Histopathology in Skeletal Muscle of Transgenic Mice
Suggests Dual-Locus of Onset (DLO) in Kennedy’s Disease

By

Mike McPhail

A thesis submitted in conformity with the requirements for the degree of Master of Science
Institute of Medical Science
University of Toronto

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Suggests Dual-Locus of Onset (DLO) in Kennedy’s Disease

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2014

Abstract
Kennedy’s Disease or Spinal-Bulbar Muscular Atrophy (KD/SBMA) is a neuromuscular disease associated with a CAG tri-nucleotide repeat expansion within the androgen receptor (AR) gene. Mouse models of KD used in this investigation expressed mutant AR either in motor neurons (HB9/AR113 mice) or skeletal muscle (HSA/AR113 mice), revealing abnormalities within the extensor digitorum longus (EDL) and soleus (SOL) muscles. Reduced oxidative metabolism was observed in both HB9/AR113 and HSA/AR113 mice, while a fiber-type switch and a reduction in cell size were only observed in HSA/AR113 mice. Deficits in fiber number and endurance strength were found in HB9/AR113 mice only. These findings support the DLO hypothesis, suggesting that onset of KD involves pathology in motor neurons, and perhaps skeletal muscle. Notably, in vivo evidence of pathology in HB9/AR113 mice is an original finding of this investigation.
Acknowledgments

I, Mike McPhail, dedicate this work first and foremost to my family, Nina, Tim, Erin and Aiden McPhail, whose generosity and support have made my academic career possible.

I am deeply indebted to Dr. Ashley Monks, my supervisor, for giving me this opportunity and relieving many of my doubts about myself along the road. A much honored thanks is also offered to Dr. Melissa Holmes for her support and guidance over my two years at the University of Toronto at Mississauga (UTM). As friends I wish them well and would be enthusiastic if we were to collaborate again in the years to come. To my Program Advisory Committee (PAC), Dr. John Yeomans and Dr. Junchul Kim, I extend warm thanks for their advice and criticism which, I hope, lends me strength. Over the years, as a pupil of theirs, I learned more than I ever have before.

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I am especially indebted to my loyal friends and mentors, Dr. Albert Wong, Dr. Bruce Bowden, Dr. Howard Mount and Dr. Mary Seeman, without whom, I may never have made this wonderful journey.
Contributions

Included in this thesis document is the work of Dr. Kaiguo Mo, whose expertise in the methods of molecular genetics is highly valued by all laboratory members. Figure 13 in the Discussion section describes the results gleaned from this work, which involved Q-PCR processing of the anterior tibialis (AT) muscle (dissected by Firyal Ramzen and myself) from both transgenic and wild-type (WT) mice. Dr. Kaiguo Mo also performed the statistical analysis of data acquired from the AT in order to represent the relative expression of mutant AR in skeletal muscle for these mice (see Rao & Monks, 2009 for a description of the Q-PCR methods undertaken). In this way, some comparisons between transgenic and WT mice using our laboratory techniques may be explained in terms of expression of mutant AR in skeletal muscle.

A special thanks is offered to Firyal Ramzen, whose help in gathering behavioral data and mouse dissections was greatly appreciated. It should be mentioned also that Felicia Phan contributed important data corroborating my own findings using the quantitative methods of this investigation.
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<tbody>
<tr>
<td>A</td>
<td>Adenine</td>
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<tr>
<td>AIS</td>
<td>Androgen Insensitivity Syndrome</td>
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<td>ALS</td>
<td>Amyotrophic Lateral Sclerosis</td>
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<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>AR</td>
<td>Androgen Receptor</td>
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<td>ArKO</td>
<td>Androgen Receptor Knock Out</td>
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<tr>
<td>AT</td>
<td>Anterior Tibialis</td>
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<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>β-NADH</td>
<td>β-Nicotinamide adenine dinucleotide, reduced dipotassium salt</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CBP</td>
<td>cAMP response element protein-Binding Protein</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxycycline</td>
</tr>
<tr>
<td>DLO</td>
<td>Dual-Locus of Onset</td>
</tr>
<tr>
<td>DHT</td>
<td>Dihydrotestosterone</td>
</tr>
<tr>
<td>DMD</td>
<td>Duchenne muscular dystrophy</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>EDL</td>
<td>Extensor Digitorum Longus</td>
</tr>
<tr>
<td>EMG</td>
<td>Electromyography</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>FG</td>
<td>Fast-Glycolytic</td>
</tr>
<tr>
<td>FO</td>
<td>Fast-Oxidative</td>
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<tr>
<td>G</td>
<td>Guanine</td>
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<tr>
<td>H&amp;E</td>
<td>Hematoxylin &amp; Eosin</td>
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<tr>
<td>HB9</td>
<td>Homeobox9</td>
</tr>
<tr>
<td>HSA</td>
<td>Human Skeletal α-Actin</td>
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<tr>
<td>HSP</td>
<td>Heat Shock Protein</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like Growth Factor</td>
</tr>
<tr>
<td>KD</td>
<td>Kennedy’s Disease</td>
</tr>
<tr>
<td>KO</td>
<td>Knock Out</td>
</tr>
<tr>
<td>K-S</td>
<td>Kolmogorov–Smirnov</td>
</tr>
<tr>
<td>LBD</td>
<td>Ligand Binding Domain</td>
</tr>
<tr>
<td>MAPK</td>
<td>Ras/mitogen-activated protein Kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>Myosin Heavy Chain</td>
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<td>MusK</td>
<td>muscle-specific Kinase</td>
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<tr>
<td>NADH</td>
<td>Nicotinamide Adenine Dinucleotide Dehydrogenase</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitro Blue Tetrazolium</td>
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<tr>
<td>NFL</td>
<td>Neurofilament Light Chain</td>
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<td>NHS</td>
<td>Normal Horse Serum</td>
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<td>NMJ</td>
<td>Neuromuscular Junction</td>
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<tr>
<td>NSE</td>
<td>Neuron-Specific Enolase</td>
</tr>
<tr>
<td>PAC</td>
<td>Program Advisory Committee</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PGC-1α</td>
<td>peroxisome proliferator-activated receptor γ co-activator 1α</td>
</tr>
<tr>
<td>polyQ</td>
<td>Polyglutamine tract</td>
</tr>
<tr>
<td>PrP</td>
<td>Prion Protein Promoter</td>
</tr>
<tr>
<td>Q</td>
<td>Glutamine</td>
</tr>
<tr>
<td>rTta</td>
<td>Reverse Tet-controlled Transactivator</td>
</tr>
<tr>
<td>SBMA</td>
<td>Spinal-Bulbar Muscular Atrophy</td>
</tr>
<tr>
<td>SDH</td>
<td>Succinate Dehydrogenase</td>
</tr>
<tr>
<td>SOL</td>
<td>Soleus</td>
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<tr>
<td>SRC-1</td>
<td>Steroid Receptor Co-activator-1</td>
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<td>SV</td>
<td>Seminal Vesicle</td>
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<tr>
<td>Tg</td>
<td>Transgenic</td>
</tr>
<tr>
<td>Tfm</td>
<td>Testicular Feminization</td>
</tr>
<tr>
<td>TMS</td>
<td>Trans-cranial Magnetic Stimulation</td>
</tr>
<tr>
<td>TRE</td>
<td>Tetracycline Response Element</td>
</tr>
<tr>
<td>UTM</td>
<td>University of Toronto at Mississauga</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-Type</td>
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Chapter 1

Introduction

1.0 Overview

KD is a rare neuromuscular disease, occurring in 1/40 000 men, for which there is currently no cure (Fu et al., 2008). This disease is associated with a polymorphism in the N-terminal transactivation domain of the AR gene, consisting of a very long polyglutamine (polyQ) repeat tract. Although the pathway from this genetic abnormality to overt pathological signs is unknown, evidence from histopathological studies suggests that KD is primarily neurogenic in origin, with degeneration of motor neurons innervating muscular structures viewed as the proximal cause (Merry, 2005). However, there is also evidence that KD may be myogenic in origin (Monks et al., 2007; Kemp et al., 2011). That is, onset of muscle weakness in KD may be due directly to muscular degeneration, rather than motor neuron pathology.

The main goal of this investigation is to contribute to the growing support for the DLO hypothesis of KD, which would provide a starting point for further research of this illness. The DLO hypothesis holds that onset of KD involves pathology in motor neurons, and perhaps skeletal muscle. In supporting this hypothesis, this work would contribute to the overall understanding of the progression of KD from genetic abnormality to acquired illness. In the following Introduction, KD will be described, followed by a discussion of the relevant tools and associated findings in previous studies of KD. The animal models used in this investigation will be discussed within the framework of this previous research. Finally, predictions will be presented that, if true, would support the DLO hypothesis.
1.1 Description of KD

Human genome studies identify a polymorphism of glutamine (Q) repeats in the N-terminal transactivation domain of Exon-1 of the AR gene (Fischbeck et al., 1999; LaSpada et al., 1991). The length of this polymorphism is associated with differences in androgen sensitivity, such that shorter repeat lengths increase AR function while longer repeat lengths decrease AR function upon ligand binding. While normal individuals show Q lengths between 9-36 residues, repeat lengths outside this range confer risk of disorders associated with the androgenic system. Individuals with shorter Q lengths incur susceptibility to benign prostate hyperplasia and prostate cancer, whereas longer Q lengths, usually between 38 and 62, are associated with symptoms of androgen insensitivity, including abnormal primary and secondary sex characteristics (e.g. gynecomastia, hypogonadism, loss of libido and erectile function, oligospermia and azoospermia), as well as progressive neuromuscular atrophy, resulting in behavioral signs of muscle weakness (La Spada et al., 1991). This broad symptom spectrum as a result of carrying longer Q lengths, that is, polyQ-AR, clinically describes individuals with KD.

KD was originally identified as being a distinct diagnosis, with its own set of symptoms by Kennedy et al. (1968). KD is a rare illness, although it is likely that it is often misdiagnosed as amyotrophic lateral sclerosis (ALS) and is thus under-estimated (Fu et al., 2008). There is a positive correlation between polyQ repeat length and disease severity and a negative correlation between repeat length and age of disease onset, a pattern similar to other polyQ expansion disorders, such as Huntington’s Disease (Fu et al., 2008).
KD has an X-linked recessive pattern of inheritance whereby males only are affected and female carriers are either asymptomatic or display only mild signs of motor dysfunction. This is also true of homozygous females, in which symptoms are limited to occasional muscle cramps and twitches (Schmidt, 2002). This circumstance of KD reflects the fact that sufficient androgen-mediated activation of polyQ-AR is necessary for muscle weakness to become manifest. For this reason, the class of symptoms related to onset of muscle weakness reflect ‘toxic gain of function’ of polyQ-AR. Notably, toxic gain of function of polyQ-AR sets this disease apart from a diagnosis of Androgen Insensitivity Syndrome (AIS) in which secondary female sex characteristics appear due to deficient function of AR but individuals typically do not express muscle weakness akin to KD patients (Galani et al., 2008).

The course of KD for males begins with muscular atrophy in the hips, followed by the shoulders and bulbar muscles of the brainstem, resulting in difficulty with walking, speech and swallowing. As muscular degeneration progresses, with loss of muscle bulk in the limbs, many KD patients use aids for ambulation with the most severely affected becoming wheelchair-bound (Douglass et al., 2010). The onset of KD usually occurs between 30-55 years of age with a one to two decade life window prior to death. As a result, life expectancy is only slightly reduced (Finsterer, 2009). Although the distal cause of KD has been localized (i.e. Q repeat expansion within the AR gene), the pathway from the genetic locus of KD to phenotypic expression of the illness is still poorly understood. Studies examining aspects of polyQ-AR and its role in KD at the level of gene expression and intracellular and membrane protein interactions are informative as they reveal possible pathways to onset. In the following
sections, biological features of both normal and polyQ-AR will be reviewed including their different functional roles in neuromuscular systems affected by KD.

