail are priceless. Chad, my brother, not only provided inspiration but has been my best friend since before I can remember; his humor, perspective and passion are some of my biggest assets. Although she has watched over me from Heaven for the past seven years, Granny was and always will be my source of strength and love. From her, I learned what it meant to work hard and how to give completely and selflessly; I know her and my Grandfather are smiling down on me and hope they are proud. My fiancé, Joseph, is my closest friend; he carried me through my ups and downs and was always there to cheer me up or talk things through. He is an irreplaceable part of my life, and I could not be more excited to know that our life together has only just begun. Perhaps my most devoted and understanding companion throughout this journey was my precious little angel, Jared Dan. More than anyone else, Jared made the sacrifice to attend graduate school with me and endured things like weekend trips to lab and the whole thesis-writing process. It wasn't always easy for either of us, but coming home to Jared made all the difference in the world; he is the best part of everyday, and I am so proud that he can call me "Dr. Mommy." And I am so happy that he will soon be a big brother and that his baby sister will only hear from him what having a Mom in graduate school really means. I must also acknowledge the love and support that I have and continue to receive from my Uncle Andy and Aunt Ginger, the Schmitt family and Darlene and Dean; all of these people are examples of some of the greatest blessings that I have ever received.

My family has understood my need for a “thesis room” (even when that room extended beyond its boundaries to the kitchen table) and that when I said I was almost done I would surely still be in graduate school another year. But most of all, my family has reminded me of what truly matters at the end of the day, and I could not be luckier to have them. Without each and every one of these people, I would not have made it to this point in my career/life. And, although there will surely be other mountains to climb and countless other obstacles ahead, it is only because of these people that I have the opportunity to face and overcome these new challenges.

1.0 INTRODUCTION

1.1 THE NERVOUS SYSTEM

The nervous system is a complex and fascinating organ system consisting of neural circuits of specialized cells that coordinate, integrate and control all body activities/functions. In humans, it consists of both central and peripheral parts (Figure 1). The central nervous system (CNS) includes the brain, spinal cord and retina while the peripheral nervous system (PNS) includes sensory (afferent) and motor (efferent) neurons, clusters of neurons called ganglia and nerves which connect these components to each other and to the CNS.

1.1.1 Cells and Synapses

Neurons are considered the functional unit of the nervous system and communicate with other cells via synapses or membrane-to-membrane junctions that allow for the rapid transmission of electrical or chemical signals. For electrical synapses, there is a direct electrical connection between neurons; chemical synapses are more common and more diverse in function (Figure 2) [1]. At these junctions, the pre-synaptic cell releases neurotransmitters that depolarize the post-synaptic cell to cause an influx of calcium (Ca^{2+}) ions. Synaptic communication results in excitatory, inhibitory or modulatory effects in the post-synaptic cell depending on the receptors to which the neurotransmitter binds.

Importantly, the nervous system includes other specialized glial cells including astrocytes, microglia, oligodendrocytes and ependymocytes in the CNS. Among the most important functions of these cells are to support neurons; to supply nutrients to neurons; to insulate neurons electrically; to destroy pathogens and remove dead neurons; and to provide guidance cues directing the migration of neurons and axons of neurons to their targets [2].

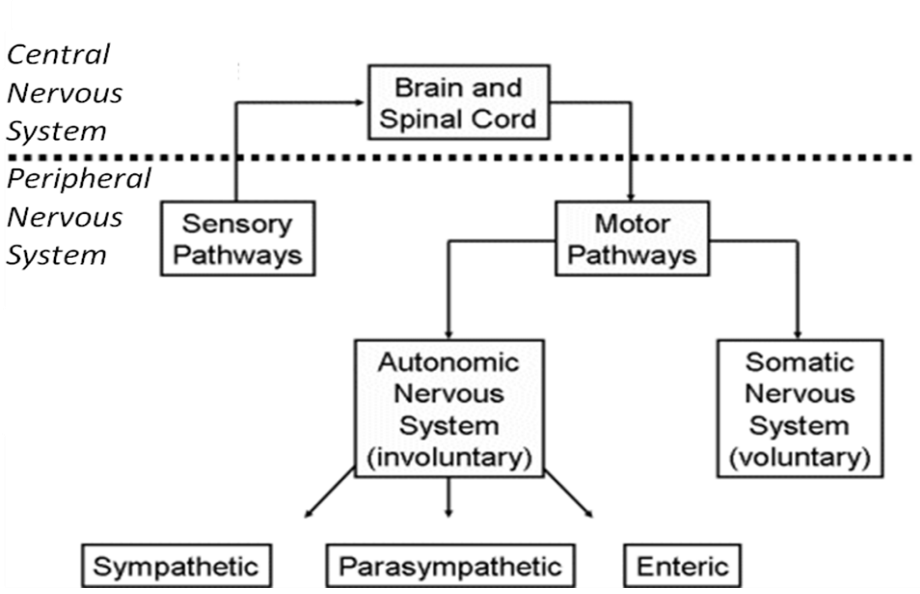


Figure 1. Schematic Overview of the Nervous System

The nervous system includes both central and peripheral parts. The central nervous system includes the brain and spinal cord while the peripheral nervous system includes both sensory (afferent) and motor (efferent) systems with further subdivisions of the motor pathway into the autonomic and somatic nervous systems. The autonomic nervous system includes sympathetic, parasympathetic and enteric systems. Figure modified from http://faculty.clintoncc.suny.edu/faculty/michael.gregory/files/bio%20102/bio%20102%20lectures/nervous%20system/nervous1.htm.

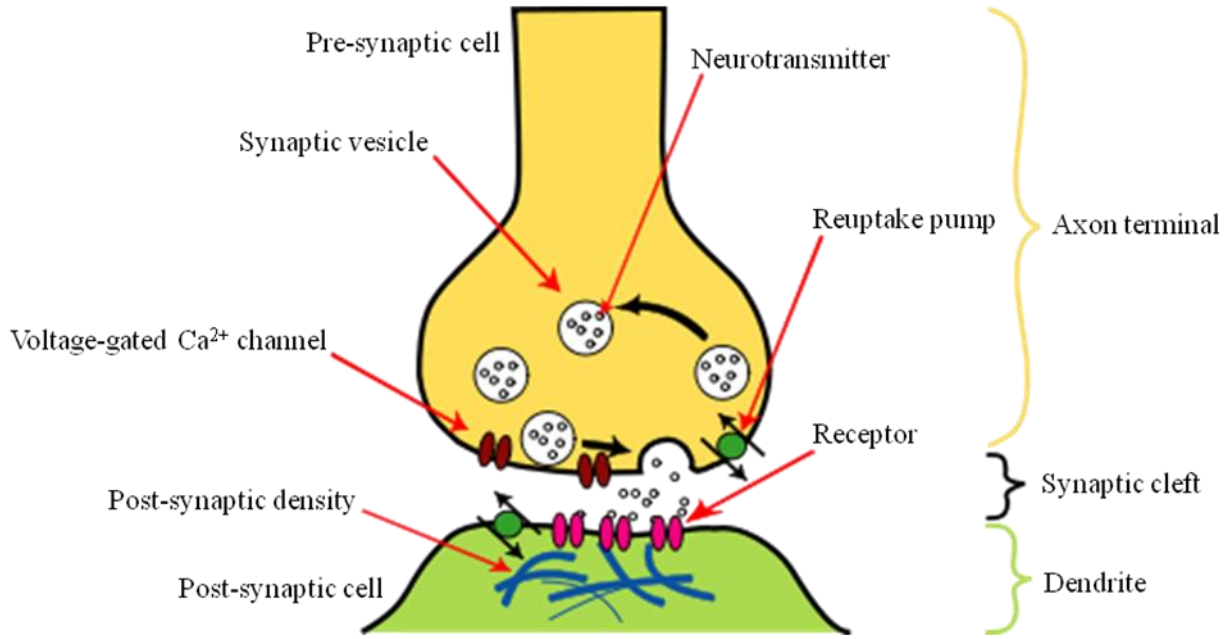


Figure 2. Neuronal Synapse

Neurotransmitters (e.g., glutamate) contained within synaptic vesicles of the pre-synaptic cell are released into the synaptic cleft. Receptors on the post-synaptic cell bind neurotransmitter molecules which respond by opening nearby ion channels which results in an influx of calcium into the post-synaptic cell (a neuron in this case) and depolarization. Reuptake pumps located on both neuronal and glial cells remove and recycle excess neurotransmitter. Figure modified from http://en.wikipedia.org/wiki/File:Synapse_Illustration_unlabeled.svg.

1.1.2 Central Nervous System

The CNS consists of the brain and spinal cord with the brainstem serving as the connection between them. While the brain controls most bodily functions including awareness, thought, speech, memory, sensation and movement, the spinal cord serves as a highway for carrying signals between the brain and PNS. In vertebrates, the brain is protected by the skull and the spinal cord by the vertebrae, and both are covered by the meninges, three continuous sheets of

connective tissue including the pia mater, the arachnoid and the dura mater. The region between the pia and arachnoid mater is filled with cerebrospinal fluid (CSF), a secretion produced by the cells of the choroid plexus. CSF surrounds the brain and spinal cord and circulates throughout the CNS through the central cerebrospinal canal of the spinal cord and through the four ventricles of the brain. Eventually, it returns to the blood through veins that drain the brain. CSF plays multiple and critical roles in that it acts as a cushion, regulates extracellular fluid, allows for the distribution of neuroactive substances and is the "sink" that collects the waste products produced by the cells of the brain and spinal cord. In addition, the brain and spinal cord are isolated by the blood-brain barrier, which restricts the movement of microscopic objects (i.e., bacteria) and large or hydrophilic molecules from the bloodstream to the interior of the CNS.

1.1.3 Brain Overview and Functions

The major structures of the brain are outlined below (Figure 3) and generally consist of the hindbrain, midbrain and forebrain. The hindbrain or rhombencephalon includes the medulla oblongata, pons and cerebellum. The medulla oblongata is interconnected with the cervical spinal cord and helps control vital processes like breathing, heart rate and blood pressure. Importantly, it carries the corticospinal tract toward the spinal cord. The pons serves as a bridge between the midbrain and the medulla oblongata and is involved in motor control and sensory analysis. The cerebellum consists of two deeply-convoluted hemispheres containing as many neurons as the rest of the brain combined, and it functions in the regulation and coordination of bodily movements, posture and balance.

The midbrain (mesencephalon) is located below the hypothalamus and includes the tectum and tegmentum. Together, the midbrain, medulla oblongata and pons are referred to as

the brainstem. This compact structure marks the convergence of multiple pathways that carry information to and from the brain to the spinal cord. Therefore, damage to the brainstem can affect multiple functions and, because these fibers do not typically regenerate, such damage can result in permanent loss of function.

The telencephalon and diencephalon make up the human forebrain (prosencephalon) which includes the largest part of the brain, the cerebrum. The cerebrum is divided into the left (controls the right side of the body) and right (controls the left side of the body) hemispheres which are linked by a mass of fibers called the corpus callosum. Each hemisphere consists of four interconnected lobes (frontal, parietal, temporal and occipital) with distinct functions. The cerebral cortex or gray matter is the external-most layer of the brain and consists predominantly of neuronal cell bodies which store and process information. Myelin-sheathed axonal projections of these cells extend to other areas of the brain and body and make up the white matter.

The limbic system, consisting of the thalamus, hypothalamus, amygdala and hippocampus, is located within the cerebrum. The thalamus is a large mass of gray matter situated deep in the forebrain with both sensory and motor functions. Axons from every sensory system (except olfaction) synapse here as the last relay site before the information reaches the cerebral cortex (parietal, temporal and occipital lobes). The hypothalamus controls automatic functions like emotion, hunger, circadian rhythms and the autonomic nervous system. In addition, it controls the pituitary gland and thereby the release of other hormones. The amygdala is located in the temporal lobe and involved in memory, emotion and fear. The hippocampus is also located within the temporal lobe and plays a critical role in learning and long-term memory.

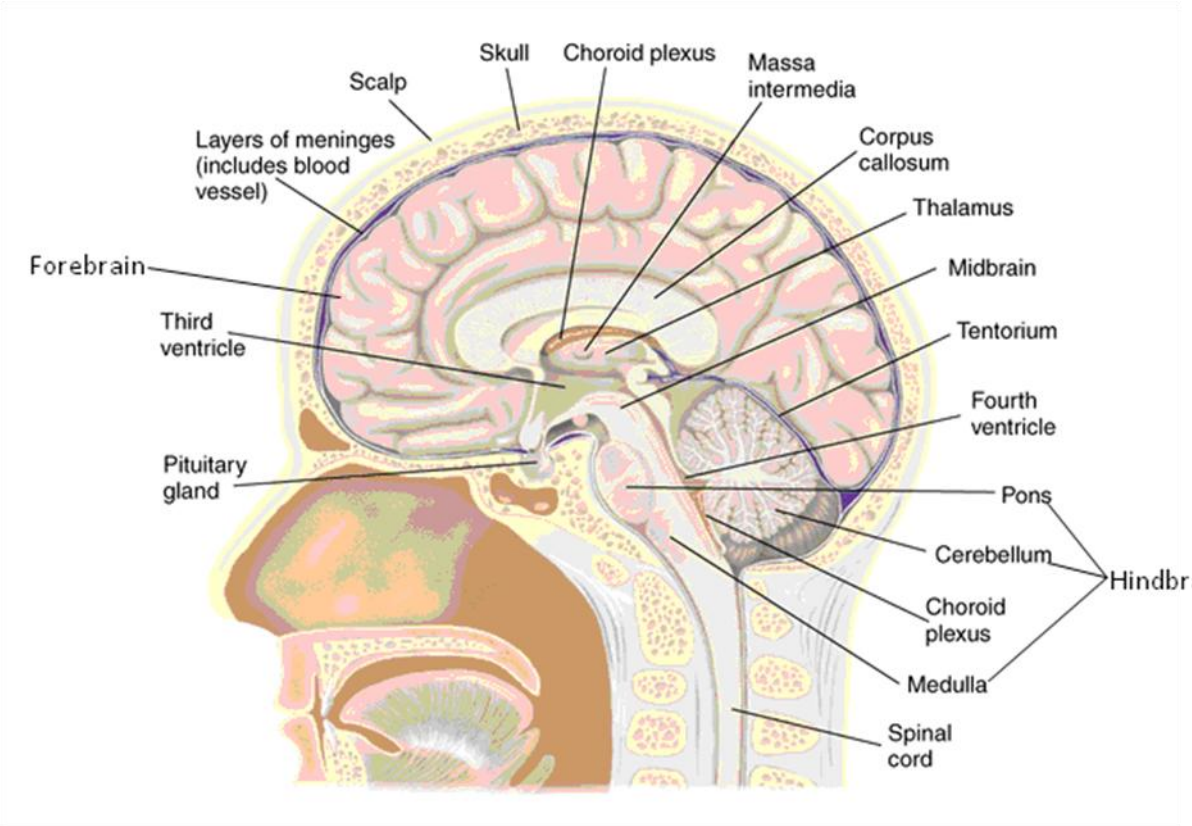


Figure 3. Midsagittal View of the Brain

The brain controls most bodily functions and can generally be divided into the hindbrain, midbrain and forebrain with distinct structures that comprise each region. Physical protection is provided by the layers of the meninges as well as the skull. Cerebrospinal fluid is synthesized by the cells of the choroid plexus. Figure modified from http://homepage.psy.utexas.edu/homepage/class/Psy332/Salinas/Neuroanatomy/Neuroanat.html.

1.1.4 Spinal Cord Overview and Functions

The spinal cord connects to the brain via the brainstem and is physically protected by the vertebral bodies that make up the spinal column and the meningeal layers (Figures 4 and 5). It conducts sensory (afferent) information from the PNS (including both somatic and autonomic

systems) to the brain and motor (efferent) information (including autonomic efferents) from the brain to muscles (skeletal, cardiac and smooth) and glands. Cranial nerves exit the brainstem and nerve roots exit the spinal cord on both sides of the vertebral body. Nerve roots are located ventral in relation to the spinal cord if efferent or dorsal if afferent. Fibers that carry motor input to limbs and fibers that bring sensory information from the limbs to the spinal cord grow together to form a mixed (motor and sensory) peripheral nerve. A portion of the lumbar and all sacral nerve roots travel downward into the spinal canal before exiting the spinal cord in a bundle called the cauda equina (horse's tail).

Within the spinal cord there are thirty-one pairs of mixed (both sensory and motor axons) spinal nerves belonging to different levels according to the region of the body innervated: 1.) cervical (8 pairs of nerves that transmit signals from or to parts of the head, neck, shoulders, arms and hands), 2.) thoracic (12 pairs of nerves that transmit signals from or to parts of the arms and the chest and abdominal areas), 3.) lumbar (5 pairs of nerves that transmit signals from or to the legs, feet and some pelvic organs), 4.) sacral (5 pairs of nerves that transmit signals from or to the lower back, buttocks, pelvic organs, genital areas and some areas in the legs and feet) and 5.) coccygeal (1 pair of nerves located at the bottom of the spinal cord). All sensory axons pass into the dorsal root ganglion where their cell bodies are located and then into the spinal cord while all motor axons pass into the ventral roots before combining with the sensory axons to form the mixed nerves.

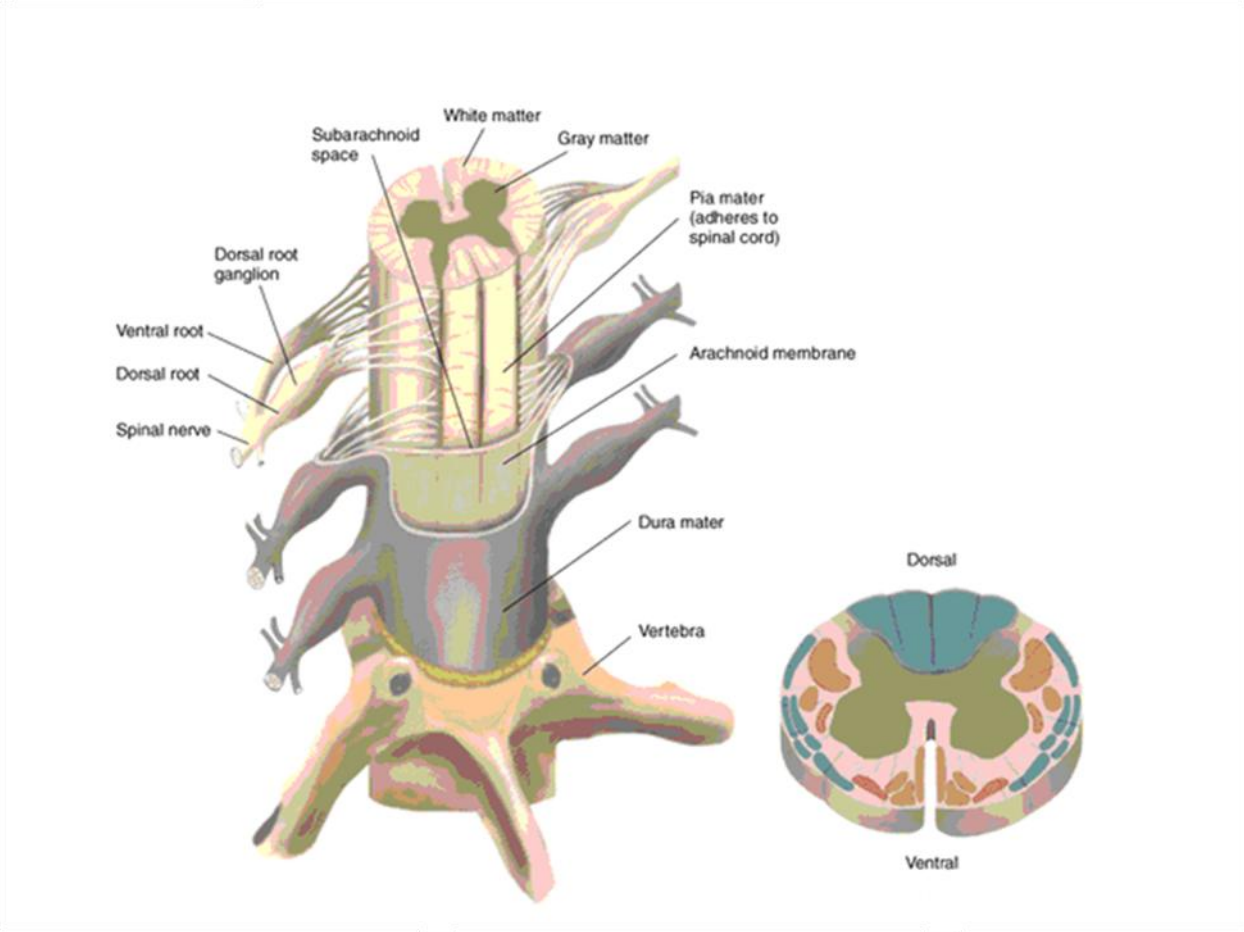


Figure 4. Spinal Cord Anatomy

The spinal cord is protected by the vertebrae and the three meningeal layers. Fibers exit both sides of the spinal cord and their location (dorsal or ventral) depends on the direction of the signals they carry (afferent or efferent). Figure modified from http://homepage.psy.utexas.edu/homepage/class/Psy332/Salinas/Neuroanatomy/Neuroanat.html.

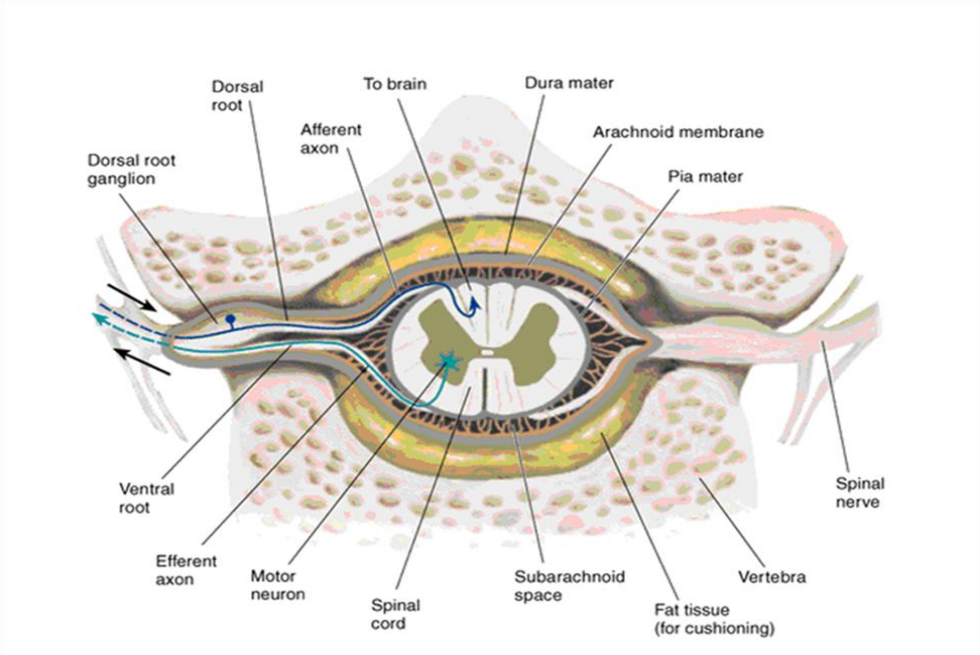


Figure 5. Cross-Sectional View of the Spinal Cord

The cross-sectional view of the spinal cord reveals the characteristic butterfly-shaped pattern resulting from the boundary between the gray matter (nerve cell bodies) and the surrounding tissue of the white matter. The location of motor neurons in the ventral horn is highlighted as well as the routes of both afferent and efferent axons. Figure modified from http://homepage.psy.utexas.edu/homepage/class/Psy332/Salinas/Neuroanatomy/Neuroanat.html.

1.2 THE MOTOR SYSTEM

The motor system is responsible for the generation and control of voluntary and reflex movements and is an efferent pathway conducting impulses from the CNS to muscle. It can be divided into (1) the complex central apparatus, including the descending tracts involved in control (i.e., the pyramidal system) and the systems involved in initiating and regulating movement (the basal ganglia and cerebellum) and (2) the peripheral apparatus, which consists of

the anterior horn cell and its peripheral axon, the neuromuscular junction and muscle. The motor innervation of the human body is often represented by a distorted human figure called the motor homunculus (Figure 6).

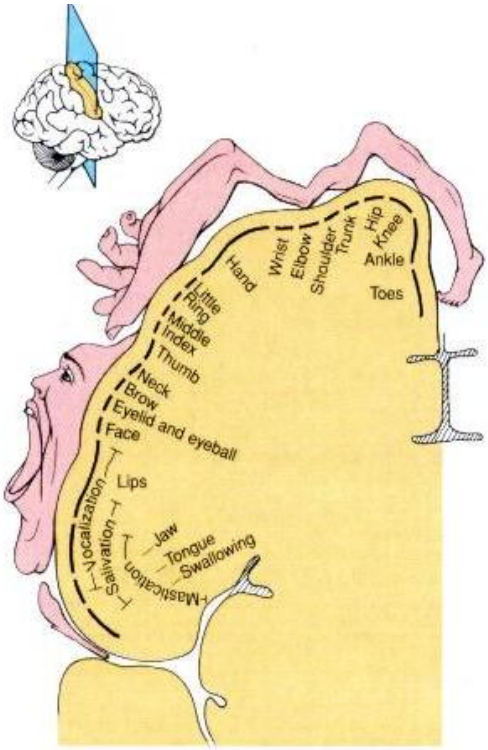


Figure 6. The Motor Homunculus

The motor homunculus is a representation of the different body parts controlled by specific regions of the motor cortex. The leg area is located medially, the head and face area laterally and the arm and hand area between the leg and face. The map was determined by stimulating different points in the cortex of conscious patients during brain surgery and recording what they said they felt. Figure modified from http://www.harmonicresolution.com/homunculus1.jpeg.

1.2.1 The Motor Pathway

The motor pathway, also called the pyramidal tract or the corticospinal tract, originates in the giant pyramidal (or Betz) cells of the precentral gyrus (motor cortex) [3-6]. Long axons extend from the motor cortex to the motor cranial nerve nuclei of the midbrain (forming the corticomesencephalic tract), pons (forming the corticopontine tract) and medulla oblongata (forming the corticobulbar tract) or down to the spinal cord (forming the corticospinal tract). Most of the corticospinal fibers (about 85%) cross over to the opposite side in the medulla oblongata, a process called pyramidal decussation, forming the lateral corticospinal tract. The remaining 15% of the fibers form the ventral corticospinal tract and cross in the spinal cord. The axons that make-up both of these tracts eventually synapse with another neuron located in the ventral horn of the spinal cord. The corticospinal and corticobulbar fibers are considered the pathways for control of voluntary movements and are affected in motor neuron diseases.

1.2.2 Motor Neurons

The motor neuron is a specialized type of neuronal cell responsible for controlling muscle movement. A single motor neuron can synapse with one or more muscle fibers; collectively, the motor neuron and the muscle fibers to which it connects are called a motor unit. Both upper and lower motor neuron populations exist. The cell bodies of upper motor neurons (UMNs) are located in the motor cortex and their axons carry motor information down the brainstem and spinal cord but are not directly responsible for stimulating target muscle (Figure 7). Rather, the axons of these neurons connect (mostly via interneurons but some through direct synapses) with lower motor neurons (LMNs). The cell bodies of LMNs are located in the ventral horn of the

spinal cord or in the motor cranial nerve nuclei of the brainstem. The LMN axonal projections leave either the brainstem or spinal cord via motor cranial nerves or anterior roots of the spinal nerves, respectively, and terminate at their muscle fiber target. They are classified as either alpha or gamma motor neurons based on the type of muscle they innervate. Alpha motor neurons innervate extrafusal muscle fibers which function in muscle contraction. Gamma motor neurons innervate intrafusal muscle fibers which are involved with muscle spindles and the sense of body position.

The distinction between types of motor neurons is of particular interest in ALS because of the observation that different types of motor neurons responsible for innervating different subsets of muscle fibers have different susceptibilities to mutated proteins that cause toxicity in familial ALS. More specifically, in two different models of familial ALS, the fast-fatiguable motor neurons were found to be affected first with denervation of their target muscles occurring prior to symptom onset. Subsequent retraction of the fast-fatigue-resistant motor neurons occurred with the slow type demonstrating partial resistance to toxicity [7, 8]. The latter motor neuron population also appeared to demonstrate the ability to reinnervate previously denervated regions.

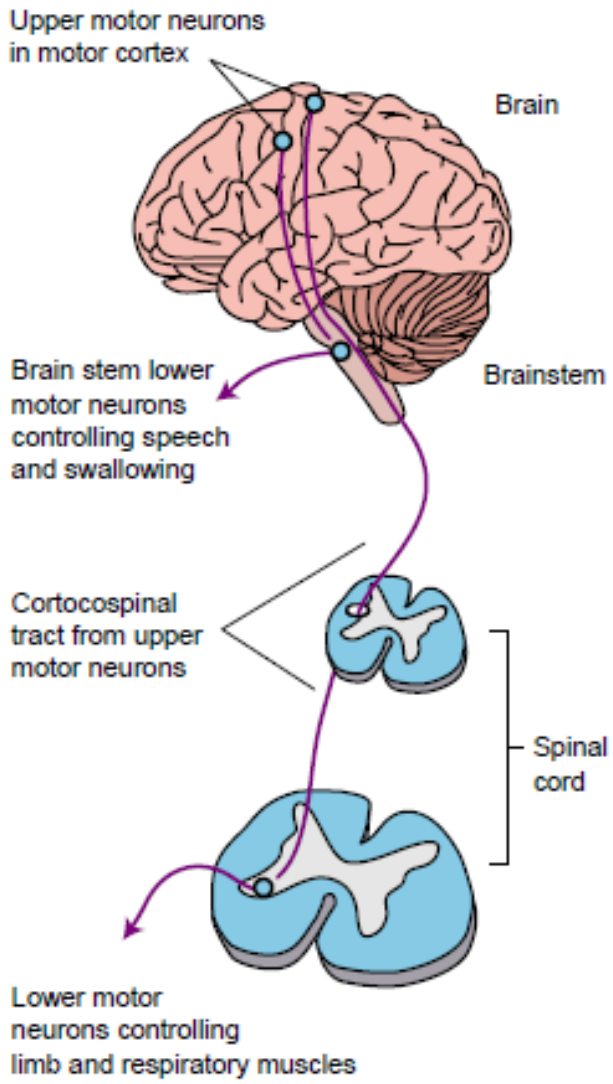


Figure 7. Upper and Lower Motor Neurons of the Human Motor System

The cell bodies of upper motor neurons originate in the motor cortex with long axons that extend and connect (either directly or via interneurons) to lower motor neurons via the corticospinal tract. Lower motor neurons directly innervate target muscle fibers. Both upper and lower motor neuron populations are affected in ALS. Figure modified from [9].

1.2.3 Neuromuscular Junction

Motor neurons communicate with skeletal muscle fibers at a specific type of neuroeffector junction called the neuromuscular junction (NMJ) (Figure 8). At this synapse, the axon terminal of the motor neuron signals to the motor end plate of a muscle fiber through the release of the neurotransmitter acetylcholine (ACh) which ultimately results in muscle contraction. More specifically, glutamate released from the UMNs triggers the depolarization of LMNs which results in an action potential that propagates along the axon of the LMN. In response to this action potential, voltage-dependent Ca^{2+} channels open to allow for the influx of Ca^{2+} into the motor neuron. This leads to vesicle docking and fusion such that ACh is released into the synaptic cleft. Nicotinic ACh receptors located on the motor end plate bind the released neurotransmitter, increasing the permeability of ion channels that cause sodium (Na^{+}) influx and potassium (K^{+}) efflux. Local depolarization of the motor end plate (end-plate potential) then spreads across the surface of the muscle fiber to transverse tubules (T-tubules), eliciting Ca^{2+} release from the sarcoplasmic reticulum and initiating muscle contraction. Importantly, remaining ACh is degraded to produce choline and acetic acid by the enzyme acetylcholinesterase (AChE). Choline is then transported back into the presynaptic terminal via reuptake receptors such that new ACh molecules can be made.

Although the effect of a neurotransmitter (i.e., whether it is excitatory, inhibitory or modulatory) is typically dependent upon the receptor it binds, the response of a muscle fiber can only be excitatory (i.e., contractile). For muscle relaxation or inhibition of muscle contraction, the motor neuron must be inhibited. It is worth noting that although one motor neuron can control multiple muscle cells, each muscle cell only responds to one motor neuron; if a motor neuron dies, however, adjacent neurons can sprout to innervate additional muscle fibers. When

this compensatory sprouting capacity is exhausted, normal skeletal muscle function and movement are compromised and disease occurs. In ALS mouse models, connection to the muscle at the NMJ is lost prior to motor neuron degeneration or death and the initiation of symptoms [7, 8, 10, 11].

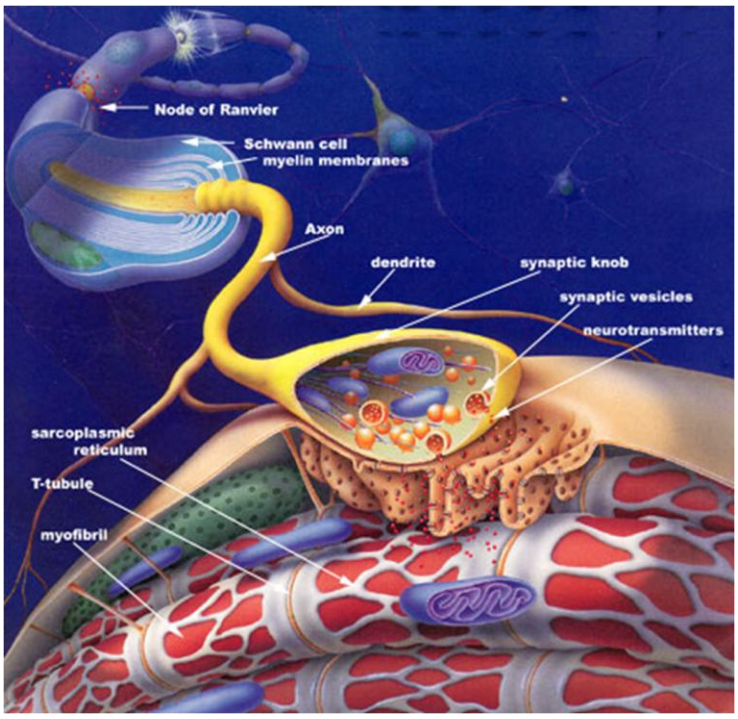


Figure 8. The Neuromuscular Junction

Motor neurons communicate with skeletal muscles at the neuromuscular junction. Release of ACh-containing vesicles from the motor neuron ultimately causes calcium release from the sarcoplasmic reticulum and muscle contraction. Figure modified from http://www.bio.miami.edu/~cmallery/150/neuro/neuromuscular-sml.jpg.

1.3 MOTOR NEURON DISEASE

Motor neuron diseases (MNDs) are a heterogeneous group of disorders involving the progressive degeneration and death of motor neurons located in the brain, brainstem and spinal cord (Table 1). As both UMNs and LMNs can be affected, this family of disorders is often classified into three major subgroups according to the neuronal population affected: 1.) primary lateral sclerosis (PLS) (pure upper (corticospinal) motor neuron involvement), 2.) progressive muscular atrophy (PMA) (pure lower (spinal) motor neuron involvement), and 3.) amyotrophic lateral sclerosis (ALS) (combined upper and lower motor neuron involvement). Further discussion will focus on ALS.

Table 1. Types of Motor Neuron Disease

Motor Neuron Disease		Affected Neuronal Population	Cause
Primary lateral sclerosis (PLS)		upper motor neurons	Idiopathic
	Pseudobulbar palsy	bulbar upper motor neurons	Idiopathic
Primary muscular atrophy (PMA)		lower motor neurons	Idiopathic
	Progressive bulbar palsy	bulbar lower motor neurons	Idiopathic
Amyotrophic lateral sclerosis (ALS)		upper and lower motor neurons	Idiopathic and Inherited Forms
Other Motor Neuron Diseases			
	Kennedy's disease or X-linked spinal and bulbar muscular atrophy (SBMA)	brainstem and spinal cord motor neurons	CAG repeat in the androgen receptor gene
	Spinal muscular atrophy	spinal cord motor neurons	Survivor of motor neuron (SMN) gene mutations

1.4 AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic lateral sclerosis (ALS), commonly referred to as Lou Gehrig's disease in North America, was first described in 1869 by the French neurologist Jean-Martin Charcot as a late-onset and progressive motor neuron disease [12]. Over 140 years later, the most common adult onset motor neuron disease remains poorly understood and effective therapeutic interventions are still needed.

1.4.1 Epidemiology

The incidence and prevalence of ALS are 1-2 cases and 4-6 cases per 100,000, respectively, and as many as 30,000 Americans have this disease at any given time [13-15]. There is a slight gender disparity toward males (1.5 to 1.0 in sporadic cases) although all races and ethnic backgrounds can be affected. The average age of disease onset is 55 years with a median survival of approximately 3 years for newly diagnosed patients, although this can vary between 1 and 20 years [16, 17]. While it is unclear what determines this difference, improved survival and slower disease progression have been associated with limb onset (muscles affecting the legs and arms are affected initially) while a poorer prognosis and more rapid disease progression typically occur with bulbar onset (muscles affecting speech and swallowing are initially affected) [18].