1.2 Description of the Biological Processes of AR

1.2.1 General Properties of normal human AR and polyQ-AR

Normal AR is a ligand activated cytosolic protein, functioning as a gene transcription factor, a common feature of the steroid receptor superfamily to which it belongs (Gallo & Leigh, 2007). The AR protein is encoded by the AR gene, composed of eight exons which reside on the q11-12 locus of the X chromosome (Chang, Kokontis & Liao, 1988). In its protein form, AR has a molecular weight of approximately 110 kDa, depending upon the number of glutamine residues (Wilson & McPhaul, 1994). The natural ligand-activators of AR are testosterone or its more potent metabolite 5α-dihydrotestosterone (DHT) (Gallo & Leigh, 2007).

Prior to ligand-binding, AR is localized in the cytoplasm, forming an inactive complex with sub-cellular chaperones. Chaperones binding to AR include the heat-shock proteins, HSP70, HSP90 and HSP56, which maintain the receptor in a conformational shape optimal for binding with androgen ligand (Gallo & Leigh, 2007). After interaction with the endogenous ligand at the ligand binding domain (LBD) in the C-terminus of the AR protein, this receptor disengages from the chaperone complex and becomes active in cellular processes (Gallo & Leigh, 2007).

In its active form, AR can affect cell function through a variety of signal transduction pathways. Upon migration to the nucleus, AR binds to androgen response elements of DNA to affect intranuclear transcription. This mechanism is responsible for most of the function of normal AR,
that is, the modulation of the expression of AR responsive genes (Onate, Tsai, Tsai, & O’Malley, 1995). Target gene expression may be up-regulated or down-regulated depending on which co-transcription factors are recruited by AR and/or the transcriptional machinery with which the receptor complex interacts. For example, co-activators involved in AR function, such as Cyclin E and steroid receptor co-activator 1 (SRC-1), up-regulate expression of target genes, while corepressors, such as Cyclin D1 and F-steroid receptor co-activator-1 (F-SRC-1), down-regulate expression (Onate, Tsai, Tsai, & O’Malley, 1995). Alternatively, AR may have distal effects via regulation of the expression of certain hormones or influence over proximal tissues via interactions with autocrine or paracrine factors (Saez, 1994). These protein products themselves may act to regulate gene expression as part of complex signal cascades. Such cellular mechanisms of AR action can occur wherever AR is localized in mammalian tissues, including the nervous system, skeletal muscle, bone, and reproductive organs (Saez, 1994).

Of relevance to KD, the sequence of the AR gene corresponding to Exon-1 (i.e. the highly translated portion of AR) contains the N-terminus (which in turn contains the polyQ sequence) responsible for the overall activity of the molecule upon ligand binding (Mhatre et al., 1993). Interestingly, the expansion of the polyQ sequence within the N-terminus to the clinically significant range decreases ligand-dependent activity by up to 40% (Mhatre et al., 1993; Chamberlain et al., 1994). This is likely to explain the mild signs of androgen insensitivity observed in KD. Despite this fact, polyQ mediated disruption of N-terminus activity cannot plausibly explain the onset of muscle weakness due to toxic gain of function of translated AR. The mechanisms of muscle weakness in response to activation of polyQ-AR by ligand binding are still unknown.
1.2.2 Normal AR and polyQ-AR in Motor Neurons

An important question with regard to onset is why pathology is selective for lower motor neurons and/or muscle. AR is present in several neuronal populations within the nervous system, including the motor neurons of the spinal cord (Sar & Stumpf, 1977; Menard & Harlan, 1993). As this receptor has a functional role in the sexual differentiation and growth of the developing nervous system; it may influence the growth and survival of motor neurons (Fargo et al., 2008). For example, among the transcriptional targets of AR are tubulin genes coding for proteins comprising the structural component of microtubules in motor neuron axons (Yu and Srinivasan, 1981; Jones et al., 1999). During adulthood, AR may have a maintenance role in motor neurons, such as promoting motor axon regeneration after axonal damage (Kujawa et al., 1989; 1991; Jones, 1994).

Other proteins related to the androgenic system are disproportionately active in motor neurons. It is a characteristic of spinal cord neurons to convert testosterone into DHT by the enzyme, 5α-reductase. The two isoforms of 5α-reductase, type 1 and type 2, are encoded by different genes, with type 2 5α-reductase having a higher affinity for testosterone. In situ hybridization analyses in rats reveal that the two isoforms of 5α-reductase are present in the spinal cord, but type 2 is exclusively expressed in lower motor neuron cell bodies of the anterior horn of the spinal cord (Pozzi et al., 2003). Thus, expression of both the receptor and type 2 5α-reductase in lower motor neurons may explain the selectivity of polyQ-AR for these cells.

One of the proposed disease mechanisms of polyQ-AR in mature motor neurons is related to transport of materials along motor axons necessary for cell function. Critical proteins are
synthesized in the neuronal cell body and transported along the axon toward the nerve terminals, and retrograde transport from nerve terminals to the cell body is also important for recycling proteins required for cytoskeletal and synaptic vesicle formation. In KD, depletion of motor proteins may act to disrupt axonal transport of these materials (Gunawardena et al., 2003). For example, accumulations of aggregated mutant AR, known as inclusion bodies, may disrupt Kinesin and Dynactin 1, proteins necessary for microtubule-mediated anterograde and retrograde transport (Piccioni et al., 2002; Katsuno et al., 2002). Thus, carriers of polyQ-AR may experience deficits in neuromuscular signalling necessary for muscle contraction as a result of initial dysfunction in motor neuron transport.

1.2.3 Normal AR and polyQ-AR in Skeletal Muscle

Onset in skeletal muscle is a possibility considering the fact that androgens interact strongly with muscle tissue. In fact, androgenic supplementation increases skeletal muscle mass in healthy men. Numerous studies report that testosterone induced increases in muscle mass is associated with hypertrophy of both slow and fast-twitch muscle fibers as well as myogenesis (Brodsky, Balagopal, & Nair, 1996; Katznelson et al., 1996; Snyder et al., 1999; Blackman et al., 2002; Bhasin et al., 1996; Bhasin, et al., 2001; Bhasin et al., 1997).

In situ hybridization and Western blot analyses confirm that normal AR is expressed in several cell types in human skeletal muscle, including satellite cells, fibroblasts, CD34+ precursor cells, vascular endothelial, smooth muscle cells, and mast cells, with satellite cells being the predominant site of AR expression (Sinha-Hikim et al., 2004). AR may have its effects in muscle via a wide range of transcriptional targets in these cells, including those involved in
muscle contraction, extracellular matrices, transcription, metabolism, proliferation, and cell signalling (Yoshioka et al., 2006). In the case of polyQ-AR, these cellular routes of normal AR action may be disrupted, making them possible candidates in the pathogenesis of KD.

1.2.4 Normal AR and polyQ-AR interactions with maintenance proteins at the Neuromuscular Junction (NMJ)

Each lower motor neuron innervates a number of muscle fibers that taken together form a motor unit. Motor unit commands for contraction ultimately occur at the NMJ, linking each motor neuron and muscle fiber. Here, presynaptic axon terminals release acetylcholine into the synaptic cleft, binding to postsynaptic receptors on the membranes of muscle fibers. This process mediates neuromuscular communication necessary for recruitment of muscle in physical activity.

At the NMJ, synaptic editing between motor neuron and skeletal muscle fibers is instrumental during development and into adulthood for functional and useful circuits to arise in the neuromuscular system. Some inputs to a target cell become gradually weakened and eliminated, while other inputs are strengthened and maintained. The general pattern of NMJ editing during development is progression from many neural inputs to skeletal muscle fibers to a single input (Personius & Balice-Gordon, 2000). This feat is accomplished by competition between presynaptic inputs for influence over muscle targets with postsynaptic muscle modulating this competition by providing nourishment for motor axons. These activity-dependent anterograde and retrograde interactions that play critical roles in developmental synaptic editing also play ongoing roles throughout life (Personius & Balice-Gordon, 2000).
The factors released by postsynaptic muscle cells that produce the most potent effects on synaptic growth and maintenance are neurotrophins, such as brain-derived neurotrophic factor (BDNF). Motor axons must compete for consumption of these neurotrophic factors in order for their survival (Personius & Balice-Gordon, 2000). At the present time, there is a growing body of literature regarding activity-dependent anterograde and retrograde signalling at the NMJ required for synaptic maintenance (Personius & Balice-Gordon, 2000).

Neuromuscular disease involving motor neuron as well as muscular atrophy, such as is hypothesized for KD, likely requires the NMJ as a bridge for neuromuscular pathology. Kemp et al. (2011) demonstrate that disrupted transport of cargo in motor neuron axons occurs when disease is triggered in muscle fibers of transgenic mice, suggesting the importance of synaptic signalling in the pathogenesis of KD. It is conceivable that various proteins, involved in cross-synaptic signalling pathways may be involved in such pathological signs either by interaction with trophic factor function or independently. A potential candidate molecule is ubiquitin ligase E3, an enzyme in the ubiquitin protein cascade, whose fellow proteins are found in AR nuclear inclusion bodies (Li et al., 1998). Ubiquitin-mediated degradation of proteins is essential for controlling precise synaptic conditions through targeted protein degradation and clearance. Disruption of this enzyme may interfere with trophic factor function that is the basis for neuromuscular symptoms (Lei, Fu, & Ip., 2012).

Another candidate molecule in anterograde signalling across the NMJ, is the protein agrin. This protein is synthesized and released from lower motor neuron axon terminals, functioning as a master organizer that governs postsynaptic receptor assembly and synchronization (Lei, Fu, &
Moreover, this protein interacts with the androgenic system, such that it may have a direct role in the spread of KD between motor neurons and skeletal muscle fibers (Joseph, 1997). Proteins activated by agrin on the muscle membrane have roles in other neuromuscular disorders. Among the proteins necessary for NMJ development and maintenance, and modulated by agrin, include muscle-specific Kinase (MusK). A sub-type of myasthenia gravis, an autoimmune disorder, causing muscle weakness and fatigue, is characterized by immune antibodies directed toward this agrin-receptor protein. Furthermore, the protein dystrophin, an important structural component within muscle tissue that provides structural stability to the cell membrane, is implicated in Duchenne muscular dystrophy (DMD), an illness involving severe muscle degeneration. Thus, the spread of KD between pre- and post-synaptic DNA containing cells, ultimately leading to denervation of motor units, and cell dysfunction/loss, may be dependent on polyQ-AR interacting with synaptic proteins involved in similar diseases.

### 1.3 Research Models of KD

Tools used to model physiological illness in the laboratory may be sorted according to the particular paradigm used to investigate pathology (e.g. clinical, molecular, systems level, behavioral). What follows is a description of the relevant findings accrued from these methods which may be useful in the characterization of the onset of KD.

### 1.4 The Clinical Paradigm: Human Studies

#### 1.4.1 Electrophysiology

Although diagnosis of KD relies on genetic testing to determine clinically significant Q repeat expansion of the AR gene, electrophysiological recordings from patients may be useful in characterizing early signs of illness (Gallo & Leigh, 2007). The presence of diffuse
disturbances in lower motor and sensory neuron activity patterns is a common indicator of KD. Such findings have been shown in studies using either sensory and motor conduction measures or electromyography (EMG).

1.4.2 Sensory and Motor Conduction Studies
Motor conduction studies are performed by electrical stimulation of a peripheral nerve and recording from muscle supplied by this nerve. Motor conduction studies reveal a modest reduction in the amplitude of electrical recordings from skeletal muscle (Sobue et al., 1989; Olney, Aminoff & So, 1991; Trojaborg & Wulff, 1994; Ferrante & Wilbourn, 1997). Somatosensory evoked potentials from both upper and lower limb stimulation have also been reported to be abnormal, as are sensory evoked potentials recorded from the brainstem. Interestingly, repetitive lower motor nerve stimulation is normal in KD but neuromuscular jitter may be very increased. Neuromuscular jitter is the variability in the time intervals between potentials generated by two muscle fibres of the same motor unit upon stimulation. This electrophysiological profile has been regarded as the reason for endurance fatigue in KD at the neuromuscular level (Meriggioli & Rowin, 2003). Although these conduction studies are performed on patients already diagnosed, they may be promising as electrophysiological markers of onset.

1.4.3 Electromyography
EMG is a technique for evaluating and recording the electrical activity produced by skeletal muscles. EMG investigations have not been typically used to identify individuals with KD, but evidence from more advanced patients show diffuse denervation with evidence of re-innervation, identifiable by long duration, large amplitude EMGs, even if there is no loss in
strength at these neuromuscular locations (Harding et al., 1982; Olney, Aminoff & So, 1991; Trojaborg & Wulff, 1994). This is informative when characterizing the onset of KD, as irregular recordings from skeletal muscle may precede signs of muscle weakness at these locations. However, an actual EMG profile during the period of onset has not yet been reported (Gallo & Leigh, 2007).

With regard to muscular fasciculations, which are common in KD, visible twitching tends to occur most frequently in perioral muscles of the facial cheeks (Huang et al., 1998). However, in KD, visible fasciculations occur with a frequency of less than 1% of patient lifetime, with muscles responsible having an EMG frequency of only 3 per minute, a frequency much lower than in ALS (Hirota, Eisen & Weber, 2000). This makes fasciculations difficult to monitor either by observation or using EMGs, making diagnosis and tracing onset based on this marker problematic.

1.4.4 Upper Motor Neuron Studies

There is virtually no evidence that pathology in KD is related to cortico-spinal motor neurons. Trans-cranial magnetic stimulation (TMS) studies show that both the brain and upper motor neurons of the spinal cord are normal in KD (Eisen, 2001). Peri-stimulus time histograms of descending volleys of select groups of upper motor neurons confirm absence of pathology in the cortico-spinal tract, making it irrelevant as either a pre-clinical marker or a possible location of onset (Weber & Eisen, 1999).
1.5 **Histopathology in Human Patients: Neural and Muscular Degeneration**

Post-mortem studies of KD patients reveal that both spinal cord and brain stem nuclei exhibit motor neuron loss, with remaining neurons showing atrophy and decreased ventral root size (Sobue et al., 1989). Moreover, sensory neuron loss has been found in the dorsal root ganglion associated with mild sensory disturbance (Sobue et al., 1989). This evidence is consistent with results from electrophysiological investigations showing pathology of the peripheral nervous system being the primary site of onset of KD. While histopathological evidence in motor neurons is common, recent studies propose that onset of KD may be due to primary pathology in muscle fibers; that is, onset of KD may be myogenic. There is autopsy evidence of fiber splitting, centralized nuclei and fiber degeneration (e.g. fibers have atrophic, angulated shape and are grouped into small clumps) typical of diseases that originate in muscle (Katsuno et al., 2006). Contrarily, findings of pathology in skeletal muscle have also been described as secondary to denervation due to motor neuron pathology. As a result, there is an ongoing debate whether onset of pathology occurs primarily in skeletal myocytes or motor neurons.

Support for the idea that onset occurs in both locations arrives from knowledge that nuclear AR-immunoreactive inclusions in KD are observed in motor neurons and other non-neuronal tissues, such as scrotal skin epidermal cells, and are found in muscle in rodent models of KD (Yu et al., 2006; Monks et al., 2007). Potential therapies that target the clearance of inclusion bodies in diseased cells have been suggested, including the use of leuprolelin, a synthetic luteinizing hormone-releasing hormone (Banno et al., 2012). Yet, whether inclusions are a proximate cause of motor neuron or muscular pathology or protection from deleterious effects of polyQ-AR protein is still unknown (Todd & Lim, 2013). If a pattern of inclusion formation
is associated with early signs of illness, it may be useful as a histological measurement for determining the location of onset of KD disorder.

1.6 The Molecular Paradigm: Cell Culture Studies

1.6.1 Measures of polyQ-AR Toxicity

*In vitro* studies are helpful in identifying molecular interactions of polyQ-AR which may explain onset. Studies typically transflect tissue samples with the polyQ-AR gene for expression in order to determine possible effects on cell function and viability. In so doing, intracellular pathways may be identified that are the cause of KD and frontline therapeutic solutions tested (Rusmini et al., 2013). One possible mechanism of polyQ mediated neuronal dysfunction and cell death involves the transcriptional co-activator CBP (cAMP response element protein-binding protein). This protein has been shown to be reduced in amount by its sequestration in nuclear inclusions or as the victim of enhanced degradation in cells free of inclusions (Nucifora et al., 2001; Dunah et al., 2002; McCampbell et al., 2000; Jiang et al., 2003). Given the importance of CBP in transcriptional regulation of neurotrophic factors, depletion of CBP may deprive motor neurons of sufficient nourishment to remain viable. CBP has a role in the expression of vascular endothelial growth factor (VEGF), a trophic factor with a demonstrated role in motor neuron survival (Freedman et al., 2002; Oosthuyse et al., 2001). Thus, a possible pathway to the KD phenotype is a polyglutamine-dependent reduction in CBP resulting in decreased VEGF production and function (Merry, 2005).

Cell culture studies of polyQ-AR expressing cells have potential in informing the design of animal models of KD. Portions of polyQ-AR (e.g. the N-terminal transactivational domain) have been identified as more toxic in inclusion bodies than other domains of AR *in vitro* (Young
et al., 2009). Such findings have led to the building of mouse models of KD expressing only truncated portions of AR in search of a more precise pathogenic process traceable to polyQ (e.g. Abel et al., 2001). This aspect of research will be revisited in subsequent sections describing animal models of KD.

1.6.2 polyQ-AR Effects on Metabolism

A much neglected aspect of KD is the interaction of polyQ-AR with intra-cellular machinery necessary for energy production and maintenance. Mitochondria are sub-cellular organelles that are the sites of energy production, making adenosine triphosphate (ATP), the fuel of cellular processes, via the electron transport chain. As oxygen is necessary as an electron acceptor in ATP production, this process can be referred to as oxidative metabolism. That this possible route to pathology has not been extensively investigated for a role in KD is surprising as skeletal muscle is necessary for gross motor function and onset of physical impairment may be traced to the mechanism of energy production in individual muscle cells.

As physical activity becomes longer in duration, skeletal muscle fibers increasingly rely on oxidative metabolism for mechanical work and less dependent on glycogen stores as a source of ATP via glycolysis. This is clinically significant for KD patients reporting excessive fatigue following physical activity as reduced oxidative capacity in skeletal muscle may underlie this symptom (Krivickas, 2003). Electron microscopy of cultured motor neurons reveals that both normal and polyQ-AR associate with mitochondria at the sub-cellular level (Ranganathan et al., 2009; Piccioni et al., 2002). There also exists some knowledge regarding the direction in which polyQ-AR expression may shift oxidative metabolism. Flow cytometry on cultured cells
reveals reduced mass of mitochondria when polyQ-AR is expressed in response to chemical treatment, with these cells also having a reduction in mitochondrial number (Ranganathan et al., 2009). Mitochondrial gene expression was also measured using Q-PCR for proteins linked to electron transport, finding among others, that mRNA for nicotinamide adenine dinucleotide dehydrogenase (NADH) protein was significantly reduced in AR-65Q cells relative to normal AR over-expressing cells. While this information is still sparse, the weight of the evidence suggests that reduced oxidative metabolism may be a characteristic feature of cellular interactions with polyQ-AR, and as such, a possible marker of illness onset. Unfortunately, cell culture studies have neglected to study metabolism effects of polyQ-AR in skeletal muscle, focusing instead on neuronal cell lines.

Possible routes whereby mutant AR may alter oxidative capacity in muscle include intracellular pathways for transcription of proteins necessary for (1) mitochondrial biogenesis, and (2) mitochondrial enzyme activity (i.e. enzymes of the electron transport chain). Increasing either of these two parameters will augment oxidative metabolism, while decreasing them will reduce oxidative metabolism (Gavrilova-Jordan & Price, 2007). Protein cascades responsible for such shifts in oxidative metabolism are influenced by motor neuron-mediated stimulation of muscle fibers as well as by local changes within muscle (Handschin et al., 2007). Among the proteins promoting activity-dependent mitochondrial biogenesis is peroxisome proliferator-activated receptor $\gamma$ co-activator 1$\alpha$ (PGC-1$\alpha$). PGC-1$\alpha$ knockout mice (KOs) have reduced oxidative capacity in skeletal muscle (Handschin et al., 2007). Also, muscle-specific over-expression of PGC-1$\alpha$ results in large increases in functional mitochondria via enhanced downstream activation of mitochondrial transcription factor A (Wu et al., 1999; Lin, Handschin &
Evidence from cell lines reveals a link between PGC-1α and polyQ-AR gene expression, as proteins activated by PGC-1α, and involved in muscle metabolism, become down-regulated upon expression of the disease causing gene (Ranganathan et al., 2009).

Another mediator of mitochondrial biogenesis, and also enhanced enzyme activity, is the Ras/mitogen-activated protein kinase (MAPK) pathway. While the role of MAPK proteins in mitochondrial biogenesis is unknown, these cellular molecules may mediate this function by activating PGC-1α and increasing PGC-1α protein stability (Knutti & Kralli, 2001; Puigserver et al., 2001). Evidence that MAPK enhances mitochondrial enzymes in electron transport is currently evolving from knowledge that both MAPK and mitochondrial enzyme activity are elevated at similar time intervals during endurance exercise and rest (Hawley, 2002).

PolyQ-AR may alter muscle metabolism by disrupting motor neuron signalling patterns necessary for PGC-1α and/or MAPK activity. Just how expression of polyQ-AR in skeletal muscle affects pathways mediating biogenesis and enzyme activity of mitochondria is unknown, although capture of PGC-1α and/or MAPK proteins in inclusion bodies is possible. More research is required to better understand how these protein cascades interact with processes of oxidative metabolism in both KD and healthy individuals.

1.7 An Integrated Paradigm: Animal Models

An animal model is a living, non-human animal used for the investigation of human disease in order to better understand the disease without the risk of harming human beings in the process (Merry, 2005). Among the benefits of animal models of disease are the many measures of
pathology that may be gleaned from their use. Transgenic deletion or insertion of a disease causing human gene into an organism and subsequent expression of an inserted gene may result in behavioral, histopathological and molecular changes that are quantifiable.

Several laboratories have developed animal models of KD since the discovery of the disease causing allele (i.e. polyQ-AR) two decades ago. In a synopsis, D.E. Merry (2005) outlines the major challenges faced by laboratories engaged in this line of research. The slowly progressing nature of the disease in contrast to the limited lifespan of animals in the laboratory, the fact that KD has a broad set of symptoms and its androgen dependent nature is difficult to replicate in one transgenic model and the difficulties in creating a model in which behavioral signs of KD are observed while maintaining the precise pathogenic process are 3 main obstacles to advances in understanding this illness. In this section, a critical history of animal models of KD is described, followed by the presentation of the transgenic mouse models used in this investigation. Merry’s review (2005) provides a useful guide to this previous work which serves as a template for the following discussion. The advantages of the techniques used in this investigation as a means of testing theories of onset will also be discussed.

1.7.1 Tfm and ArKO as possible Animal Models of KD

There is a substantive literature describing the various transgenic animal models displaying the phenotype of androgen insensitivity. The two models most widely used are Testicular feminization (Tfm) and androgen receptor KO (ArKO) mice. The Tfm mice bear a single nucleotide deletion in Exon-1 of the AR gene, disrupting translation (Gaspar et al., 1991). As a result, Tfm male mice are infertile and their testes are smaller compared with WT mice. In
addition, the testes are located in the inguinal region, and fail to descend as a result of pubertal inguinoscrotal migration (Hutson et al., 1994; Couse & Korach, 1999).