Although the incidence of ALS is regarded as regionally uniform, there are a few regions where an elevated occurrence of ALS has been observed. The largest is the area of Guam where the Chamorro people have had an incidence as high as 143 cases per 100,000 people per year of a condition called Lytico-Bodig disease which is a combination of ALS, Parkinsonism and dementia [19]. Two other areas with increased incidence are the Kii peninsula of Japan and West Papua [20, 21]. In addition, although there have been reports of "clusters" of cases including three San Francisco 49ers players, over fifty Italian soccer players and husband and wife pairs [22-29], these appear to be chance events.

1.4.2 Clinical Presentation and Affected CNS Regions

The earliest symptoms of ALS are obvious weakness and/or muscle atrophy followed by twitching, cramping or stiffness of affected muscles. The twitching and cramping associated

with dying motor neurons must be accompanied by clinical weakness or atrophy for a diagnosis of ALS to be made.

The site of disease onset determines which part of the body is affected by early symptoms. Approximately 75% of cases experience limb onset disease which manifests as awkwardness when walking or running, an increase in the frequency of tripping or stumbling or difficulty performing simple tasks that require manual dexterity. The remaining 25% initially notice difficulty speaking clearly (slurred and/or nasal speech, decreased volume) and have bulbar onset disease. Regardless of the location of initial onset, muscle weakness and atrophy spread to other parts of the body as the disease progresses. Patients typically have increasing difficulty moving, swallowing (dysphagia) and speaking or forming words (dysathria), and denervation of the respiratory muscles and diaphragm is typically the cause of death.

Clinical features of ALS combine progressive lower motor neuron dysfunction characterized by focal and multifocal weakness, muscular atrophy, cramps and fasciculations with symptoms of upper motor neuron or corticospinal tract dysfunction (enhanced and pathological reflexes and spasticity) [17, 30]. Approximately 15-45% of patients also experience what is known as a pseudobulbar affect or emotional lability. This is characterized by uncontrollable laughter, crying or smiling and attributed to the degeneration of bulbar upper motor neurons. It was thought that proper cerebellar and sensory functions were preserved in individuals with ALS such that intellect, memory and personality remain intact. However, it is now recognized that patients with ALS may also be affected by cognitive deficits that culminate to frontotemporal dementia (FTD) [31]. Control of the eye muscles is the most preserved function although this can also be affected in patients with longer disease duration, and bladder

and bowel control are usually preserved in ALS. Some of the pathological features associated with ALS and their descriptions are outlined below (Table 2; modified from [9]).

Table 2. Pathological Features Observed in ALS

Feature		Description/Location
Motor neuron loss		Observed in the cortex, brainstem and spinal cord
Axonal spheroids		Neurofilament aggregates in proximal axons of motor neurons
Bunina bodies		Eosinophilic bodies found in the soma of anterior horn cells
Ubiquitinated inclusions		Found predominantly in lower motor neurons
	Skein-like inclusions	Threads/filamentous bodies
	Lewy body-like inclusions	Compact, dense bodies
Hyaline inclusions		Large aggregates containing neurofilaments and other proteins located in the soma of motor neurons

1.4.3 Diagnosis of Probable ALS

Currently, no test can provide a definitive diagnosis of ALS; however, the clinical diagnosis of probable ALS is made when both upper and lower motor signs are present [32-34]. Diagnosis is based primarily on the signs and symptoms observed by the neurologist along with a battery of tests to rule out other (more treatable) diseases. These can include electromyography (EMG) to measure electrical activity in muscles, nerve conduction velocity (NCV) tests to rule out peripheral neuropathy or myopathy and magnetic resonance imaging (MRI) to determine

whether other conditions like spinal cord tumors, multiple sclerosis or a herniated disk are responsible for the symptoms. Blood and urine samples as well as muscle biopsies may also be taken because of the prognosis associated with ALS and the variety of other diseases that can mimic ALS at its early stages.

1.4.4 Proposed Underlying Mechanisms

Several mechanisms have been implicated in the initiation and propagation of motor neuron degeneration (overview in Figure 9) including excitotoxicity, immune activation, mitochondrial dysfunction, oxidative stress, altered proteosomal function and apoptosis [16]. In addition, prominent disruptions in the neuronal cytoskeleton and the formation of intraneuronal protein aggregates have been noted [35]. The most extensively studied of these processes is excitotoxicity defined as the "phenomenon whereby the excitatory action of glutamate and related excitatory amino acids becomes transformed into a neuropathological process that can rapidly kill central nervous system neurons" [36]. Although precise details of this neuronal insult are not completely understood, enzymatic cascades and oxidative stress have been implicated [37]. However, questions pertaining to the relationship between alterations in these pathways and disease onset and progression remain unanswered. Importantly, non-neuronal neighboring cells can enhance damage to motor neurons (reviewed in [38]); contributing to excitotoxicity, causing inadequate growth factor signaling and initiating/exacerbating an inflammatory response are examples of how these cells can accelerate disease progression.

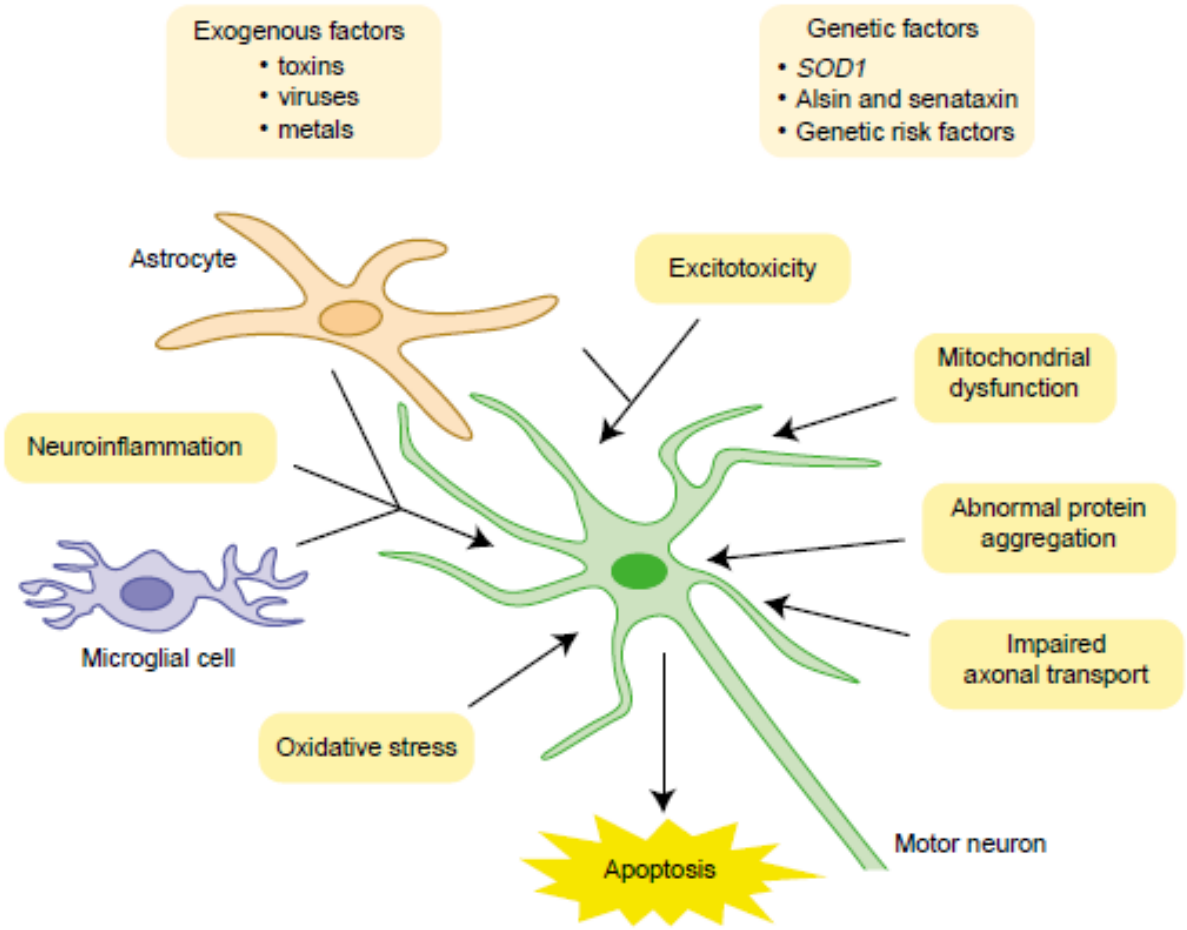


Figure 9. Proposed Mechanisms Underlying ALS

A variety of mechanisms are hypothesized to contribute to the motor neuron degeneration and death that occurs in ALS. Exogenous and genetic factors along with a number of other processes are believed to play a role. Non-neuronal cells including both astrocytes and microglia contribute to motor neuron cell death as well. Figure taken from [9].

An underlying theme to many of the aforementioned mechanisms is the susceptibility of the motor neuron population (Figure 10) which likely arises from a combination of factors. For example, motor neurons are large cells with long axonal processes (up to 1 meter long) that extend to distal lower limb muscles [39]. This creates high energy demands and the need for a robust cytoskeleton which translates to high mitochondrial activity and a high intracellular

content of neurofilament proteins, respectively. In addition, the lack of certain calcium buffering proteins like parvalbumin as well as the low expression of the GluR2 subunit of the AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) receptor may result in increased vulnerability to calcium-mediated toxic processes following activation of cell surface glutamate receptors [40, 41]. Motor neurons also have a high perisomatic expression of the excitatory amino acid transporter 2 (EAAT2) and very high intracellular expression of the free radical-scavenging enzyme SOD1 [42]. This may increase vulnerability in the presence of genetic or post-translational alterations that interfere with the proper function of these proteins.

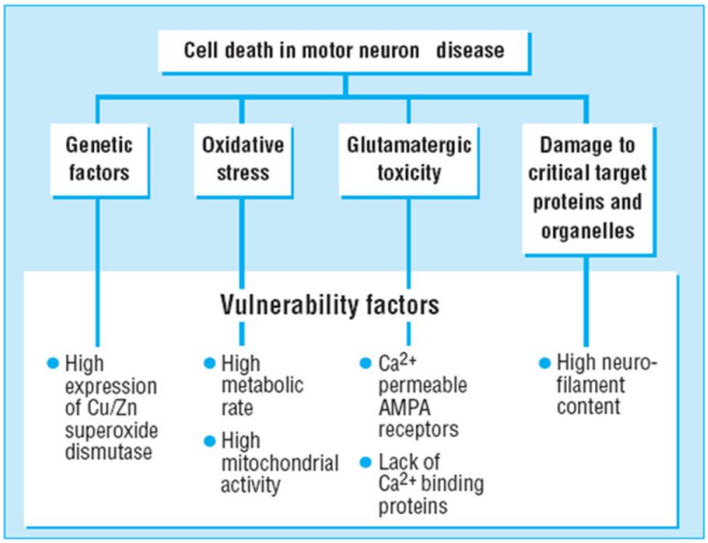


Figure 10. Susceptibility of Motor Neurons

Specific features of motor neurons may explain their susceptibility to some of the mechanisms thought to underlie the motor neuron cell death that occurs in motor neuron diseases like ALS. Figure modified from [42].

1.4.5 Genetics

Although the majority of ALS cases (over 90%) are idiopathic in nature, the list of genes associated with the 10% of cases with a genetic component (familial ALS) continues to grow (reviewed in [38] and [43]). Inheritance in familial ALS is typically autosomal dominant, but some autosomal recessive pedigrees have been described [44-49]. A current list of the genes in which mutations cause motor neuron degeneration is provided in Table 3. The chromosomal loci associated with ALS-like motor neuron diseases have been defined as ALS1 through ALS11; ALS with frontotemporal dementia (ALS-FTD) and ALS-FTD coupled with Parkinson's disease (ALS-FTDP) are included. As described in the references listed below, the majority of these mutations are rare and most will not be discussed further.

The 17-year old discovery linking mutations in the gene encoding copper/zinc superoxide dismustase (SOD1) to ALS had a major impact in the field [50]. Although SOD1 mutations (of which there are over 122) are the most common form of inherited ALS (accounting for approximately 1 in 10 familial cases), they account for less than 2% of total cases. Intensive research efforts have focused on these mutations with various *in vitro* and transgenic animal models developed as the clinical features of *SOD1*-linked ALS are indistinguishable from ALS without *SOD1* mutations (with the exception that the former has a slight tendency to begin in the limbs rather than the bulbar region) [51]. Although the pathways leading to the specific motor neuron degeneration in the presence of SOD1 mutations have not been fully identified, this enzyme is highly conserved throughout evolution, ubiquitously expressed (constituting approximately 1% of total cytosolic protein) and responsible for converting superoxide radicals to hydrogen peroxide which is subsequently converted to water by either catalase or glutathione peroxidase. The proposed pathogenetic mechanisms underlying the toxic gain of function

associated with *SOD1* mutations include oxidative stress, glutamate excitotoxicity, neurofilament disorganization, copper toxicity and apoptotic cell death [52].

Table 3. Reported Genes in Familial ALS/MND

Disease Name	Gene	Chromosome	References
Adult onset dominant typical ALS			
ALS1	*SOD1**	21q	[50, 53]
ALS3	Unknown	18q	[54]
ALS6	*FUS/TLS*	16p	[55-59]
ALS7	Unknown	20p	[59]
Adult onset dominant atypical ALS			
ALS8	*VAPB*	20q	[60, 61]
ALS9	*Angiogenin*	14q	[62, 63]
ALS10	*TDP-43*	1p	[64]
ALS11	*FIG4*	6q	[65]
ALS-FTD1	Unknown	9q	[66]
ALS-FTD2	Unknown	9p	[67]
ALS-FTD3	*CHMP2B*	2p	[68]
ALS-FTDP	*MAPT*	17q	[69]
Progressive LMN disease	*DCTN1*	2p	[70]
Juvenile onset dominant ALS			
ALS4	*Senataxin*	9q	[71, 72]
Juvenile onset recessive ALS			
ALS2	*Alsin*	2q	[47, 49, 73]
ALS5	Unknown	15q	[48]

*SOD1, superoxide dismutase 1; FUS/TLS, fused in sarcoma/translocated in liposarcoma; VAPB, vesicle-associated membrane protein B; TDP-43, transactive response DNA-binding protein 43; FIG4, factor-induced gene 4 protein; FTD, frontotemporal dementia; CHMP2B, charged multivesicular body protein 2B; FTDP, frontotemporal dementia/parkinsonism; MAPT, microtubule-associated protein tau; DCTN1, dynactin1; *, the D90A SOD1 mutation can be either recessive or dominant [74]*

The most recent discovery in ALS genetics was the identification of mutations in FUS (Fused in Sarcoma, ALS6) or TLS (translocated in liposarcoma) as the cause of approximately 1 in 20 cases of familial ALS [55, 56]. This protein is involved in RNA processing and normally

localized to the nucleus as is observed with transactive response DNA-binding protein 43 (TDP-43). Interestingly, mutant forms of both FUS/TLS and TDP-43 are found in the cytoplasm rather than the nucleus suggesting a similar pathogenetic mechanism. That is, alterations in RNA processing may be a common theme in the pathogenesis of ALS [75].

Several genome-wide association studies have also been conducted in ALS [76-82] although inconsistent and/or negative results have been obtained potentially as a result of small sample sizes. In spite of this limitation, two themes in underlying pathogenesis were further supported. That is, identification of polymorphisms in the kinesin-associated protein 3 (*KIFAP3*) and elongator protein 3 (*ELP3*) genes implies that axonal transport and RNA metabolism, respectively, impact the disease process [80, 82].

1.4.6 Model Systems

As may be expected from the idiopathic nature of the majority of ALS cases, the model systems utilized to study this disease are based on the above-mentioned genetic linkages. In particular, the lack of mechanistic differences between *SOD1*- and non-*SOD1*-linked forms of ALS has provided rationale for the most commonly-used animal models of the disease. Overexpression of mutant SOD1 protein in rodents leads to a neurodegenerative disease that is similar to what is observed in the human condition [83-88]. Interestingly, each model of ALS is consistent and distinct with a given mutation (although some variation due to the number of copies of the transgene in the animal model does occur), and they vary in their age at onset, disease progression and particular histopathological features, thereby mimicking the diversity of phenotypes observed in human cases [43]. It was thought that effective therapies in the SOD1 animal models would translate to the sporadic condition in humans. Unfortunately, this has not

been the case as there are many instances in which agents that have modified the disease process in animal models have failed in clinical trials. Nevertheless, the importance of this model system cannot be overlooked particularly with respect to the finding that the toxicity of *SOD1* mutations is non-cell autonomous such that motor neuron toxicity requires damage from mutant SOD1 acting within non-neuronal cells [89-92].

A number of other models have also been generated. For instance, two groups generated an alsin knockout mouse, but no major motor deficits consistent with ALS or MND in general were observed [93, 94]. No rodent models for vesicle-associated membrane protein B (VAPB) have been generated although male mutants in *Drosophila melanogaster* (*D. melanogaster*) die in early larva stage and show severe motor deficits along with severely compromised synaptic microtubule assembly [95]. Both microtubule-associated protein tau (MAPT) and dynactin1 (DCTN1) models exist. Transgenic mice overexpressing different human tau isoforms in neurons experience age-dependent CNS pathology including axonal degeneration in the brain and spinal cord, progressive motor disturbances and behavioral impairments [96-99]. Transgenic mice overexpressing the p150 subunit of dynactin develop a late onset, progressive motor neuron disease [100] and studies in *D. melanogaster* indicate dynactin promotes synapse stability at the NMJ [101]. Finally, gain and loss of function studies using TDP-43 mutations linked to ALS are being performed in zebrafish models and will undoubtedly provide novel insight into the role of this protein in ALS/MND [102] as will a recently established transgenic rat model [103].

1.4.7 Current and Developing Therapies

There is currently only one Food and Drug Administration (FDA)-approved pharmacologic agent designed for the treatment of ALS. Riluzole (or rilutek) functions as a glutamate

antagonist and was first approved for clinical use over ten years ago [104]. Since that time, a modest increase (about 3 months) in survival has been demonstrated [104-106] and mortality rates were reduced by 23% and 15% at 6 and 12 months, respectively [107]. Recent studies have shown that patients receive the maximal benefit when the drug is initiated early in the course of disease [108]. In addition, riluzole may be most useful with initial and less severe cases of ALS as no effect on overall survival or rate of deterioration of muscle strength were noted in patients with a forced vital capacity less than 60% of predicted or over 75 years of age [109, 110]. Riluzole side effects exist, though these are not common and generally reversible after stopping the drug [111], while long-term neurotoxic effects remain unclear.

The predictability with respect to disease onset, duration and endpoint times in SOD1 transgenic animals makes them ideal (at least in theory) models for evaluating potential ALS therapeutics. In fact, over 150 different potential therapeutic agents or strategies have been tested in this model according to published trials (outlined in [112]). These include antioxidants, anti-glutamatergic agents, anti-aggregation compounds, anti-apoptotics, immunomodulatory drugs, neurotrophic factors, compounds that target non-glutamatergic neurotransmission, gene and antisense therapies (both viral and non-viral delivery methods), cell replacement strategies and combination treatments. Unfortunately, agents that modified the disease in the animal model have failed to successfully translate to patients, most likely for a multitude of reasons [113-115]. In an attempt to standardize the translational research conducted in ALS transgenics, a set of international guidelines addressing study design, conduct and interpretation have been established [116, 117]. Recommendations include matching of gender, litter, genetic background, transgene copy number, clinical onset and endpoint criteria. Importantly, studies involving animals treated at pre-symptomatic stages are to be distinguished from those with

direct applications to humans (those involving treatment after symptom onset) [116]. The following paragraphs will highlight some of the more current and/or ongoing trials for ALS with particular emphasis on those that have advanced to later clinical phases.

Although previous growth factor therapies have failed to prolong survival in transgenic ALS mice and patients with ALS (potentially as a result of limitations related to CNS delivery and sustained quality), reduced expression of vascular endothelial growth factor (VEGF) has been shown to cause motor neuron degeneration in mice [118] and systemic delivery delayed onset and death in mutant SOD1 mice [119]. In addition, intracerebroventricular (i.c.v.) administration of recombinant VEGF was able to attenuate disease in the mutant SOD1 rat model of ALS [120]. In this study, VEGF was shown to have direct neuroprotective effects on motor neurons *in vivo* and was able to preserve NMJs after being anterogradely transported following i.c.v. delivery. Safety trials in humans are currently ongoing.

An antisense oligonucleotide (ASO) therapy for patients with *SOD1*-linked familial ALS is also currently in Phase I clinical trials (ClinicalTrials.gov Identifier: NCT01041222). In rodent models, this treatment was shown to double survival following symptom onset [121]. In primates, the potential target cell effect (uptake by microglia) was identified 13 weeks after the ASO was administered. In the human study, an intrathecal infusion using an external pump is being used.

Increasing interest in stem cell-based therapies has developed particularly as the role of non-neuronal cells has become apparent. In theory, replacing these supporting cells may be more feasible than replacing motor neurons. In animal models, the transplantation of glial progenitor cells had beneficial effects [122] although the impact of migroglial cells in this model is still somewhat unclear [123-125]. However, there is currently a Phase I clinical trial to

determine the safety of neural progenitor cell transplantation into the spinal cord as tumor formation was not observed in animal models [126]. Similarly, the differentiation of induced pluripotent stem (iPS) cells to motor neurons using fibroblasts from an 82-year old patient with familial ALS has provided additional possibilities for disease modeling, drug discovery and autologous cell replacement therapies [127].

In a previous Italian study, a marked delay in disease progression (as assessed by a quality of life scale and measures of muscle strength and pulmonary function) was observed in patients with ALS with daily doses of lithium [128]. However, a combination trial of riluzole and lithium was recently terminated early as the dramatic effects observed in this study could not be repeated [129]. However, other drug trials remain promising. Ceftriaxone is currently being evaluated for its effect on human ALS in a multi-center clinical trial sponsored by Massachusetts General Hospital and the National Institute of Neurological Disorders and Stroke (NINDS) after showing promise in mutant SOD1 mice [130]. This antioxidant may increase the expression of excitatory amino acid transporter 2 (EAAT2), the glial glutamate transporter found to be down-regulated or lost in patients with ALS and in rodent models [130].

In addition, KNS-760704 developed by Knopp Neurosciences, Inc. is now moving to Phase III clinical trials after the first dose-dependent effect in patients was observed in Phase II trials that included over 100 subjects (ClinicalTrials.gov Identifiers: NCT00647296 and NCT00931944). This small molecule is the optical enantiomer of pramipexole but has reduced dopamine receptor affinity [131, 132]. This has allowed the dopamine receptor-independent neuroprotective properties of KNS-760704 to be evaluated over a broader dose range with exciting results.

The number of ongoing clinical trials in sporadic ALS is encouraging, and the results of these and other trials will be highly anticipated by scientists, clinicians and especially the patients and families affected by this disease.

1.5 PROTEOMICS AND BIOMARKERS

1.5.1 Proteomics

The field of proteomics has progressed rapidly in recent years as it seeks to identify the functional consequences of disease-related genetic aberrations at the level of the protein. Defined as the study of the total protein complement of an organelle, cell, tissue or entire organism [133], proteomics is a multi-faceted approach used to investigate protein expression and post-translational modification as well as protein interactions, organization and function [134]. Although limitations and technical hurdles have been encountered, proteomic studies have provided unparalleled information with regard to cellular biology and underlying disease mechanisms [134]. Additionally, the application of specific techniques such as two-dimensional (2-D) gel electrophoresis and/or mass spectrometry promises to uncover biomarkers critical to differentiating normal from disease states. In combination with computer-based algorithms, these characteristic biomarkers can, in turn, be used to objectively measure and evaluate normal biological processes, pathological conditions or pharmacologic responses [135]. However it must be stressed that proteomics has yet to achieve these outcomes in validated studies for any pathological condition.

1.5.2 Samples and Techniques for Biomarker Identification

Most proteomics-based research utilizes accessible biological fluids like blood, saliva, synovial fluid or urine. In addition, CSF has been analyzed for proteins to detect diseases ranging from Creutzfeldt-Jakob disease [136] to leptomeningeal metastases in breast cancer patients [137]. For identification of biomarkers of neurodegenerative diseases, it is thought that the use of CSF may better reflect proximal disease-specific molecular events.

In general, the types of techniques used for biomarker discovery can be classified into gel-based, mass spectrometry-based and antibody/protein-based approaches. However, multidimensional fractionation techniques, including various combinations of two dimensional gel electrophoresis (2D-GE), liquid chromatography (LC) and hybrid mass spectrometry are increasingly more common. As our laboratory has utilized predominantly mass spectrometry-based technologies for initial biomarker discovery, this method will be elaborated on below.

Mass spectrometry (MS) is a sensitive and analytical technique used for a variety of applications including identification of unknown compounds and analysis of biological samples. All MS instruments consist of three parts: an ion source which ionizes the sample, a mass analyzer which sorts ions by mass using an electromagnetic field and a detector which measures the time-of-flight and the abundance of each ion present. This technique is based on the principle of sample desorption and ionization with subsequent detection based on mass-to-charge (m/z) ratios.

Two of the ion source techniques commonly for the analysis of liquid and solid biological samples are matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). For MALDI, samples are mixed with an energy-absorbing matrix (EAM) and applied to a metal plate where crystals form once dried. Laser energy is then used to ionize the EAM which

transfers protons to the sample and protects it from the energy of the laser [138]. Once ionized, the samples are desorbed from the plate and in the gas phase prior to being injected into the mass analyzer. Mass analyzer technologies include time-of-flight (TOF), quadrupole (Q), ion trap and combinations of these (i.e., Q-TOF). For the TOF mass analyzer, an electric field is used to accelerate ions through the same potential. The time to reach the detector (or time-of-flight) is then measured. Particles with the same charge (and same kinetic energy) will be identical such that the velocity at which they travel will depend only on their mass. In other words, mass is directly proportional to the speed at which the ions travel with lighter ions reaching the detector first. Ultimately, the detector records when an ion reaches a surface to produce a mass spectrum.

A modified version of MALDI-TOF-MS is surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS). In SELDI-TOF-MS, the solid-phase chromatographic surfaces are modified either chemically or biochemically to retain proteins according to specific properties such as hydrophobicity or charge [139, 140]. Retained proteins are then ionized and detected by TOF-MS and the resulting spectra displayed as peak intensities of various molecular masses ranging from 0-200 kilodaltons (kDa) as with MALDI-TOF-MS. A key feature of these mass spectrometry-based techniques is their ability to provide a rapid protein expression profile from a variety of biological and clinical samples [141]. If used in combination with computer-based algorithms, a panel of potential biomarkers can be selected and used for diagnostic purposes [142].

1.5.3 Value of Biomarkers

In general, biomarkers could be used to accurately detect the presence of disease, provide mechanistic disease information, identify landmarks of disease progression, indicate the

effectiveness and overall response to treatment and allow for comparisons between human and animal models (Figure 11). In addition, protein biomarkers could be generated to predict either prognostic or therapeutic outcomes and allow for customized therapeutic interventions. With discovery efforts that include mass spectrometry and antibody-based arrays like the enzyme-linked immunosorbent assay (ELISA), multiple potential clinical uses for biomarkers have been suggested (Figure 12).

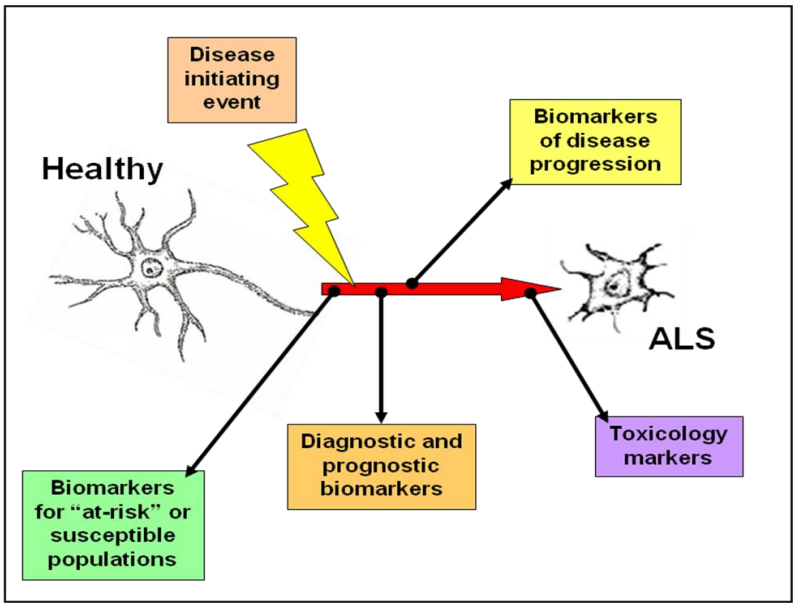


Figure 11. Potential Uses of Biomarkers

The progressive degeneration and subsequent loss of motor neurons that occurs with ALS begins with the initiation of the disease process. As depicted in the schematic, biomarkers may be identified at all stages of the disease process and used for multiple purposes.

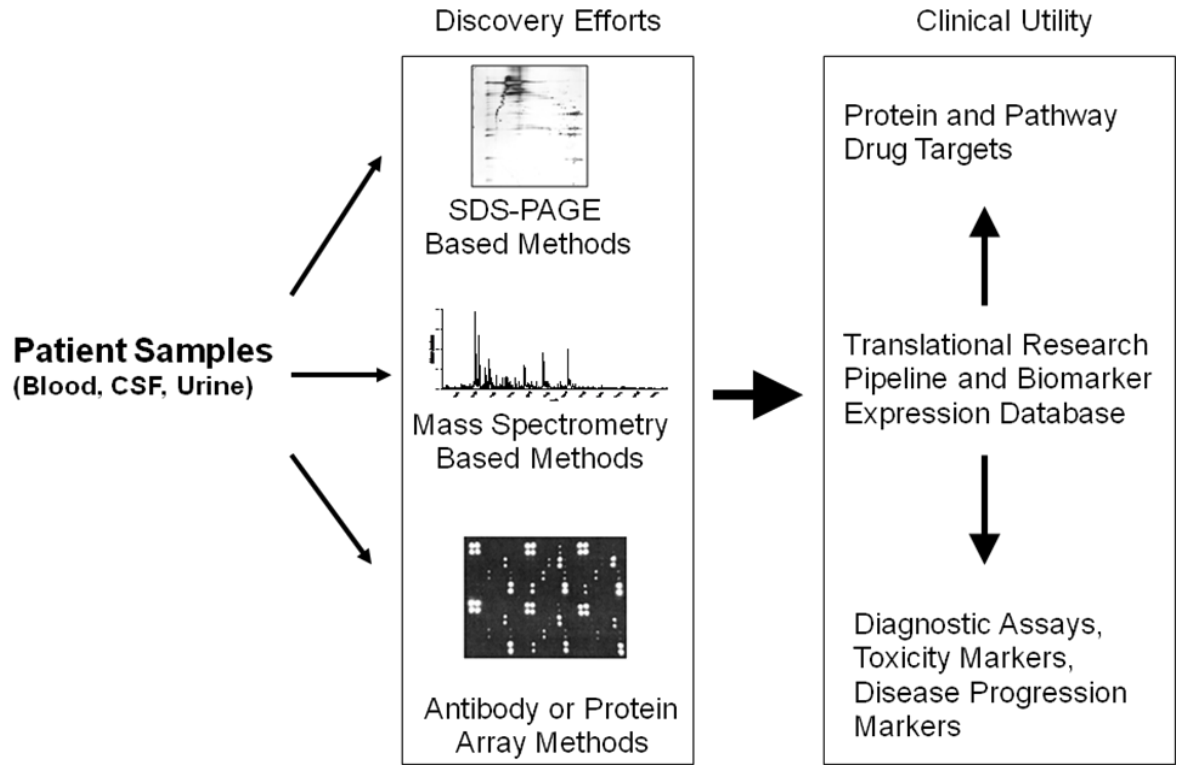


Figure 12. Proteomic Biomarker Discovery Efforts for ALS

Patient samples can be examined by a number of proteomic technologies including sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), mass spectrometry and array profiling. These results are analyzed to uncover putative disease-specific biomarkers which are then validated for measures in diagnostics, toxicity or disease progression. Protein biomarkers are also used to identify proteins or pathways for development as therapeutic targets.

Significant research efforts have been made to identify biomarkers of neurodegenerative diseases such that earlier diagnoses and therapeutic interventions can be made. In the case of ALS, inadequate diagnostic tools and disease complexity cause, on average, a 13-month interval between symptom onset and diagnosis [143]. Currently, the clinical diagnosis of probable ALS is made only when both upper and lower motor signs are present [32-34]. However, UMN involvement can be missed when LMN involvement is severe, thereby prolonging the pre-diagnosis testing period and increasing a patient's period of uncertainty regarding diagnosis.

More sensitive and objective diagnostic methods are indeed necessary, particularly to improve detection of UMN involvement.

In the context of drug discovery, disease-specific biomarkers and/or biomarker panels have become an attractive option for identifying therapeutic targets for which specific agents could be designed. This is of particular interest in the treatment of ALS where current drug treatment options are limited and impart minimal life-prolonging benefits.

Protein biomarkers may also help define subtypes of ALS or determine which patients will more rapidly progress. Specifically, the use of protein biomarkers may enable researchers to link specific pathogenic mechanisms to either bulbar or limb onset ALS. In addition, proteomic profiling has the potential to greatly impact the field of toxicology by identifying biomarkers that can be used as measures of toxicity related to a particular drug or chemical. Protein profiling to uncover "safety biomarkers" related to drug-induced toxicity [144, 145], called toxicoproteomics [146], provides the opportunity to rapidly assess risk/hazard affects of new drugs or chemicals and could enable drug companies or academic investigators to make quick decisions with regard to drug development activities.

Finally, protein biomarkers may be useful to bridge model systems and provide translational biomarkers to assess efficacy or toxicity in animal models [147]. This would be quite valuable for a neurologic disease such as ALS where current drugs successful in slowing or delaying disease during preclinical trials in the SOD1 transgenic mouse model for ALS have typically failed when tried in humans. While it remains unclear how accurately the rodent models for ALS mimic the human disease, protein biomarkers common to both the rodent model and human disease would be quite valuable and informative in identifying key biochemical pathways to target for drug screens. Drugs that modulate protein biomarkers common to animal

models and human ALS patients that are demonstrated to be effective in the animal model would have increased justification for use in human clinical trials. The use of protein biomarkers that bridge the model systems may reduce the time from bench to bedside for effective treatments. Targeted proteomic studies that examine protein levels of specific proteins in control and disease states have led to the generation of ELISA-based diagnostic assays for a number of conditions. There are currently no validated and FDA-approved diagnostic tests using newer proteomic technologies like mass spectrometry, suggesting that while these technologies hold great promise there is still much work remaining to translate the findings to the clinic. Other proteomic methodologies such as protein arrays or antibody arrays promise to uncover pathogenic mechanisms in neurologic diseases as well.

1.5.4 Important Considerations in Biomarker Discovery

Improvements in diagnosis, monitoring of disease progression and evaluation of drug efficacy or toxicity are needed for ALS. Similarly, markers enabling patient stratification and treatment selection remain unmet medical needs, prompting many to turn to biomarker discovery. Although the proteomics field has exploded in recent years, few, if any, of the potential biomarkers identified have held up to subsequent validation studies. Although unfortunate, these failures indicate that efforts must be made to reduce both pre-analytical and analytical variables as much as possible.

Pre-analytical variables including patient health and diet, time of day, fasting, position during sample collection, collection tubes, temperatures, centrifugation times and temperatures, shipping and storage times and processing times, temperatures and conditions can all affect the results of proteomic analyses [148]. Therefore, strict adherence to stringent protocols is required

to minimize sample-to-sample variability. Prospectively collected samples are also required as banked samples have most likely been collected or stored inconsistently, or their viability has been compromised due to long storage periods. For instance, Carrette and colleagues found that cystatin C in the CSF undergoes cleavage at its amino terminus during long-term storage at -20^{o}C but not when stored at -80^{o}C [148]. As noted by these authors, this "artifactual, storage-related truncation" could erroneously be considered of biological significance if it is not carefully assessed prior to additional testing [148].

Similarly, experimental design is critical in that correctly collected samples from the proper patient cohorts (ALS, healthy controls, neurologic disease controls including diseases with symptoms that mimic ALS) are utilized within a well-characterized analytical system. Although seemingly obvious, proper controls, matched to case samples in sufficient numbers, must be included to reduce chance and overfitting and to provide statistical significance. In addition, there must be sufficient power within the experimental design to identify true positives and eliminate false positives. Finally, randomization along with other methods can and should be used to further reduce bias during data analysis [149].

It is also critical to properly validate any putative biomarkers using a distinct patient cohort and multiple methodologies. In these validation studies, patients with clinical features similar to those observed with the disease of interest must be included. For example, when diagnosing ALS patients the biomarker must appropriately distinguish individuals with ALS from those with multiple sclerosis, Kennedy's syndrome or multifocal motor neuropathy. A higher level of disease specificity (>95%) may be required to warrant use or approval of the biomarker in the clinical setting. As indicated in Figure 13, mass spectrometry-based diagnostics may be capable of such patient classification/differentiation. CSF mass spectra between 4,500 –

15,000 Da are shown for individual healthy control, MS and ALS subjects. Specific peak patterns are common or unique amongst each of the subject types that upon proper validation may lead to biomarker proteins. It may also be necessary to combine results from proteomic, genomic and metabolomic studies to uncover pathways relevant to disease. This systems biology approach is only beginning to be applied to various human diseases but may ultimately provide the best means to uncover biomarkers of pathogenic disease mechanisms.

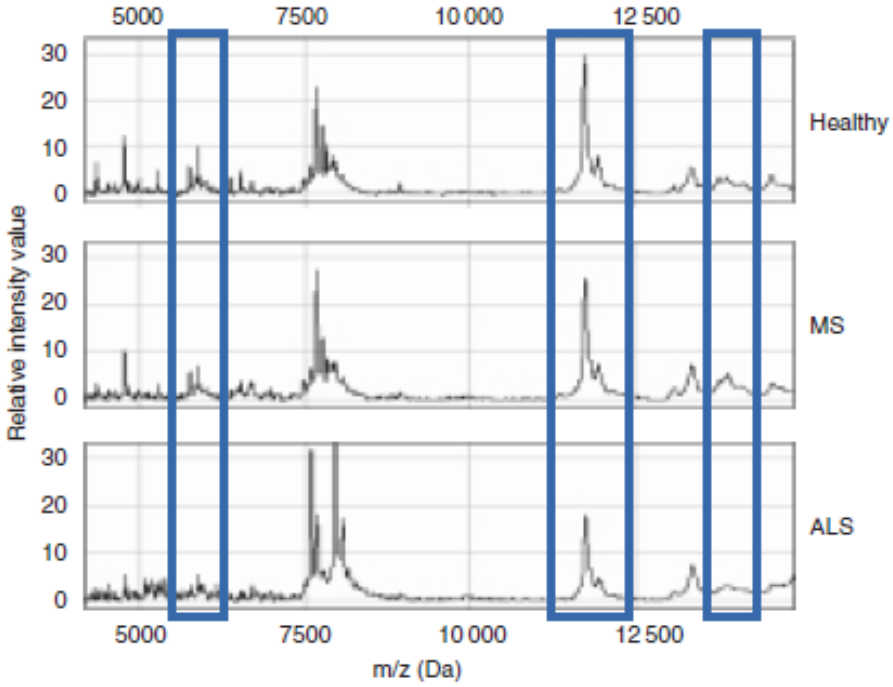


Figure 13. Deciphering ALS from Healthy and Disease Controls by Mass Spectrometry

Mass spectrometry of CSF isolated from healthy control (top), multiple sclerosis (MS) (middle) and ALS (bottom) subjects. Representative spectra from 4500-15000 Daltons (Da) are shown. Boxed areas highlight differences in peak pattern and intensity observed in these patient populations. Mass (m/z) peak alterations enable ALS patients to be distinguished from those with MS or healthy controls using various computer algorithms.

Finally, while many of the recent SDS-PAGE proteomic studies have pre-fractionated the biologic samples prior to proteomic analysis, the mass spectrometry-based proteomic studies for ALS have yet to pre-fractionate samples. In turn, most of the potential protein biomarkers identified are abundant proteins, and the analysis of low abundance proteins that may likely yield the best potential for disease-specific biomarkers is hindered. Future proteomic studies should focus efforts on pre-fractionated samples in order to delve more deeply into the proteome.

Regardless of the experimental approach used to identify putative biomarkers for ALS, an understanding of their pathological relevance will help identify and characterize disease mechanisms. Ideally, proteins that function in the initiation of disease or in early pathologic events will be revealed along with factors that increase disease susceptibility. In addition, protein identification and characterization is necessary for the development of independent validation assays and ultimate regulatory approval as a diagnostic tool.

2.0 EVALUATION OF TRANSTHYRETIN AS A BIOMARKER OF ALS

2.1 ABSTRACT

Currently, objective molecular markers that allow for the diagnosis and monitoring of disease progression are lacking for ALS. Our laboratory previously identified transthyretin (TTR) as a candidate protein biomarker for ALS and observed decreased levels of TTR in the CSF of ALS patients when compared with healthy and other neurological disease controls [150]. To further characterize the role of TTR and its utility as a biomarker, the frequency of TTR genetic polymorphisms and post-translational protein modifications in the ALS population were examined using MALDI-TOF-MS analysis of TTR obtained from the CSF of ALS and control subjects. In addition, enzyme-linked immunosorbent assays (ELISA) specific for total TTR were used to independently measure protein levels. Preliminary results regarding genetic polymorphisms combined with data from single nucleotide polymorphism (SNP) studies conducted by other groups indicated that genetic polymorphisms were not a factor in the ALS population. However, alterations in specific post-translationally modified forms of the TTR protein were observed. In particular, oxidative modifications at the sole cysteine residue were altered. No significant change in TTR protein level was observed over the course of the disease suggesting that altered post-translational protein modifications as opposed to total protein levels are important. Temperature-dependent changes to these protein modifications were observed; as

these modifications are critical for stabilization of the tetrameric forms of TTR, preliminary studies to determine the impact of these alterations on protein aggregation were performed. My results indicate that post-translational modifications and high molecular weight species of TTR are important factors in ALS pathology with further characterization required to determine the biological significance of each.

2.2 INTRODUCTION

2.2.1 Identification of TTR

Our laboratory used SELDI-TOF-MS to identify a panel of protein biomarkers in the CSF of ALS patients. Using CSF from a total of 52 ALS and control subjects along with a machine learning algorithm, a panel of predictive biomarkers was created and applied to a coded set of CSF samples for diagnostic predictions. Three measures were used to assess the diagnostic utility of this biomarker panel including: 1.) the proportion of correctly predicted ALS cases out of all ALS cases (sensitivity), 2.) the proportion of correctly predicted control cases out of all control cases (specificity) and 3.) the proportion of correctly predicted cases out of all cases (accuracy). Using these parameters, analysis of the small test set provided 80% sensitivity, 100% specificity and 91% overall accuracy in predicting ALS [150]. Importantly, the 19 protein mass peaks included in the panel best predicted ALS in patients near the time of symptom onset. Subsequent studies have focused on these putative biomarkers in an attempt to maximize the chance of finding proteins relevant to disease pathogenesis (as opposed to those that are simply a consequence of the disease process or end-stage effects). Further characterization of the

biomarker peaks using peptide mass fingerprinting or direct peptide sequencing with tandem mass spectrometry identified transthyretin (TTR) as one of the candidate biomarkers. TTR was found to be decreased in the CSF of ALS patients when compared to healthy and other neurological disease controls [150].

2.2.2 Biological Functions

TTR is a tetrameric protein composed of identical 13.7 kilodalton (kDa) subunits each 127 amino acids long [151]. It is secreted into the CSF and plasma by epithelial cells of the choroid plexus and liver cells, respectively [152], but it is also expressed by neurons and motor neurons [153, 154]. Both the expression and synthesis of TTR are independently regulated in the blood and CSF [155] although TTR from the CSF is catabolized by the liver, muscle and skin following reabsorption by arachnoid villi [156]. While TTR comprises only a small percentage of plasma, it is one of the most abundant proteins in the CSF comprising 12% of the total protein synthesized by the choroid plexus [152, 157].

Within the CNS, TTR is involved in many important biological processes with roles in regulating thyroid function and modulating immune response [153, 158]. In addition, TTR functions as a protein chaperone with the ability to sequester proteins and prevent amyloid formation by binding to proteins such as the amyloid β peptide (Aβ) [159]. TTR also regulates retinoic acid signaling, a pathway critical for neural development/specification and neural plasticity and regeneration [160, 161]. Through its interaction with retinol-binding protein (RBP), TTR impacts vitamin A metabolism and prevents glomerular filtration of the low-molecular-weight RBP from the kidneys [162]. Decreased TTR protein in the CSF may directly impact the amount of retinol available to cells of the CNS and therefore affect signaling and

regenerative capacities during neurologic injury or disease. Additional work in a transgenic mouse model of Alzheimer's disease (AD) demonstrated a lack of neurodegeneration in animals with increased levels of the TTR transcript in the hippocampus [154] suggesting that TTR may also be neuroprotective although this function is not fully understood.

2.2.3 Links to Disease

Mutations in the TTR gene have been identified as causal for multiple pathological conditions including TTR-related amyloidosis (ATTR), a disorder characterized by the extracellular systemic deposition of mutated or wild-type TTR as amyloid fibrils [163, 164]. Similarly, genetic variants of the TTR gene have been shown to induce familial amyloidosis in both cardiac and neural tissues [165, 166]. In these amyloidoses, pathological deposits composed of TTR β-structured fibrils accumulate and cause organ dysfunction and death [167]. Recently, the formation of these TTR fibrils was correlated both *in vitro* and in a cell culture system to the loss of TTR quaternary structure [168, 169], suggesting that tetramer disassembly may promote protein aggregation and subsequent fibril formation [170].

2.2.4 Post-translational Protein Modifications

Various post-translational protein modifications of TTR are known including sulfation, glutathionylation, phosphorylation, cysteinylation and cysteinylglycine addition. Covalent adducts of TTR with sulfite (TTR-SO_3H), cysteine (TTR-Cys), cysteinylglycine (TTR-CysGly) or glutathione (TTR-Glutathione) involve the thiol group of the sole cysteine residue of TTR

(Cys^{10}) and are oxidative post-translational modifications of TTR [171, 172]. This cysteine residue plays a key role in the stabilization of the tetrameric form of TTR [173].

In the generally accepted model of TTR fibrillogenesis, tetrameric TTR dissociates into monomers which in turn undergo subtle conformational changes that lead to protein aggregation, fibril formation and cytotoxicity [170, 174]. Therefore, altered post-translational modifications at the Cys^{10} residue would be expected to compromise the function of TTR. This has been observed in familial amyloid polyneuropathy (FAP) where the level of Cys adducts with the mutant TTR chain was significantly higher in the plasma of symptomatic patients when compared to the wild-type protein [171]. In patients with AD, oxidized forms of TTR (i.e., TTR-Cys and TTR-CysGly) were significantly less abundant [156]. Differential modification at this residue has also been shown to alter the ability of TTR to prevent Aβ aggregation and toxicity in mouse models of AD [175].

2.2.5 Study Objectives

The goals of this study were to determine if individuals with ALS have a higher incidence of TTR genetic variants that alter protein function and thus constitute a novel susceptibility factor for the disease. Concurrently, differential post-translational protein modifications of TTR were also investigated in the ALS population. Importantly, previous studies by other laboratories have successfully utilized mass spectrometry to identify genetic polymorphisms in the TTR gene [176, 177] and to investigate differential post-translational modifications of the protein [156, 178].

2.3 MATERIALS AND METHODS

2.3.1 Collection and Storage of Cerebrospinal Fluid

Patient samples were obtained from the ALS/Multidisciplinary and Muscular Dystrophy Association Clinics at the University of Pittsburgh Medical Center (UPMC). ALS subjects included in this study were clinically diagnosed using the revised El Escorial criteria [179, 180], and the age range for all individuals was 20-80 years old. All subjects were evaluated using accepted diagnostic principles and standards.

CSF was obtained following IRB-approved patient consent. CSF was collected through lumbar puncture and centrifuged immediately after collection at 1000 rpm (450*g*) for 5 minutes at 4^{o}C. Processed CSF was stored at -80^{o}C until needed or as outlined in Table 4 to determine the effects of storage conditions. Mass spectrometry was used to confirm the lack of blood contamination within each CSF sample obtained.

2.3.2 ELISA

Each 96-well Immulon 4HBX ELISA plate was coated with 100 μL of 1:1000 capture antibody (rabbit anti-human TTR, Dako, Carpinteria, CA) in phosphate buffered saline (PBS) overnight at 4^{o}C. After washing, plates were blocked using Super Block (ScyTek Laboratories, Logan, Utah) with 0.05% Tween-20 for 30 minutes. After further washing, optimized dilutions of protein standards and CSF samples were added and allowed to bind for 1 hour with gentle shaking at room temperature. The plates were then washed again and 100 μL of 1:10000 detection antibody (horseradish peroxidase-conjugated goat anti-human TTR, EY Laboratories, Inc., San

Mateo, CA) in PBS with 0.1% Tween-20 was added to each well and allowed to bind for 1 hour with gentle shaking at room temperature. Detection was performed with 3,3', 5,5''-tetramethylbenzidine (TMB, Sigma, St. Louis, MO) and stopped with 0.1 M hydrochloric acid (HCl). Plates were read in a μQuant micro-plate reader (BioTek Instruments, Inc., Winooski, VT) at 450 nm.

2.3.3 Identification of Genetic Polymorphisms

CSF from a total of 32 different patients (27 ALS, 5 healthy controls) was used to determine the frequency of genetic variants. TTR was immunoprecipitated from human CSF with rabbit anti-human TTR antibody (Dako) overnight at 4^{o}C using antibody-coated Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). Disulfides were reduced with 100 mmol/L tris(2-carboxyethyl)phosphine (TCEP) for 20 minutes at 55^{o}C prior to analysis with mass spectrometry. TCEP treatment was used to resolve protein mass peaks and further enable mass shifts caused by amino acid substitutions to be identified. Wild-type TTR appears as a single spectral peak, but mass differences (typically between +/- 6-40 Daltons) are observed when an amino acid substitution occurs as a result of a polymorphism in the TTR gene.

2.3.4 Detection of Post-Translational Protein Modifications

For desalting with PepClean C18 Columns (Thermo Fisher Scientific, Rockford, IL) the solid-phase material was activated using multiple washes with acetonitrile (ACN)/water (1:1) and then equilibrated in buffer containing 0.5% trifluoroacetic acid (TFA)/5% ACN. CSF samples (20 μL) were acidified by addition of 0.5% TFA/10% ACN (20 μL) prior to loading onto the

column. Columns were washed multiple times with 0.5% TFA/5% ACN with an additional spin at 1500*g* for 1 minute to ensure dryness. Samples were eluted from the columns with 0.5% TFA/70% ACN. The eluate was then dried in a Speed Vac on medium heat for approximately 30 minutes and resuspended in 0.5% TFA/50% ACN. Protein modifications were analyzed and quantified by determining the area under the curve (AUC) of spectral peaks [181] and the amplitude for each mass-to-charge (m/z) position [182] and the level of each post-translational modification compared in ALS and control subjects.

2.3.5 MALDI-TOF-MS Analysis

Bovine β-lactoglobulin A and bovine ubiquitin were purchased from Ciphergen and used as molecular mass calibrators. Both were dissolved in 0.1% TFA in deionized water. The MALDI matrix used was a saturated solution of 3,5-dimethoxy-4-hydroxy-cinnamic acid (sinapinic acid) purchased from Fluka in 50% ACN and 0.5% TFA. All MALDI analyses were performed using a Voyager-DE PRO MALDI-TOF mass spectrometer (Applied Biosystems, Carlsbad, CA). Samples were irradiated with a nitrogen laser, and the ion source and flight tube evacuated by turbo pumps to a pressure lower than 6×10^{-7} mbar. Instrument parameters were tuned to obtain the highest resolution and sensitivity in the mass range 12000-14000 Daltons (Da). All spectra were acquired in the linear mode, and the laser power was modulated between 10 and 30% to obtain similar ion counts for each sample.

2.3.6 Immunoblotting

CSF from ALS patients and healthy controls was analyzed on 12% gels by non-denaturing gel electrophoresis. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (PerkinElmer, Boston, MA) and blocked with 5% non-fat milk/Tris-buffered saline with Tween-20 (TBST). Blots were probed with primary antibody overnight at 4^{o}C in 5% milk/TBST and then washed with TBST. Goat anti-rabbit horseradish peroxidase-conjugated secondary antibody was added for 2-3 hours at room temperature after which blots were washed with TBST. The final reaction products were visualized using enhanced chemiluminescence (PerkinElmer).

2.3.7 Statistical Analysis

Comparisons between any two groups of data were accomplished using the unpaired t test at the 95% confidence interval. A p value ≤ 0.05 was considered statistically significant.

2.4 RESULTS

2.4.1 Genetic variations can be detected via mass spectrometry

Initially, I demonstrated the ability to reduce immunoprecipitated TTR from the CSF. In the presence of TCEP, the multiple peaks associated with the modified forms of TTR were reduced to one predominant peak (Figure 14) indicative of free (unmodified) TTR.

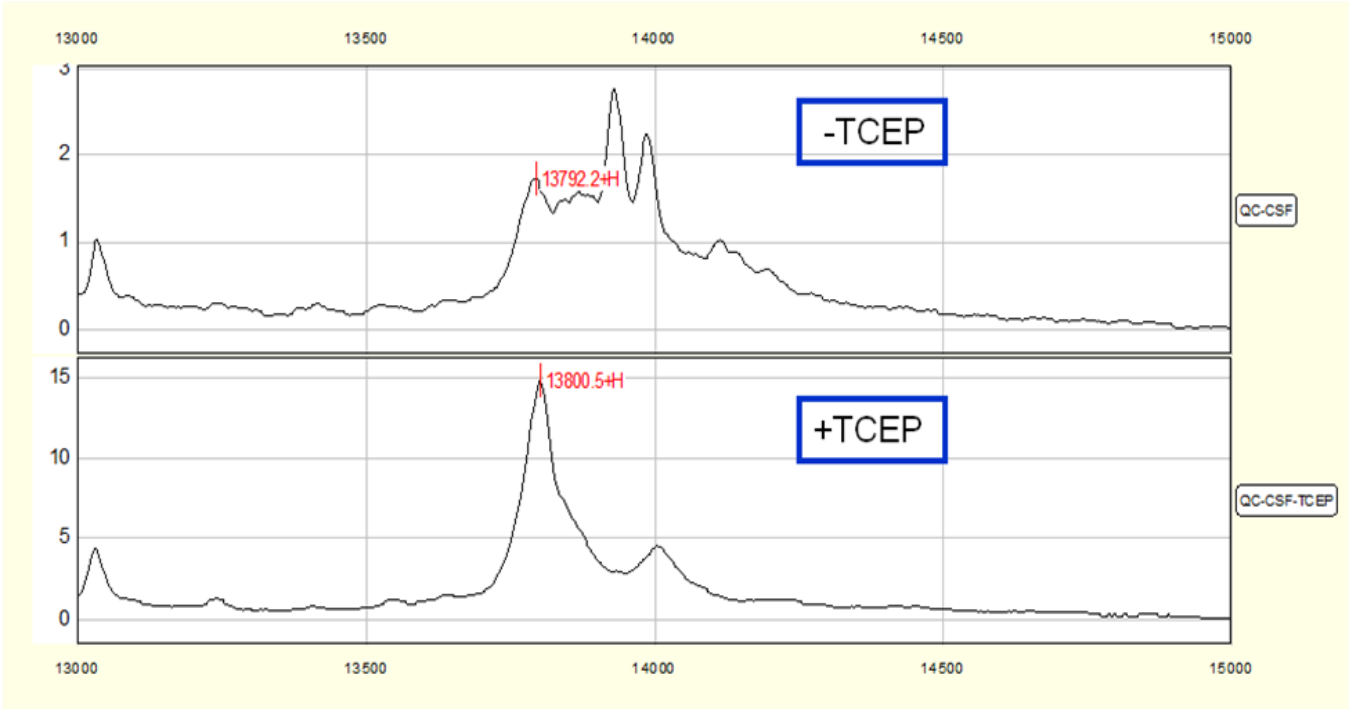


Figure 14. Reduction of TTR Protein Modifications with TCEP

TTR was immunoprecipitated from a pooled CSF sample (QC-CSF) and analyzed via MALDI-TOF-MS. The top panel represents the mass spectral pattern without TCEP treatment while the bottom panel represents the mass spectral pattern following TCEP treatment for 20 min at 55°C.

TCEP-reduced TTR immunoprecipitated from the CSF from ALS patients and healthy controls was then analyzed to determine the frequency of a "split peak" pattern indicative of genetic variants of free TTR. For these studies, both ALS patients and controls were analyzed and example spectra are provided (Figure 15). Of the 32 CSF samples analyzed, only 4 (3 ALS, 1 healthy control) exhibited a "split peak" pattern with a corresponding mass shift of approximately +30 Da. The remaining 28 CSF samples (24 ALS, 4 healthy controls) exhibited only one peak indicative of free TTR. Importantly, samples from the same patient obtained at different points in the disease course were consistent in the presence or absence of the additional TTR peak.

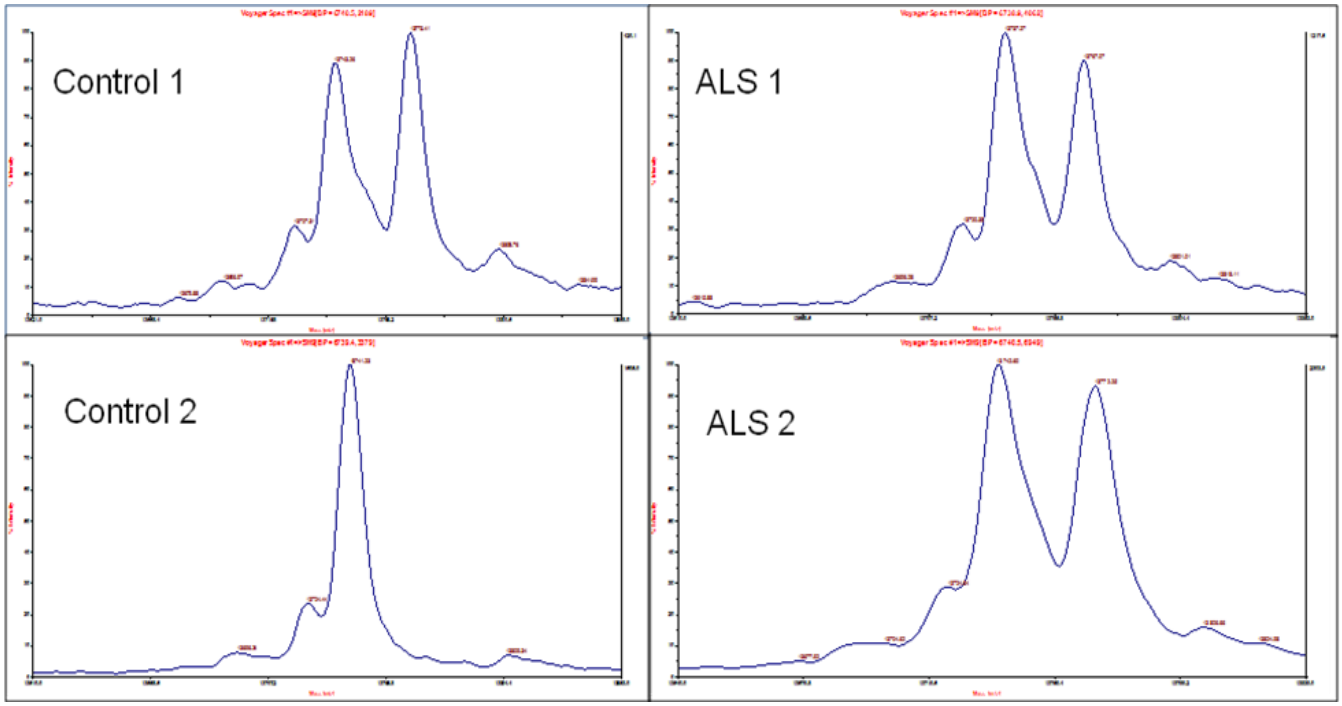


Figure 15. TTR Spectra Following TCEP-Reduction

TTR from the CSF of healthy controls and ALS patients was immunoprecipitated and reduced with TCEP prior to MALDI-TOF-MS analysis. Resulting spectra were examined for those with two peaks indicating the presence of genetic variations in the TTR gene which were observed in both healthy controls and ALS patients.

2.4.2 Total TTR levels do not change over the course of the disease

To validate the decreased levels of TTR observed via mass spectrometry, ELISA specific for total TTR protein was performed using longitudinal CSF samples from healthy controls (n = 8) and ALS patients (n = 8) obtained over an approximately two-year time period (initial and two-year time points from healthy controls; CSF from ALS subjects was collected every 4-6 months for the two-year time period). Over the course of the disease, the level of total TTR did not change significantly for either group (Figure 16).

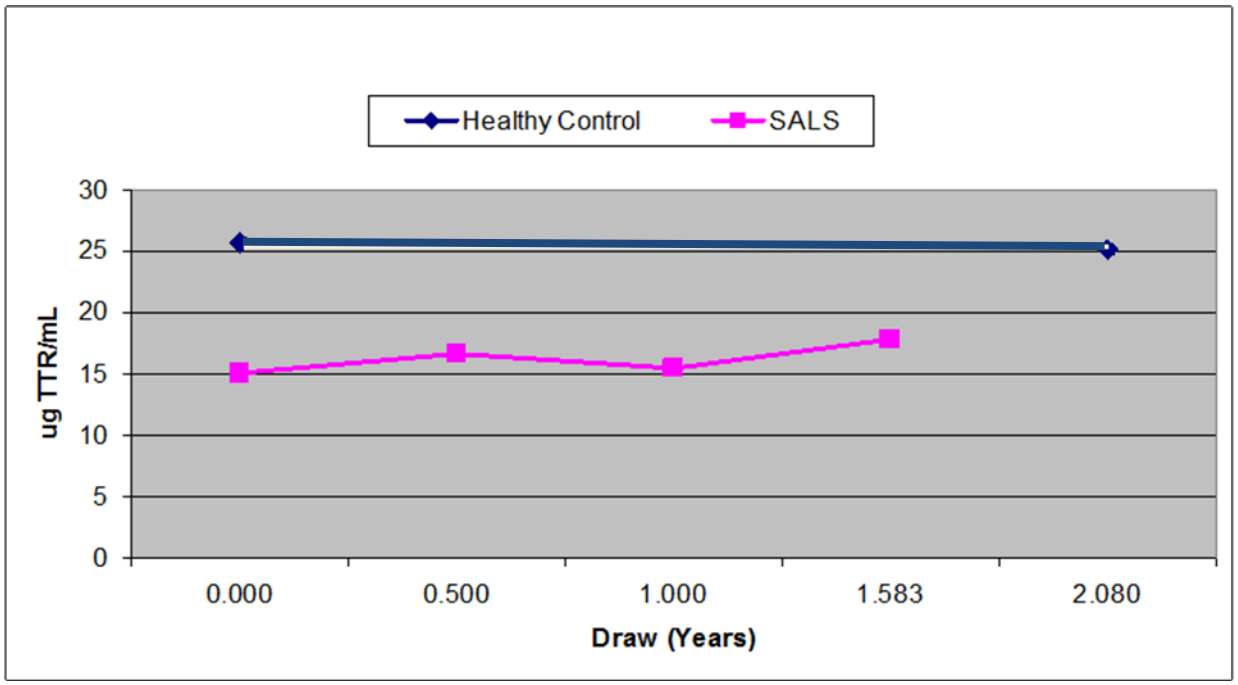


Figure 16. Overall Levels of TTR Do Not Change Over the Course of the Disease

Longitudinal analysis of CSF from ALS patients (n = 8) and healthy controls (n = 8) shows similar levels of TTR protein over time. A representative healthy control (top line) and ALS patient (bottom line) are shown. Similar results were obtained when plasma was analyzed in these patients.

2.4.3 Oxidative modifications of TTR can be detected via mass spectrometry and are altered with different storage conditions

We next examined TTR immunoprecipitated from human CSF to determine if altered protein processing occurs. Although the spectral patterns observed in the absence of reducing agent indicated that protein modifications could be detected, we confirmed the presence of additional TTR peaks indicative of post-translational modifications in the CSF and observed alterations in

the pattern of these peaks when CSF from ALS patients and healthy controls was compared (Figure 17).

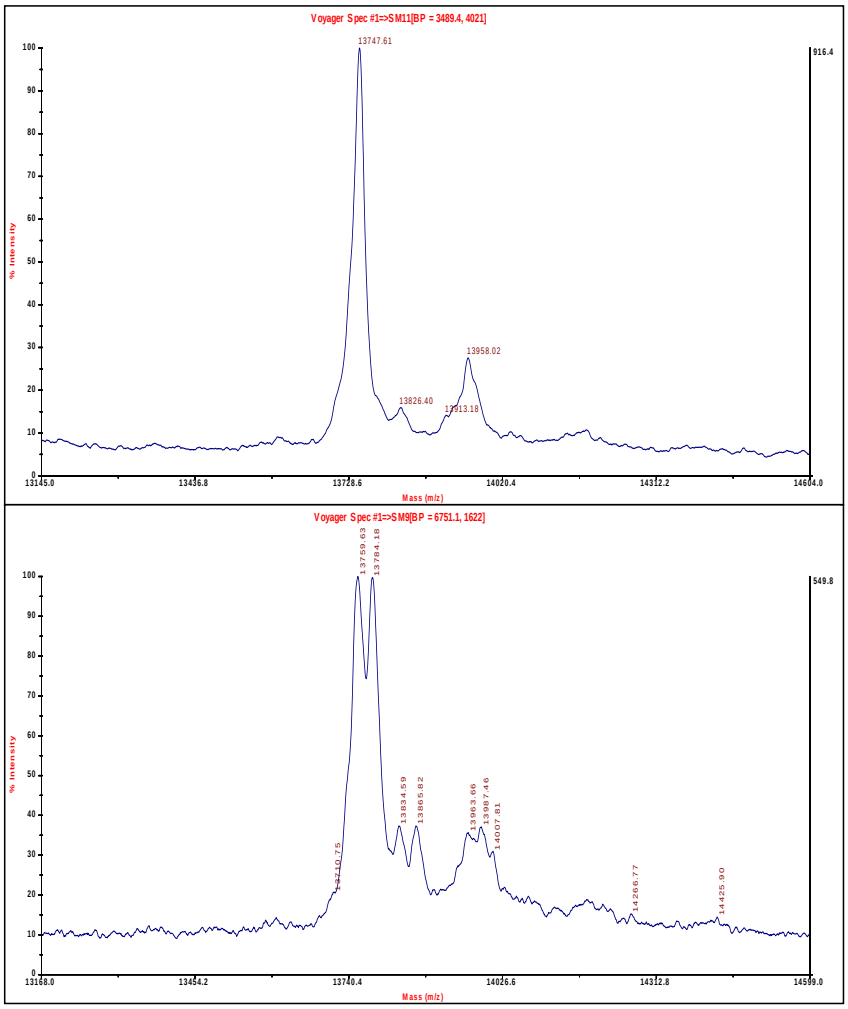


Figure 17. Post-translational Modifications of TTR are Detectable via Mass Spectrometry

MALDI-TOF-MS analysis indicates that peaks corresponding to the modified forms of TTR are altered in ALS patients. Representative spectrograms from a healthy control (top) and ALS patient (bottom) are provided.

PepClean C18 columns were then utilized to remove salt from the CSF prior to MALDI-TOF-MS analysis. Based on initial observations, we sought to further characterize and then compare the post-translational protein modifications of TTR that occur in ALS patients including sulfation, phosphorylation, cysteinylation, cysteinylglycine addition and glutathionylation. With

the exception of phosphorylation, these additions occur at the single cysteine residue and often comprise the majority of species found in circulation [176]. These studies indicated that these modified forms of the protein could be detected via mass spectrometry and that specific oxidative modifications were decreased in ALS patients when compared to healthy controls (Figure 18).

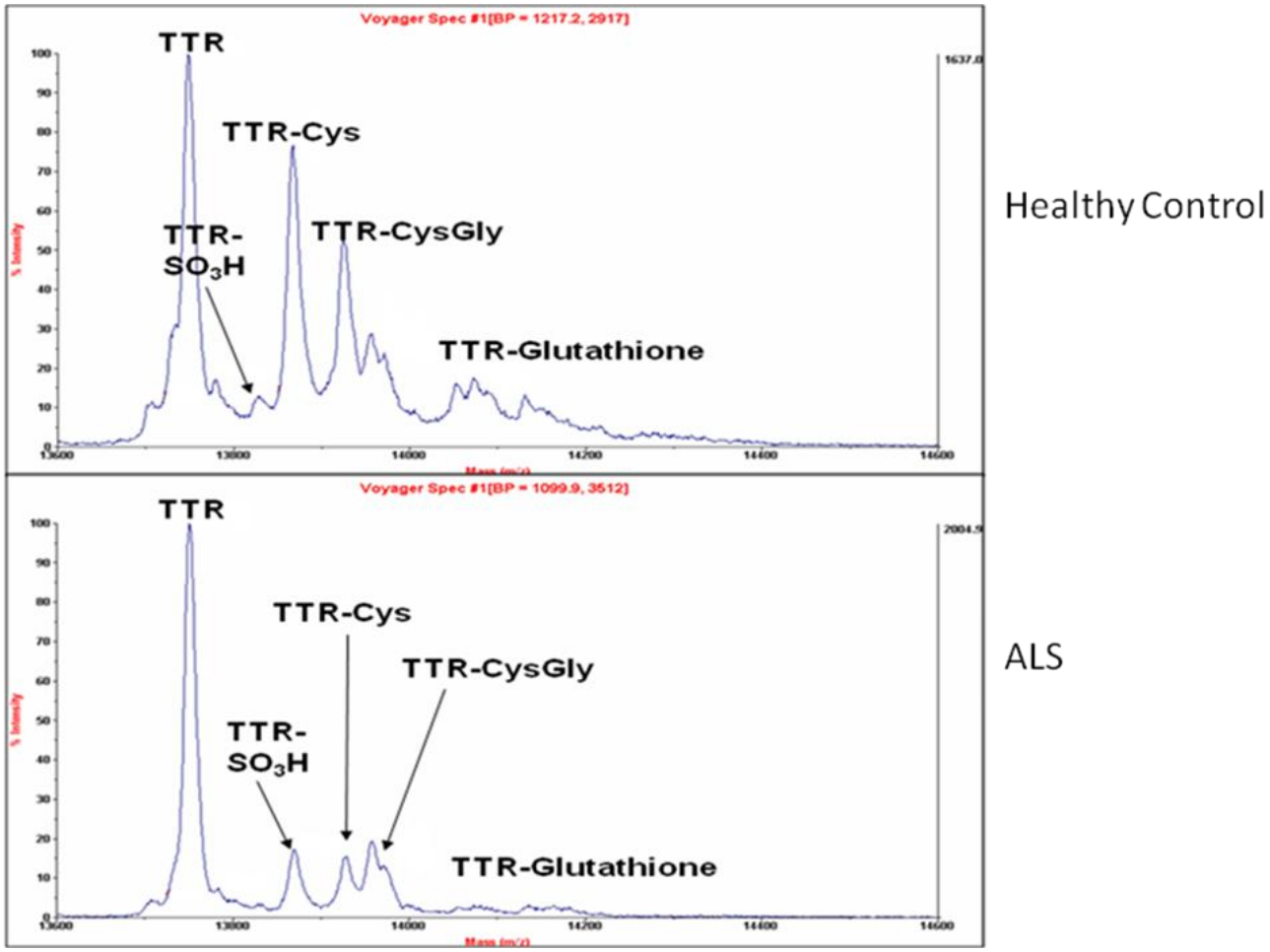


Figure 18. Oxidative Post-translational Protein Modifications are Altered in ALS Patients

MALDI-TOF-MS analysis of CSF from a healthy control (top) and ALS subject (bottom) are shown. Reduced levels of specific oxidative post-translational protein modifications (i.e., sulfonated and cysteinylated TTR) can be observed in patients with ALS.

Subsequent analyses to quantify these differences using a much larger sample set and longitudinal CSF samples indicated that modified forms of TTR were altered depending on the temperature at which they were stored prior to analysis. In an attempt to determine which modifications were reflective of fresh CSF samples, a variety of storage conditions were compared (outlined in Table 4) to fresh CSF and CSF snap frozen in liquid nitrogen. The results of these experiments are shown in Figures 19 and 20.