Besides the Tfm model, there are several transgenic mouse models (i.e. ArKO s) with a global knockout of AR. ArKO mouse models are created using two mutant strains: a transgenic mouse that expresses Cre recombinase ubiquitously, often using the cytomegalovirus (CMV) promoter and a mouse strain in which part of the AR gene is flanked by loxP sites (Kerkhofs, Denayer, Haelens, & Claessens, 2009). After breeding, offspring bearing the double transgene undergo excision of the floxed regions, preventing any transcriptional activity of AR. The phenotype of ArKO males is similar to the phenotype of male Tfm mice. They all have a female-like external appearance, testes are reduced in size, located intra-abdominally and Wolffian duct derived structures are absent (Kerkhofs, Denayer, Haelens, & Claessens, 2009). While these animal models reproduce many of the phenotypic markers of human AIS and even the androgen insensitivity experienced in KD, there are difficulties in using them as holistic exemplars of onset. Tfm and ArKO models fail to reproduce the muscle weakness of KD. The failure to demonstrate this phenotypic aspect of KD is logical due to the fact that KD is very much a disease of functioning AR causing muscle weakness, a circumstance not replicated when the AR gene is functionally ablated. The attempts to develop better models representing the neuromuscular signs of KD, and informative of disease onset, are described further below.

1.7.2 Drosophila models of KD

Drosophila models of KD, although not often used as an investigative tool, have illuminated intracellular pathways perhaps involved in the pathogenesis of KD. With co-expression of polyQ alone or in full-length AR, neural degeneration akin to KD has been observed (Chan et
al., 2002; Takeyama et al., 2002). Furthermore, a Drosophila model engineered to express the full-length polyQ-AR, demonstrated the role for androgen binding in the pathogenesis of KD, as well as specific details regarding the role of AR-immunoreactive inclusions (Takeyama et al., 2002). For example, various genetic modifications and pharmacologic manipulations were introduced to Drosophila to determine that cytosolic location of inclusion bodies were benign, while intra-nuclear inclusions were more strongly associated with neural degeneration (Takeyama et al., 2002). However, such Drosophila models of KD are limited in explanatory power, given the fact that histological and behavioral signs of this disease are difficult to measure in insects using current methods. This fact is reflected by the greater amount of research devoted to engineering a KD phenotype in mouse species.

1.7.3 Early Transgenic Mouse Models of KD

The first attempts to develop transgenic mouse models of the neuromuscular symptoms of KD informed researchers of difficult obstacles to be overcome in order to observe the signs of pathology. Early mouse models in which the clinically significant repeat number for the human AR gene (i.e. >44 CAG repeats) was expressed, failed to reproduce the disease phenotype in these animals after 2 years of life. Bingham et al. (1995) produced several transgenic mouse lines in which the human AR gene carrying 45 repeats is expressed upon induction by systemic treatments. One line expressed polyQ-AR throughout the CNS using the interferon-inducible antiviral Mx promoter and the other used a neuron-specific enolase (NSE) promoter to drive the transgene. However, these models could not induce expression of polyQ-AR and naturally did not find any indication of translation of the AR transgene in the targeted tissues of interest (Bingham et al., 1995).
Other transgenic models were qualified in that they succeeded at expression of the AR transgene, but still could not reproduce the KD phenotype. These mice used both neurofilament light chain (NF-L) and NSE promoters to drive expression of 65 CAG repeat AR (Merry et al., 1996). This second generation of animal models of KD was developed based on clinical knowledge of the inverse correlation between repeat length and age of onset in KD patients. While the disease did not develop in mice carrying a 45 repeat transgene, it would more likely develop in mice carrying a transgene containing the longest CAG repeat found in KD patients (Merry et al., 1996). Despite expression levels that reached two to five times endogenous AR levels, no evidence of disease developed.

This early experience with transgenic mouse models of KD led to several hypotheses regarding the animal modelling of the illness. The fact that a disease phenotype could not be induced over the course of the 2 year lifespan of mice, coupled with the knowledge of the inverse correlation of disease onset with Q repeat length, led to the idea that eliciting onset in a mouse may require drastic exaggeration in expression levels and repeat lengths. Furthermore, onset of symptoms were deemed more likely to occur if only the most toxic segments of AR were to be expressed, making transgenic mouse models more efficient.

1.7.4 Mouse Models using Toxic Truncated AR

Besides the shrewdness of investigators in recognizing the need for vast expansion of Q repeat lengths, cell culture studies informed subsequent animal models of KD, as they suggested that truncated forms of AR (i.e. expression of segments of AR bound in inclusion bodies) may confer greater toxicity than conventional polyQ-AR transgenes. These insights led to successes
in eliciting a KD phenotype in transgenic mice for the first time. The truncated, expanded AR gene in these transgenic mouse lines, responsive either to the NFL or prion protein promoter (PrP), respectively, represented a fragment similar to that found in nuclear inclusions, and known to form aggregates (Abel et al., 2001; Diamond et al., 2000; Merry et al., 1998). Moreover, the repeat length used (112 residues) was much longer than that previously used, which may explain how researchers were able to elicit signs of muscle weakness. Soon after 12 months of age, NFL-AR112 animals were unable to withstand more than one or two rotations on a rotarod, a behavioral task in which mice attempt to remain on a rotating beam with ‘time to fall’ taken as a measure of muscular endurance. In a gait analysis of NFL transgenic mice, using paint to mark the paws, an abnormal weight-bearing pattern on the hind feet was observed. While non-transgenic animals placed only the most distal part of the hind paw on the ground during a footfall, transgenic animals touched the ground with the entire paw, a sign of disturbance in hind limb musculature.

Compared with NFL-112 mice, PrP-112 mutant mice showed even greater signs of muscle weakness on strength measures and failure to thrive with significantly decreased body weight and earlier death. However, both of these animal models failed to reproduce the specificity of KD for lower motor neuron illness and the selectivity of the disease for the male gender. The abnormal gait observed in these transgenic mice showed substantial spasticity, a feature of upper motor neuron disease, as in ALS, but not in KD (Abel et al., 2001). Also, the truncated AR gene lacked both the hormone-binding and DNA-binding domains of AR such that female transgenic mice also showed the disease phenotype (Abel et al., 2001).
Included in this second generation of transgenic mouse models of KD, Adachi et al. (2001) used the human AR promoter to drive expression of a very long polyQ tract (239 residues) rather than the AR gene itself. These transgenic mice demonstrated muscle weakness with locomotion impaired by dragging of the hind legs, decreased walking speed, incoordination and progressive impairment on the rotarod task. They had a marked decrease in body weight compared with their WT littermates and earlier death (10-16 weeks after birth). Despite displaying signs of muscle weakness, this model also failed to reproduce the motor neuron and gender selection of KD. The brains of both genders of transgenic mice demonstrated widespread nuclear inclusions, an atypical location for this histopathological sign. It was now apparent that expression of the full-length AR gene may be required to reproduce key aspects of the disease.

1.7.5 Full-Length Expanded polyQ-AR Transgenic Mouse Models

Learning from previous models, the third cohort of transgenic models of KD involved expression of full-length polyQ-AR expanded well beyond the norm for KD patients (i.e. longer than 65 residues) (McManamny et al., 2002; Sopher et al., 2004; Katsuno et al., 2002; Chevalier-Larsen et al., 2004). The major success of this disease cohort to emerge was the replication of the androgen-dependent nature of muscle weakness. Pre-symptomatic transgenic males of this cohort were spared of developing any signs of illness following surgical castration (Katsuno et al., 2002). Furthermore, while female transgenic mice were asymptomatic, systemic treatment of females with testosterone could initiate disease in a manner comparable to that observed in males (Katsuno et al., 2002).
The third transgenic cohort succeeded in eliciting some of the behavioral signs of muscle weakness, but whether they had the precise histopathological underpinnings of KD was less certain. Histopathological analysis of the spinal cord of PrP promoter-AR112 mice with widespread expression revealed no neuron loss (Chevalier-Larsen et al., 2004). Despite this inability to replicate the motor neuron loss found in human patients, male, but not female, animals showed muscle weakness as defined by shorter performance of the rotarod task, decreased vertical activity in open-field locomotion and decreased grip strength. Katsuno et al. (2002) failed to observe any neuron loss with expression of AR with 97 Q repeats in non-castrated transgenic mice using the chicken β-actin promoter. However, measurements from the ventral roots of the lumbar region of the spinal cord revealed that the diameter of the largest axons and soma size of these motor neurons were reduced. These structural changes in motor neurons were accompanied by progressive emaciation, shorter endurance on the rotarod, reduced cage activity, ataxia and reduced survival rate compared to castrated AR-97Q and WT littermates.

In the YAC transgenic mice designed by Sopher et al. (2004) in which the expression patterns closely resemble those of endogenous AR, motor neuron number was reported as reduced, although they did not deliver a quantitative analysis. Regarding behavioral signs of muscle weakness, these 100 CAG repeat transgenic mice had reduced body weight, decreased stride length and shorter “time to fall” when trying to keep bodies suspended from an inverted cage lid (i.e. the hang test). Perhaps the best success in this transgenic cohort was described by McManamny et al. (2002) in which significant motor neuron loss and cross-sectional area of surviving motor neurons was reduced in mice expressing AR with 120 CAGs using the CMV
promoter. These mice demonstrated reduced cage activity as measured by a locomotor activity test and disturbances in total body weight. Impaired endurance strength was determined by their shorter duration to fall on a narrow bar hang test. Notably, female transgenic mice showed similar deficits but on a time lag of ~ 2 months following presentation of muscle weakness in males.

Despite the gains made in representing KD more holistically, the full-length vastly expanded AR cohort only partially replicated the motor neuron loss experienced by human patients. It remains a possibility that onset of muscle weakness in transgenic mice is dependent on other factors amounting to cell dysfunction rather than cell death. It is also likely that some of the strength deficits exhibited may be due to pathology originating in skeletal muscle, which was not as extensively investigated.

Another challenge faced by the third cohort of transgenic animals is the presentation of intracellular inclusion bodies. While all transgenic mouse lines experienced behavioral pathology not all transgenic mice displayed nuclear inclusions. The CMV promoter AR120 mice of McManamny et al. (2002) and the YAC AR100 mice of Sopher et al. (2004) did not display inclusions, but both the actin-AR97 transgenic mice of Katsuno et al. (2002) and the PrP-AR112 transgenic mice of Chevalier-Larsen et al. (2004) successfully developed them. This evidence suggests several possibilities regarding the link between inclusion bodies and pathology. It may be the case that intracellular inclusions are toxic in transgenic mouse models of KD, or alternatively they may appear as protection from disease. A third possibility exists that inclusions appear in transgenic animals due to cellular compensation for expression of
polyQ-AR transgenes, a separate process from the link to disease in humans. Notably, none of these animal models searched for inclusion bodies in skeletal muscle, which may be related to the behavioral abnormalities observed in some cases.

1.7.6 A Mouse Model shows Onset may be Myogenic

Monks et al. (2007) were the first investigators to describe onset as myogenic, rather than the traditional view of KD as being a motor neuron disease. Monks et al. created transgenic mice that over-express AR with a WT number of repeats (22 residues) exclusively in skeletal muscle fibers using a Human skeletal a-actin (HSA) promoter. Compared to WT littermates, HSA-AR mice have markedly reduced body weight and spinal curvature (kyphosis) indicative of advanced muscular atrophy. As measured by paw prints during gait testing, HSA-AR transgenic males have reduced hind limb stride length and reduced endurance on the hang test. Loss of motor function is androgen-dependent as castration of transgenic males dramatically improves muscle strength. Also, treating normally asymptomatic females with exogenous testosterone induces the weight loss and motor deficits akin to transgenic males.