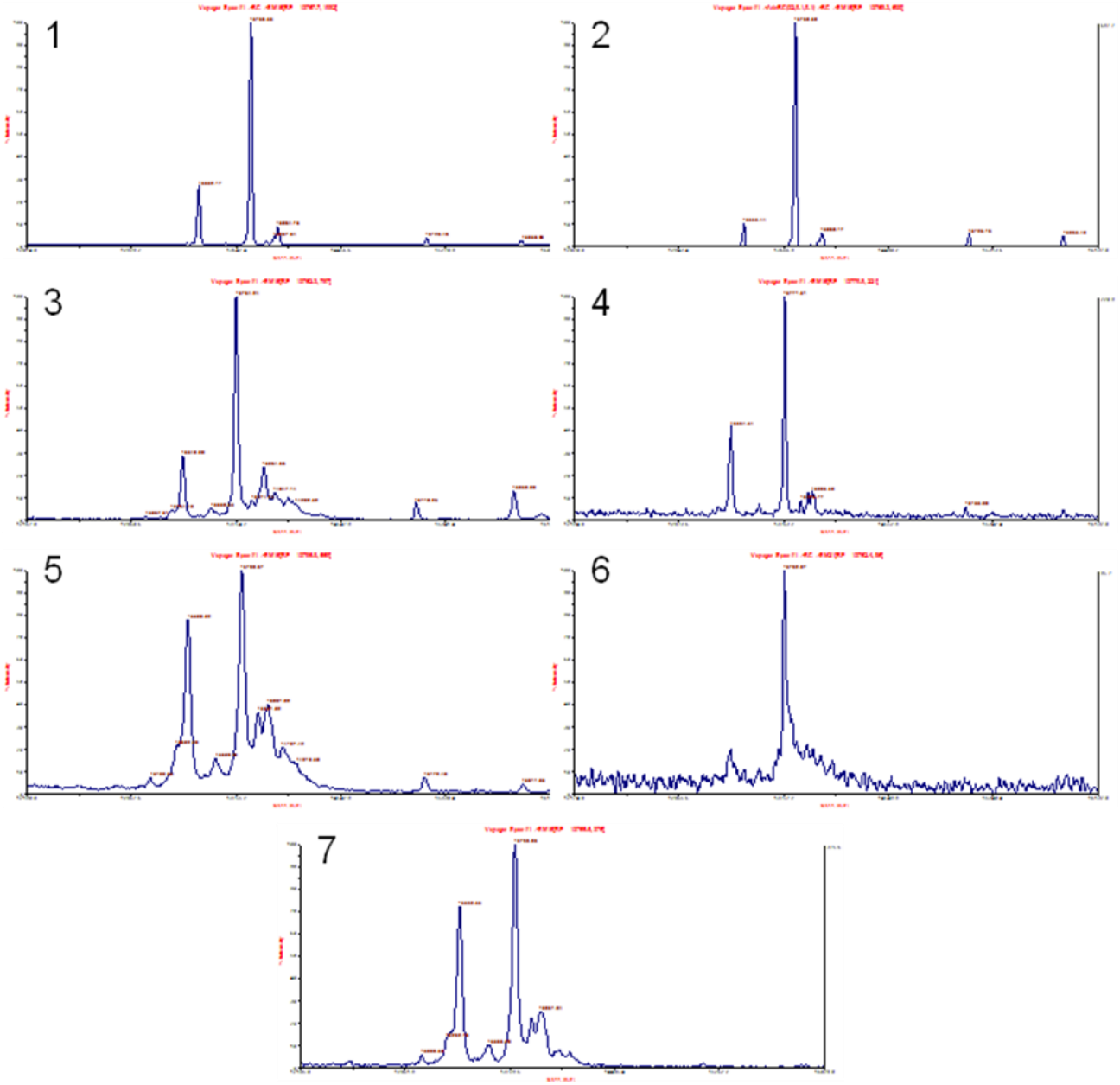


Figure 19. MALDI-TOF-MS of CSF Stored at Various Temperatures

CSF samples were desalted and then analyzed via MALDI-TOF-MS to determine the effects of storage conditions on modified forms of the protein. Only spectra from samples stored at -80^{o}C initially were obtained as this is standard processing. Differences in peak patterns were observed when storage conditions varied. Numbers correspond to the samples listed in Table 4.

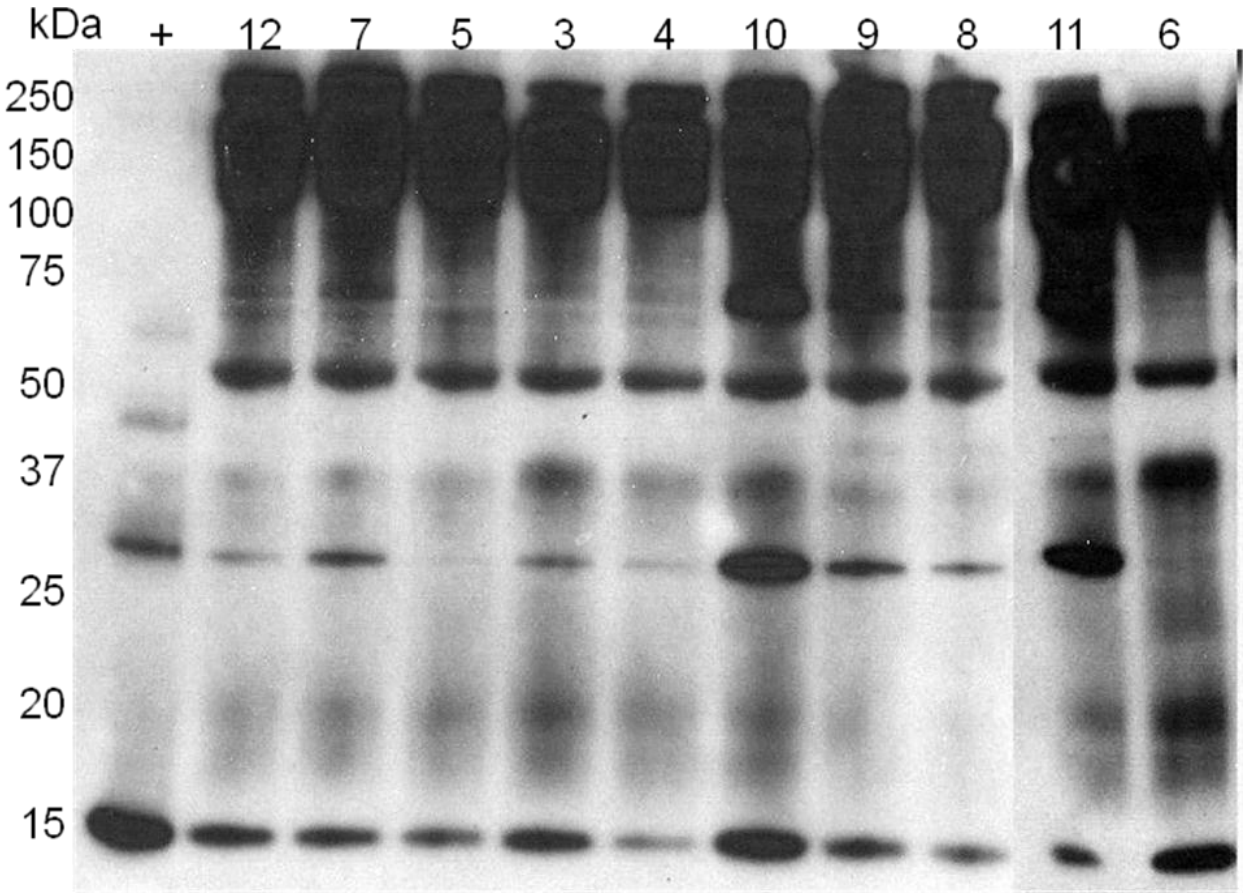


Figure 20. Immunoblot Analysis of CSF Stored at Different Temperatures

CSF samples were analyzed using non-denaturing gel electrophoresis to determine if storage temperature and conditions affected the high molecular weight bands immunoreactive for TTR. The intensity of many bands is altered. Numbers correspond to the samples listed in Table 4 and "+" denotes purified TTR.

Table 4. Storage Conditions Utilized to Determine the Relationship of Temperature to Protein Modifications

Sample Number	Storage Conditions Prior to Analysis
1	Fresh (unfrozen)
2	Snap frozen in liquid nitrogen
3	-80^{o}C only
4	-80^{o}C followed by -20^{o}C for 24 hours
5	-80^{o}C followed by -20^{o}C for 72 hours
6	-80^{o}C followed by a thaw at room temperature with -80^{o}C storage for 4 days
7	-80^{o}C followed by a thaw at room temperature with -20^{o}C storage for 4 days
8	-20^{o}C only
9	-20^{o}C followed by -80^{o}C for 24 hours
10	-20^{o}C followed by -80^{o}C for 72 hours
11	-20^{o}C followed by a thaw at room temperature with -20^{o}C storage for 4 days
12	-20^{o}C followed by a thaw at room temperature with -80^{o}C storage for 4 days

2.4.4 High molecular weight forms of TTR are present in the CSF of ALS patients

Finally, we sought to further characterize a TTR-immunoreactive species that did not enter the gel (Figure 21A) and was present in ALS samples by performing non-denaturing gel electrophoresis using CSF from ALS and healthy control subjects. Our preliminary analyses indicate that levels of this high molecular weight TTR species are increased in ALS patients and that particular high molecular weight bands are present only in ALS (Figure 21B).

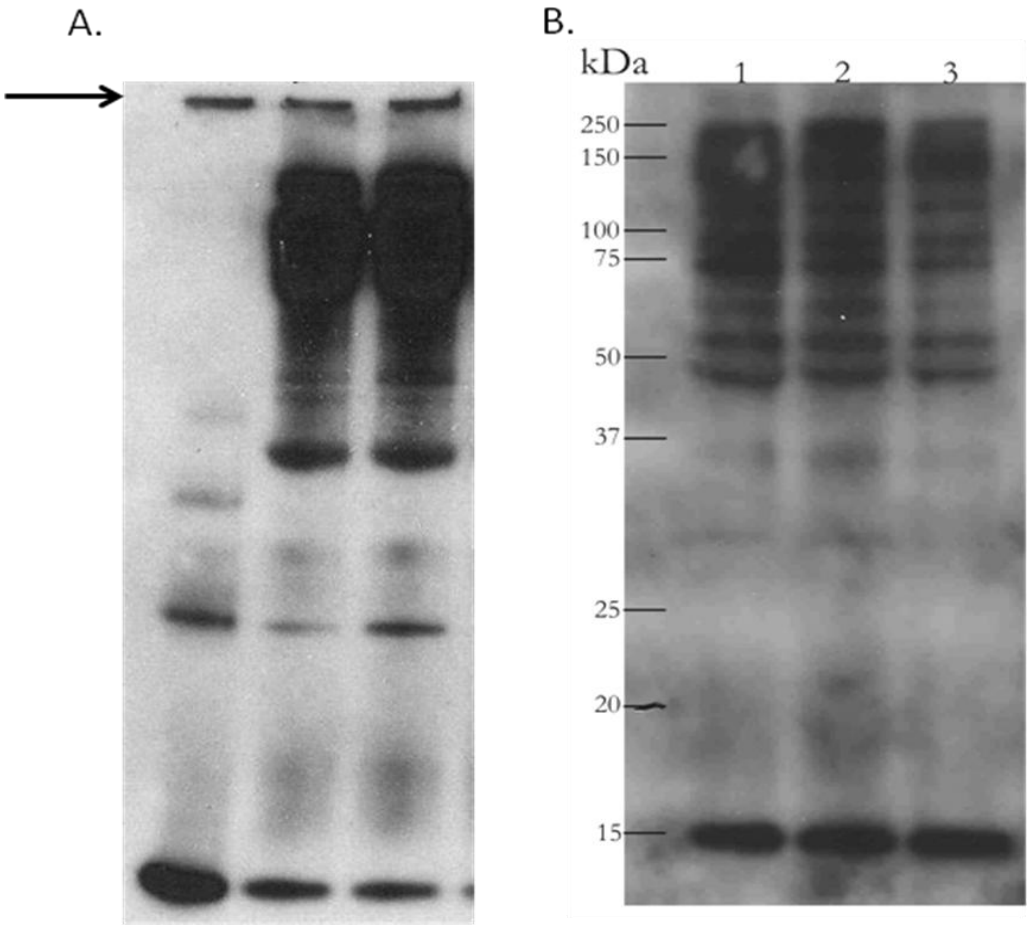


Figure 21. Denaturing and Non-Denaturing Gel Electrophoresis for TTR

*CSF was analyzed under denaturing (**A**) or non-denaturing (**B**) conditions. **A**: TTR immunoreactive bands that did not enter the gel were observed. **B**: CSF from 2 ALS (lanes 1 and 2) and 1 healthy control (lane 3) were subjected to non-denaturing gel electrophoresis and TTR immunoreactive species identified. Monomeric TTR is observed around 14 kDa. High molecular weight species were positive for TTR, and many of the bands corresponding to these protein species were more intense in the ALS patients when compared to the healthy control.*

2.5 DISCUSSION

Our laboratory has previously demonstrated decreased levels of TTR in the CSF of patients with ALS, and we sought to further characterize the potential of this protein as a biomarker of ALS. This study utilized mass spectrometry to distinguish between wild-type and variant forms of TTR and to identify oxidative post-translational modifications of the protein. Although genetic variations in the TTR gene could be detected at the protein level, they did not appear to be more abundant in the ALS population. However, specific oxidative post-translational protein modifications were altered in the ALS patient population despite the fact that the total TTR protein level did not change over the course of the disease as measured by ELISA. In studies to further investigate these alterations, temperature-dependent changes were observed using both mass spectrometry and immunoblotting techniques. In addition, preliminary studies suggest that high molecular weight protein species are more abundant in the CSF of patients with ALS when compared to healthy controls. Additional studies are required to validate these initial findings and to determine if these high molecular weight TTR species contribute to extracellular or intracellular protein aggregation in ALS patients.

Approximately 110 known variants of the TTR protein exist, and the pathogenic variants have been associated with protein plaques (amyloid) that cause organ dysfunction and death [183]. Although we were able to detect variant forms of TTR, my results suggest that genetic variants in the TTR gene do not contribute to ALS pathogenesis. However, the mass spectrometry-based system used was not sensitive enough to resolve mass differences less than 6 Daltons. These variants could result from a single amino acid change, a double variant or a variant that does not circulate [184]. In addition, mass differences that are isobaric (e.g., Leu to Ile) would not be detected. However, the speed with which we could perform the study

warranted its use as an initial screen of our ALS population. In addition, the known TTR variants with mass shifts less than 10 Daltons are not common, and they constitute only 10% of the known variants [176]. In fact, if all potential single-amino-acid variants are examined, 48 of 380, or 12.6%, would have a variant that is less than 10 Daltons, giving a theoretical minimum sensitivity of approximately 87% [176]. Nevertheless, in combination with data from single nucleotide polymorphism (SNP) studies covering the TTR gene performed by other groups [185], we are confident that this does not represent a novel susceptibility factor for this disease.

The mass shift that we observed (+30 Da) likely corresponds to the Gly6Ser polymorphism which is present in the Caucasian population with a 12% prevalence [176]. Although DNA sequence analysis was not used to confirm this observation, it has previously been reported that the circulating concentrations of variant forms of TTR are lower in abundance than those of wild-type TTR [186, 187]. The exception is the Gly6Ser variant which is usually present in close-to-equal abundance. The spectral patterns observed in our analyses indicates an approximate equal abundance of the wild-type and variant forms of TTR suggesting that this common variant is present in our patient population.

Our ELISA data confirmed that decreased TTR protein levels are present in the ALS population although these levels did not appear to change with disease progression. A decrease in TTR could negatively affect retinoid signaling, a pathway critical during neural development/specification, with evidence supporting a role for this signaling pathway during adulthood as well. More specifically, RA-dependent signaling cascades have been suggested to play an important role in the intrinsic regenerative capacity of the CNS following injury or degeneration [188]. As the importance of the interaction between TTR and retinol-retinol binding protein (retinol-RBP) is well-documented [153, 162, 189, 190], decreased levels of TTR

could prevent or at least decrease the amount of retinol available to cells of the CNS. In the context of ALS, this could translate to a diminished regenerative response in degenerating motor neurons attempting to promote their own survival. Additional characterization of proteins of this signaling pathway is addressed in Chapter 3.

The observation that post-translational modifications of the TTR protein are altered in the CSF of patients with ALS suggests that these changes may also be of great importance in disease pathogenesis. As TTR is known to function as a protein chaperone with the ability to sequester proteins like amyloid β [191], the impact of these modifications on the formation of amyloid fibrils/deposits is of great interest. It is worth noting that in transgenic mice overexpressing a mutant form of amyloid precursor protein, the TTR gene was found to be the most relevant overexpression change in a microarray screen of over 12,000 mouse genes [154]. This differential expression pattern was associated with activation of cell survival pathways and a lack of neurodegeneration [154]. This same group demonstrated reversal of this protective phenotype upon treatment with a TTR-neutralizing antibody [192] although it is unclear how the presence (or absence) of thiol adducts modulates these neuroprotective abilities. The observation that altered levels of modified TTR protein are present in the ALS CSF combined with preliminary data on high molecular weight and TTR-immunoreactive protein species in ALS indicates that these two phenomena might have a common biochemical link.

Previous studies have utilized similar methods to those employed in this study to investigate the differential oxidation of TTR thiols in serum [171] and CSF [156]. Interestingly, the percentage of conjugated (modified) TTR to unconjugated (free) TTR in these studies correlated with disease but the predominant form of TTR differed depending on the biofluid analyzed. That is, in the serum of familiar polyneuropathy (FAP) patients, the percentage of

conjugated to unconjugated TTR was higher in FAP symptomatic patients than in asymptomatic carriers [171]. However, in the CSF of patients with Alzheimer's disease (AD), the thiol conjugated forms of TTR were much less abundant than observed in control patients [156]. This difference may be explained by the fact that serum TTR is regulated and expressed by the liver while CSF TTR is produced predominantly by the cells of the choroid plexus. Therefore, it is possible that the post-translational protein modifications observed in the CSF depend, at least in part, on the redox balance of the CNS. In fact, it has been suggested that this balance might determine disease progression by impacting the propensity of TTR to form fibrils [156]. If this is found to be the case in ALS, TTR post-translational modifications may be useful biomarkers for disease progression.

The relationship between TTR levels and the nutritional status of patients with ALS is another consideration. As the half-life of TTR is relatively short (approximately 2 days in the plasma), it is more sensitive to changes in protein-energy status and its concentration closely reflects recent dietary intake [193, 194]. Therefore, serum levels of TTR which normally range from 15.7-29.6 mg/dL are commonly used to assess the degree of malnutrition as mild (12-15 mg/dL), moderate (8-10 mg/dL) or severe (<8 mg/dL). As it is well-established that deficiencies in energy intake/malnutrition are associated with a poor prognosis in ALS patients [195, 196], the impact of post-translational modifications on TTR half-life could be examined further to determine their impact.

The effects of pre-analytical factors on the stability of the proteome in mass spectrometry-based research is well-documented [197], and our laboratory has certainly contributed to these findings [198]. While artifactual and storage-related truncations have been reported previously [148], the observed temperature-dependent changes in modified forms of the

TTR protein are still somewhat intriguing. For certain high molecular weight TTR-immunoreactive bands, there appears to be a "reversal" of the formation of these protein species when samples are moved from -20^{o}C to -80^{o}C (Figure 20). Although this phenomenon cannot currently be explained, a recent mass spectrometry-based validation study performed in our laboratory using CSF from a total of 241 subjects [100 ALS, 53 Alzheimer's disease (AD), 41 healthy controls (HC), 18 multiple sclerosis (MS), and 29 other neurologic diseases (DC)], confirmed the presence of TTR-CysGly in patients with ALS. The observed increase in this modified form of TTR suggests that thiol-conjugated TTR plays a role in ALS disease pathogenesis although further studies are necessary to understand the impact of these modifications and to determine whether they contribute to or occur as a result of the disease process.

Intracellular and extracellular protein aggregates have been observed in ALS, and the contribution of TTR to these aggregates warrants further study. As modifications of TTR at the Cys^{10} residue are critical for stabilization of the tetrameric (and functional) form of TTR, alterations or a lack thereof at this residue could lead to disassembly of the functional form of TTR into its monomeric components. Further conformational changes could result in the formation of protein aggregates. As we have observed both intracellular and extracellular TTR in the lumbar spinal cord of patients with ALS [150], we hypothesize that a variety of connections between TTR and ALS are plausible (outlined in Figure 22). The exact role of TTR in ALS disease pathogenesis is being further investigated using a variety of techniques including high performance liquid chromatography (HPLC), liquid chromatography-tandem mass spectrometry (LC-MS/MS) and primary motor neuron cultures.

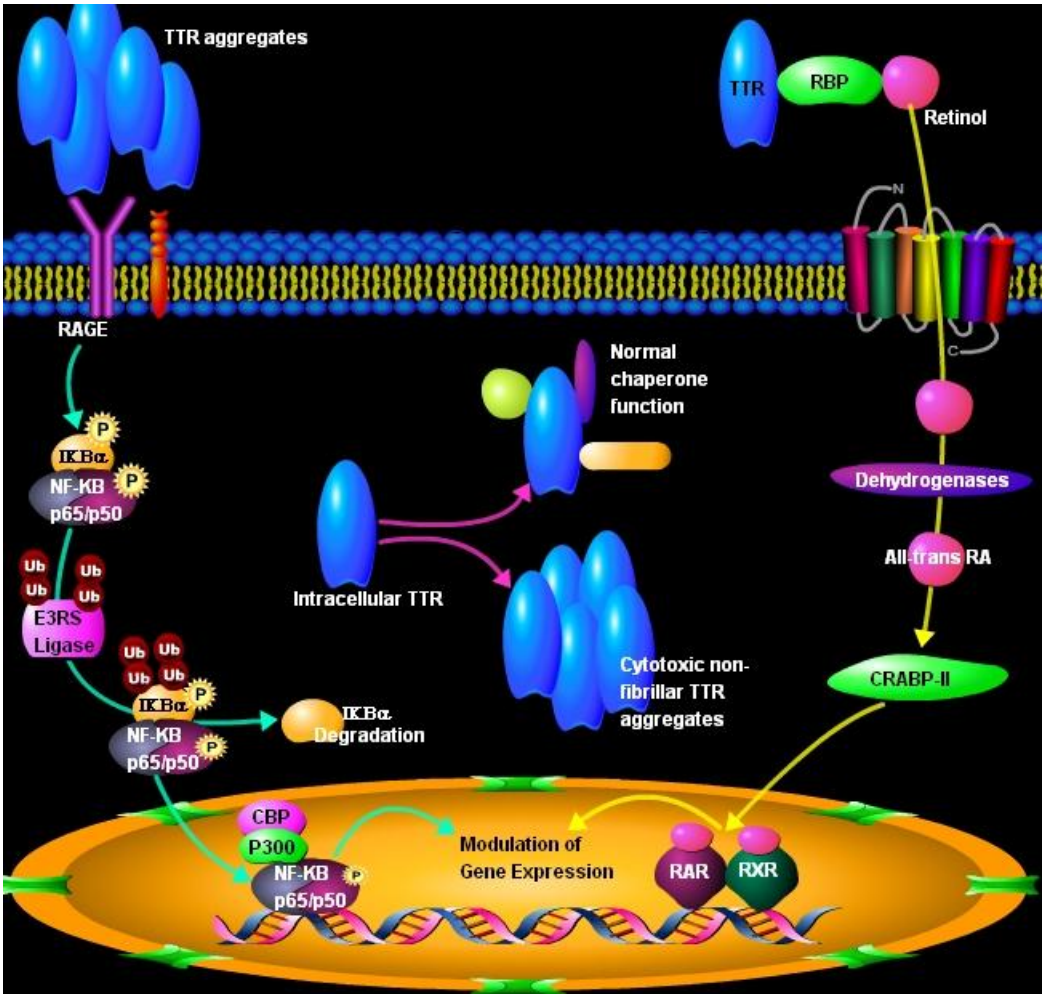


Figure 22. Potential Roles of TTR in ALS

The hypothesized potential roles of TTR in ALS pathogenesis are depicted above. TTR forms extracellular aggregates (non-fibrillar and fibrillar) that impact gene expression by binding to receptors like the receptor for advanced glycation end products (RAGE) and modulating NF-κB-dependent gene expression (left). Intracellular TTR can function as a chaperone for other proteins thereby preventing misfolding and aggregation but under conditions of stress or injury the formation of TTR aggregates would be cytotoxic (middle). TTR impacts retinoic acid signaling thereby affecting the expression of downstream genes containing retinoic acid response elements (RAREs) (right).

3.0 RETINOID SIGNALING ALTERATIONS IN ALS PATIENT TISSUE

3.1 ABSTRACT

ALS is a fatal neuromuscular disease for which effective therapeutic interventions and an understanding of underlying disease mechanism are lacking. A variety of biochemical pathways are believed to contribute to the pathophysiology of ALS that are common to both sporadic and familial forms of the disease [9]. Evidence from both human and animal studies indicates that expression of retinoid signaling genes is altered in ALS and that deficiencies in this signaling pathway may contribute to motor neuron loss [199-206]. My objective was to examine the expression and distribution of proteins of the retinoid signaling pathway in spinal cord samples from patients with sporadic and familial ALS. In sporadic ALS, the cytoplasmic binding protein that facilitates nuclear translocation of retinoic acid, cellular retinoic acid binding protein-II (CRABP-II), was localized to the nucleus and retinoic acid receptor β (RARβ) was significantly increased in motor neuron nuclei when compared to either familial ALS patients or non-neurologic disease controls. Motor neurons with increased nuclear RARβ were negative for markers of apoptosis. Differences in protein-protein interactions between CRABP-II and the nuclear receptors were not detected; however increased binding at the retinoic acid response element was observed in spinal cord extracts from ALS patients. This data suggests retinoid

signaling is altered in ALS and increased nuclear RARβ occurs in motor neurons of sporadic ALS patients.

3.2 INTRODUCTION

3.2.1 Overview of the Retinoid Signaling Pathway

Within the cell, the level of free versus bound retinol and retinoic acid (RA) are regulated by cellular retinol-binding protein (CRBPI) and cellular retinoic acid-binding proteins (CRABP-I and II), respectively [207] (Figure 23). CRBPI binds retinol in the cytoplasm and promotes its metabolism to retinaldehyde [208] and esterification to the retinyl ester for storage [209]. CRABP-I has been suggested to promote the catabolism of RA [210] whereas CRABP-II is thought to mediate the transport of RA to the nucleus for association with its receptors [211]. RA mediates its effects on gene transcription through the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) [212, 213]. Each receptor has three isotypes (α , β and γ) with multiple isoforms of each subtype generated by alternative splicing and differential promoter usage [212]. RARs are activated by either all-*trans* retinoic acid (ATRA) or 9-*cis*-retinoic acid (9-*cis*-RA) and mediate gene expression by forming heterodimers with RXRs whereas RXRs are activated only by 9-*cis*-RA and modulate gene expression either as homodimers or by forming heterodimers with RARs or a variety of orphan receptors such as peroxisome proliferator-activated receptor (PPAR), nerve growth factor receptor-induced orphan receptor (NGFI-B) and thyroid hormone receptor [214-216].

These retinoic acid nuclear receptors function as ligand-activated transcription factors and allow retinoic acid to serve as a transcriptional activator for a large number of other, downstream regulatory molecules including enzymes, transcription factors, cytokines and cytokine receptors [161]. Although less studied, retinoic acid receptors can also function as transcriptional repressors. That is, in the absence of ligand, receptors remain bound to the response element within the gene promoter where they can act as strong inhibitors of gene transcription [161]. In addition, RA has been shown to impact phosphatidylinositol-3-kinase (PI3K) and extracellular signal-regulated kinase 1/2 (ERK1/2) mitogen-activated protein kinase (MAPK) signaling pathways through a rapid, non-genomic mechanism [217-221]. Finally, interactions between mitochondria and both cytoplasmic binding proteins and nuclear receptors of the retinoic acid signaling pathway indicate a role for this signaling molecule in mitochondrial function [222-224].

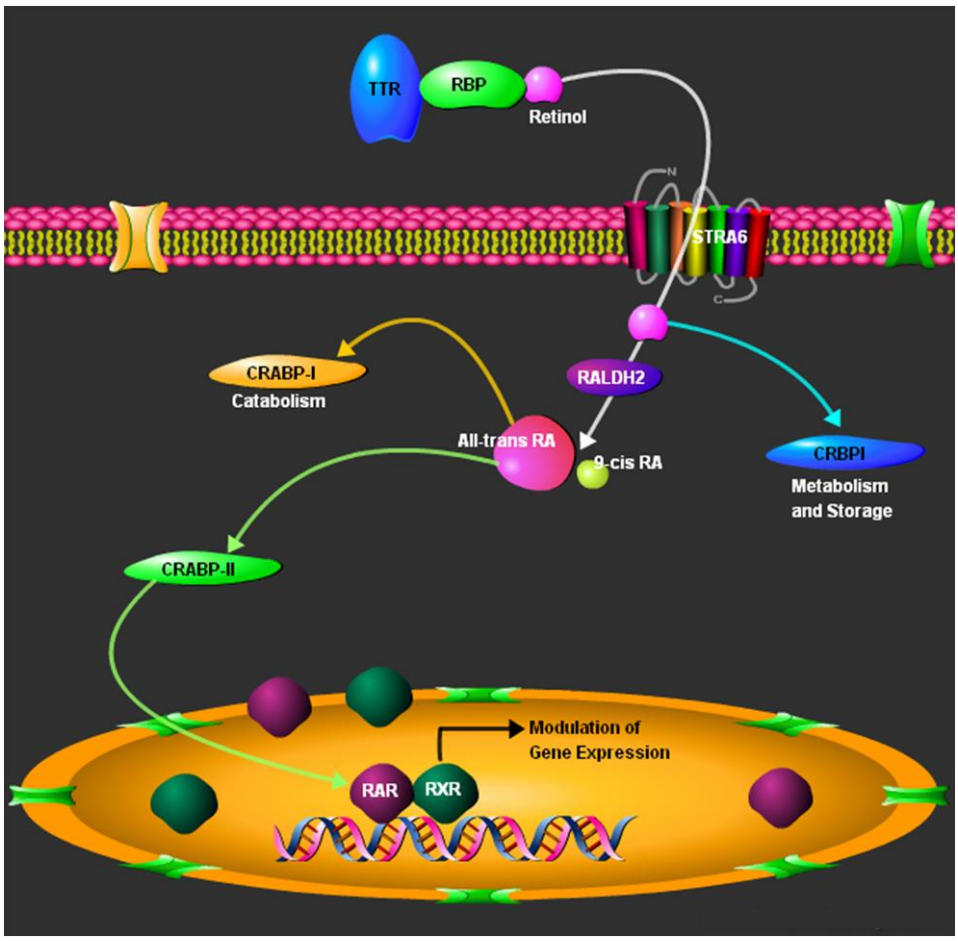


Figure 23. Retinoid Signaling Overview

Retinol is transported throughout the body in complex with transthyretin (TTR) and retinol binding protein (RBP). At the cell surface, receptors such as STRA6 transport retinol into the cell where it is converted to all-trans RA and 9-cis RA through the action of dehydrogenases (including RALDH2). Interactions with cytoplasmic binding proteins (CRBPI, CRABP-I and CRABP-II) determine downstream fate. CRABP-II facilitates interactions with the nuclear receptors (RARs and RXRs) which function as ligand-activation transcription factors that modulate target gene expression via binding at retinoic acid response elements.

3.2.2 Role in CNS Development

RA is a member of the retinoid family of lipids and mediator of vitamin A activity. It is an essential morphogen in vertebrate development and is critical for neural development/specification and neural plasticity and regeneration [160, 161]. During neural development, RA is thought to act after neural induction by noggin, follistatin and chordin [225]. RA, along with fibroblast growth factors (FGFs) and Wnts, simultaneously induce neurogenesis and create the anteroposterior (AP) pattern of the CNS. For AP patterning, RA up-regulates a variety of posterior genes (*Krox20*, *Wnt1*, *En*, *Pax2*, *XIF-3*, *Xlim-1* and Hox genes) while down-regulating anterior genes (*Otx2*, *XCG-1*, *XAG-1*, *XA-1*, *Emx1*, *Emx2*, *Dlx1* and *XINK-2*). This generates pattern in the posterior hindbrain and anterior spinal cord [225]. A similar role for RA occurs during neurogenesis in that it up-regulates some (*X-ngnr-1*, *X-MyT1*, *X-delta-1* and *N-tubulin*) and down-regulates other (*Zic2* and *X-shh*) identified genes.

During spinal cord development, RA is required at multiple times for a series of inductive events (outlined in Figure 24). First, it acts as a posteriorizing factor during neuronal induction along with Wnts and FGFs to pattern the anterior spinal cord. It is then required for the induction of a subset of ventral interneurons known as V0 and V1 which are characterized by the expression of Dbx1, Dbx2 and En1 proteins. The induction of neural differentiation throughout the spinal cord via the gene *NeuroM* also requires RA. Then, in combination with sonic hedgehog (Shh), RA initiates motor neuron differentiation via the gene *Olig2* for the production of lateral motor column (LMC) neurons which innervate limb muscles. For each of these events, RA synthesized in the adjacent mesoderm/somites diffuses into the overlapping neural plate/neural tube in a paracrine fashion [226]. Later stages of motor neuron differentiation involve the migration of a subset of later-born motor neurons through a RA signaling region

consisting of earlier-born LMCs to differentiate into the lateral part of the lateral motor column (LMCL). This highlights the crucial role of RA signaling in development of the motor neuron complement as well as the inductive nature of RA in spinal cord development, neuronal differentiation and patterning.

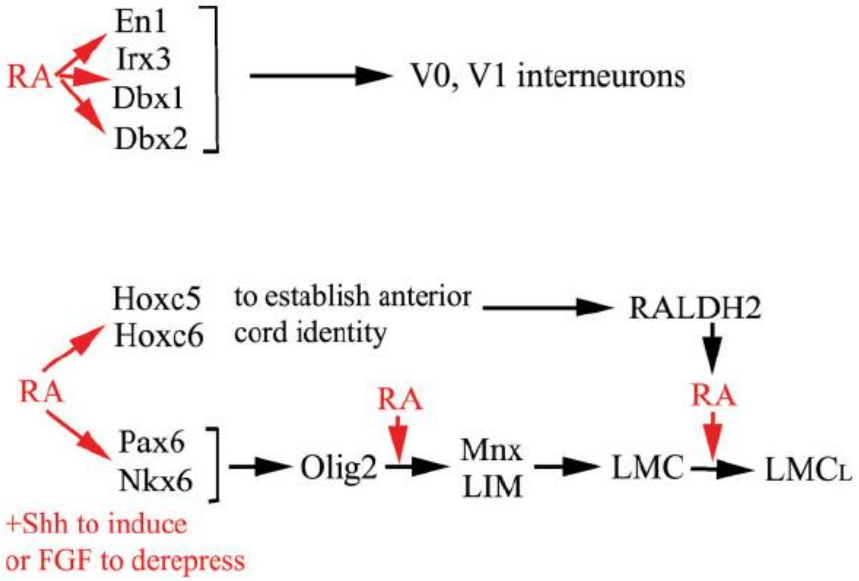


Figure 24. Retinoic Acid in Interneuron and Motor Neuron Development

The gene pathways involved in the production of V0 and V1 interneurons and motor neurons as well as the points of action of RA (retinoic acid) are outlined. After Shh (sonic hedgehog) from the floor plate has established the general conditions for motor neuron differentiation, RA signaling from the somites initiates further differentiation steps. Figure taken from [226].

3.2.3 Roles in Adulthood

Experimental evidence indicating that vitamin A-mediated signaling continues to play a role in adulthood comes from studies in animal models of vitamin A deprivation [206, 227] with additional studies suggesting that retinoid signaling can promote regeneration of the adult rodent

nervous system [228-230] or impact the process of neurodegeneration [231, 232]. Interestingly, retinoid-sensitive sequences have been described in the regulatory regions of over 500 genes, some of which have a neuronal expression pattern and encode for enzymes involved in the biosynthesis and degradation of neurotransmitters, ligand-gated ion channels, G protein-coupled receptors, neurotransmitter transporters, voltage-gated ion channels, cytoskeletal proteins and intracellular signaling molecules [233]. RA-mediated gene expression has also been detected in different cell types throughout the adult neuraxis (including the spinal cord) [234-237] with an up-regulation of molecular factors involved in RA-mediated responses following injury or degeneration in the adult CNS (outlined in Figure 25). This suggests that RA-dependent signaling cascades could play an important role in the intrinsic regenerative capacity of the CNS.

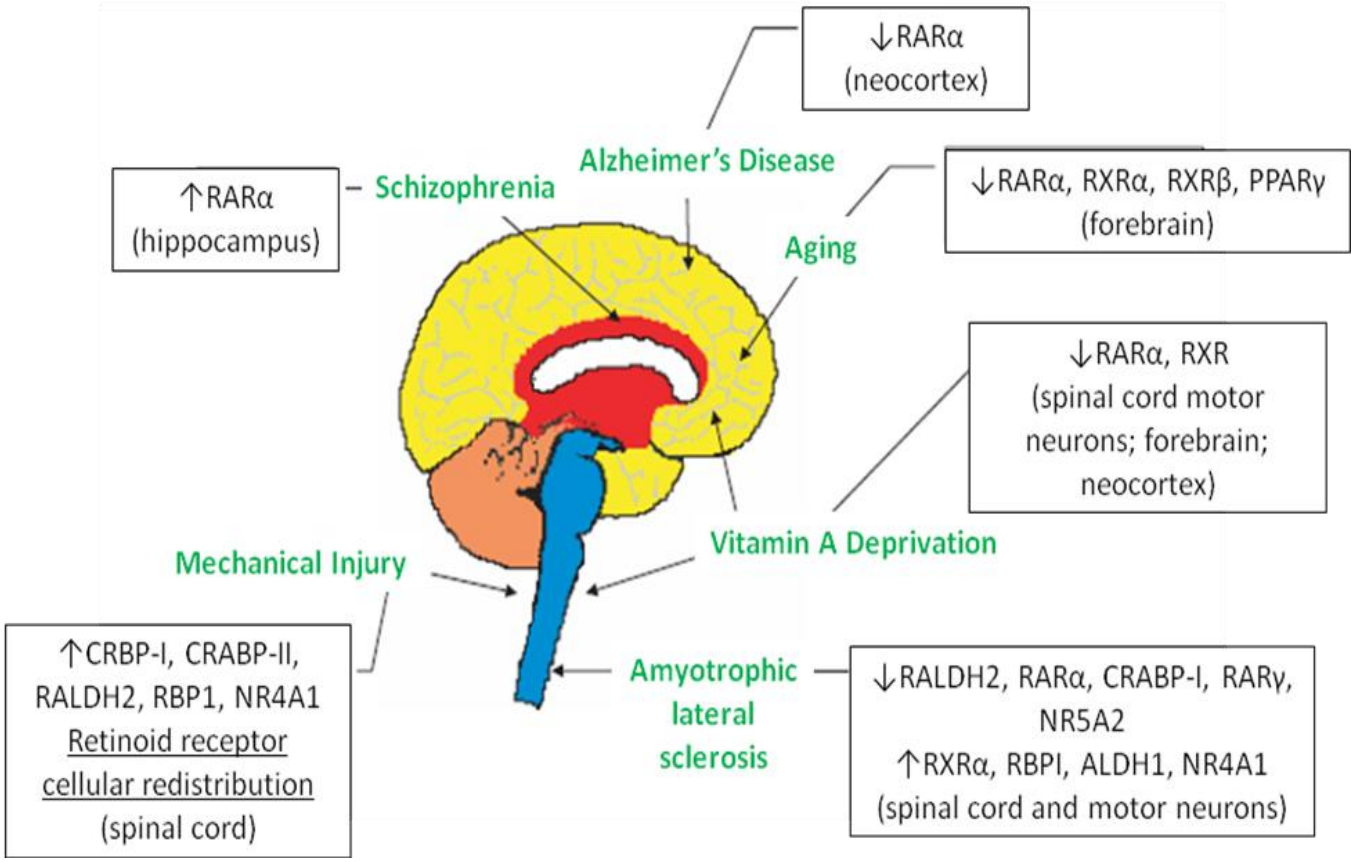


Figure 25. RA-Mediated Responses Associated with Injury or Degeneration in the Adult CNS

In response to CNS injury or degeneration, a number of RA-related responses are altered. The arrows indicate the main anatomic location where the changes have been described with the condition/pathology in green. The figure is color-coded to denote brain hemispheres (yellow), cerebellum (orange), brainstem and spinal cord (blue) and subcortical structures (red). Figure taken from [188].

3.2.4 Connections to ALS

RA has been shown to increase peroxisome proliferator-activity binding to the peroxisome proliferator-response element that participates in the induction of the rat SOD1 gene [238] and to reduce susceptibility to oxidative stress in chick embryonic neurons [239, 240], PC12 cells [241]

and mesangial cells [242] although the mechanism for this antioxidant effect remains unclear. Similarly, Ahlemeyer and colleagues found that treatment with RA prevented staurosporine-induced decreases in SOD1 and SOD2 protein levels and reduced oxidative stress and apoptotic damage in primary cultures from neonatal rat hippocampus [243]. Taken together, these studies suggest that RA may improve the antioxidant defense system and that deficiencies may exacerbate or cause cell damage/death.

In addition to the observation that RA can modulate SOD1 gene expression, multiple studies have demonstrated that genes for retinoid pathway proteins and genes regulated by retinoid signaling are differentially expressed in post-mortem tissues from ALS patients [199-203]. Moreover, in transgenic animal models of familial ALS, spinal cord gene expression profiling has revealed altered expression of genes of the retinoid signaling pathway [204]. A recent study using the mutant G93A SOD1 transgenic rat model of ALS indicates that changes in the retinoid receptor expression profile occur in the spinal cord of early pre-symptomatic and terminal stage disease both at the RNA and protein levels [205]. Furthermore, Corcoran and colleagues observed astrocytosis, accumulation of neurofilaments and motor neuron loss in the lumbar spinal cord of vitamin A deficient animals [206]. Finally, single nucleotide polymorphisms in the anaplastic lymphoma kinase (ALK) gene are correlated to sporadic ALS [244]. As ALK is modulated by midkine (a heparin-binding growth factor with putative functions in neurogenesis, neurodifferentiation and cell survival) through 9-*cis*-RA and the RXRs, this serves as additional evidence suggesting a role for polymorphisms in genes modulated by the retinoid receptors in ALS. However, as a systemic vitamin A deficiency has not been observed in patients with ALS [245], it remains unclear how this signaling pathway plays a role in the disease process.

3.2.5 Study Objectives

The goal of this study was to investigate the role of proteins involved in the RA signaling pathway in ALS. My overall hypothesis was that retinoid signaling is altered in ALS and thereby contributes to motor neuron cell death. I analyzed post-mortem lumbar spinal cord tissue from individuals with both sporadic and familial ALS and non-neurologic disease controls to determine the levels and subcellular localization of proteins involved in this pathway. Specific protein-protein interactions were examined as well as protein-DNA interactions.

3.3 MATERIALS AND METHODS

3.3.1 Subjects

Lumbar spinal cord tissue was obtained from the ALS Tissue Bank at the University of Pittsburgh Medical Center (RB, Director). Approval was obtained from the University of Pittsburgh Institutional Review Board. In selecting cases for this study, efforts were made to control for age, gender and post-mortem interval with cases with available frozen spinal cord tissue whenever possible. Sporadic ALS subjects (n = 20) included in this study were clinically diagnosed using the revised El Escorial criteria. Familial ALS patients (n = 4) had a family history of ALS. Control subjects (n = 9) lacked clinical or neuropathological evidence of neurologic disease. The age range for all individuals was 40-95 years old. The average age at death was 60.25 ± 12.66 years for sporadic ALS (range, 40 to 82 years) and was not significantly different from familial ALS (52.75 ± 8.02 years; range, 45 to 63 years; p = 0.27) or control

subjects (64.78 ± 15.60 years; range, 51 to 95 years; p = 0.41). The average post-mortem interval time for sporadic ALS subjects was 5.50 ± 2.12 hours (range, 2 to 11 hours) and not significantly different from familial ALS (6.50 ± 5.07 hours; range 3 to 14 hours; p = 0.51) or control subjects (7.89 ± 5.71 hours; range 2 to 20 hours; p = 0.11).

3.3.2 Antibodies

Monoclonal antibodies were used to detect CRBPI (G4E4; Santa Cruz Biotechnology, Santa Cruz, CA), CRABP-I (C-1; Novus Biologicals, Littleton, CO), RARα (763; Chemicon International, Temecula, CA) and RARγ (1371; Chemicon International). Polyclonal antibodies were used to detect CRABP-II (K-13; Santa Cruz Biotechnology), RARβ (Chemicon International), cleaved caspase-3 (Asp175; Cell Signaling Technology, Boston, MA) and TDP-43 (BC001487; ProteinTech Group, Chicago, IL). For immunohistochemistry, these antibodies were used at dilutions of 1:50 (RARα and RARβ), 1:100 (CRABP-I, RARγ and cleaved caspase-3), 1:200 (TDP-43), 1:250 (CRBPI) and 1:2500 (CRABP-II). Western blots were probed with the antibodies listed above at 1:500 (CRABP-II) or alternate polyclonal antibodies to detect RARα (C-20; Santa Cruz Biotechnology) and RARβ (C-19; Santa Cruz Biotechnology) used at a dilution of 1:500.

3.3.3 Tissue Homogenates and Protein Extraction

Lumbar spinal cord tissue homogenates were prepared from a subset of patients (9 sporadic ALS; 4 familial ALS; 5 control) for co-immunoprecipitation and DNA-binding studies. The age

range and post-mortem interval for this patient subgroup did not differ from that of all subjects nor across the subgroups.

Spinal cord frozen tissue samples from control and both sporadic and familial ALS cases were homogenized and analyzed as previously described [246]. For total cell lysates, spinal cord tissue samples were homogenized using an Omni Tissue Homogenizer (Omni International, Marietta, GA) set at 15,000 rpm for 45 seconds in lysis buffer containing 25 mmol/L HEPES (pH 7.4), 50 mmol/L NaCl, protease inhibitor cocktail II (Sigma, St. Louis, MO) and 1% Triton X-100. The homogenized product was spun at 14,000 rpm at 4^{o}C and the supernatant saved as the total cell lysate. Nuclear and post-nuclear extracts were prepared as described previously [247]. Following homogenization with a Potter-Elvehjem grinder (Omni International) in buffer containing 10 mmol/L Tris (pH 8.0), 10 mmol/L $MgCl_2$, 15 mmol/L NaCl, 0.5 mmol/L phenylmethyl sulfonyl fluoride (PMSF), 2 μg/mL pepstatin A and 1 μg/mL leupeptin, nuclei were collected via low-speed centrifugation at 800*g* for 5 minutes. The resulting supernatant was saved as the post-nuclear extract and the nuclei further extracted with high-salt buffer (20% glycerol, 20 mmol/L HEPES (pH 7.9), 0.42 mol/L NaCl, 0.1% Nonidet P-40, 0.5 mmol/L PMSF, 2 μg/mL pepstatin A and 1 μg/mL leupeptin) on ice for 10 minutes. Remaining insoluble material was removed via centrifugation at 14,000 rpm for 5 minutes. The resulting supernatant fraction was collected as the nuclear-enriched fraction. Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL) per the manufacturer's instructions.

3.3.4 Immunohistochemistry and TUNEL staining

Paraffin-embedded tissue sections (8 μM) were deparaffinized and a hydrogen peroxide/methanol mixture used to block endogenous peroxidase activity. Sections were steamed for 20 minutes in Antigen Retrieval Citra (pH 6.0) (BioGenex, San Ramon, CA) or Target Retrieval Solution (pH 9.0) (Dako North America, Carpinteria, CA). After washing, blocking was performed with Protein Blocking Agent (Thermo Fisher Scientific) or Power Block Universal Blocking Reagent (BioGenex, San Ramon, CA). Primary antibodies were diluted in phosphate-buffered saline (PBS) or Super Block (ScyTek Laboratories, Logan, Utah) and incubated for 1 hour at room temperature in a humidified chamber. After washing, sections were incubated with the appropriate biotinylated secondary antibody (1:200 dilution; Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature in a humidified chamber. The signal was amplified using Vectastain ABC Reagent (Vector Laboratories) according to the manufacturer's protocol. NovaRED (Vector Laboratories) served as the chromagen and all sections were counterstained with hematoxylin.

For terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL), sections (4 μM) were deparaffinized, treated with proteinase K and endogenous peroxidase activity blocked with a hydrogen peroxide/methanol mixture. TdT enzyme was then added for 25 minutes at 37^{o}C. Anti-digoxigenin conjugate (rhodamin) was added for 30 minutes at room temperature. After washing, sections were incubated in 3,3'-diaminobenzidine (DAB) solution and counterstained with hematoxylin.

Motor neurons located within the ventral horn of the spinal cord were counted for each case. The number of motor neurons with nuclear CRABP-II or RARβ was reported as a percentage of the total number of motor neurons counted for each case (Table 5) and by grouping

(i.e., control, sporadic ALS or familial ALS). Only motor neurons for which the nucleus could be observed were counted. One slide was counted for each case although consistent staining patterns were observed over multiple slides.

3.3.5 Protein-Protein Interactions

Co-immunoprecipitation (co-IP) of nuclear-enriched fractions was performed using the MultiMACS Protein A/G Kit and μMACS Protein A/G MicroBeads (Miltenyi Biotech, Inc., Auburn, CA). Lysates were incubated with primary antibody for two hours at 4^{o}C with gentle rocking. Protein A/G MicroBeads were then added to the immune complex. After washing, extracts were added and the magnetically labeled protein retained on the column. The eluted immunoprecipitates were analyzed by immunoblot as described below.

3.3.6 Immunoblot

Co-immunoprecipitated nuclear-enriched fractions were fractionated on 12% gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (PerkinElmer, Boston, MA) and blocked with 5% non-fat milk/Tris-buffered saline with Tween-20 (TBST). Blots were probed with primary antibody overnight at 4^{o}C in 5% milk/TBST and then washed with TBST. Horseradish peroxidase-conjugated secondary antibodies specific for each primary antibody were added for 2-3 hours at room temperature after which blots were washed with TBST. The final reaction products were visualized using enhanced chemiluminescence (PerkinElmer) and the density of bands measured using NIH ImageJ software (National Institutes of Health).

3.3.7 Electrophoretic Mobility Shift Assay

Nuclear-enriched fractions from human lumbar spinal cord samples were prepared as described above. ^{32}P-labeled DNA probes corresponding to the binding site for the RAR receptors were generated by annealing templates containing the protein binding motifs to 10-nucleotide primers complimentary to the 5' end of the template. Both consensus (5'–TCG AGG GTA GGG TTC ACC GAA AGT TCA CTC GGG ATC C–3') and mutant (5'–TCG AGG GTA GGG AAC ACC GAA AGA CTC GGG ATC C–3') templates were used. Overhangs were filled using Klenow in the presence of [α-^{32}P]-labeled dATP and 5mM each of dCTP, dGTP, dTTP and 5-bromo-2'-deoxyuridine-5'-triphosphate (BrdU). Electrophoretic mobility shift assays were performed as previously described [248]. Briefly, ^{32}P-labeled probes were combined with 50-100 mM KCl, 5 mM dithiothreitol, 0.25 mg/mL bovine serum albumin, 1 μg of poly (dI-dC), 20 mM HEPES (pH 7.9), 20% glycerol and 0.2 mM EDTA. For competition assays, unlabeled consensus or mutant probes were added and the binding reactions incubated for 10 minutes at room temperature. Protein-DNA complexes were subsequently resolved via 8% polyacrylamide gel electrophoresis under non-denaturing conditions in a Tris borate/EDTA buffer. The gels were dried and exposed to Classic X-Ray Film, Blue Sensitive (Laboratory Products Sales, Rochester, NY) at -80^{o}C for between 1 hour to 1 week.

3.3.8 Statistical Methods

Comparisons between any two groups of data were accomplished using the unpaired t test at the 95% confidence interval. Multi-group comparisons (sporadic ALS vs. familial ALS vs. controls)

were performed using ANOVA followed by the Tukey post test. A p value ≤ 0.05 was considered statistically significant.

Table 5. Cases Utilized for the Human Tissue-Based Study

	Age	Gender	PMI (hours)	Percentage of Motor Neurons with Nuclear CRABP-II Localization	Percentage of Motor Neurons with Increased RARβ Nuclear Localization
SALS1	45	M	11	40% (4 of 10)	55% (6 of 11)
SALS2	51	M	7	50% (4 of 8)	60% (6 of 10)
SALS3	45	M	7	82% (9 of 11)	64% (7 of 11)
SALS4	62	M	7	70% (7 of 10)	14% (1 of 7)
SALS5	73	M	6	82% (9 of 11)	55% (6 of 11)
SALS6	80	F	4	64% (9 of 14)	88% (22 of 25)
SALS7	82	M	5	64% (9 of 14)	73% (22 of 30)
SALS8	72	F	9	50% (4 of 8)	70% (7 of 10)
SALS9	56	M	3	63% (5 of 8)	62% (8 of 13)
SALS10	63	F	5	67% (6 of 9)	18% (2 of 11)
SALS11	59	M	4	67% (8 of 12)	33% (5 of 15)
SALS12	43	M	6	70% (7 of 10)	86% (6 of 7)
SALS13	67	M	4	41% (7 of 17)	13% (2 of 16)
SALS14	51	M	4	78% (7 of 9)	58% (7 of 12)
SALS15	40	M	6	38% (3 of 8)	44% (4 of 9)
SALS16	77	F	4	89% (8 of 9)	50% (5 of 10)
SALS17	69	M	5	80% (8 of 10)	56% (5 of 9)
SALS18	60	F	2	70% (7 of 10)	27% (3 of 11)
SALS19	50	F	7	88% (7 of 8)	73% (8 of 11)
SALS20	60	F	4	78% (7 of 9)	50% (5 of 10)
FALS1	45	M	3	17% (2 of 12)	8% (1 of 12)
FALS2	48	F	5	17% (2 of 12)	20% (2 of 10)
FALS3	55	M	14	56% (5 of 9)	20% (2 of 10)
FALS4	63	F	4	50% (4 of 8)	20% (2 of 10)
CON1	54	M	6	26% (6 of 23)	13% (2 of 15)
CON2	51	F	5	38% (3 of 8)	18% (2 of 11)
CON3	76	M	13	31% (4 of 13)	29% (9 of 31)
CON4	57	M	2	25% (3 of 12)	4% (2 of 47)
CON5	58	F	5	73% (8 of 11)	25% (6 of 24)
CON6	82	F	5	11% (1 of 9)	19% (4 of 21)
CON7	57	F	11	ND*	21% (7 of 34)
CON8	95	M	20	50% (5 of 10)	0% (0 of 7)
CON9	53	F	4	20% (2 of 10)	6% (1 of 16)

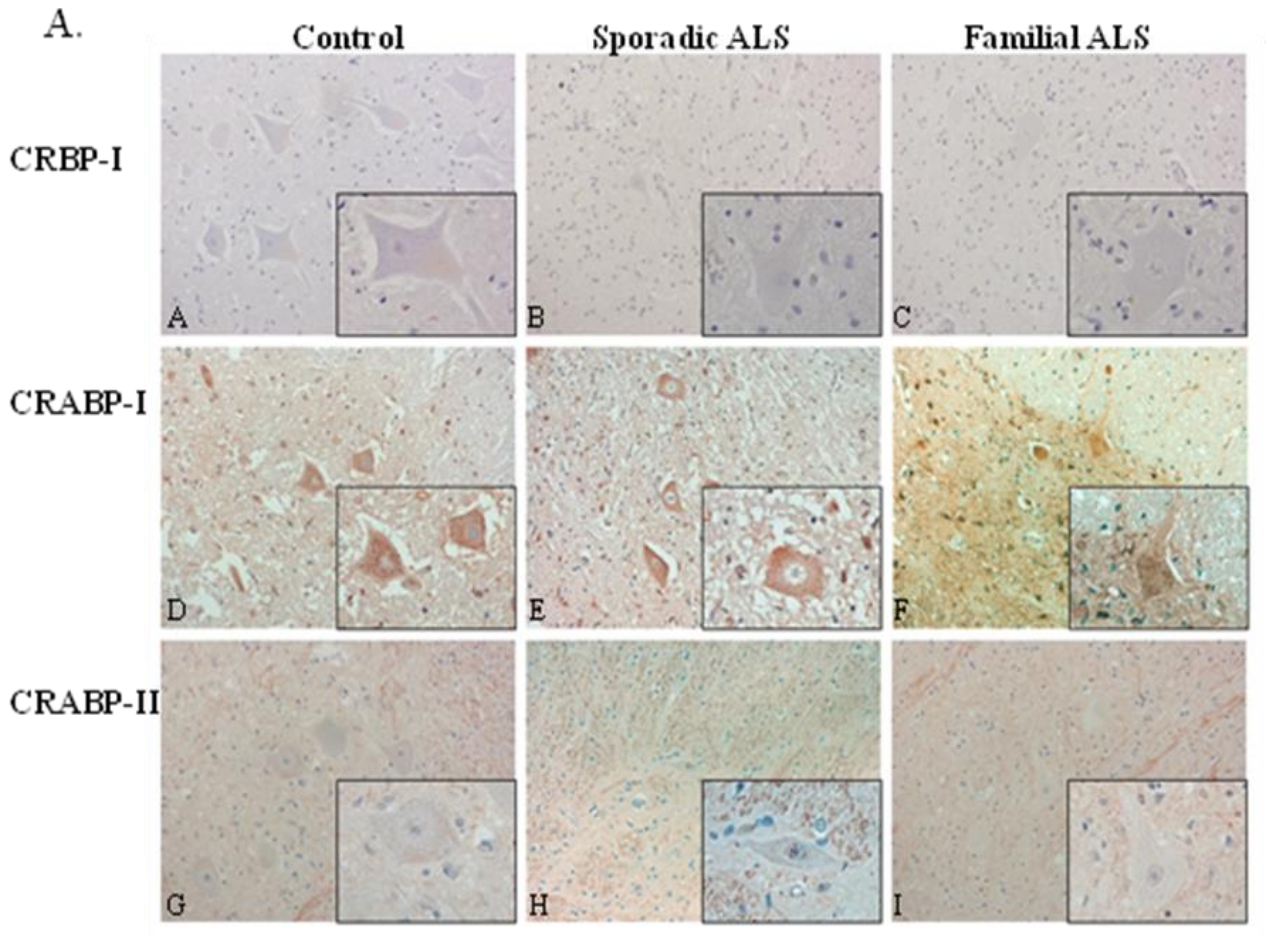
*The age and post-mortem interval (PMI) in hours (average SALS 5.50 hours; average FALS 6.5 hours; average control 7.89 hours) are indicated for the SALS (60.25 years), FALS (52.75 years) and control (64.78 years) cases. 17 of 20 SALS cases exhibited increased nuclear localization of RARβ as compared to controls and FALS. *ND = not determined*

3.4 RESULTS

3.4.1 Subcellular distribution of CRABP-II is altered in sporadic ALS spinal cord motor neurons

I first examined the localization of cytoplasmic retinol and RA binding proteins in the lumbar spinal cord of ALS and control subjects using commercially available antibodies. CRBPI, the cytoplasmic protein that mediates metabolism and storage, was not detected in spinal cord motor neurons of sporadic ALS, familial ALS or control subjects (Figure 26A, A-C). CRABP-I was noted in all three subject groups although the immunostaining pattern and intensity were not significantly different across all subject groups (Figure 26A, D-F). However, CRABP-II, the protein responsible for mediating nuclear translocation and facilitating nuclear receptor interactions, exhibited enhanced nuclear and peri-nuclear immunoreactivity in spinal cord motor neurons of sporadic ALS cases (Figure 26A, H). CRABP-II was observed in a more diffuse, cytoplasmic pattern in both familial ALS and control subjects (Figure 26A, G and I). When morphologically distinct motor neurons from multiple lumbar spinal cord sections of the sporadic and familial ALS and control cases were counted, the percentage of motor neurons with nuclear staining for CRABP-II summed across cases was 66% (135 of 205) for sporadic ALS

cases, 32% (13 of 41) for familial ALS cases and 33% (32 of 96) for control cases. These differences were statistically significant between and across all subject groups (Figure 26B). The percentage of motor neurons with punctate nuclear CRABP-II for each individual subject is shown in Table 5.



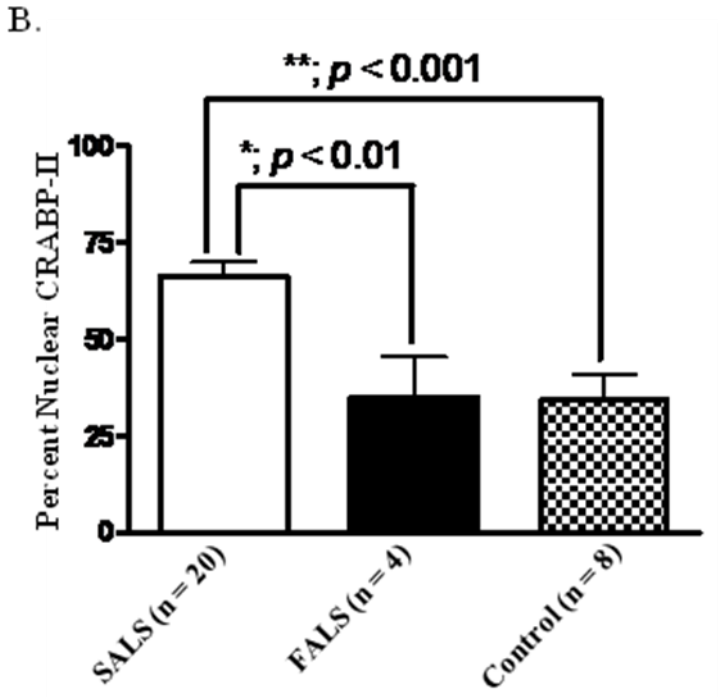


Figure 26. Immunohistochemistry Analysis of Cytoplasmic Binding Proteins in Human Spinal Cord Tissue

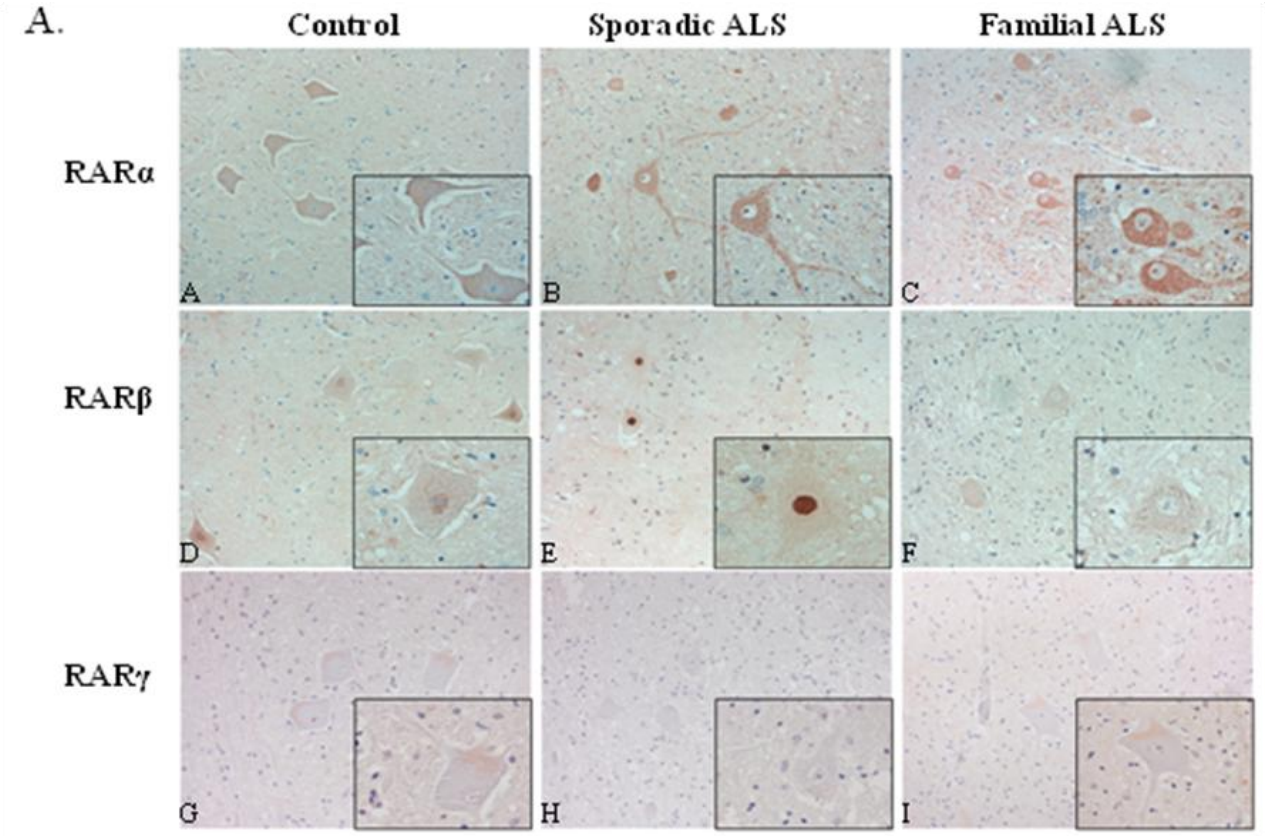
***A**: Lumbar spinal cord sections were immunostained for CRBPI (**A-C**), CRABP-I (**D-F**) and CRABP-II (**G-I**) in red and counterstained with hematoxylin. Insets represent a high power magnification of each panel. Original magnifications: 200X (**A** to **I**); 400X (insets in **A** to **I**). **B**: The localization of CRABP-II was assessed for morphologically distinct motor neurons from each patient group. The percent of motor neurons with nuclear CRABP-II was significantly greater in patients with sporadic ALS (white bars) as compared to familial ALS (black bars; $p < 0.01$) or controls (stippled bars; $p < 0.001$). There was no significant difference between familial ALS patients and controls ($p > 0.05$).*

3.4.2 RARβ immunoreactivity is significantly increased in motor neuron nuclei of sporadic ALS patients and does not correlate with TUNEL, activated caspase-3 or TDP-43 staining

I next examined the levels and localization of the three retinoic acid receptor (RAR) isotypes (RARα, RARβ and RARγ) in lumbar spinal cord motor neurons as these receptors are activated by the predominant RA isoform, all-*trans* RA. RARα was observed in motor neurons of all ALS cases as well as controls and was localized predominantly to the cytoplasmic compartment (Figure 27A, A-C). The level of this protein did not differ significantly between motor neurons of the three patient groups but was noted in distal processes of motor neurons of sporadic ALS and, to a lesser extent, control subjects. For RARβ, there was a significant increase in the immunostaining of motor neuron nuclei in sporadic ALS subjects (Figure 27A, E). In familial ALS and non-neurologic disease controls, RARβ exhibited a predominantly cytoplasmic distribution in lumbar spinal cord motor neurons (Figure 27A, D and F). The percentage of motor neurons with nuclear RARβ immunostaining summed across cases was 55% (137 of 249) for sporadic ALS cases, 17% (7 of 42) for familial ALS cases and 16% (33 of 206) for control cases (Figure 27B). This difference in nuclear RARβ was statistically significant when motor neurons from sporadic ALS patients were compared to those of familial ALS patients ($p < 0.01$) and controls ($p < 0.001$) (Figure 27B). The percentage of motor neurons with increased nuclear RARβ per subject is shown in Table 5. RARγ was not detected in spinal cord motor neurons of sporadic ALS, familial ALS or control subjects (Figure 27A, G-I).

RARβ has been implicated in molecular mechanisms regulating cell death in multiple cell types [249-251]. Conversely, this RAR isotype has been shown to mediate both neurotrophic and neuritogenic effects of retinoic acid [252]. To determine whether this response was

apoptotic or pro-survival, I used immunohistochemistry to evaluate the co-localization of TUNEL with RARβ in motor neurons. Serial sections of lumbar spinal cord from sporadic ALS patients were immunostained for RARβ and TUNEL, and the same motor neurons evaluated from both sections as denoted by tissue landmarks. I did not observe TUNEL staining in motor neurons with RARβ nuclear localization (Figure 28A) but did observe TUNEL-positive nuclei in motor neurons lacking nuclear RARβ (representative image in Figure 28B). Approximately 79% (26 of 33) of motor neurons with nuclear RARβ immunostaining were TUNEL negative across multiple sporadic ALS subjects (21% (7 of 33) were both RARβ and TUNEL positive). Similar results were obtained when activated caspase-3 was used as a marker of apoptosis (Figure 29). In addition, this RARβ nuclear localization did not correlate with cytoplasmic inclusions of 43 kDa TAR DNA-binding protein (TDP-43) (Figure 30).



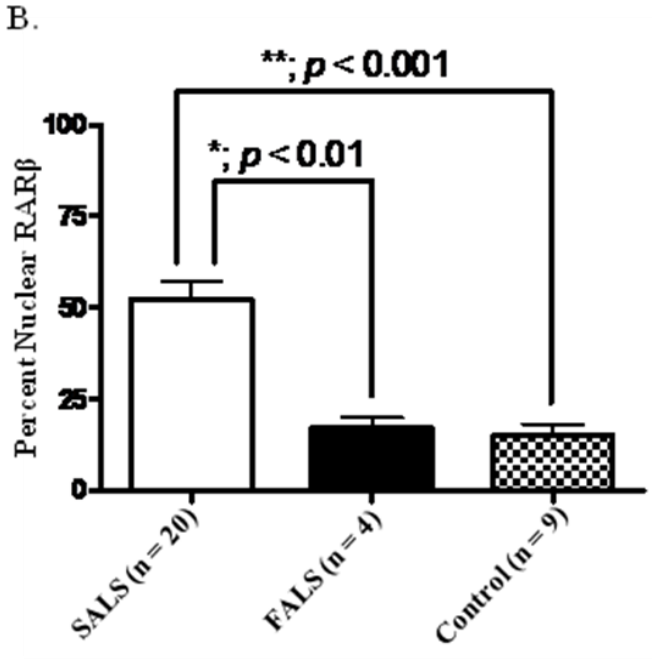


Figure 27. Immunohistochemistry Analysis of RAR Proteins in Human Spinal Cord Tissue

***A**: Lumbar spinal cord sections were immunostained for RARα (**A-C**), RARβ (**D-F**) and RARγ (**G-I**) in red and counterstained with hematoxylin. Insets represent a high power magnification of each panel. Original magnifications: 200X (**A** to **I**); 400X (insets in **A** to **I**). **B**: Nuclear RARβ immunoreactivity was determined by counting morphologically distinct motor neurons from all three patient groups. The percent of motor neurons with increased nuclear RARβ was significantly greater in patients with sporadic ALS (white bars) when compared to familial ALS (black bars; $p < 0.01$) or controls (stippled bars; $p < 0.001$). There was no significant difference between familial ALS patients and controls ($p > 0.05$).*

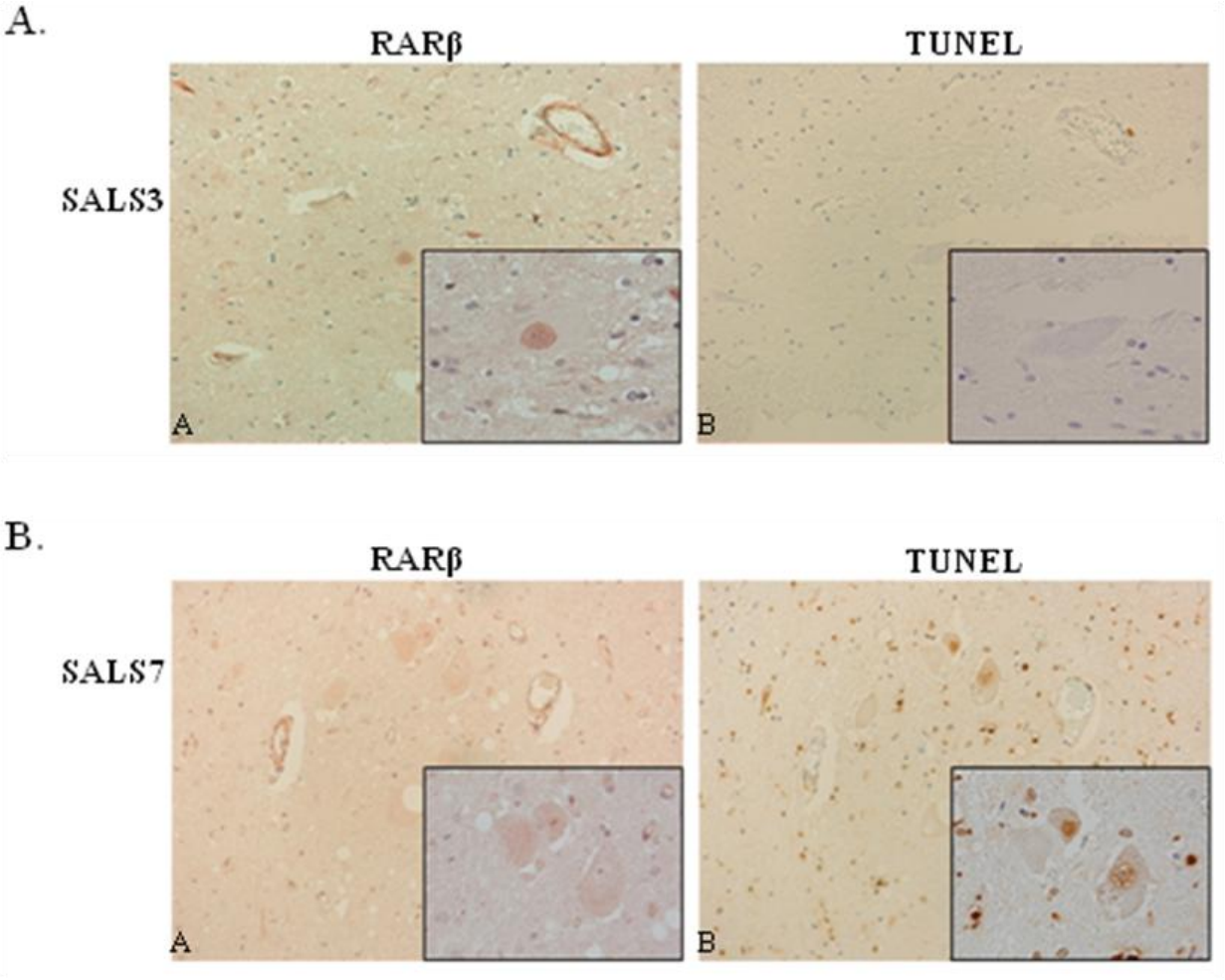


Figure 28. Immunohistochemical Analysis of RARβ and TUNEL in Human Spinal Cord

Serial lumbar spinal cord sections from sporadic ALS cases were immunostained for RARβ and TUNEL and representative cases are shown. Identical motor neurons were imaged as determined by anatomical hallmarks and location within the ventral horn. ***A****: Motor neurons with nuclear RARβ immunostaining (****A****) lacked TUNEL staining (****B****).* ***B****: Motor neurons within the spinal cord without nuclear RARβ immunostaining (****A****) were TUNEL positive (****B****). Insets represent a high power magnification of each panel. Original magnifications: 200X; 400X (insets).*