For measurement of histopathological symptoms, cross-sections of the EDL of male transgenic mice were stained using the hematoxylin and eosin (H&E) technique for visualizing cellular changes and counting cell number. In addition, an NADH assay was performed for measuring changes in oxidative metabolism. These mice show muscular characteristics consistent with the KD phenotype, including atrophic and hypertrophic fibers, and fiber splitting. They also show markedly reduced number of muscle fibers and motor axon loss in L4 and L5 of the spinal cord, although neuron cell body number is unaffected. While these mice do show increased
oxidative metabolism in the EDL, whether this replicates disease processes in human KD is uncertain.

While mouse models prior to Monks et al. (2007) tended to emphasize the relation between neural abnormalities and strength deficits, there existed some evidence of a role for muscular changes in disease onset. Katsuno et al. (2002) found that neural pathology was accompanied by signs of muscular atrophy and abnormal fiber-type grouping in the gastrocnemius muscle. Sopher et al. (2004) observed fiber-type grouping in the quadriceps of their transgenic mice and McManamny et al. (2002) also described signs of muscular degeneration, including reduced number of muscle fibers with remaining fibers showing atrophic, condensed appearance in the medial gastrocnemius of CMV-AR120 mice. The findings of the Monks et al. study suggest that muscular pathology observed in these previous transgenic mice may result from disease processes originating in skeletal muscle, rather than being secondary to motor neuron pathology.

1.7.7 Transcriptional Changes in Mouse Models of KD

There have only been a small number of studies that have examined alterations in gene transcription in mouse models expressing polyQ-AR. Despite this fact, such studies are important as they provide useful insights into the pathogenic processes that are the basis for KD. Regarding the idea that ‘toxic gain of function’ may occur via VEGF depletion in neuromuscular tissues, Sopher et al. (2004) performed Q-PCR of the spinal cords of their YAC-AR100 mice, finding depletion of one VEGF isoform (VEGF 164) with an associated increase in CBP/AR interaction. Conversely, Monks et al. (2007) reported decreased expression of VEGF isoform 164 and 188 in limb muscle of severely affected mice. As skeletal muscle
showed signs of denervation (e.g. atrophic fibers, reduced motor neuron number), investigators proposed that motor axon atrophy may be secondary to primary changes in muscle. That is, a decrease in muscle-derived VEGF may trigger axonopathy with expression of polyQ-AR in myocytes. Whether the site of toxic gain of function of polyQ-AR lies in motor neurons or skeletal muscle, via transcriptional dysregulation of VEGF, is unresolved.

1.7.8 Muscle Metabolism and Fiber-typing

Representations of disturbances in metabolism and fiber-typing are relatively sparse in animal models of neuromuscular symptoms, despite being important indicators of pathology. Briefly, skeletal muscles differ in their contractile properties whereby those with high maximal shortening velocity are considered “fast-twitch” while those with low shortening velocity are “slow-twitch”. These muscle fibers express different isoforms of Myosin Heavy Chain (MHC), a component of each mechanical sarcomere necessary for muscle contraction. Muscles that contract relatively slowly express MHC I (slow MHC) within muscular sarcomeres. Those that contract more rapidly (usually twice the rate of slow-twitch fibers) express MHC II (fast MHC) in muscular contractile machinery. This information is useful as application of an antibody for fast or slow MHC to muscle samples permits classification of individual fibers as either fast-twitch or slow-twitch.

While a myosin assay is useful for determining the twitch properties of muscle fibers, classification based on metabolism parameters requires a different technique. Muscles contracting rapidly, but not for prolonged periods, require transiently high levels of ATP. Therefore, they typically comprise fibers that rely on glycolysis to fuel activity. By contrast,
muscles that contract more slowly, but for longer periods, have fibers that rely on oxidative metabolism. The degree of oxidative metabolism performed by transgenic muscle can be revealed using a biochemical assay for the metabolic enzymes, NADH and succinate dehydrogenase (SDH), both of which are linked by an early enzyme (ubiquinone) in the electron transport chain. When both a myosin and an SDH (or NADH) stain are typically used in conjunction, classification of fibers as either, slow, fast-glycolytic (FG) or fast-oxidative (FO) is possible. In commonly used muscles for fiber-typing, also useful in this analysis, the EDL and SOL, proportions of each of these 3 fiber-types are given below:

<table>
<thead>
<tr>
<th></th>
<th>EDL</th>
<th>SOL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slow</td>
<td>~0.5%</td>
<td>~37%</td>
</tr>
<tr>
<td>FG</td>
<td>~66%</td>
<td>~6%</td>
</tr>
<tr>
<td>FO</td>
<td>~33.5%</td>
<td>~57%</td>
</tr>
</tbody>
</table>

Note: These fiber-type proportions are estimated based on histochemical results of Augusto, Padovani & Campos (2004) for C57Bl6J mice.

Previous generations of mouse models of KD have investigated disturbances in fiber-typing and muscle metabolism using similar histological techniques to the methods described above. Abel et al. (2001) performed myosin and NADH stains on quadriceps, gastrocnemius, masseter, levator ani and bulbocavernosus muscles, but did not report any changes in fiber-type for their truncated-AR expressing mice. Sopher et al. (2004) report fiber-type grouping, with darker NADH stained myocytes clustering together in YAC-AR100 mice, but no evidence of a fiber-type switch with polyQ-AR expression. Similarly, Chevalier-Larsen et al. (2004) performed an
NADH stain but did not report any changes in fiber-typing or in oxidative metabolism. McManamny et al. (2002) performed an NADH stain of their muscle tissue also finding no changes in proportion of fiber-types despite reduced fiber number and slow fibers showing a disproportionate degree of hypertrophy. Thus, these animal models of KD failed to show a strong link between metabolism and fiber-type changes with onset of muscle weakness.

Using HSA-AR mice, Monks et al. (2007) found strong evidence of an increase in oxidative metabolism in the EDL based on NADH staining. Electron microscopy revealed that HSA-AR mice also show increased mitochondrial content in the EDL (Musa et al., 2011). Notably, HSA-AR transgenic females treated with testosterone also show increased mitochondrial volume density and size relative to vehicle-treated transgenic females. These findings demonstrate the androgen-dependent nature of increased oxidative metabolism when normal AR is over-expressed selectively in muscle. Despite increased oxidative metabolism, this switch was not related to a change in fiber-type proportion in the AT muscle, as measured by an overlay of fast-twitch myosin and SDH (Musa et al., 2011). A fiber-type switch was also not revealed in rats bearing HSA-AR using the same procedure, but FG fibers showed hypertrophy in the EDL (Fernando et al., 2010). Taken together, the overall pattern of evidence gleaned from studies of selective over-expression of normal Q-length AR in skeletal muscle demonstrates a shift toward enhanced oxidative metabolism in muscle. However, this does not preclude a fiber-type switch in the opposite direction, toward glycolytic metabolism in polyQ-AR mice. It remains a possibility that onset in animal models of KD may be related to such a switch, first discovered in cell cultures.
Towards an Inducible Mouse Model of Onset

A weakness of the transgenic mouse models discussed thus far is the fact that none of these laboratories could effectively portray onset of muscle weakness in a time-dependent manner. For such a description to arrive, an inducible animal model is required whereby initiation of expression of polyQ-AR is performed in the course of life rather than previous attempts to model KD in which transgene expression began from perinatal periods. Rao & Monks (2009) were the first investigators to integrate aspects of previous generations of transgenic mice in a single model in a style better permitting the testing of hypotheses regarding onset (see Figure 1 on p.33-34 for a description of the transgenic mouse model). Spatiotemporal specificity of expression was achieved by Rao & Monks (2009) by using a triple transgene method. The first transgene uses the HSA promoter to drive expression of the reverse tetracycline-controlled transactivator (rtTA) selectively in skeletal muscle. In the presence of tetracycline, rtTA molecules activate a second transgene bearing Cre recombinase under control of the tetracycline responsive element (TRE). These inactive double transgenic animals are crossed with mice carrying a human AR transgene (113 CAG repeats) with a LoxP-flanked transcriptional STOP sequence (preventing unwanted expression of the AR gene). Offspring carrying all three transgenes can now engage in expression of AR113 in the presence of tetracycline as Cre, once activated, can bind LoxP sites, removing the STOP sequence, permitting unconstrained transcription.

Crosses of HSA-rtTA/TRE-Cre founders with LoxP/LacZ reporter mice reveal that Cre expresses efficiently and is restricted to skeletal muscle following doxycycline (DOX; synthetic tetracycline) treatment. It is also shown that Cre expression in the absence of this chemical is
negligible (Rao & Monks, 2009). In other words, the triple transgene method of expression in skeletal muscle is empirically robust.

The novel method created by Rao & Monks for expression of polyQ-AR in skeletal muscle is adapted for expression selectively in motor neurons upon exposure to DOX treatment. Instead of carrying an HSA-rtTA/TRE-Cre double transgene, founders are established carrying a homeobox9 (HB9) promoter for rtTA. When HB9-rtTA/TRE-Cre mice are crossed with mice carrying the human AR transgene, offspring bearing all three transgenes are available. In this way, two experimental groups (i.e. HB9/AR113 and HSA/AR113) with spatiotemporal specificity for expression in either motor neurons or skeletal muscle are available for investigating onset at the main sites where illness is hypothesized to originate.
Figure 1: *Summary of Transgenes.* (A) For expression of human AR113 in skeletal muscle, the HSA promoter for rtTA is used in transgenic mice. (B) For expression in motor neurons, the HB9 promoter for rtTA is used in transgenic mice. Shown in (C) is the CMV/AR transgene with human AR bearing 113 Q repeats and loxP flanked STOP cassette (Rao & Monks, 2009).
1.8 **Objective, The DLO Hypothesis and Predictions**

The goal of this investigation was to test the DLO hypothesis. According to the DLO, an idea built upon the work of previous researchers, onset of KD involves pathology in motor neurons, and perhaps skeletal muscle. Towards testing the DLO, it was predicted that both HSA/AR113 and HB9/AR113 mice would show abnormalities in skeletal muscle after a short duration of transgene expression. Also, a secondary prediction was tested for further evidence of the likelihood of the DLO hypothesis. Given the fact that neuromuscular symptoms of KD have onset in middle adulthood, abnormalities observed in young adult transgenic mice were expected to be consistent across age when an older adult cohort of transgenic and WT mice was used.

Towards testing the general prediction, it was expected that there would be a reduction in cell number in HSA/AR113 and HB9/AR113 mice in the EDL and/or SOL muscles stained using the H&E technique. This result was expected as both clinical and post-mortem evidence reveal that the polyQ-AR gene is associated with muscular degeneration, as well as neuropathy. Also, a reduction in number was predicted for HSA/AR113 mice as this finding would be consistent with previous evidence that over-expression of AR in HSA-AR mice results in reduced number in the EDL (Monks et al., 2007). A reduction in number was also suspected for HB9/AR113 mice as previous work performed by McManamny et al. (2002) revealed reduced number of muscle fibers in the medial gastrocnemius of CMV-AR120 mice, expressing mutant AR globally.
Next, it was expected that there would be a shift in oxidative metabolism in the EDL and SOL muscles of both HSA/AR113 and HB9/AR113 mice. This idea was investigated using both an NADH and SDH stain and a fiber-typing analysis based on an image-overlay of myosin and SDH stains. Evidence to support the predicted switch in oxidative metabolism in transgenic mice arose from knowledge that selective over-expression of normal AR in skeletal muscle and polyQ-AR in cell cultures result in such changes (Monks et al., 2007; Ranganathan et al., 2009). Despite this knowledge, the direction of the switch in oxidative metabolism was too difficult to envision, with increased oxidative metabolism found in HSA-AR mice and reduced oxidative metabolism found in cell cultures.