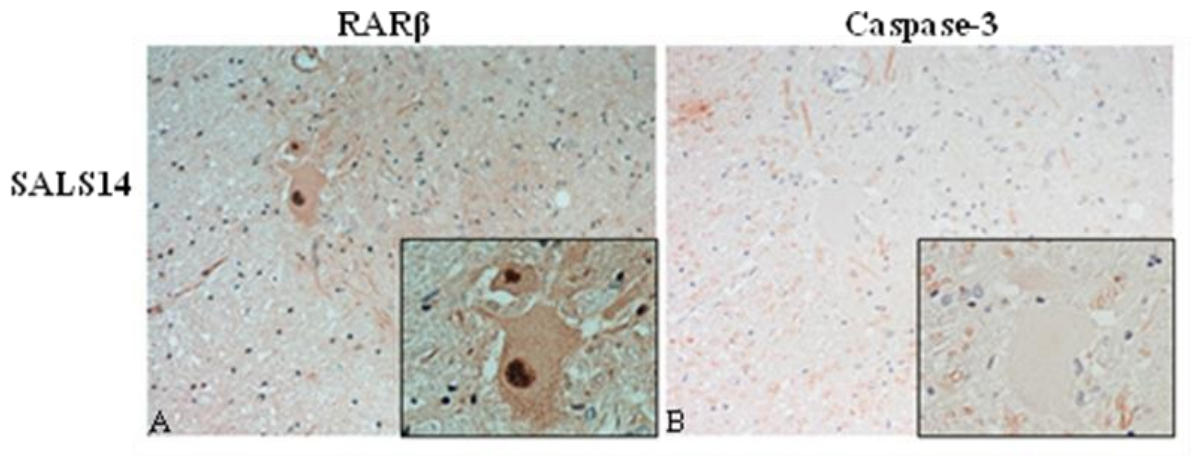


Figure 29. Immunohistochemical Analysis of RARβ and Activated Caspase-3 in Human Spinal Cord

*Serial lumbar spinal cord sections from sporadic ALS cases were immunostained for RARβ and activated caspase-3 and representative images are shown. Identical motor neurons were imaged as determined by anatomical hallmarks and location within the ventral horn. Motor neurons with nuclear RARβ immunostaining (**A**) lacked activated caspase-3 staining (**B**). Insets represent a high power magnification of each panel. Original magnifications: 200X; 400X (insets).*

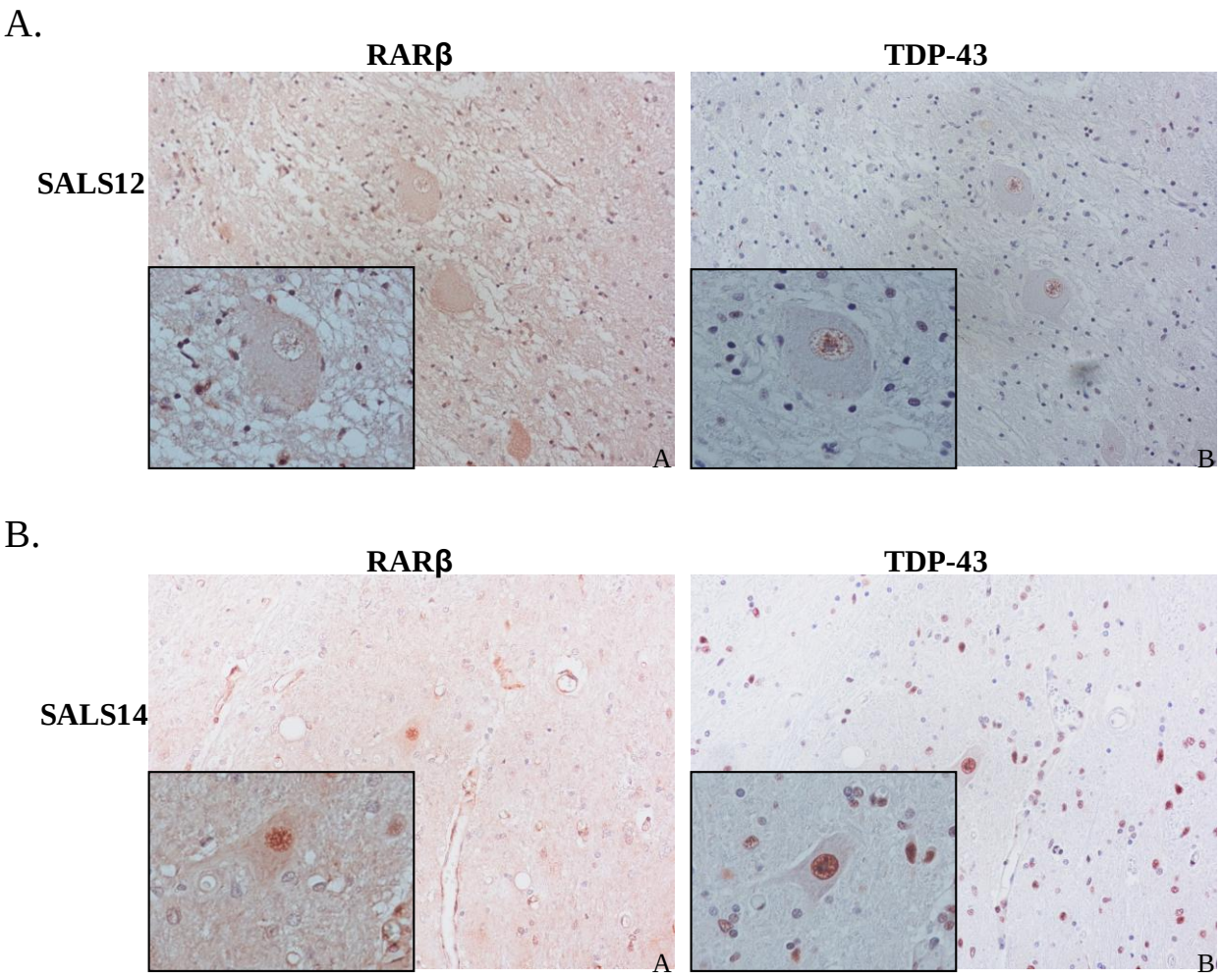
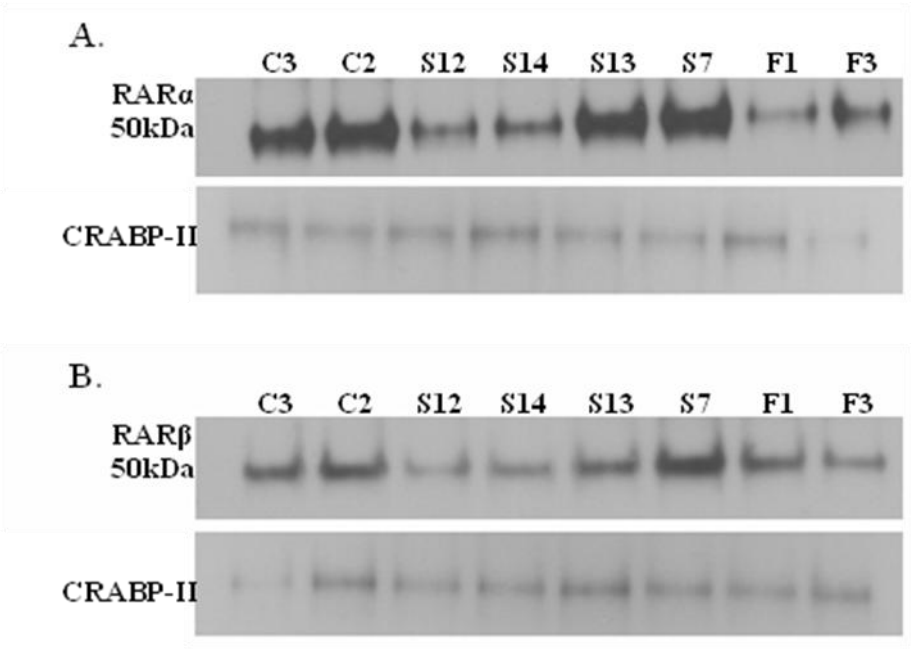


Figure 30. Immunohistochemical Analysis of RARβ and TDP-43 in Human Spinal Cord

Serial lumbar spinal cord sections from sporadic ALS cases were immunostained for RARβ and TDP-43 and representative cases are shown. Identical motor neurons were imaged as determined by anatomical hallmarks and location within the ventral horn. ***A****: Motor neurons that lacked nuclear RARβ immunostaining (****A****) were positive for nuclear TDP-43 (****B****).* ***B****: Motor neurons within the spinal cord with nuclear RARβ immunostaining (****A****) also had nuclear TDP-43 immunostaining (****B****). Insets represent a high power magnification of each panel. Original magnifications: 200X; 400X (insets).*

3.4.3 Protein interactions between CRABP-II and the nuclear receptors do not differ between ALS and control groups

I next investigated interactions between cytoplasmic components of this signaling pathway and the nuclear receptors in lumbar spinal cord tissue. More specifically, I examined protein-protein interactions between CRABP-II and either RARα or RARβ to determine the functional consequences of CRABP-II localization to the nucleus in ALS. I immunoprecipitated CRABP-II from nuclear-enriched fractions and determined whether the nuclear receptors co-immunoprecipitated with CRABP-II. I observed an interaction between CRABP-II and both RARα (Figure 31A) and RARβ (Figure 31B) although there was no significant difference between ALS and control groups (Figure 31C; $p = 0.9042$ for RARα in ALS versus controls; $p = 0.8881$ for RARβ in ALS versus controls).



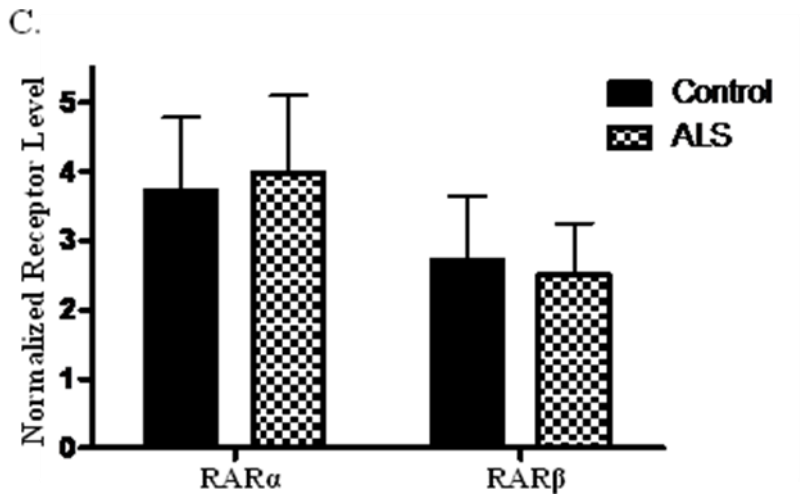


Figure 31. Protein-Protein Interactions in Nuclear-Enriched Fractions from Human Lumbar Spinal Cord

*Protein extracts from ALS and controls were prepared as described in the Materials and Methods. Protein (500 μg) from each extract was immunoprecipitated with CRABP-II and resulting blots were probed with antibodies specific to RARα (**A**) or RARβ (**B**). **A**: Immunoblots of nuclear-enriched fractions from lumbar spinal cord co-immunoprecipitated with CRABP-II and probed for RARα. **B**: Immunoblots of nuclear-enriched fractions from lumbar spinal cord co-immunoprecipitated with CRABP-II and probed for RARβ. **C**: Graph representing the results from densitometry quantification. Receptor levels were normalized to that of CRABP-II for each sample. Black bars represent control cases (n = 2) and checkered bars are ALS cases (n = 6). Statistical analyses were performed using an unpaired t-test with a 95% confidence interval. The p values for RARα and RARβ were 0.9042 and 0.8881, respectively. Letters and numbers correspond to patients in Table 5.*

3.4.4 Retinoic acid response element binding in spinal cord tissue homogenates is increased in ALS as compared to controls

Finally, I examined the DNA-binding activity of nuclear-enriched fractions from ALS patients and controls as a measure of RAR functional activity. Electrophoretic mobility shift assays (EMSAs) were performed using nuclear-enriched fractions from spinal cord tissue. Nuclear extracts were incubated with [α-^{32}P]-labeled probes corresponding to the consensus RAR binding element (RARE), and retardation in protein mobility due to DNA-protein interaction was visualized by autoradiography.

I first demonstrated that nuclear RARE binding could be detected using increasing amounts of spinal cord extract (Figure 32A). Numerous bands exhibit increased intensity with increasing amounts of nuclear extract. I then used competition with either unlabeled consensus or mutant probes to determine the specificity of the bands. The unlabeled consensus probe but not the mutant oligo was able to compete specific bands (data not shown). I next examined nuclear fractions from patients for RARE DNA-binding activity (Figure 32B). Bands representing the protein:DNA complex indicative of binding at the RARE were absent in all of the control extracts analyzed (n = 5). However, this protein:DNA complex was observed in approximately 77% (10 of 13) of patients with ALS (Figure 32B and data not shown). The nuclear extracts from subjects that lacked this band corresponded to patients that had less than 30% of motor neurons with increased nuclear RARβ (SALS18, SALS13 and FALS1 from Table 5).

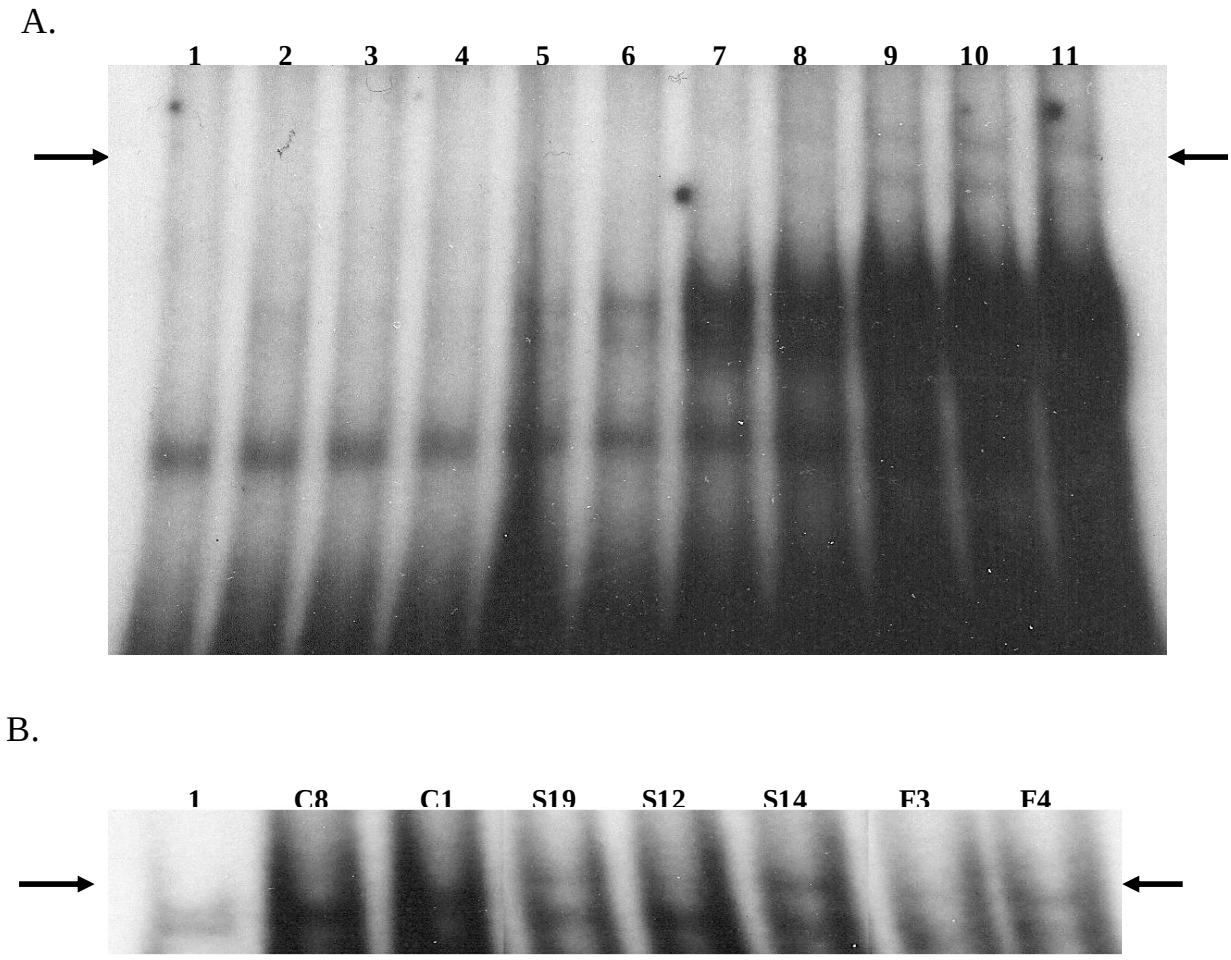


Figure 32. RARE Binding in Spinal Cord Tissue Homogenates

Nuclear-enriched fractions from lumbar spinal cord tissue of 9 sporadic ALS, 4 familial ALS and 5 controls were used for EMSA. ***A****: EMSA using radiolabeled RARE and increasing amounts of spinal cord nuclear extract. Lane assignments:* ***lane 1****, labeled probe only;* ***lanes 2*** *to* ***11****, increasing spinal cord nuclear extract (100 ng, 250 ng, 500 ng, 1 μg, 2 μg, 5 μg, 10 μg, 15 μg, 20 μg, or 25 μg).* ***B****: EMSA using radiolabeled oligo and spinal cord nuclear extracts (25 μg). Lane assignments:* ***lane 1****, labeled probe only;* ***lanes 2*** *to* ***3****, control cases (Con8 and Con1 from Table 5);* ***lanes 4*** *to* ***6****, sporadic ALS cases (SALS19, SALS12 and SALS14 from Table 5);* ***lanes 7*** *to* ***8****, familial ALS cases (FALS3 and FALS4 from Table 5).*

3.5 DISCUSSION

The retinoid signaling pathway is critical for neuronal development with increasing evidence that it has equally important functions in adulthood. Although gene expression studies suggest that the RA signaling pathway and genes regulated by this pathway are altered in ALS, the role of this pathway in ALS remains unclear. The goal of the current study was to examine cytoplasmic binding proteins and nuclear receptors of the retinoid signaling pathway in lumbar spinal cord motor neurons of control and ALS patients. Interestingly, I noted differences between sporadic and familial forms of ALS. More specifically, I observed increased nuclear immunoreactivity for both CRABP-II and RARβ in motor neurons of sporadic ALS patients. In sporadic ALS cases with increased CRABP-II in motor neuron nuclei, 85% (17 of 20) also had increased nuclear RARβ in motor neurons. However, I did not detect any differences with respect to protein-protein interactions between CRABP-II and either of the two nuclear receptor isotypes detected in motor neurons (RARα or RARβ). In terms of binding at the nuclear response element, I observed increased binding in ALS patients. My data indicates that there is increased nuclear targeting/retention of RARβ with a functional impact on transcription. The lack of RARβ nuclear immunostaining in familial ALS suggests differences in underlying disease mechanism between familial and sporadic forms of the disease. However, further studies are necessary to validate these initial findings.

I did not observe alterations between ALS and controls with respect to CRABP-II and nuclear receptor interaction via co-immunoprecipitation. Due to low cellular CRABP-II levels and multiple RAR binding partners, the reverse co-immunoprecipitation experiment was not successful. I suggest that downstream interactions between RA (ligand), RARβ (receptor) and

nuclear response element (RARE) may explain the altered subcellular distribution we observed in motor neurons of sporadic ALS patients rather than upstream protein-protein interactions.

Although RA signaling has been shown to mediate apoptosis in a variety of cells [249, 253-255], this pathway has also been shown to inhibit apoptosis in specific cell types [239, 240, 242]. I propose that redistribution of RARβ to motor neuron nuclei represents a regenerative response as RARβ has been shown to mediate neurite outgrowth and neurotrophic effects of RA [252]. The observed negative correlation between nuclear RARβ and TUNEL or activated caspase-3 suggests that RA signaling through RARβ is a pro-survival response. In fact, RARβ-mediated pro-survival and regenerative responses have been reported in models of spinal cord and axonal injury [228, 230, 256, 257]. More specifically, neurite outgrowth and functional recovery were demonstrated from peripheral nervous system (PNS) tissue (dorsal root ganglia) following laminectomy [228]. In the adult rat spinal cord, neurite outgrowth could be stimulated by transfection with RARβ2, one of the RARβ isoforms [230].

The downstream effects of RA occur via an RAR isotype- and isoform-specific manner as nuclear receptor diversity is at least partially responsible for the pleiotrophic effects of RA. Several genes known to be modulated by RA have been implicated in motor neuron disease including various cytokines, growth factors and the amyloid precursor protein (APP) [258]. Studies in ALS patients indicate that APP is overexpressed in deltoid muscle biopsies and that Aβ42 accumulation occurs in lumbar spinal cord motor neurons [259, 260]. Amyloid deposition and plaque formation have been reported in post-mortem brain and spinal cord tissues of ALS patients [261]. In addition, matrix metalloproteases (MMPs), which are modulated by RA, have been shown to be increased in both the serum and cerebrospinal fluid of ALS patients [262-264]. Treatment with an MMP-inhibitor extended survival in the transgenic G93A mutant SOD1

mouse model of ALS [265]. In addition, the more rapid non-genomic effects of RA signaling and interaction of retinoid signaling proteins with mitochondria further link retinoid signaling with mechanistic pathways associated with ALS. The interplay between these proteins and pathways and their connection to ALS warrants further investigation. The downstream signaling cascades initiated by RARβ in motor neurons are also of great interest as they may provide novel insights into the molecular mechanisms underlying ALS.

The intracellular distribution of the RARs is an indicator of their transcriptional activity [266, 267]. This localization is believed to be determined, in part, by the presence of ligand. Previous reports suggest that approximately 80% of unliganded RARβ was localized to the nucleus while over 95% of fluorescent-tagged RARβ translocated to the nucleus in the presence of RA [268]. Moreover, nucleocytoplasmic shuttling of RARβ is energy-mediated and shuttling out of the nucleus was prevented by co-expression of RXR. Although both ligand binding and receptor heterodimerization are required for transcriptional activation, only heterodimerization is necessary to ensure proper binding to retinoic acid response elements. Based on the tissue-specific expression of the RXRs [269], I expect RXRβ (expressed in most tissues) and possibly RXRγ (expressed mostly in muscle and brain) to heterodimerize with RARβ in the spinal cord. I did not observe differences in the localization of RXRβ in spinal cord motor neurons between control and ALS subjects (data not shown). Additional studies are required to define the nuclear receptor binding partners of RARβ in motor neurons. However, it is possible that, in the absence of RA, RARβ remains bound to the response element within the gene promoter where it functions as a strong transcriptional repressor [161].

Recent studies have demonstrated that the nucleic acid binding proteins TDP-43 (43 kDa TAR DNA-binding protein) [270, 271] and FUS (fused in sarcoma) or TLS (translocation in

liposarcoma) [56, 272] exhibit altered nucleocytoplasmic distribution in motor neurons of many ALS patients. Therefore, I examined lumbar spinal cord motor neurons from sporadic ALS patients to determine if increased nuclear RARβ occurs in motor neurons harboring TDP-43 inclusions. We failed to co-localize increased nuclear RARβ in motor neurons with cytoplasmic TDP-43 inclusions suggesting that these two pathologies occur independently.

Prior studies to investigate the role of retinoid signaling proteins, RARs and RXRs in chronic spinal cord degeneration failed to identify the alterations in subcellular distribution described in this report (comparison outlined in Figure 33). For example, Jokic and colleagues observed diffuse RARα and RARβ immunostaining in lumbar spinal cord motor neurons at pre-symptomatic stages which declined in end-stage disease in a rat model of ALS [205]. A similar decline in RARα immunoreactivity was reported in rats fed a vitamin A-deficient diet [206]. Although I was unable to perform a longitudinal study of RARs in human tissue samples, I did not observe a difference between ALS and control subjects with respect to the level or subcellular localization of RARα. I believe this is due, at least in part, to mechanistic differences between the transgenic SOD1 animal models of ALS and the human disease. Such differences in basic biologic pathways between ALS animal models and the human disease may help explain the many failed human clinical trials when the drug candidate successfully modified the disease in animal models. While acknowledging the limitations to this human tissue-based study, I feel that observations made in ALS tissue samples are vital and necessary to help interpret findings in transgenic models and facilitate future *in vitro* studies.

I also acknowledge that protein extraction from spinal cord tissue includes glial cells that contribute to both the immunblotting and DNA-binding studies. However, these cells impact motor neuron survival [273] and are able to respond to RA signaling [274]. In fact, cells within

the white matter were immunopositive for RARα and RARβ suggesting that they were responding to the disease process. The issue of cell type heterogeneity will be addressed in future studies using a combination of laser capture microdissection and quantitative real-time polymerase chain reaction to determine protein and mRNA levels on an individual cell basis.

The shared clinical and pathological features of sporadic and familial forms of ALS are well-established and indicate common underlying disease mechanism(s). Most unexpectedly, my study of the retinoid signaling pathway has identified an instance in which sporadic and familial forms of the disease may differ. In particular, altered localization of two key components of the retinoid signaling pathway (CRABP-II and RARβ) were observed in spinal cord motor neurons of patients with sporadic ALS. This difference in localization also appeared to translate to an increase in binding at the RARE in spinal cord extracts in ALS. Increased nuclear RARβ in lumbar spinal cord motor neurons may increase the ability of these cells to survive in the spinal cord microenvironment during ALS as it did not correlate to markers of apoptosis. My findings add to the growing amount of evidence supporting a role for retinoid signaling in the pathophysiology of ALS. Additional work is required to elucidate the exact function of the aforementioned nuclear receptors in motor neuron survival and death and to determine if pharmacologic modulation of specific receptor activity (i.e., stimulating RARβ-mediated signaling) can protect motor neurons and serve as effective therapeutic targets.

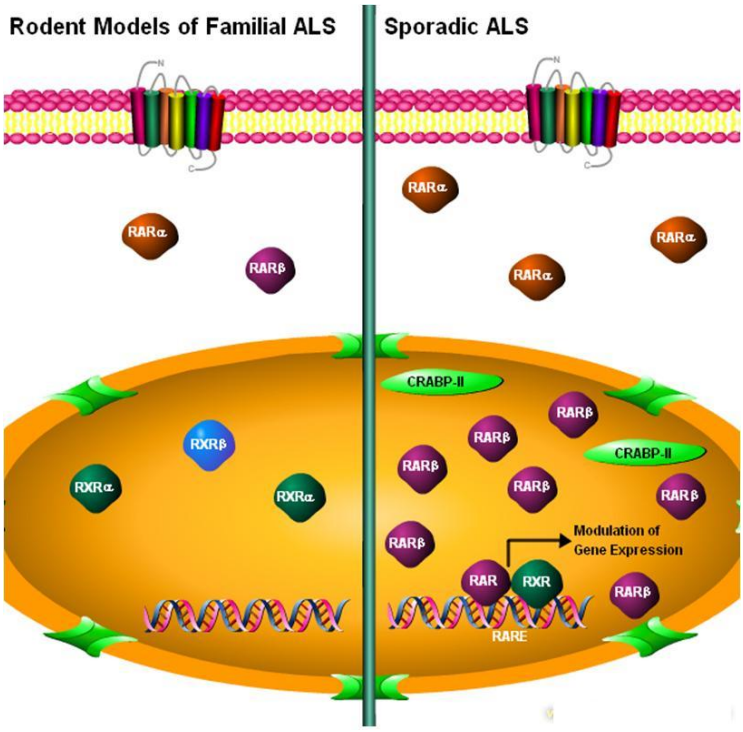


Figure 33. Schematic Representation of Retinoid Signaling in Familial and Sporadic ALS

In rodent models of familial ALS, an increase in RXRα has been noted along with declines in both RARα and RARβ in end-stage disease. RXRβ has also been detected in the lumbar spinal cord. In the current study, I noted an increase in the nuclear localization of RARβ in patients with sporadic ALS. Nuclear CRABP-II and cytoplasmic RARα localization were also observed. Finally, spinal cord tissue homogenates from patients with ALS were able to bind at the consensus RARE suggesting modulation of downstream gene expression. Pharmacologic agents that stimulate this receptor may be promising therapeutic targets that could delay the motor neuron cell death that occurs in ALS, thereby slowing the progression of this devastating disease.

4.0 RETINOIC ACID RECEPTOR-MEDIATED SIGNALING IN MOTOR NEURONS

4.1 ABSTRACT

The work of our group and others suggests that alterations in the retinoid signaling pathway may contribute to the motor neuron cell death that occurs in ALS. More specifically, the increase in nuclear localization of RARβ in motor neurons of patients with sporadic forms of ALS (described in Chapter 3) warrants further investigation to determine the functional significance of this alteration. As the RARs are well-established and critical transcription factors, the need for a system in which the subcellular localization and downstream effects of these proteins can be evaluated in longitudinal studies is necessary. For these endeavors, I established a primary motor neuron-enriched cell culture system. Initially, mRNA and protein expression were characterized with polymerase chain reaction (PCR) and immunofluorescence, respectively. Hydrogen peroxide (H_2O_2) was then used as a model of oxidative-induced cell damage to determine the effects of modulating the retinoid signaling pathway on motor neuron cell survival/death. Both RARα and RARβ were expressed in this *in vitro* system as was observed in human lumbar spinal cord. RARα was localized to processes while RARβ localization was observed at the cell body and nucleus. When motor neurons were cultured in the absence of retinoic acid (RA), cell viability was decreased and increased cell death was demonstrated in the

presence of toxic insult. Pre-treatment with a pan-RAR or RARβ-specific agonist decreased motor neuron cell death associated with oxidative injury/stress while an RARβ-specific antagonist resulted in increased cell death. Delays in motor neuron cell death were also observed when these agonists were administered after H_2O_2 treatment. Modulation of downstream targets of the RARs (specifically RARβ) has been confirmed using quantitative PCR. My results suggest that signaling through RARβ can promote motor neuron cell survival in the presence of a toxin relevant to ALS.

4.2 INTRODUCTION

4.2.1 Retinoid Signaling and Limitations of Human Study

The genomic effects of the retinoid signaling pathway are mediated by the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) [212, 213]. These nuclear receptors function as ligand-activated transcription factors, modulating gene expression via binding at retinoic acid response elements (RAREs) in the promoter regions of downstream genes [161]. Although both all-*trans* retinoic acid (ATRA) and 9-*cis*-retinoic acid (9-*cis*-RA) serve as ligands, ATRA is the predominant isomer and activates only the RARs (9-*cis*-RA activates both RARs and RXRs). This signaling pathway is further outlined in Figure 23 and in Chapter 3.

While the alterations in the retinoid signaling pathway observed in ALS patients discussed in Chapter 3 suggest a role for this pathway in ALS disease pathogenesis, the clinical samples analyzed provide only a snapshot of the motor neuron degeneration and death that occurs at end-stage ALS as post-mortem samples were utilized. As this remains a complex and

incompletely understood process, further characterization of retinoid signaling, particularly in the context of motor neuron cell biology, is required. More specifically, deciphering the impact of the RARs on motor neuron cell survival/death and the downstream genes modulated by each of the RAR isotypes is of interest. For these studies, primary motor neuron-enriched cultures from rats offered many advantages including: 1.) increased embryo size as compared to mice; 2.) established protocols for isolation and culture; and 3.) the option to utilize transgenic animals (i.e., mutant SOD1 and mutant TDP-43 animal models).

4.2.2 Oxidative Stress

Motor neuron damage as a result of oxidative stress is a key hypothesis underlying ALS. The fact that oxidative damage increases with age is consistent with the average middle-life onset of the disease. In addition, several studies have confirmed the presence of elevated oxidative metabolism in ALS including the detection of increased biochemical markers of oxidative injury in post-mortem samples from patients [275]. Increased lipid peroxidation products have also been detected in both the plasma [276, 277] and CSF [278] of ALS patients. In mutant SOD1 (mSOD1) transgenic mice, elevated levels of protein and lipid oxidation at pre-symptomatic and post-symptomatic stages has been observed [279], and mSOD1 can catalyze the production of reactive oxygen species (ROS) like superoxide anions (O_2^-), peroxynitrite ($ONOO^-$) and hydroxyl radicals (OH-) [9].

Oxidative stress may also overlap with other mechanisms thought to contribute to ALS. For example, excitotoxicity results in increased levels of intracellular calcium which leads to increased nitric oxide (NO) formation. The reaction of superoxide anions (O_2^-) with nitric oxide generates peroxynitrite (ONOO-) which can lead to oxidative damage [280]. Nitration may also

target neurofilament proteins, disrupting their phosphorylation status and negatively affecting axonal transport [281].

Connections between vitamin A and its derivatives and oxidative stress are suggested by a number of studies including those describing their role as antioxidants and radical scavengers [282, 283]. For example, RA has been shown to reduce susceptibility to oxidative stress in a variety of cell types [239-242] and treatment with RA reduced oxidative stress and apoptotic damage in primary cultures from neonatal rat hippocampus [243]. When vitamin A is depleted, the resulting mode of cell death has been characterized by increased ROS formation [284, 285] and activation of poly-(ADP-ribose) polymerase (PARP)-1, a nuclear protein that acts as a sensor of DNA damage [286] and regulator of transcription, cell cycle and cell death [287]. In addition, inhibition of RA synthesis in human astrocytoma cells suggests that glutamate metabolism, lipid metabolism, mitochondrial function and oxidative stress responses are altered in the absence of RA [288]. Taken together, these studies suggest that RA plays a role in the antioxidant defense system and deficiencies can exacerbate or cause cell damage/death.

4.2.3 Study Objectives

As described in Chapter 3, we have observed alterations in the retinoid signaling pathway in patients with ALS. The increase in the amount of CRABP-II and RARβ localized to the nucleus of motor neurons of sporadic ALS patients coupled with an increase in RARE binding in ALS patients suggests that the impact of these alterations occurs at the level of transcription. Therefore, we sought to establish a model system with which to further study the retinoid signaling pathway and its impact on motor neuron survival/death. In particular, we sought to

investigate the functional consequences of modulating the RARs and the ability of these agents to promote motor neuron survival in the presence of a toxic insult relevant to ALS.

4.3 MATERIALS AND METHODS

4.3.1 Motor Neuron-Enriched Culture System

Motor neurons were isolated from the spinal cords of Sasco Sprague-Dawley rat embryos at gestation day 14 (E14) as previously described [289-291]. Briefly, following removal of the spinal cords, tissue was trypsinized and the dissociated cells spun over a metrizamide density gradient. Cells above the metrizamide layer (larger, dense motor neurons) were collected and brought up in serum-free growth medium. Neurobasal base media (Invitrogen, Carlsbad, CA) was supplemented with B27 (variations +/- RA) (Invitrogen), BDNF, CNTF and GDNF (Peprotech, Rocky Hill, NJ), and as described [289, 290]. Motor neurons were plated in 24-well culture trays coated with poly-D lysine (PDL) and then human placental laminin (HPL) at a density of approximately 12,000 cells/well. Cultures were incubated at 37^{o}C at 5% CO_2 and half of the culture supernatant exchanged with fresh medium after 24 hours and every 2 to 3 days thereafter.

4.3.2 RA Pathway Modulators

All-*trans* retinoic acid (ATRA) was obtained from Sigma (St. Louis, MO). Adapalene (RARβ and RARγ agonist) and LE-135 (RARβ antagonist) were obtained from Tocris Bioscience

(Ellisville, MO). Stock solutions were prepared in dimethyl sulfoxide (DMSO) such that a constant concentration of 0.1% DMSO was present for all treatment groups. Motor neuron-enriched cultures were either pre-treated with these agents for approximately 1 hour prior to the addition of hydrogen peroxide (H_2O_2) or treated with these agents approximately 30 minutes after the addition of H_2O_2.

4.3.3 RNA Extraction

Motor neurons were plated on 100-mm dishes coated with PDL and HPL as described above. Cells were cultured in media with and without RA in the B27 supplement. For RARβ stimulation, cells were treated with 2.5 μM adapalene for 0, 4 or 6 hours prior to RNA extraction. After a brief wash with PBS, RNA was extracted using the Qiagen RNeasy Kit (Valencia, CA) per the manufacturer's instructions. RNA (1.0 μg) was reverse-transcribed to complementary DNA (cDNA) using SuperScript III reverse transcriptase (Invitrogen) prior to analysis by real-time PCR. Products were resolved on 1.5% agarose gels and visualized with ethidium bromide.

4.3.4 Quantitative Polymerase Chain Reaction

Reactions were carried out in triplicate using 2.5 μL of cDNA/reaction. RNA was analyzed using primers specific to the gene of interest (Table 6) and SYBR Green master mix (Applied Biosystems, Carlsbad, CA) in 25 μL reactions. All PCR reactions were performed using an Applied Biosystems 7300 system. Expression of cyclophilin was used as the endogenous control and RAR expression at 1 day *in vitro* in the absence of retinoic acid served as the reference

sample (1 DIV –RA). The JM2 cell line was a gift from G. Michalopoulos; RNA from these cells was used as a positive control.

4.3.5 Immunofluorescence

At the designated time points, cells were washed briefly with PBS and fixed in ice cold methanol for 2 minutes. Non-specific binding was blocked with 10% serum after which primary antibodies diluted in 2.5% serum were added for approximately 1 hour. Primary antibodies include β3-tubulin (TU-20; 1:300; mouse monoclonal; Santa Cruz Biotechnology, Santa Cruz, CA), glial fibrillary acidic protein (GFAP; 1:250; mouse monoclonal; Sigma), RARα (C-20; 1:300; rabbit polyclonal; Santa Cruz Biotechnology) and RARβ (C-19; 1:300; rabbit polyclonal; Santa Cruz Biotechnology). After a brief wash with PBS, fluorophore-conjugated secondary antibodies (goat anti-mouse Alexa Fluor 555 and goat anti-rabbit Alexa Fluor 488) diluted in 2.5% serum were added for approximately 1 hour and DAPI (4', 6-diamidino-2-phenylindole) used as the nuclear stain.

4.3.6 Cell Viability/Live Cell Imaging

After 24 hours in culture, motor neurons were treated with the RA pathway modulators described above (ATRA final concentrations of 1.0, 10 or 100 μM; adapalene final concentrations of 0.25, 2.5 or 25 μM; LE-135 final concentrations of 1.0, 10 or 100 μM) in combination with 400 μM H_2O_2. Propidium idiodide (PI) was added at a final concentration of 1 μL/500 μL medium. Cells were kept in 24-well plates in a previously described time-lapsed microscopic imaging system (Automated Cell, Pittsburgh, PA) where bright-field and fluorescent images were

acquired every 10 minutes [292]. These images were analyzed using NIH Image J software and the number of cells in the bright-field and PI-positive cells quantified at 0, 4, 6, 12, 18, 24, 36, 48, 60 and 72 hour time points. The percentage of cell death at these time points was then determined for each treatment. For a typical experiment, each treatment was added to three wells and three separate fields averaging 10-20 cells each were counted per well. The percentage of cell death was determined for each field by binning at the indicated time points, and each experiment repeated at least twice.

4.3.7 Statistical Analysis

Comparisons between treatment groups was accomplished using two-way analysis of variance (ANOVA) followed by Bonferroni post-tests. A p value ≤ 0.05 was considered statistically significant.

4.4 RESULTS

4.4.1 Expression of RARs in primary cultures

The procedure for purifying spinal motor neurons was modified from that of Camu and Henderson [293, 294] and utilized a density gradient to separate low-density cells from the spinal cord cell suspension. The purity of these preparations was assessed after 48 hours in culture with β3-tubulin, a neuron-specific cytoskeletal marker and determined to be 79.5 ± 5.4% neuronal cells (Figure 34). The remaining cells were glial fibrillary acidic protein (GFAP)-positive

astrocytes and cluster of differentiation 68 (CD68) was not detected at either the mRNA or protein level.

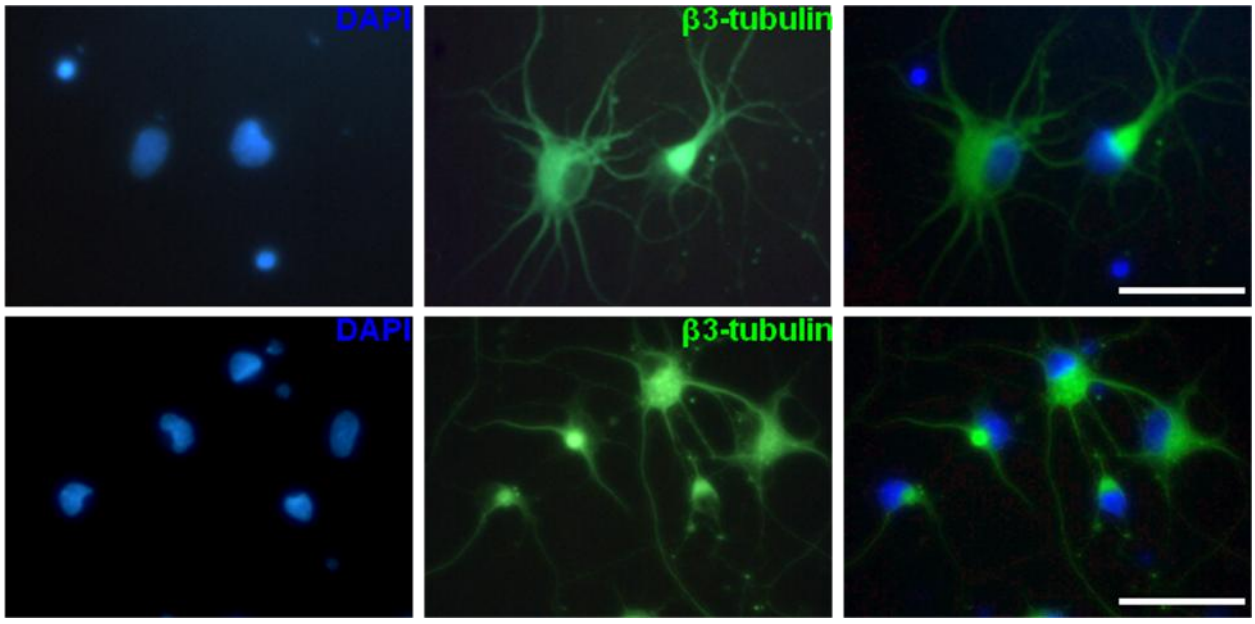


Figure 34. β3-tubulin Expression and Motor Neuron Culture Purity

Immunofluorescence images of primary motor neuron-enriched cultures established from E14 rats after 1 day in vitro (1 DIV; top panel) or 4 DIV (bottom panel). β3-tubulin was used as a neuronal cytoskeletal marker and to assess the purity of the cultures. Scale bars represent 10 μm.

Initially, it was necessary to determine which of the RARs were expressed in this *in vitro* system, and PCR and immunofluorescence were used to characterize mRNA and protein expression, respectively. Using the primers listed in Table 6, RARα and RARβ but not RARγ expression was detected (Figure 35A). When motor neurons were cultured in media with and without RA in the B27 supplement for 1, 4 or 7 days *in vitro* (DIV), the levels of these receptors differed as determined by Q-PCR. That is, expression of RARα was increased at 4 and 7 DIV even in the absence of RA (Figure 35B) while RARβ expression decreased with time (Figure 35C). The expression of these two nuclear receptors was also confirmed at the protein level

(Figure 36) with RARα localization observed in processes (Figure 36, top panel) and RARβ protein expression localized predominantly to the cell body and nucleus (Figure 36, bottom panel).

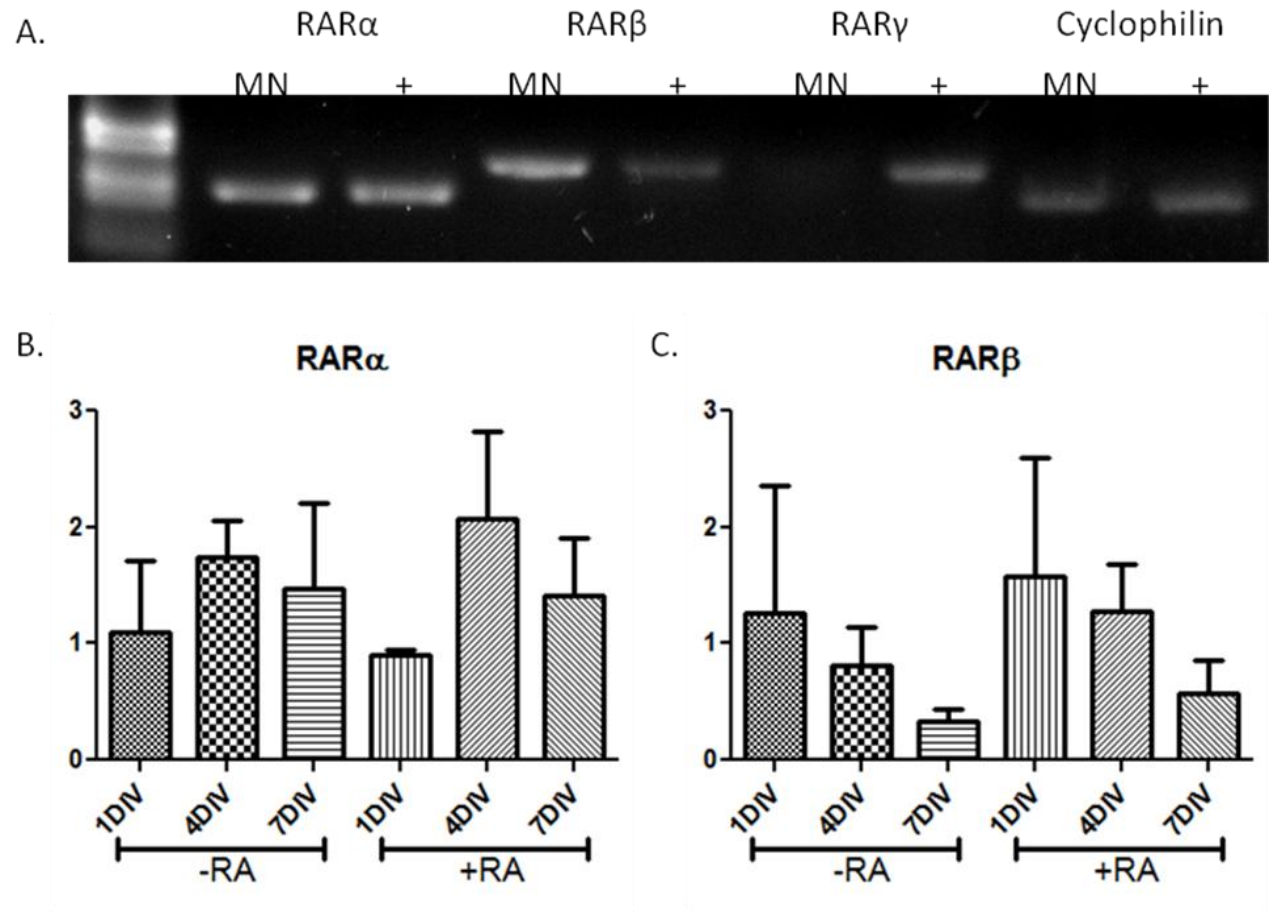


Figure 35. ***In Vitro*** **RAR Expression**

Expression of the three RAR isotypes was evaluated in the motor neuron-enriched cell culture system. ***A****: Polymerase chain reaction (PCR) analysis of RNA isolated from primary motor neuron cultures after 24 hours in culture. RARα and RARβ but not RARγ were expressed.* ***B*** *and* ***C****: Quantitative PCR (Q-PCR) analysis of RARα (****B****) and RARβ (****C****) expression at 1, 4 and 7 days in vitro (DIV) in media with and without RA. RARα expression is increased at 4 and 7 DIV while RARβ expression decreases with time and in the absence of media containing RA. RAR expression was normalized to expression at 1 DIV in the absence of RA and cyclophilin*

expression used as an endogenous control. "MN" denotes RNA from primary motor neurons; "+" denotes RNA isolated from JM2 cells used as a positive control. Error bars represent the mean ± standard deviation for triplicate wells of one experiment.

Table 6. Primers Utilized for *In Vitro* Experiments

	Forward Primer	Reverse Primer
RARα	5'-GGCTACCACTATGGGGTTAGCGC-3'	5'-GGTTCCGGGTCACCTTGTTGAT-3'
RARβ	5'-AGTCATCGGGCTACCACTATGGC-3'	5'-AGCACTTCTGCAGGCGGCA-3'
RARγ	5'-CATTTGAGATGCTGAGCCCCA-3'	5'-CATACAAAGCACGGCTTATAGACCC-3'
Cyp26a1	5'-GCAAGGACGCACTGCAGCTCTT-3'	5'-GTCCTCGTGATGGCTCTTGCAA-3'
CREB	5'-AGACAACCAGCAGAGTGGAGATGC-3'	5'-GAGACTGAATAACTGATGGCTGGGC-3'
HoxA1	5'-TAACTCCTTATCCCCTCTCCACGC-3'	5'-AACTCCTTCTCCAGCTCTGTGAGCT-3'
HoxB1	5'-AACTTTGCATCAGCCTACGATCTCC-3'	5'-GGCGCGGCTCAGGTATTTGTT-3'
Cyclophilin	5'-CCCCACCGTGTTCTTCGACA-3'	5'-CCCAGTGCTCAGAGCACGAAA-3'

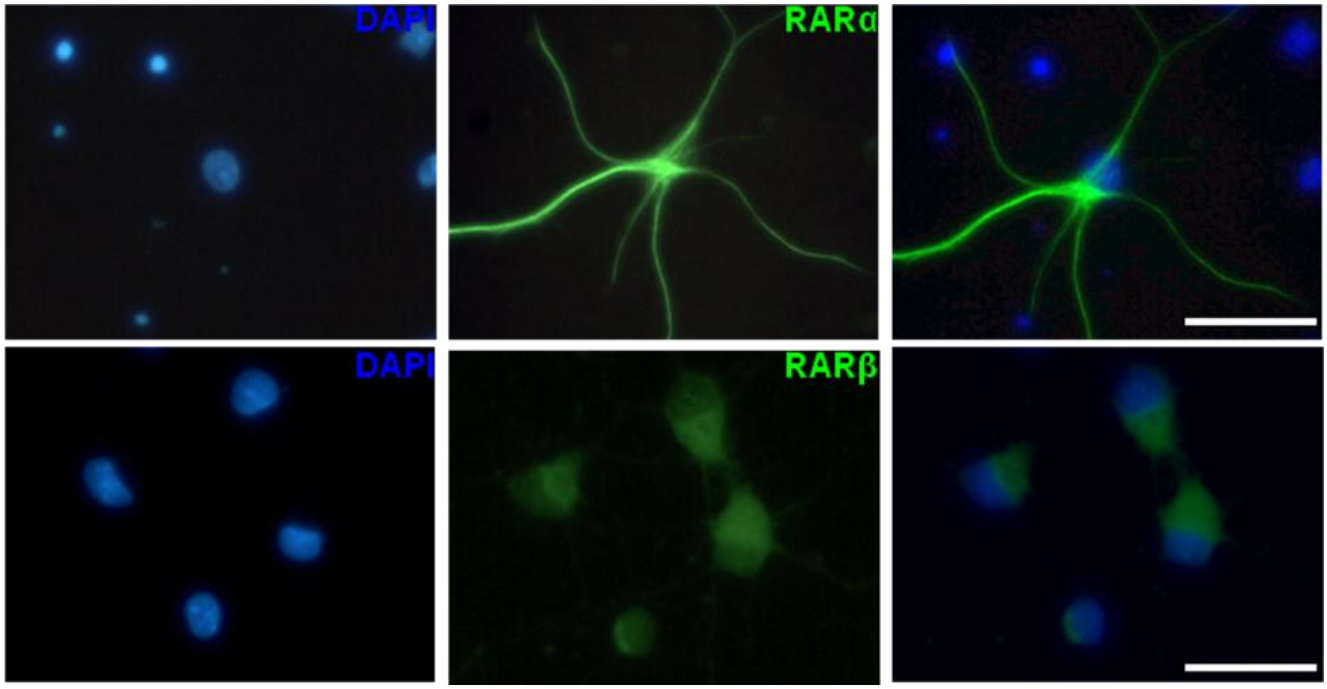


Figure 36. Characterization of RAR Protein Expression

Immunofluorescence images of primary motor neuron-enriched cultures established from E14 rats after 4 days in vitro (4 DIV). RARα (top panel) is expressed and protein localization observed in processes. RARβ (bottom panel) protein expression is localized predominantly to the cell body with some nuclear staining. Scale bars represent 10 µm.

4.4.2 Absence of RA in media results in decreased motor neuron cell viability

As vitamin A deprivation has been characterized by increased ROS formation and oxidative stress implicated in ALS disease pathogenesis, H_2O_2 was used as a model of oxidative stress/injury to determine the effect of stimulating the RARs on motor neuron cell survival. After 24 hours in culture, cells were treated with increasing concentrations of toxin and a dose-response curve established (Figure 37). The concentration used for subsequent experiments (400 µM H_2O_2) resulted in approximately 50% cell death after approximately 24 hours.

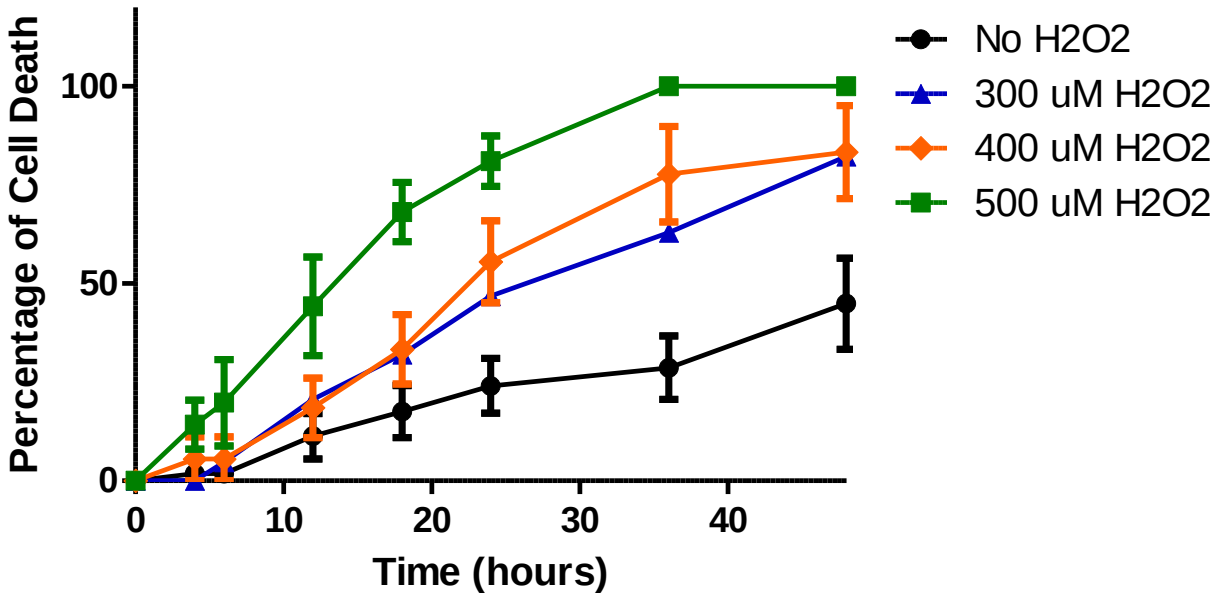


Figure 37. Hydrogen Peroxide Dose Response Curve

Motor neuron cultures were treated with various concentrations of H_2O_2 and cell death monitored using live cell imaging and propidium iodide staining over time. Subsequent experiments utilized the concentration that resulted in 50% cell death at approximately 24 hours (400 µM). Error bars represent the mean percentage of cell death ± the standard error of the mean from at least 10 motor neurons in 6 different fields from at least 2 different wells for at least 2 separate experiments.

The impact of the presence of RA in the B27 used as a supplement to the media was then evaluated. Although all other media components were included, eliminating RA resulted in decreased cell viability at the 24 hour time point and thereafter (Figure 38A) as assessed by live cell imaging/propidium iodide staining. This increased cell survival in the presence of RA was

also demonstrated in the presence of toxin as the absence of RA resulted in statistically significant increases in the percentage of cell death throughout the time course (Figure 38B).

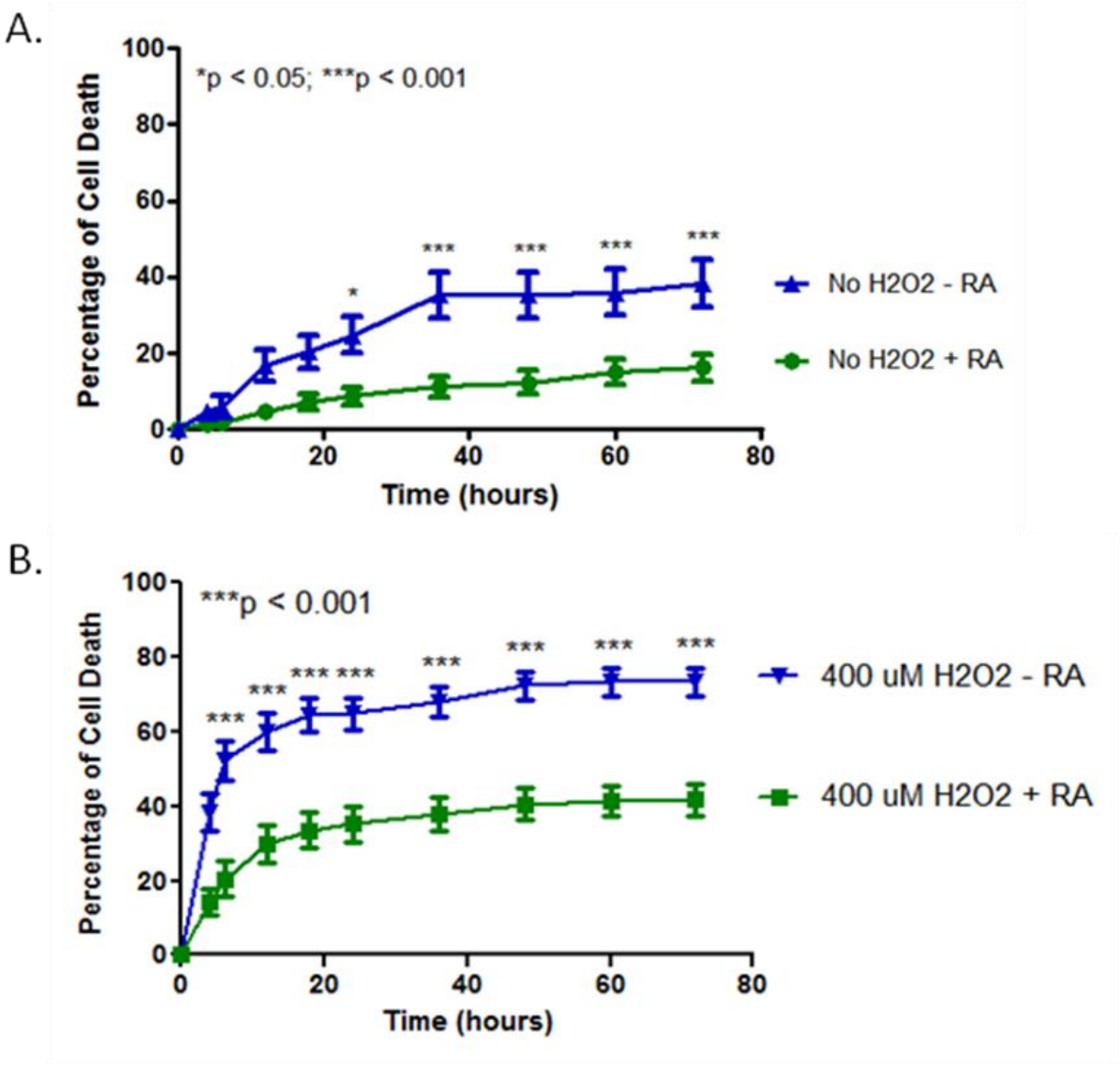


Figure 38. Decreased Cell Death in the Presence of RA

The percentage of motor neuron cell death was evaluated using live cell imaging and propidium iodide staining over 72 hours. ***A****: The presence of RA in the B27 media supplement resulted in decreased cell death as compared to motor neurons cultured in the absence of RA.* ***B****: When treated with H_2O_2 as a model of oxidative stress/injury, the absence of RA resulted in*

*significantly increased percentages of cell death. Error bars represent the mean percentage of cell death ± the standard error of the mean from at least 10 motor neurons in 6 different fields from at least 2 different wells for at least 2 separate experiments. *p < 0.05; ***p < 0.001.*

4.4.3 Stimulating the RARs significantly delays oxidative-induced cell death

The ability of ATRA, a pan-RAR agonist, to protect motor neurons from toxin-induced cell death was then evaluated. As only RARα and RARβ are expressed in this *in vitro* system, we sought to evaluate the ability of these receptors to prevent motor neuron cell death. When cells were pre-treated with 10 μM ATRA, the percentage of cell death was significantly decreased as compared to the control-treated cells (Figure 39). Protein expression for RARα and RARβ for this treatment was also assessed, and the co-localization of each of these nuclear receptors with β3-tubulin is shown (Figure 40). RARα appears to be localized to cells that lack β3-tubulin expression (Figure 40, A and B, top panels) while co-localization between this neuronal marker and RARβ is observed (Figure 40, A and B, bottom panels). There does not appear to be a difference in RAR protein localization when motor neurons are treated with ATRA.

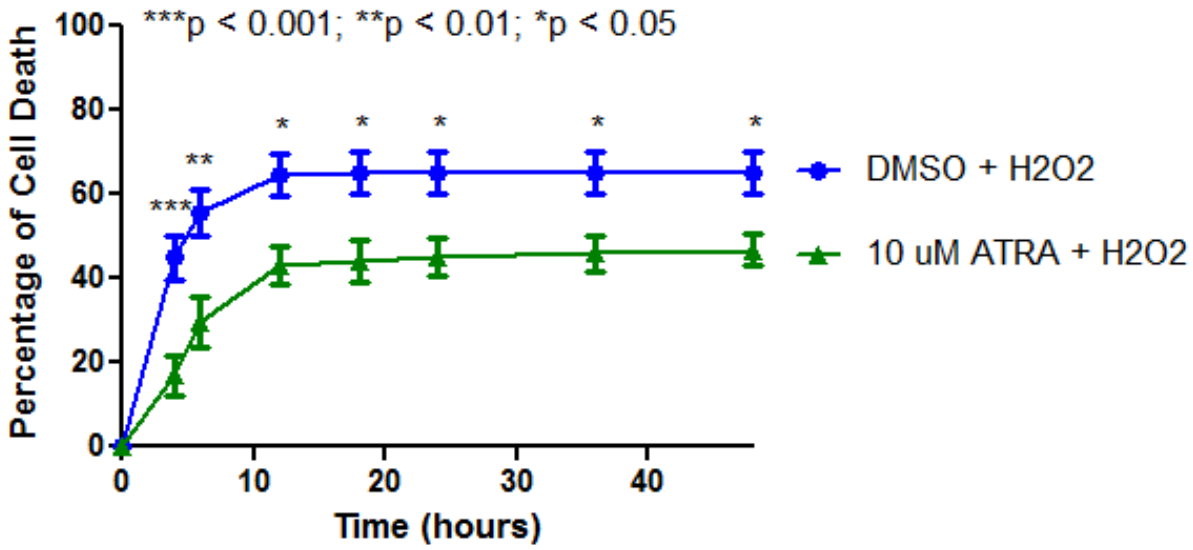


Figure 39. ATRA Treatment Decreases Oxidative-Induced Motor Neuron Cell Death

*Motor neuron-enriched cultures were pre-treated with ATRA for approximately 1.0 hour and then H_2O_2 administered to cause cell death. ATRA was able to significantly decrease the percentage of cell death as compared to the control treatment. Error bars represent the mean percentage of cell death ± the standard error of the mean from at least 10 motor neurons in 6 different fields from at least 2 different wells for at least 2 separate experiments. *p < 0.05; **p < 0.01; ***p < 0.001.*

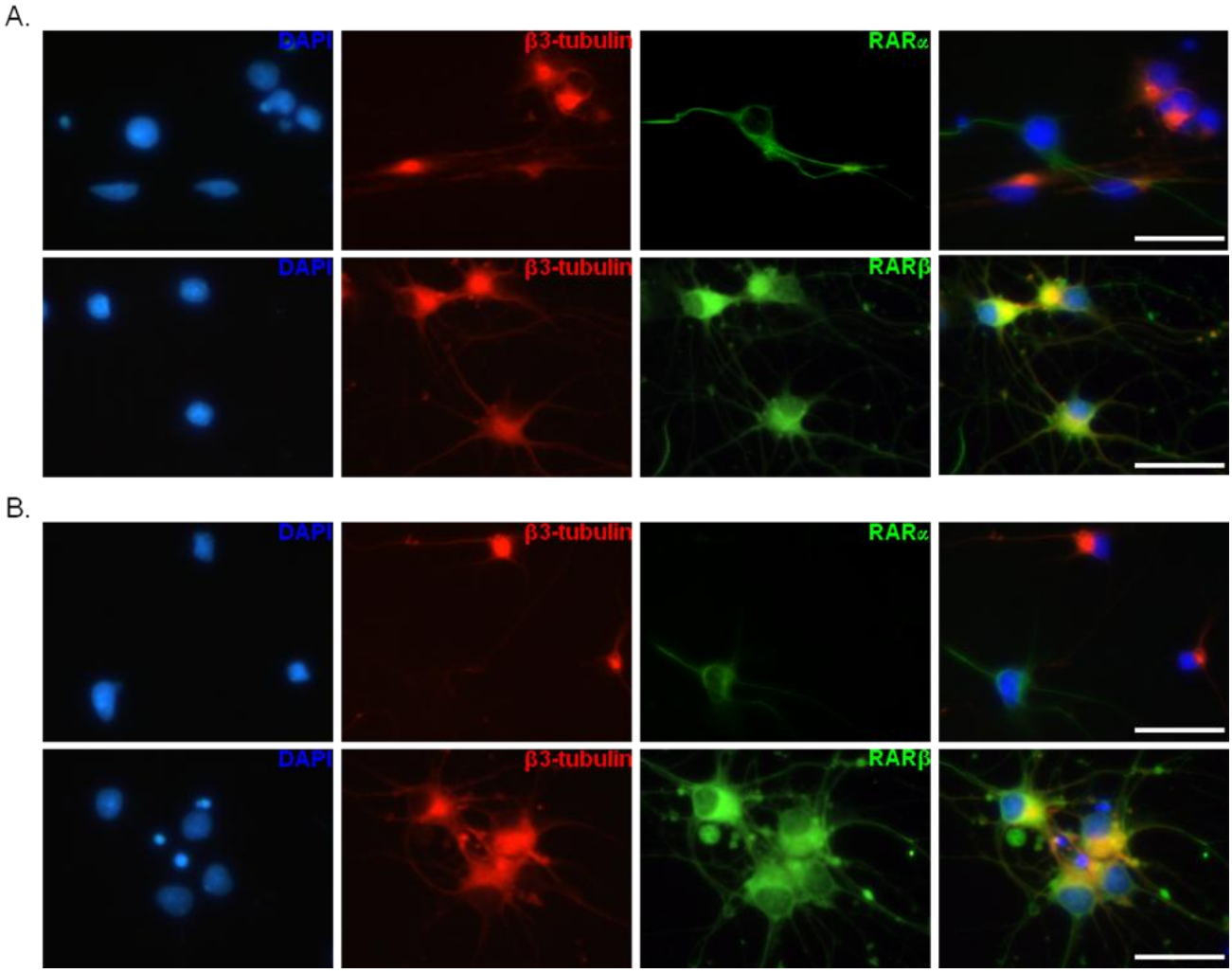
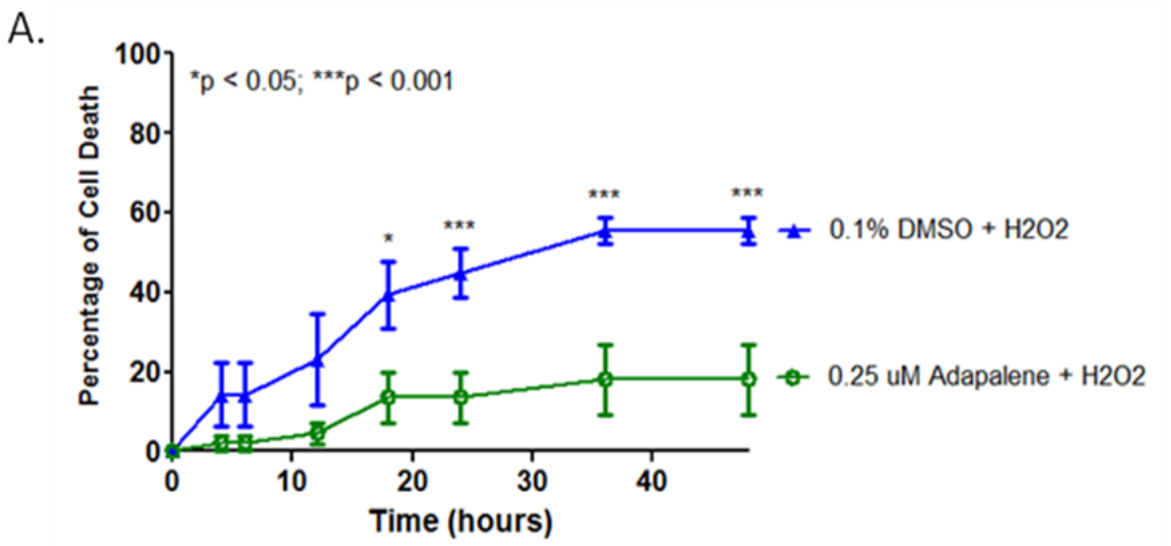


Figure 40. Localization of RARα and RARβ with and without ATRA Treatment

Motor neurons cultured in the absence or presence of ATRA. ***A****: In the absence of ATRA, RARα (top panel) and RARβ (bottom panel) protein localization was evaluated.* ***B****: When treated with 10 µM ATRA for 24 hours, RARα (top panel) and RARβ (bottom panel) protein localization was assessed. Although both nuclear receptors are expressed, RARα appears to localize predominantly to non-neuronal cells (β3-tubulin negative). RARβ and β3-tubulin co-localization is observed for the majority of cells. No difference is protein localization is observed when 0 µM are 10 µM ATRA treatments are compared. Scale bars represent 10 µm.*

Specific agonists and antagonists of RARβ were then used to determine if modulating only this receptor could recapitulate the protective effects observed with a pan-agonist that stimulated both RARα and RARβ. When pre-treated for 1.0 hour with adapalene (an RARβ (and RARγ) agonist) prior to the addition of H_2O_2, the percentage of cell death was again significantly decreased as compared to the control treatment (Figure 41A). However, when motor neuron-enriched cultures were pre-treated for 1.0 hour with an RARβ-selective antagonist (LE-135), the percentage of cell death was significantly increased over control treatment (Figure 41B).



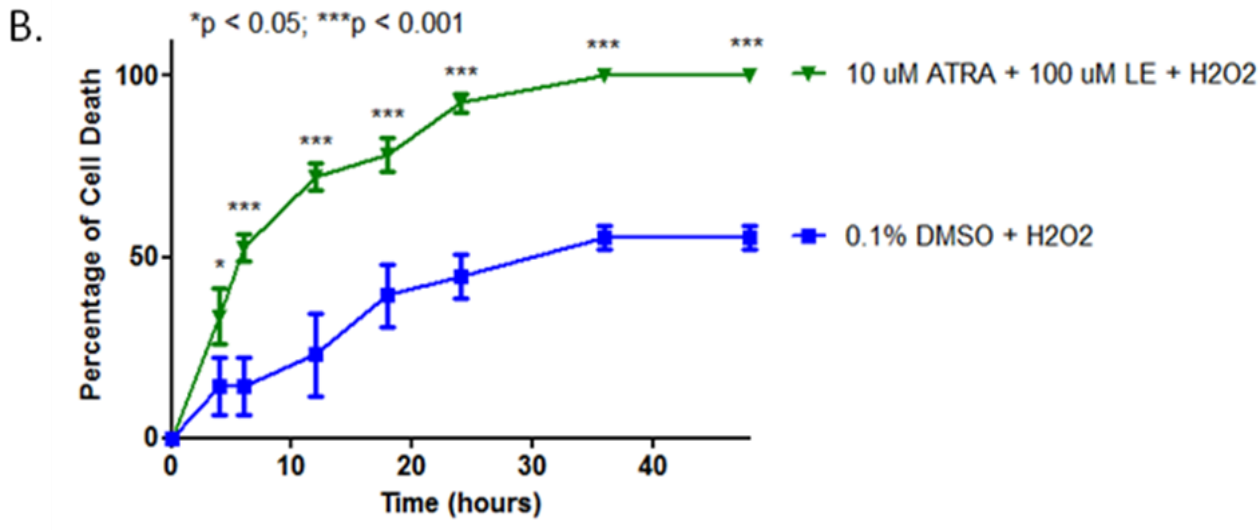


Figure 41. RARβ Modulation

Motor neuron cultures were pre-treated for approximately 1.0 hour with agents that specifically target RARβ prior to the addition of toxin. ***A****: In the presence of adapalene, an RARβ agonist, the percentage of cell death was decreased.* ***B****: When cells were pre-treated with a combination of ATRA and LE-135, an RARβ-selective antagonist, the percentage of cell death was significantly increased. Error bars represent the mean percentage of cell death ± the standard error of the mean from at least 10 motor neurons in 6 different fields from at least 2 different wells for at least 2 separate experiments. *p < 0.05; ***p < 0.001.*

To address a clinically-relevant treatment which would be administered after the initiation of disease-causing events, agonists and antagonists were administered after toxin treatment. In these experiments, there were also significant delays in motor neuron cell death at early time points for ATRA and adapalene (Figure 42). However, in the presence of the RARβ-specific antagonist, cell death was increased as compared to the control (Figure 42) indicating that cell death is exacerbated when signaling through this receptor is inhibited.