Thirdly, the general prediction of this investigation suggested that there would be changes in cell size dependent on fiber-type in HSA/AR113 and HB9/AR113 mice compared with their WT littermates. Once again, whether this manipulation would result in hypo- or hypertrophy of certain fiber-types was more difficult to envision given the evidence. While studies report muscle atrophy in post-mortem KD patients and in animal models using global expression of polyQ-AR (Katsuno et al., 2002; Sopher et al., 2004; McManamny et al., 2002), both hypo-trophic and hypertrophic fibers were found in the EDL for HSA-AR mice and hypertrophy of FG fibers was reported in HSA-AR rats (Fernando et al., 2010). It was further deemed probable that transgenic mouse lines would show signs of muscle weakness as measured by a behavioral test battery. Supporting this idea, behavioral signs of muscle weakness were found previously with global expression of polyQ-AR (Katsuno et al., 2002; McManamny et al., 2002; Sopher et al., 2004) and selective over-expression of normal AR in muscle (Monks et al., 2007).
A secondary prediction was tested in this investigation towards finding results consistent with the DLO hypothesis. That is, abnormal phenotypes exhibited by transgenic mice would be consistent across age. This prediction would be shown to be true if the same histopathological and behavioral features exhibited by young adult transgenic mice (i.e. 90-100 days old) were also observed in an older cohort of transgenic mice (i.e. 250-350 days old). In the course of testing the secondary prediction of this investigation, there was the opportunity to glean new insights into the early mechanisms of KD. Previous mouse models of KD have not been used to investigate the relationship between aging and histopathological signs. This is likely due to early mortality in these animals (Adachi et al., 2001; Katsuno et al., 2002; Sopher et al., 2004; Monks et al., 2007). Yet, there is potential for aging to have an effect on muscle weakness in transgenic mice, given the knowledge that neuromuscular symptoms in KD occur in middle adulthood. If this is the case, this investigation, though mainly aimed at testing the DLO hypothesis, would also lead to new ideas of the adult onset of KD.
Chapter 2

Materials and Methods

2.0 Overview

This investigation consisted of a series of histological and behavioral experiments designed to test both the general and secondary predictions. Towards this aim, each experiment using young adult transgenic males and their WT littermates was repeated for an older aged cohort. The EDL and SOL muscles were dissected from hind limbs and stained for histopathological signs resulting from timed activation of expression of 113 Q-repeat AR. Measurements were taken from both the EDL and SOL muscles as these muscles serve as mutual positive controls for fiber-typing: the EDL, responsible for movement of the phalanges of the feet, consists mainly of FG and FO fibers, while the SOL, required for standing posture and walking, consists mainly of slow and FO fibers (Cartee, 1995). Upon completion of muscle staining, all histological analyses were carried out using ImageJ software (NIH). Statistical significance of results was performed using SPSS version 20 (IBM).

2.1 Subjects

All animal subjects were males generated from crosses of 3 transgenic mouse lines on a C57BL6J background. Breeding pairs included males of an Inducer strain bearing both of the HSA-rtTA and TRE-Cre transgenes or the HB9-rtTA and TRE-Cre transgenes shown above in Figure 1. Inducers were paired with females carrying the LoxP-AR113 transgene. Male offspring of these crosses were used as experimental mice if they owned the appropriate triple transgene combination or were WTs (i.e. if they were null for all 3 transgenes). In all experiments, both groups of transgenic mice, HSA/AR113 and HB9/AR113 males, were
compared with a combined control group consisting of their WT littermates. After 30 days spent in their birth-litter, these animals were separated and housed in cages shared by same-sex siblings. Animals were then genotyped using epithelial tissue from ear punches for PCR. Next, mice performed baseline strength testing prior to exposure to DOX treatment for 5 days. DOX treatment began at 90-100 days old for the younger cohort and 250-350 days old for the older cohort. DOX was dissolved in drinking water at a concentration of 2 mg/ml supplemented with 5% sucrose and provided ad lib to transgenic and WT mice in light-proofed water bottles. Provided in this way, DOX solution was expected to initiate transgene expression.

At the end of the 5 day period of DOX treatment, all animals underwent Post-DOX strength testing on the same day, once a week, and dissected 4 weeks later. Due to time constraints and an excess in number of dissected animals available for this study, only a sufficient number of animals were used in each condition for histological analysis. During the life cycle, all animals were housed in controlled laboratory conditions with ad libitum access to food and water. Ambient temperature was 24°C, and the light-dark cycle was set at 12 hours.

### 2.2 Dissections and Sectioning

All mice underwent live dissections under anesthesia using 10% Avertin (0.5-0.7 ml i.p.). During anesthesia, the EDL and SOL muscles were rapidly removed from both hind limbs. Dissected muscles were then oriented in cryomolds containing Tissue-tek, flash frozen in liquid nitrogen, and subsequently stored at -80°C until further processing. All frozen muscles were later sectioned at 12 μm thickness using a microtome cryostat system (Bright Instruments
5030). Only sections near or approximately at the belly of each muscle were mounted and then stored again at -80°C until commencement of the histological analysis.

2.3 H&E Staining

*Mice Used for H&E Stains:* Muscle cross-sections of the EDL were taken from a total of 23 mice (6 HSA/AR113; 7 HB9/AR113; 10 WT littermates) and from 26 mice (6 HSA/AR113; 8 HB9/AR113; 12 WT littermates) in the case of the SOL, for determining cell number in the younger cohort (Cohort 1). Among the older cohort (Cohort 2), muscle cross-sections were included in the analysis from a total of 21 mice (5 HSA/AR113; 6 HB9/AR113; 10 WT littermates) in the case of both the EDL and SOL muscles. Dissected muscle samples were excluded from the analysis in the event of freezing artifact preventing accurate cell counting in ImageJ. Freezing artifact occasionally appeared in dissected muscle, appearing as perforations in individual fibers when viewed under the microscope. Presumably, this issue arose due to difficulty in orientation of some dissected muscle tissues in cryomolds prior to flash freezing in liquid nitrogen (Bancroft, 2008).

*Staining Protocol:* H&E staining was performed in this investigation as this stain provides sufficient differentiation between myocytes for counting cell number (Monks et al., 2007). Slides were first brought to room temperature over a period of 20 min. They were then fixed in 4% paraformaldehyde dissolved in phosphate buffered saline (PBS) for 15 minutes. In order to remove formaldehyde, slides were then washed in distilled water. Next, slides were immersed in hematoxylin for 5 minutes. This was followed by 30 sec incubation in eosin working solution. Slides were next taken through a graded series of ethanols dissolved in dH₂O (first
70%, then 95%, followed by 100% ethanol). Then slides were immersed in xylene and finally cover-slipped with Permount.

*Light Microscopy and Cell Counting:* Two images of the entire cross-section of both the EDL and SOL were photographed at either 40X or 100X magnification. Cross-sections deemed to be of the best quality were selected for photography. Images were then imported into ImageJ for counting. Following counting of all cells within each cross-section, an estimate of cell number was determined for each muscle and subjected to a statistical analysis.

*Statistics:* Both the EDL and SOL were analysed in separate between subjects ANOVAs with Genotype (HSA/AR113; HB9/AR113; WT) serving as the between subjects factor and number serving as the dependent variable. Post-hoc Tukey HSD tests followed in order to reveal which groups differed according to genotype.

### 2.4 NADH Staining

*Mice Used for NADH Staining:* Muscle cross-sections of the EDL from a total of 19 mice (5 HSA/AR113; 5 HB9/AR113; 9 WT littermates) and from 21 mice (5 HSA/AR113; 6 HB9/AR113; 10 WT littermates) in the case of the SOL were useable following NADH staining. Among the older cohort, muscle samples were included in the analysis from a total of 22 mice (5 HSA/AR113; 6 HB9/AR113; 11 WT) in the case of both the EDL and SOL muscles. Mice were excluded from the analysis in the event of freezing artifact of samples preventing accurate counting in ImageJ following NADH staining.
Staining Protocol: To begin NADH staining, slides were first allowed to thaw for 20 minutes at room temperature. Slides were then incubated in 250 ml of dH₂O containing one dissolved tablet (10 mg) of Nitroblue tetrazolium (NBT) and 20 mg of β-nicotinamide adenine dinucleotide, reduced dipotassium salt (β-NADH) at 37°C for 2 hours. Following incubation, slides were washed in dH₂O and then bathed in a graded series of acetones dissolved in dH₂O (30%, 60%, 90%, 60%). After another wash in dH₂O to remove remaining acetone, slides were cover slipped using polyvinyl alcohol mounting medium (Sigma). It is important to note that muscle samples from each animal were stained using a procedure whereby both muscles (i.e. EDL and SOL) from each condition (HSA/AR113; HB9/AR113; WT littermates) were represented on each run. This procedure was undertaken as a precaution against variation in staining quality occurring between each run, hindering accurate comparisons of oxidative metabolism between conditions. It is also noted that incubation of tissue slides in the staining mixture, introduces β-NADH to mounted muscle fibers. Thus, NADH staining actually assesses mitochondrial enzyme activity responsible for oxidation of naturally occurring NADH for oxidative metabolism (Ying, 2006).

Light Microscopy and Measurement of Oxidative Metabolism: Two cross-sections of each NADH stained muscle were selected based on overall quality for photography under 100X magnification. Two images were then acquired of each cross-section that combined encompassed the whole muscle and then imported into ImageJ for analysis. Individual myocytes in each image were counted as either dark or light, a decision based on whether cells were pigmented or not. This analysis was used to determine the number of cells that require or do not require NADH-dependent oxidative metabolism, respectively. The proportion (%) of fibers that were light in each muscle was then subjected to an overall statistical analysis.
Statistics: Separate between subjects ANOVAs were run for each muscle (i.e. EDL, SOL) with Genotype (HSA/AR113; HB9/AR113; WT) being the between subjects factor and proportion of light fibers serving as the dependent variable. Post-hoc Tukey HSD tests followed this analysis. Since proportions of fibers that were light served as statistical data, values were transformed using an arcsine function prior to statistical analysis.

2.5 Fiber-typing

Mice Used for Fiber-typing: Muscle cross-sections of the EDL from a total of 21 mice (6 HSA/AR113; 6 HB9/AR113; 9 WT littermates) and from 18 mice (4 HSA/AR113; 5 HB9/AR113; 9 WT littermates) in the case of the SOL were useable following SDH and myosin staining in the younger cohort. Among the older cohort, muscle samples of the EDL were included in the analysis from a total of 20 mice (4 HSA/AR113; 6 HB9/AR113; 10 WTs) and from 21 mice (5 HSA/AR113; 6 HB9/AR113; 10 WTs) in the case of the SOL muscle. Mice were excluded from the analysis in the event of freezing artifact of muscle samples preventing accurate measurements in ImageJ.

2.5.1 SDH Staining

Staining Protocol: As part of the fiber-typing analysis, an SDH stain was performed of muscle cross-sections for placement in an image-overlay with fast-myosin stained tissue. SDH was also used to determine proportion of myocytes requiring glycolytic or oxidative metabolism. In this way, SDH was useful in confirming the reliability of results gleaned from NADH staining.
To begin SDH staining, slides were first allowed to thaw for 20 min. Slides were then incubated in a 250 ml aqueous solution containing 3 tablets (30 mg) of NBT and 3 g of sodium succinate at 37°C for 2 hours. Slides were then rinsed and dehydrated through a series of ethanols dissolved in dH₂O (70%, 95%, 100%). After emersion in xylene, slides were then cover-slipped with polyvinyl alcohol mounting medium. Again, both muscles (i.e. EDL and SOL) from each condition (HSA/AR113; HB9/AR113; WT littermates) were represented on each run.

2.5.2 Fast-Twitch Myosin Staining

Staining Protocol: On day 1 of immunohistochemical treatment of muscle samples, slides were first allowed to thaw for 20 min and then fixed in 4% PO₄-buffered paraformaldehyde. Slides were then washed in PBS working solution followed by incubation in 10% Normal Horse Serum (NHS) in PBS. Next, muscle samples were incubated in monoclonal anti-skeletal fast-myosin antibody (1:1000 4% NHS) for 45 hours at 4°C. Staining with this primary antibody was used for labelling individual myocytes as either fast-twitch or slow-twitch. This was possible as only cells that express the fast isoform of MHC associate with the myosin primary antibody used.