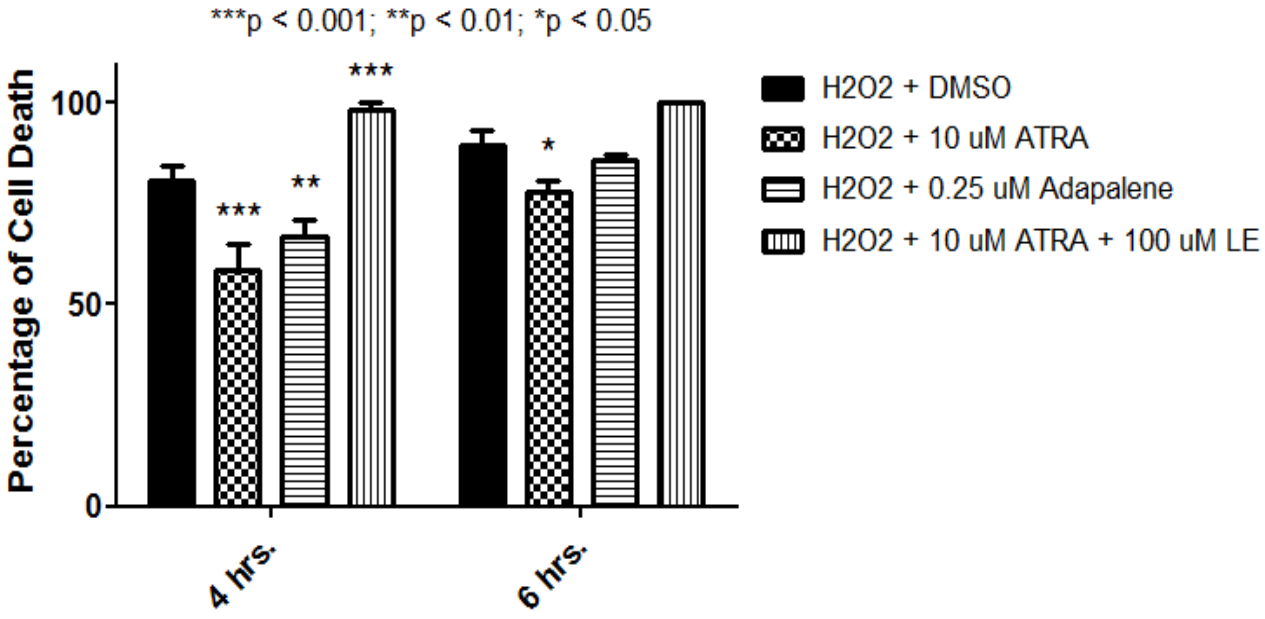


Figure 42. Treatment with RA Pathway Modulators after Toxin

*Motor neurons were treated with agonists and antagonists of the RA signaling pathway approximately 30 minutes after toxin. Cell death was monitored with live cell imaging using propidium iodide staining over time. When treated with pan-agonist (10 μM ATRA), motor neuron cell death was delayed (stippled bars). In the presence of adapalene, the percentage of cell death was also decreased (horizontal-striped bars). When an RARβ antagonist was administered following toxic insult, the percentage of cell death was significantly increased (vertical-striped bars) compared to untreated controls (solid bars). Error bars represent the mean percentage of cell death ± the standard error of the mean from at least 10 motor neurons in 6 different fields from at least 2 different wells for at least 2 separate experiments. *p < 0.05; **p < 0.01; ***p < 0.001.*

4.4.4 Downstream targets are modulated by agonists

Finally, to confirm that alterations in gene expression occurred following stimulation with RARβ agonists, mRNA expression of downstream targets of the different RAR isotypes was assessed (Figure 43; primers listed in Table 6). After 24 hours in culture, motor neurons were treated with adapalene and RNA extracted at 0, 4 and 6 hour time points. Expression of cyclic AMP (cAMP) response element-binding (CREB), a downstream target of RARβ, was increased over this time period (Figure 43). A slight increase in cytochrome P450 26A1 (Cyp26a1) expression was also observed at 4 and 6 hours. However, expression of homeobox B1 (HoxB1), a downstream target of RARα, was unchanged while homeobox A1 (HoxA1), a downstream target of RARγ, was not detected.

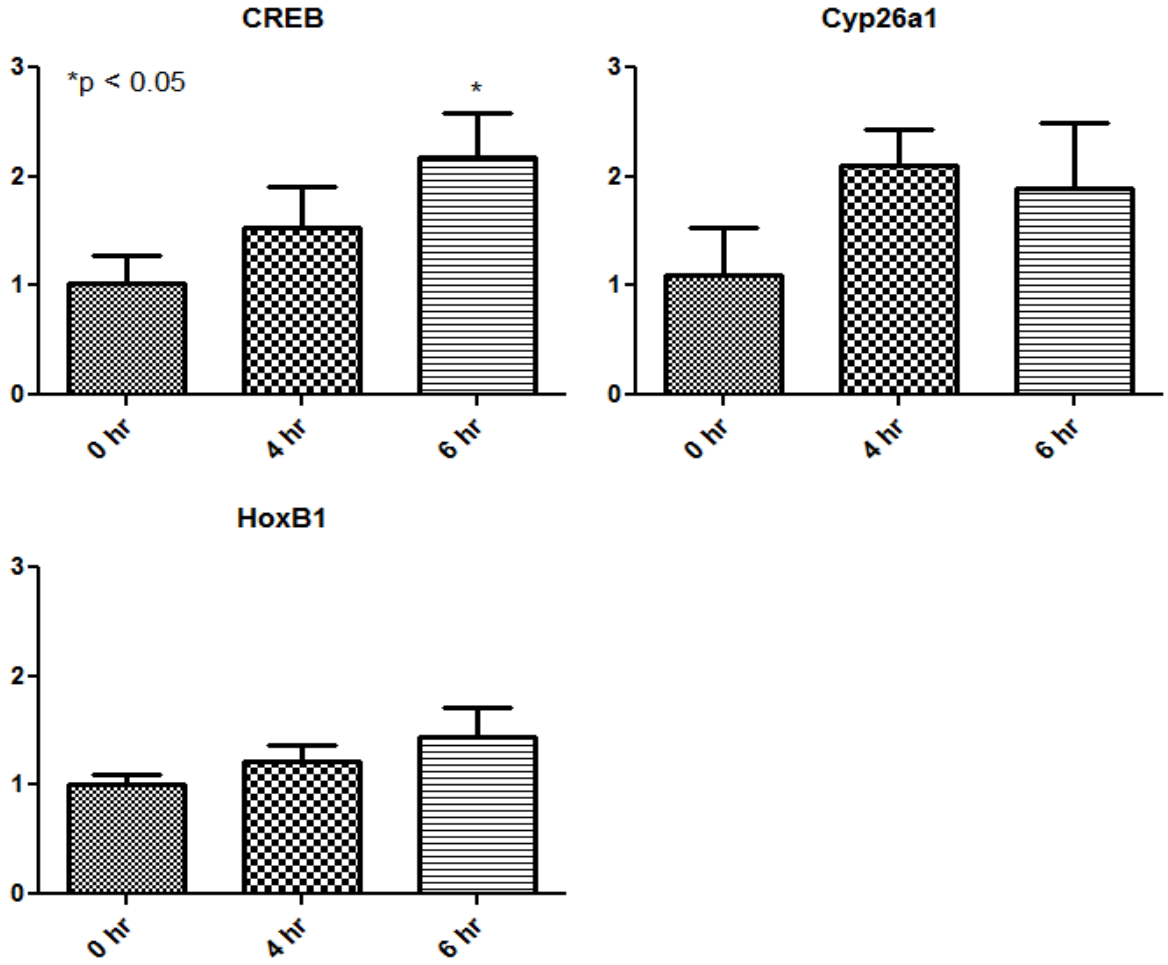


Figure 43. Q-PCR Analysis of Downstream Genes of the RARs

*Q-PCR was used to assess the expression of genes modulated by the RARs. After treatment with 2.5 μM adapalene, an RARβ-specific agonist, for 0, 4 or 6 hours, RNA was extracted and primers specific for each downstream target used to determine mRNA expression (Table 6). Expression of CREB was increased over this time period and Cyp26a1 expression was also increased after 4 and 6 hours of treatment. Expression of HoxB1 (a downstream target of RARα) did not change with adapalene treatment and HoxA1 (a downstream target of RARγ) was not detected. Error bars represent the mean ± standard error of the mean for wells run in triplicate. *p < 0.05.*

4.5 DISCUSSION

Evidence supporting a role for altered retinoid signaling in ALS continues to accumulate in both animal models of the disease [204-206] and ALS patients [199-203]. Although our work using spinal cord tissue from ALS patients and control subjects supports these findings, it is unclear how this signaling pathway contributes to motor neuron degeneration and/or death. In addition, the role of this signaling pathway, particularly the nuclear receptors, in motor neuron cell biology post-development has not been delineated. To address these unknowns, we utilized a primary motor neuron-enriched cell culture system which we combined with live cell imaging to investigate the role of the RARs which mediate the genomic effects of RA.

Initially, it was necessary to determine whether retinoid receptors were expressed in this motor neuron culture system. Obviously, it was critical that the nuclear receptor expression observed in human lumbar spinal cord was recapitulated in this *in vitro* system. This was demonstrated at both the mRNA and protein levels and suggests that this system serves as an accurate representation for the current and future studies designed to determine the impact of these nuclear receptors. Importantly, the localization of these proteins was consistent with what we have observed in human lumbar spinal cord motor neurons. That is, RARα appears to localize to processes and, to a lesser extent, the cell body while RARβ localization is observed mainly in the cell body and nucleus. The fact that RARα and RARβ were expressed by non-neuronal (β3-tubulin negative) cells is also consistent with what we have observed in human spinal cord tissue.

Interestingly, an increase in the expression of RARα was observed from 1 to 7 DIV using Q-PCR. Although we have enriched for motor neurons, GFAP-positive astrocytes account for approximately 15-20% of the cells in this system. As we have also observed RARα protein

expression predominantly in non-neuronal cells, this increase in expression is likely due to proliferation of these cells (while the population of post-mitotic motor neurons decreases as a result of cell death). The impact of RAR-mediated signaling in these cells has not been characterized extensively although a recent study in which RA synthesis was inhibited in a human astrocytoma cell line found altered expression of proteins involved in glutamate metabolism, lipid metabolism, mitochondrial function and oxidative stress response [288]. Astroglial cells have also been shown to activate RA synthesis [295] and to be RA-responsive [296, 297]. In addition, astroglia-derived RA has been shown to play a key role in glial-induced neurogenesis [298]. As astrocytes regulate the extracellular environment, stabilize cell-cell communications in the CNS and interact closely with neurons during development and in adulthood, the role of these cells can be further studied by modulating the percentage of astrocytes in co-culture with motor neurons.

The fact that motor neuron cell viability was decreased in the absence of RA was surprising considering the multitude of growth factors and supplements included in the media. However, this suggests that RA is critical for motor neuron survival and that the neuroprotective properties of RA can prevent cell death in the presence of oxidative damage. Extending these studies to include pan-RAR and RARβ-specific agonists indicates that RARβ is responsible for mediating these protective effects. As RARβ was localized predominantly to neuronal cells, this RAR isotype may more directly impact motor neuron cell survival. This protective effect may be mediated, at least in part, by cAMP-dependent signaling pathways as had been observed in adult dorsal root ganglion neurons in which expression of a particular isoform of this RAR isotype (i.e., RARβ2) modulated cAMP-protein kinase A (PKA) pathways [228]. As elevated cAMP concentrations [299-301] as well as the activation of CREB protein [302] have been

shown to correlate with an enhanced regenerative capacity in the adult CNS, RARβ-mediated gene expression may initiate or contribute to a pro-survival response. Moreover, inhibition of RARβ exacerbated motor neuron cell death, suggesting that up- or down-regulation of specific downstream targets may prevent this response. A microarray study to determine which genes are downstream of both RARα and RARβ would be useful in understanding the role of each of these RAR isotypes.

The results of other studies suggest that decreased RARα may more directly impact motor neuron cell survival at end-stage disease. For example, in post-mortem lumbar spinal cord motor neurons of patients with ALS, loss of RARα expression [206, 230] and transcriptional down-regulation of RARα have been reported in the remaining/surviving motor neurons [202]. In a rat model of ALS, this RARα down-regulation was associated with the selective loss of large ($> 600\ \mu m^2$) motor neurons. While we have not distinguished between motor neurons based on size as previously described [303], RARα is localized predominantly to non-neuronal cells in our *in vitro* system which may represent a difference between this model and what is observed in patients or animal models. Further characterization of both isotypes is worthwhile as specific effects and/or cell types are likely influenced by each.

The fact that both ATRA and adapalene could extend motor neuron survival following toxic insult suggests that these agents could be useful therapeutically. Although the effects were modest and occurred at early time points, it may be that stimulating the RARs (or RARβ specifically) may be most effective when initiated early in the disease process as has been suggested with riluzole. It is also possible that this type of treatment would best protect motor neurons that have not yet been affected by the disease. In addition, this therapeutic intervention would likely be used in combination with other pharmacologic agents such that increased RAR-

mediated signaling would be only one aspect of an optimal treatment. Although adapalene (an FDA-approved agent) has been used in humans with few and relatively minor side effects, this has been as a topical treatment for acne [304]. However, an internal adapalene delivery system has been used for the treatment of cervical intraepithelial neoplasia without side effects [305] although an ALS-relevant therapy would require an analog that could cross the blood-brain barrier. This has yet to be evaluated although a variety of RARβ agonists exist that could be modified for these purposes. An intraventricular delivery system similar to the pump utilized in the ceftriaxone trial would be another possibility.

In addition to the classical modulation of gene expression, receptors and proteins of the retinoid signaling pathway have other more rapid, non-genomic effects which could be further investigated [217, 306]. The three cytoplasmic binding proteins discussed in Chapter 3 (CRBPI, CRABP-I and CRABP-II) are expressed in this system (data not shown), and these proteins have been shown to associate with the mitochondria [224]. As the mitochondria has been suggested as a target of vitamin A regulation [285] and mitochondrial dysfunction implicated in ALS disease pathogenesis, the role of this organelle and its connection to RA signaling could be evaluated using co-localization studies.

Several additional studies concerning the differential regulation of retinoid-regulated genes support a role for this signaling pathway in the pathogenesis of ALS. For example, increased levels of the amyloid precursor protein (APP) have been detected in deltoid muscle biopsies from patients with ALS and in the muscle of G93A SOD1 transgenic mice at pre-symptomatic stages [260]. The gene that encodes APP is modulated by RA [258], and vitamin A has been shown to be anti-amyloidogenic *in vitro* [307]. In addition, expression of SOD1 (under basal conditions) and cytokines like vascular endothelial growth factor (VEGF) are modulated by

the retinoid receptors [258, 308]. Finally, we have observed decreased levels of transthyretin (TTR), a protein involved in the transport of retinol through its interaction with retinol-binding protein, in the cerebrospinal fluid of ALS patients [150]. Therefore, TTR impacts the amount of retinol available to cells of the CNS and decreased levels may translate to diminished transport function. Interestingly, RXRs have been shown to interfere with nuclear factor-kappa beta (NF-κβ) binding to DNA although this transrepression is RXR ligand-dependent [309]. The observed up-regulation of the p65 subunit of NF-κβ (RelA) in lumbar spinal cord motor neurons of ALS patients [202] may indicate increased NF-κB-dependent transcription in the absence of RXR ligand and an indirect role for the retinoid receptors in immune modulation.

These *in vitro* studies indicate the importance of retinoid signaling in motor neuron cell survival and highlight the ability of agonists of the nuclear receptors to protect motor neurons from cell death in the presence of oxidative stress/damage. Importantly, the primary motor neuron-enriched culture system utilized can be used to accurately model RAR-mediated signaling as the nuclear receptors expressed *in vitro* are the same as those observed in human lumbar spinal cord motor neurons. In addition and in combination with live cell imaging, time course studies as well as morphological assessments can be captured without the "black box" phenomenon often encountered with cell culture work. Although many additional studies can be pursued using this experimental system, the current results are encouraging. By stimulating RARβ, we were able to increase motor neuron cell survival in an environment relevant to ALS. This suggests that this nuclear receptor could be of value in the treatment of ALS most likely by delaying motor neuron degeneration and death, thereby slowing disease progression. Future drug screens to identify compounds that activate RARβ could be performed first in NSC-34 cells with confirmation in this primary culture/live cell imaging system.

5.0 FUTURE DIRECTIONS AND CONCLUDING REMARKS

5.1 ADDITIONAL TTR STUDIES

Our observations concerning oxidative post-translational protein modifications of TTR and the relation of these modifications to storage conditions are somewhat puzzling. It is difficult to rationalize how the apparent oxidation of TTR can be "reversed" to alter the amount of particular modified forms of the protein after a sample is moved from -20^{o}C to -80^{o}C. As this phenomenon was observed in CSF, it would be worthwhile to further investigate these post-translational protein modifications in spinal cord tissue homogenates from ALS patients and controls. As with the CSF studies, TTR can be immunoprecipitated from tissue extracts and analyzed via mass spectrometry. In addition to MALDI-TOF-MS, liquid chromatography-tandem mass spectrometry (LC-MS/MS) can be used for the identification of post-translational modifications to peptide fragments. High-performance liquid chromatography (HPLC) could also be used to isolate TTR prior to mass spectrometry-based analyses. It is also possible that these changes occur as a result of aggregation state and that this can be reversed by temperature. To investigate this possibility, urea could be used to denature the protein or purified TTR could be added to determine if it is retained in the aggregates.

Additional studies of the observed high molecular weight TTR species is also of interest. As protein aggregation is known to occur in motor neurons of patients with ALS, determining

the role and contribution of these TTR species to the formation of such aggregates is warranted. Following immunoprecipitation or HPLC-mediated isolation of TTR from CSF and spinal cord tissue extracts, non-denaturing gel conditions could be used to determine if molecular weights higher than expected (55 kDa for the native homotetramer) exhibit TTR immunoreactivity. In formalin-fixed human tissue, immunoelectron microscopy can be used to determine if TTR aggregates or fibrils are present and if these aggregates/fibrils are located within motor neurons. Paraffin-embedded sections could also be used for immunohistochemistry. In this case, Congo red would be used to detect fibrillar proteins enriched in the β-sheet conformation. Serial sections would then be immunostained for TTR to determine if co-localization of TTR and Congo red-labeled fibrils occurs and if this is increased in spinal cord tissue of patients with ALS as compared to controls.

Complementary studies in the animal models of familial ALS can also be pursued, and we are currently collaborating with a group in Wisconsin that investigates TTR in mouse models of AD. In these studies, our goals are to determine if TTR post-translational modifications and/or protein aggregates are detectable in different transgenic SOD1 mutant mouse models. Plasma and spinal cord tissue from animals with the G93A, G85R or H46R/H48Q mutations will be obtained at disease onset and terminal stage disease. Initially, the plasma will be analyzed via mass spectrometry to determine if the modifications observed in humans are present in the animal model. The level of total TTR protein will also be quantified using ELISA to determine if TTR is decreased in animal models of familial ALS as observed in ALS patients and if changes in total TTR protein amount occur over time. Paraffin-embedded spinal cord tissue will be used for immunohistochemistry and tissue extracts used for TTR protein isolation followed by immunoblotting and mass spectrometry.

Finally, the effect of TTR on motor neuron survival can be examined using our primary motor neuron-enriched cell culture/live cell imaging system. TTR immunopreciptated or otherwise purified from the CSF or spinal cord tissue extracts of ALS patients and healthy controls can be added to motor neurons and the effects on cell survival monitored over time. Purified recombinant TTR would serve as a control. The effects of modulating intracellular TTR levels can also be examined using a combination of adenoviral-mediated transduction and lentivirus-mediated short hairpin RNA (shRNA) gene knock-down. These studies can be performed in the presence or absence of toxic insults including H_2O_2-induced oxidative damage or glutamate-mediated excitotoxicity. The neuroprotective properties of intracellular TTR could then be evaluated (i.e., its protein chaperone function) along with the downstream signaling pathways modulated by TTR (i.e., retinoid signaling). The interaction between NF-κB-dependent and RA-mediated gene expression could be another focus of these studies as RXRs have been shown to interfere with NF-κB binding to DNA [309], and we hypothesize that extracellular TTR could impact both transcription factors (see Figure 22 above). The ligand-dependent transrepression observed between NF-κB and RXR could impact a variety of biological processes including immune modulation [309]. For example, if extracellular TTR remains in complex with RBP-retinol, the transport of retinol and subsequent cellular metabolism to 9-*cis*-RA would preserve RXR ligand availability and result in the formation of a transcriptionally inhibitory complex and down-regulation of NF-κB-dependent gene transcription. Conversely, if TTR is confined to protein aggregates and transport function diminished, the lack of RXR ligand coupled with activation of Toll-like receptors (TLRs) could lead to increased NF-κB-dependent transcription and an increased or exacerbated immune response.

5.2 TISSUE-BASED WORK

The increased nuclear localization of RARβ in motor neurons of patients with sporadic ALS is certainly worthy of further pursuit. Laser-capture microdissection and Q-PCR could be used to determine if RA-responsive genes are up-regulated in motor neurons with increased nuclear RARβ. These studies would also address the issue of isotype specificity allowing for the identification of downstream signaling cascades modulated by RARβ and providing novel insight into the role of this transcription factor in ALS and in other conditions (i.e., spinal cord and peripheral nerve injuries) in which RARβ-mediated signaling has been shown to play a role.

Obviously, additional cases should be analyzed to determine if the alterations that we have observed in sporadic cases are unique to a subset of patients and to determine if familial forms of the disease lack these changes. A critical aspect of these studies would include genotyping of all cases included in the tissue bank. As TDP-43 and SOD1 pathologies do not appear to overlap, it may be that increased nuclear RARβ is indicative of another subtype of ALS. It has been postulated that ALS comprises a spectrum of diseases, and the ability to define these subgroups could have clinical implications that impact management, treatment and outcomes.

Immunofluorescence and confocal microscopy could be used to identify the RXR isotypes expressed in motor neurons and for co-localization studies with RARβ. In addition, immunoelectron microscopy could be used to determine if components of this signaling pathway localize to particular subcellular compartments (i.e., the mitochondria) as these interactions have been observed in other cell types. Extracellular and intracellular localization of TTR can be evaluated in these patients to determine if changes in TTR correlate with altered CRABP-II and RARβ localization. Similarly, cellular (uptake) receptors as well as metabolizing enzymes can

be further investigated although our results suggest that alterations occur at the level of transcription (and not upstream).

5.3 IN VITRO STUDIES

5.3.1 Investigation of other ALS-relevant toxins

In addition to H_2O_2-mediated oxidative injury, a number of other toxins can be tested to determine if modulating the retinoid signaling pathway is protective. The most obvious choice would be to investigate glutamate-mediated excitotoxicity as this is the disease mechanism targeted by riluzole therapy. We have established dose-response curves in our preliminary studies and could utilize the available therapy alone or in combination with retinoid pathway modulators to determine if there is synergy between these agents.

CNS injury directly leads to the release of inflammatory signals [310] and RA has been shown to have direct neurotrophic effects on neurons with anti-inflammatory effects on microglia and astrocytes demonstrated in cell culture experiments [252]. More specifically, signals shown to be down-regulated by retinoids include IL-1α, IL-1β, IL-6, TNFα, IL-8 prostaglandin E_2, production of reactive oxygen species and release of lysosomal enzymes [310]. Therefore, testing the effects of immune modulation (i.e., pro- and anti-inflammatory cytokines, lipopolysaccharide) using motor neurons co-cultured with glial cells would be of interest as these supporting cells can contribute to motor neuron cell death in ALS and have been shown to respond to retinoid signaling [274].

5.3.2 Hypoxia

The oxygenation state of cultured cells is another area of interest, and we have performed preliminary experiments to determine if localization of the retinoid receptors is altered as a result of non-physiologic oxygen conditions. In particular, we evaluated the localization of RARα and RARβ in 1 DIV motor neurons cultured in the presence or absence of RA after 0, 6, 24, 48 or 72 hours in a 0% oxygen chamber. In these studies, there did not appear to be a difference in protein localization as a result of anoxia although neuronal processes were largely absent at later time points (24 hours and later). Subsequent studies looking at earlier time points could be performed to determine if changes in protein localization and gene expression occur. If axonal processes are affected, agents that stimulate neurite outgrowth could be tested as could established or potential neuroprotectants including RARβ agonists. In addition, low (i.e., 1%, 3%, 10%, etc.) oxygen levels and intermittent periods of hypoxia could be examined instead of anoxia.

These studies are of increasing interest as a number of genes downstream of hypoxia inducible factor-1 (HIF-1) have been linked to ALS. Genetic variations in both vascular endothelial growth factor (VEGF) and angiogenin (ANG) have been identified. In humans, variations in the VEGF gene have been shown to increase the risk of ALS [62] while studies in the SOD1 mutant mouse model demonstrated prolonged survival and slower rates of disease progression with VEGF treatment [120, 311]. Missense mutations in the *ANG* gene have also been identified in patients with ALS [312], and ANG has been shown to protect motor neurons against hypoxic injury [313]. Interestingly, the neuroprotective properties of ANG were lost when ALS-associated SOD1 mutants were expressed in a motor neuron-like cell line indicating that an abnormal response to hypoxia occurs in patients with ALS [313].

Erythropoietin (EPO) can also be induced by hypoxia and has been shown to have neuroprotective effects in hypoxic-ischemia brain injury in neonatal rats [314]. A study comparing EPO levels in normoxemic and hypoxemic (oxygen arterial blood pressure below the age-dependent minimal reference value) ALS patients and controls found increased levels of EPO in hypoxemic ALS patients compared to normoxemic ALS patients and controls [315]. This study proposed that EPO was acting at multiple levels including limiting production of tissue-damaging molecules like reactive oxygen species (ROS) and stimulating angiogenesis [315].

Connections between RNA processing and hypoxia may also impact the disease process. Hypoxia has been shown to up-regulate VEGF expression by increasing transcription and via post-transcriptional regulation of VEGF mRNA. The identification of a 126-base hypoxia stability region (HSR) in the 3' untranslated region (UTR) of the human VEGF gene suggests that hypoxia-inducible mRNA-protein complexes contribute to increased VEGF expression [316]. In addition, interactions between the VEGF HSR and heterogeneous nuclear ribonucleoprotein L (hnRNP L), a global regulator of alternative splicing, play an important role in hypoxia-induced regulation of VEGF mRNA stability [317].

The interdependence of immune and hypoxic responses to infection and tissue damage is another area of interest. HIF expression is up-regulated through pathways involving the key immune response regulator, NF-κB [318], and studies in patients with ALS have questioned whether elevated levels of certain cytokines indicate inflammation or hypoxia [319]. Two additional studies provide a link between mechanisms thought to underlie ALS, hypoxia and the immune system. In the first, chronic hypoxia was shown to suppress carrier-mediated glutamate uptake in astrocytes by suppressing EAAT1 and EAAT2 (2 major glutamate transporters)

expression [320]. Inhibition of transporter expression was later shown to require NF-κB activation and to be modulated by TNFα with TNFα production enhanced by hypoxia [321]. As dysfunction of EAAT2 has been implicated in ALS, the relationship between hypoxia and glutamate-mediated excitotoxicity could be further studied in our *in vitro* system.

5.3.3 Neurite outgrowth and regeneration

RA signaling is implicated in neurite regeneration [256, 257], and several mediators of the RA signaling pathway are induced after peripheral nerve injury including CRBPI, CRABP-II and RALDH2 [322]. Interestingly, local activation of the RARE was reported in regenerating nerves of transgenic reporter mice following sciatic nerve injury suggesting that RA-mediated gene expression is induced during this regenerative process [322]. The same group demonstrated increased expression of the nuclear receptors (RARs and RXRs) at both the mRNA and protein levels following sciatic nerve crush [323]. Cell culture experiments indicate that RARβ is required for this axonal regeneration [257, 324], and further studies to characterize the effects of RARβ on neurite outgrowth could be performed in this *in vitro* system. In our preliminary experiments using all-*trans* retinoic acid (ATRA) as a pan-RAR agonist, we assessed a number of morphological characteristics on a per cell basis including: 1.) number of neurites, 2.) number of branch points, 3.) length of the longest neurite and 4.) total neurite area. We observed a statistically significant decrease in the number of branch points/cell with increasing concentrations of ATRA. While intriguing, these studies need to be repeated ideally with green fluorescent protein (GFP)-tagged markers of neuronal cells (i.e., β3-tubulin) to enable identification of neuronal processes.

5.3.4 Non-genomic impact of retinoid signaling

In addition to the classical modulation of gene expression, receptors and proteins of the retinoid signaling pathway have other more rapid, non-genomic effects [217, 306]. Specifically, the impact on phosphatidylinositol-3-kinase (PI3K) and extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) signaling pathways could be determined by measuring phosphorylation of Akt kinase and ERK1/2, respectively. Interactions between retinoid signaling components and the mitochondria [224] as well as modulation of mitochondrial gene expression by retinoids (reviewed in [223]) have also been observed. As mitochondrial dysfunction is one mechanism thought to play a role in ALS disease pathogenesis, it would be interesting to perform co-localization studies and to assess these interactions in the presence of toxins or when specific agonists/antagonists are administered.

5.3.5 Microarray analysis of downstream genes

As discussed for the human tissue-based study, the genes regulated by the retinoid receptors could be investigated in this *in vitro* system. This would enable the identification of genes downstream of the RARs in an isotype-specific manner. In addition, a microarray study would be useful for determining which genes are up- or down-regulated and could uncover novel pathways underlying ALS or support further research into previously-identified mechanisms. As a great deal remains to be determined regarding the role of this signaling pathway post-development, this information would extend beyond ALS.

5.3.6 Role and effects of glial cells

It is well-established that ALS is a disease of not only the motor neurons but their non-neuronal neighbors. In our culture system, the predominant non-neuronal cell is the glial fibrillary acidic protein (GFAP)-positive astrocyte. Therefore, studies utilizing varying percentages of these cells in co-culture with the motor neurons could help determine the contribution of these cells in the context of retinoid signaling.

5.3.7 Investigation of other potential biomarkers

The overall goal of the Bowser laboratory is to identify proteins that are differentially-expressed or modified in the CSF (or plasma) of patients with ALS. Although the retinoid signaling pathway is obviously one avenue worth pursuing, there are multiple other candidate biomarkers in need of study. A few examples identified via mass spectrometry-based proteomics include: the complement system (part of the innate immune system) with components shown to be up-regulated in the CSF of ALS patients; ceruloplasmin (an enzyme involved in copper binding and oxidation of iron) which was shown to be up-regulated in ALS CSF; antithrombin (a serine protease inhibitor involved in coagulation) which is decreased in ALS CSF; cystatin C (a cysteine protease inhibitor) which is decreased in ALS CSF [150, 325]; and the carboxy-terminal protein fragment of the 7B2 protein (7B2-CT; an indirect inhibitor of hormone and neuropeptide maturation) shown to be increased in ALS CSF [150]. As multiple pathways and categories of proteins have been identified in these studies with unclear roles in motor neuron cell function, this *in vitro* system will enable the impact of these alterations to be investigated and understood at the cellular level.

5.3.8 Primary cultures from transgenic mutant SOD1 or mutant TDP-43 rats

Finally, the differences we have observed between sporadic and familial ALS patients are ones for which we still do not have a definitive explanation. In an attempt to provide insight into how retinoid signaling impacts at least a fraction of the familial forms of the disease, transgenic mutant SOD1 or mutant TDP-43 rats can be used to generate primary motor neuron-enriched cultures. Initial characterization of the localization of RARα and RARβ would determine if the presence of the mutated proteins alone results in altered subcellular distribution. Subsequent studies would focus on the impact of either mutant protein in the presence of the toxins discussed above and the ability (or inability) of RARβ agonists to protect toxin-exposed cells. In this way, we can begin to define the relationship between SOD1, TDP-43 and the retinoid signaling pathway and to rationalize why increased nuclear localization of CRABP-II and RARβ does not occur in motor neurons from patients with familial forms of the disease.

5.4 CONCLUDING REMARKS

Ultimately, my goal was to establish an experimental system with which to expedite the transition from bench discoveries to the bedside. Although all experimental systems and approaches have limitations, the approach we have taken begins where it will eventually end—with the patient. Identification of protein alterations that occur in patients with a predominantly idiopathic disease combined with the ability to determine the functional consequences of these alterations at the level of the motor neuron could enable the translation of these findings to early

stages of clinical trials once drugs that target proteins/pathways of interest are developed and proven in our *in vitro* system.

The data presented suggest that the retinoid signaling pathway is altered in patients with ALS and that modulating this pathway can promote motor neuron survival in the presence of oxidative-induced cell death. The latter experiments are especially encouraging as protection was observed at low doses of RARβ agonist and indicate that RARβ-mediated signaling is neuroprotective. Importantly, the increase in RARβ nuclear localization observed in spinal cord motor neurons of patients with sporadic ALS could result in transcriptional repression (and lack of neuroprotection) in the absence of ligand (outlined in Figure 44). Therefore, agonists of this nuclear receptor could be used therapeutically to delay the motor neuron degeneration and death that occurs in ALS thereby slowing disease progression in patients with this devastating disease.

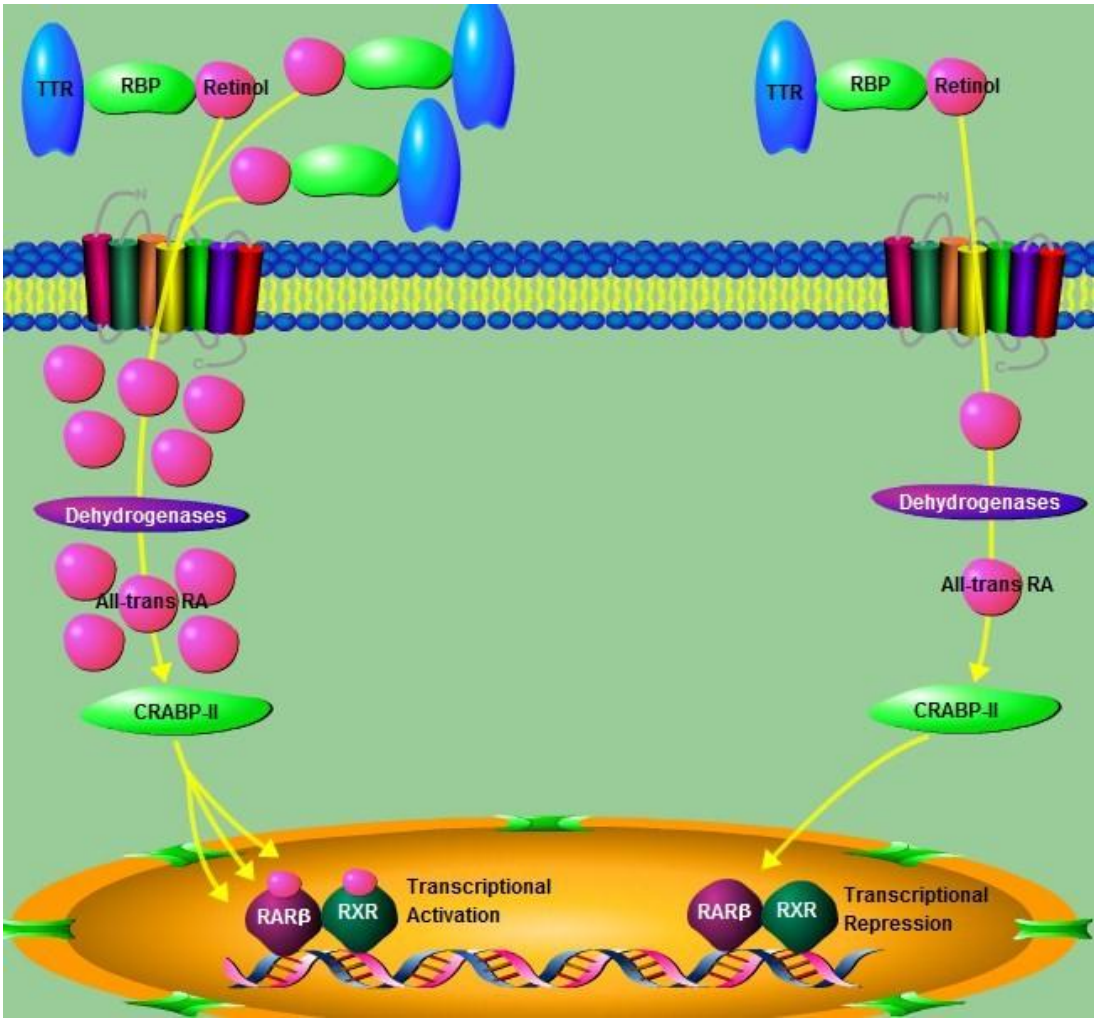


Figure 44. TTR and the Transcriptional Impact of Nuclear RARβ

Alterations in the amount of TTR in patients with ALS impact the amount of ligand available to the retinoid receptors. ***Left****: When TTR is abundant, retinol (which circulates in complex with TTR and RBP) is available for uptake at the cell surface. Once inside the cell, the action of dehydrogenases converts available retinol to (predominantly) all-trans retinoic acid which binds to RARβ to activate transcription of downstream targets.* ***Right****: Decreased levels of TTR in the CSF would translate to a lack of retinol available for cellular uptake. Without retinol and in the absence of ligand, nuclear RARβ remains bound to retinoic acid response elements where it acts as a transcriptional repressor.*

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