On Day 3 of staining for fast-twitch myosin, mounted sections were washed in PBS and then incubated in rat-adsorbed biotinylated horse anti-mouse 2⁰ antibody (1:200 10% NHS in PBS) for one hour. Following an intermediate wash in PBS solution, mounted sections were then treated with avidin-biotin-peroxidase complex (ABC) and then immersed again in PBS. Biotinylated horse anti-mouse 2⁰ antibody and ABC were visualized with diaminobenzidine (DAB) and nickel chloride. As reaction time with DAB can be variable between runs, 2 test
slides of WT muscle cross-sections were used in which the 1° antibody was either applied or omitted to mounted tissue, respectively, on Day 1. All experimental slides were removed from DAB solution upon confirmation that fast-myosin labelling was completed by visualizing the progress of staining on the test slides. This was useful also in determining the specificity of the protocol for labelling fast-twitch fibers as the no primary antibody condition should be free from specific staining. All slides were then quickly washed in PBS, dehydrated through a series of ethanols and immersed in xylene before cover slipping with normal Permount.

*Light Microscopy and Fiber-typing*: Two photomicrographs were taken of both SDH and myosin stained cross-sections, respectively under 100X magnification. These images were then overlaid for fiber-typing the entire muscle. Only the matching sections of best quality were used. The proportion of each fiber-type in the entire EDL and SOL was determined based on cell counts in ImageJ followed by an overall statistical analysis. Classification of fiber-types was performed according to the scheme shown in Figure 2 on p.46-47.
Figure 2: Fiber-typing. SDH stain of WT SOL (left) fast-myosin stain of WT SOL (right).

Method: An image-overlay of fast-myosin and SDH staining reveals 3 different fiber-types. Myocytes that stain dark for fast-myosin and light for SDH are FG; those that stain dark for fast-myosin and dark for SDH are FO; slow fibers stain light for fast-myosin (see labels below for examples).
The same images of SDH stained tissue used for fiber-typing were also useful for measuring oxidative capacity of skeletal muscle. Myocytes were counted as either dark or light to determine the number of cells that require or do not require SDH-dependent oxidative metabolism. The proportion of fibers that were light in each muscle was then determined and subjected to an overall statistical analysis.

Statistics: A repeated measures ANOVA was performed in order to determine any differences between conditions in the proportion of slow, FG or FO fibers in the EDL and SOL. Genotype (HSA/AR113; HB9/AR113; WT) served as the between subjects factor and Fiber-type served (slow; FG; FO) as the within subjects factor in the analyses. Since the proportions of each fiber-type served as statistical data, values were transformed using an arcsine function prior to the ANOVA. If the Genotype X Fiber-type interaction was determined to be significant, the post-hoc Tukey HSD statistic was used to determine instances where groups differed based on genotype.

A between subjects ANOVA was run for measurement of oxidative capacity in the EDL and SOL using the SDH staining method. Genotype (HSA/AR113; HB9/AR113; WT) was included as the between subjects factor while proportion of fibers that were light served as the dependent variable. Post-hoc Tukey HSD tests were used to determine which groups differed based on genotype. As the proportion of myocytes that were light served as the dependent variable, values were transformed using an arcsine function prior to the statistical analyses.
Mice Used for Cell Size Estimates: Muscle cross-sections of the EDL from a total of 19 mice (6 HSA/AR113; 4 HB9/AR113; 9 WT littermates) and of the SOL from 17 mice (4 HSA/AR113; 4 HB9/AR113; 9 WT littermates) were used to determine physical size of fiber-types in the younger cohort. Among the older cohort, muscle samples were included in the analysis from a total of 18 mice (5 HSA/AR113; 5 HB9/AR113; 8 WT littermates) in the case of the EDL and from 18 mice (5 HSA/AR113; 6 HB9/AR113; 7 WT littermates) in the case of the SOL muscle. Mice were excluded from the analysis in the event that freezing artifact of samples prevented accurate measurements of size in ImageJ.

Protocol for measuring Cell Size: In order to estimate size of cells classified by fiber-type, an overlay of H&E, myosin and SDH stains was used, with fiber-typing performed using myosin and SDH stained sections as before and corresponding H&E stained tissue sections serving as the template for measurement of size in ImageJ. All images were generated from light microscopy under 100X magnification. Measurements were taken from H&E stained sections as these other staining methods tend to manipulate the structure of myocytes, skewing accuracy.

In ImageJ, size was measured by taking the minor ellipse (i.e. shortest width) of each fiber-typed myocyte, as previous researchers have performed (Fernando et al., 2010). The minor ellipse was an appropriate dependent variable for determining cell size as the angle of sectioning of muscle may misrepresent the actual diameter of fibers, preventing accurate quantification. The minor ellipse was taken from a total of 25-60 myocytes of each fiber-type in
each image of H&E stained muscle. The average minor ellipse of each fiber-type was then determined for each muscle and subjected to an overall statistical analysis.

**Statistics:** Cell size was analysed using separate repeated measures ANOVAs for the EDL and SOL, with Fiber-type (slow; FG; FO) serving as the within subjects factor and Genotype (HSA/AR113; HB9/AR113; WT) serving as the between subjects factor. In the event that the interaction of Genotype X Fiber-type was significant, post-hoc testing using the Tukey HSD statistic was then performed to determine instances where groups differed based on genotype.

### 2.7 Behavioral Measures

**Mice Used for Behavioral Testing:** A total of 31 mice (7 HSA/AR113; 9 HB9/AR113; 15 WT littermates) underwent behavioral testing in the younger cohort. In the older adult cohort, a total of 31 mice (10 HSA/AR113; 8 HB9/AR113; 13 WT littermates) were used for behavioral testing. Prior to DOX exposure, all animals experienced a habituation period to the test battery on 3 consecutive days. To begin all testing sessions, mice were brought into the testing room and allowed to acclimate to the environment in their cages for 10 min. Habituation consisted of a 2 min hang test, an escalation in speed per day (5, 10, 15 rotations per min) on the constant-speed rotarod test and familiarity with the gait testing chamber. On the day following the habituation period, animals performed the entire behavioral test battery as a baseline test of muscle strength. This was followed by DOX exposure for 5 days in their cage water. One week after day 1 of DOX exposure, animals began weekly performance of the behavioral test battery for 3 weeks. On the fourth week (Post-DOX), all animals were dissected.
2.7.1 *Endurance Strength Tests*

*Rotarod:* To test for muscular endurance and motor coordination, mice were tested with a rotarod (Columbus Instruments; axle diameter 3.6 cm; speed of 16 rpm). Each animal was given three trials per session with 10 min in between each trial. The test was stopped after 480 sec. Only an animal’s best rotarod test score per session was included in the analysis.

*Statistics:* For measuring performance on the rotarod, ‘time to fall’ served as the dependent variable. A repeated measures ANOVA was performed with Test Session (Pre-DOX; Post-DOX1; Post-DOX2; Post-DOX3) as the within subjects factor and Genotype (HSA/AR113; HB9/AR113; WT) serving as the between subjects factor. In the event that the interaction of Test Session X Genotype reached significance, post-hoc Tukey HSD tests were then run in order to determine which groups differed based on genotype on specific test days. In the event that Test Session was alone significant, independent samples t-tests were performed to determine general trends across testing intervals for the rotarod test.

*Hang Test:* Mice were tested further for their muscular endurance using the hang test. Mice were placed on a wire grid cage top and turned upside down 40 cm above cage bedding, and the latency to fall up to 120 sec was measured. Each mouse was given three trials per session with 10 min in between each trial. Only the best hang test score on each session for each mouse was included in the statistical analysis.

*Statistics:* A repeated measures ANOVA was performed with time to fall serving as the dependent variable. In the ANOVA, Test Session served as the within subjects factor and
Genotype (HSA/AR113; HB9/AR113; WT) served as the between subjects factor. In the event that the interaction of Test Session X Genotype reached significance, post-hoc Tukey HSD tests were run in order to determine which groups differed based on genotype on specific test days. In the event that Test Session was alone significant, independent samples t-tests were performed to determine general trends across testing intervals for the hang test.

2.7.2 Peak-Force Measure

*Grip Strength:* A grip strength meter (Columbus Instruments) was used to measure forelimb grip strength. Mice were held by the tail and lowered toward the apparatus. Mice were allowed to grasp the metal triangular pull bar with their forelimbs only and then were pulled backward. The force applied to the bar at the moment the grasp was released was recorded as the peak tension (kg). Each mouse was given three trials per session with 10 min in between each trial. The test was repeated 3 consecutive times within each trial, and the highest value arising from the 3 trials was recorded as the grip strength for that mouse.

*Statistics:* A repeated measures ANOVA was performed with peak tension serving as the dependent variable. In the ANOVA, Test Session served as the within subjects factor and Genotype (HSA/AR113; HB9/AR113; WT) was the between subjects factor. In the event that the interaction of Test Session X Genotype reached significance, post-hoc Tukey HSD tests were run in order to determine which groups differed based on genotype on specific test days. In the event that Test Session was alone significant, independent samples t-tests were performed to determine general trends across testing intervals for grip strength.
2.7.3 Gait Analysis

Paw print analysis: Prior to testing, forepaws were painted with non-toxic acrylic red paint and hind paws painted with blue. Mice were placed at one end of a chamber with a sheet of paper along its length and guided to walk along it. Stride length (mm) was measured from fore and hind limbs and averaged to yield a single estimate per mouse.

Statistics: A repeated measures ANOVA was performed with stride length measured as the dependent variable. In the ANOVA, Test Session served as the within subjects factor and Genotype (HSA/AR113; HB9/AR113; WT) was the between subjects factor. In the event that the interaction of Test Session X Genotype reached significance, post-hoc Tukey HSD tests were then run in order to determine which groups differed based on genotype on specific test days. If only Test Session was significant in the ANOVA, independent samples t-tests were performed, determining which sessions differed.
Chapter 3

Results

3.0 Overview

The goal of this investigation was to provide an adequate test of the DLO hypothesis, the idea that onset of KD involves pathology in motor neurons and perhaps skeletal muscle. In order to perform this task, the general prediction was made that both HSA/AR113 and HB9/AR113 mice would show abnormalities over the course of a brief 4-week period of transgene expression (see Table 1 on p.56 for a summary of results). A further means of testing the DLO hypothesis was envisioned by generating the secondary prediction, the idea that abnormalities observed in 90-100 day old transgenic mice would be replicated among an older cohort of transgenic mice (250-350 days old). If both the general and secondary predictions were confirmed, findings of this investigation would be consistent with the DLO hypothesis of KD.

Among both the young and older adult cohorts of transgenic and WT mice, some abnormalities were observed as a result of transgene activation. The young adult HB9/AR113 mice of Cohort 1 were alone in showing reduced numbers of myocytes. However, reduced cell numbers in these mice were limited to the SOL. In older adult mice, this finding was not replicated. Next, both histological measures of oxidative metabolism (i.e. NADH and SDH) revealed reduced oxidative capacity in both groups of transgenic mice relative to WTs. However, this effect was not as pronounced in older mice as in young adult mice. An overlay of fast-myosin and SDH stains better characterized the fiber-type switch in young adult HSA/AR113 mice as resulting from FO fibers turning to the FG fiber-type in the EDL.
Measurements of cell size in young adult HSA/AR113 mice showed reduced myocyte size in the case of FG fibers in the EDL. Behavioral measures of muscle strength revealed that young adult HB9/AR113 mice showed deficits on the hang test following DOX exposure. When older adult mice were tested they failed to show this same deficit. Interpretations of these results in terms of the hypothesis of this investigation are addressed further in the Discussion section.
Table 1: Summary of Results for both Young Adult HSA/AR113 and HB9/AR113 Mice from Cohort 1.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number</th>
<th>NADH</th>
<th>SDH</th>
<th>Fiber-typing</th>
<th>Size</th>
<th>Behavior</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA/AR113</td>
<td>No change</td>
<td>Reduced</td>
<td>Reduced</td>
<td>Fiber-type switch observed</td>
<td>Reduced in FG fibers</td>
<td>No change</td>
</tr>
<tr>
<td></td>
<td></td>
<td>staining</td>
<td>staining</td>
<td>FO $\rightarrow$ FG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HB9/AR113</td>
<td>Reduced in SOL</td>
<td>Reduced</td>
<td>Reduced</td>
<td>No change</td>
<td>No change</td>
<td>Impaired on hang test</td>
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3.1 Muscle Cell Number

In order to determine an effect of transgene expression on skeletal muscle health, the number of cells in both the EDL and SOL for HSA/AR113 and HB9/AR113 were compared with the number observed in their WT littermates. One-way ANOVAs, run for both muscles separately, revealed a significant effect of Genotype in the SOL (F=4.754; p=0.019), but not in the EDL (see Figure 3 on p.58-59 for results). Post-hoc analysis revealed that HB9/AR113, but not HSA/AR113 mice, were reduced in number relative to WTs in the SOL (p=0.015). In the older cohort, the EDL and SOL were analysed separately in between subjects ANOVAs. Both analyses failed to reveal a main effect of Genotype (see Figure 4 on p.60-61 for results in the older cohort).
Figure 3: Cell Number. (A) H&E stains of the EDL and SOL for both young adult transgenic and WT mice. (B) Cell counts within the EDL (left) and SOL (right) for young adult transgenic and WT mice.
Figure 4: *Cell Number*. (A) H&E stains of the EDL and SOL for both older adult transgenic and WT mice. (B) Cell counts within the EDL (left) and SOL (right) for older adult transgenic and WT mice.
3.2 NADH Stain

In order to measure differences in oxidative capacity between transgenic mice and their WT littermates based on transgene expression, between subjects ANOVAs were run on statistical data gleaned from NADH stained EDL and SOL muscles (see Figure 5 on p.63-64 for results). In this analysis, there was a main effect of Genotype in the EDL (F=9.798; p=0.002). Post-hoc tests revealed that both HSA/AR113 and HB9/AR113 mice showed reduced oxidative capacity in the EDL relative to WT mice (p=0.01; p=0.004, respectively). In the SOL, there was also a main effect of Genotype (F=18.703; p<0.001), with HSA/AR113 and HB9/AR113 showing reduced oxidative metabolism relative to WT mice (p=0.013; p=0.001, respectively).

In the older cohort, this study replicated findings recorded for younger mice (see Figure 6 on p.65-66 for these results). When both muscle types were analysed separately, there was a main effect of Genotype (F=3.952; p=0.037) in the EDL, however, both HSA/AR113 and HB9/AR113 mice did not differ compared to WTs (p=0.097; p=0.068, respectively). In the SOL, there was a main effect of Genotype (F=11.126; p=0.001), with both HSA/AR113 and HB9/AR113 mice showing significantly reduced NADH-dependent oxidative metabolism relative to WT mice (p=0.002; p=0.005, respectively). Thus, it appears that reduced oxidative capacity of skeletal muscle is consistent across age. Of note, percentage data gleaned from NADH staining in Cohort 1 and 2 was deemed to be generally normally distributed both before and after arcsine transformation as determined by the Kolmogorov–Smirnov (K-S) test. It is suggested then that statistical transformation of percentage data using this method was acceptable for quantifying results for NADH staining.
Figure 5: *NADH Assays.* (A) NADH staining of the EDL and SOL for both young adult transgenic and WT mice. (B) Percentage (%) of light fibers within the EDL (left) and SOL (right) upon NADH staining (young adult transgenic and WT mice).
Figure 6: *NADH Assays.* (A) NADH staining of the EDL and SOL for both older adult transgenic and WT mice. (B) Percentage (%) of light fibers within the EDL (left) and SOL (right) upon NADH staining (older adult transgenic and WT mice).
3.3 SDH Stain

In order to confirm the reliability of the effects found using the NADH stain (i.e. reduced oxidative metabolism in transgenic mouse groups), proportions of light fibers after SDH staining in the EDL and SOL were compared with WTs (see Figure 7 on p.68-69 for results). When the proportion of light fibers were compared between groups in the EDL and SOL separately, there was a main effect of Genotype in both the EDL ($F=6.876; p=0.006$) and the SOL ($F=4.342; p=0.033$). Post-hoc tests revealed that both HSA/AR113 and HB9/AR113 mice showed reduced oxidative capacity compared with WT mice in the EDL ($p=0.008; p=0.039$, respectively) and only HB9/AR113 mice showed a difference in the SOL ($p=0.032$).

In older adult mice, a one-way ANOVA performed on light fibers in the EDL revealed a main effect of Genotype ($F=5.406; p=0.015$). Post-hoc tests revealed reduced oxidative metabolism in HSA/AR113 mice ($p=0.026$). In the SOL, all between subjects comparisons were non-significant (see Figure 8 on p.70-71 for results in Cohort 2). Overall, results for NADH were supported by evidence of reduced oxidative metabolism using the SDH staining method. Percentage data gleaned from SDH staining in Cohort 1 and 2 was deemed to be generally normally distributed both before and after arcsine transformation as determined by the K-S test. Transformation of percentage data using the arcsine formula was thus acceptable for quantification of SDH staining.
Figure 7: *SDH Assays.* (A) SDH staining of the EDL and SOL for both young adult transgenic and WT mice. (B) Percentage (%) of light fibers within the EDL (left) and SOL (right) upon SDH staining (young adult transgenic and WT mice).
Figure 8: *SDH Assays.* (A) SDH staining of the EDL and SOL for both older adult transgenic and WT mice. (B) Percentage (%) of light fibers within the EDL (left) and SOL (right) upon SDH staining (older adult transgenic and WT mice).
3.4 Fiber-typing

In order to investigate whether transgene expression had an effect on fiber-type proportions in skeletal muscle, the proportions of slow, FG and FO fibers in the EDL and SOL were compared between HSA/AR113, HB9/AR113 and WT mice. A repeated measures ANOVA was performed with Genotype as the between subjects factor and Fiber-type as the within subjects factor in the EDL and SOL separately (see Figure 9 on p.74-75 for results). In the EDL, Fiber-type was significant (F=5.390; p=0.032) as well as the Genotype X Fiber-type interaction (F=10.856; p=0.001).

In order to further investigate the interaction of Genotype X Fiber-type within the EDL for Cohort 1, post-hoc Tukey HSD testing was performed. For slow fibers, post-hoc tests revealed no significant differences between groups. This was not surprising as only 2 mice displayed slow fibers in HSA/AR113 mice, while slow fibers were not observed in HB9/AR113 or WT mice in the EDL. When FG fibers were compared between groups, HSA/AR113 mice showed a larger proportion of FG fibers than WT mice (p<0.001). For FO fibers, HSA/AR113 mice showed less FO fibers than HB9/AR113 and WT mice (p=0.039; p<0.001, respectively). In the SOL, Fiber-type was significant (F=5.777; p=0.008), but Genotype and their interaction were not. These results in the younger cohort suggest the presence of a fiber-type switch in HSA/AR113 mice from FO to FG metabolism in the EDL muscle. This finding was not accompanied by a switch between slow–twitch and fast-twitch fiber-types.

In older adult mice, Fiber-type was significant (F=42.994; p<0.001), but Genotype and the interaction of Genotype X Fiber-type was not in the EDL (see Figure 9 on p.74-75 for results). The repeated measures ANOVA performed on the SOL, revealed a within subjects effect of
Fiber-type (F=191.760; p<0.001), but Genotype, and the interaction of Genotype X Fiber-type were non-significant. Thus, results from older adult mice did not support a fiber-type switch in the EDL as was found in young adult HSA/AR113 mice as measured by the fiber-typing paradigm. Of note, fiber-type proportions in both Cohort 1 and 2 underwent K-S testing for normality revealing that data was generally normally distributed both before and after arcsine transformation.

### 3.5 Cell Size

In order to investigate whether transgenic mice differ from WT mice in cell size over the course of transgene expression, measurements of cell size according to fiber-type within the EDL and SOL were included in the histopathological analysis. Separate repeated measures ANOVAs were run for the EDL and SOL muscles (see Figure 9 on p.74-75 for cell size results). In the EDL, size of slow fibers was not included in the analysis due to their absence in HB9/AR113 and WT mice. When only FG and FO fibers were measured, there was an effect of Fiber-type (F=89.333; p<0.001), a main effect of Genotype (F=5.120; p=0.019) and their interaction was significant (F=10.480; p<0.001). Post-hoc testing revealed that HSA/AR113 mice had smaller FG fibers than WTs and HB9/AR113 mice (p=0.005; p=0.023, respectively). In older adult mice, except for Fiber-type in the EDL (F=7.616; p=0.015), all comparisons based on cell size were non-significant (see Figure 9 for results in Cohort 2).
Figure 9: *Fiber-typing and Cell Size.* (A) Fiber-typing and Cell Size Results from Cohort 1. % of each fiber-type is given for the EDL (Top) and SOL (second from Top); average minor ellipse of fiber-typed cells is provided for the EDL (third from Top) and SOL (Bottom). (B) Fiber-typing and Cell Size Results from Cohort 2 (provided in the same style as for Cohort 1).
3.6 Dissection and Behavioral Measures

3.6.1 Seminal Vesicle and Total Body Weight

Over the course of dissections, both the left and right seminal vesicles (SVs) were removed from anesthetized experimental mice and scale weighed (g). SV weight was taken as a measure of the health of systemic machinery devoted to testosterone production in transgenic and WT mice. Figure 10 on p.77-78 shows results for SV weight. In Cohort 1, a between subjects ANOVA was non-significant for SV weight. However, within the older cohort, there was a main effect of Genotype (F=4.790, p=0.17), with HSA/AR113 mice owning significantly increased SVs (p=0.019).

In order to measure total body weight (g) prior to each testing session, all animals were scale weighed and this value was then recorded. Upon completion of testing on Post-DOX3, a repeated measures ANOVA was performed on body weight data to determine any differences incurred by polyQ-AR transgene expression (see Figure 10 on p.77-78 for results). In the young adult cohort, Test Session was significant (F=32.509; p<0.001), but Genotype and the interaction between these factors were not. In order to learn from weight data across Pre- and Post-DOX sessions, independent samples t-test comparisons were made between each interval of testing. T-tests revealed that there was a general increase in body weight from Pre-DOX1 to Post-DOX2 (p=0.013) and Pre-DOX1 to Post-DOX3 (p=0.009). In the older cohort, all comparisons were non-significant.
Figure 10: Dissection and Weight measures. (A) SV weight (g) for Cohort 1 (left) and Cohort 2 (right). (B) Total Body Weight (g) for Cohort 1 (left) and Cohort 2 (right).
3.6.2 Rotarod

In order to determine whether transgenic mice would display abnormalities in neuromuscular endurance, mice were measured on a rotarod test (see Figure 11 on p.80-81 for results on this measure). When the younger cohort was tested for performance on the rotarod, Test Session was significant (F=420.455; p<0.001), but Genotype and the interaction between these variables were not. Upon testing using the t-statistic, none of the test sessions differed. In the older cohort, Test Session was significant (F=253.869; p<0.001), but Genotype and the interaction between these variables were not. As was the case for the younger cohort, overall performance on each session did not differ with any other test sessions. From these results, trends in performance on the rotarod did not improve or decline across the 3-week testing period.

3.6.3 Hang Test

Performance on the hang test, measured as time to fall, was used to examine neuromuscular endurance in transgenic and WT mice (see Figure 11 on p.80-81 for results). Test Session was significant (F=10.078; p<0.001), along with a main effect of Genotype (F=5.095; p=0.013). Post-hoc Tukey HSD analysis revealed that only HB9/AR113 mice showed a deficit on this measure, differing from WT mice on Post-DOX1 (p=0.007) and Post-DOX3 (p=0.042). These results on the hang test in young adult mice may be interpreted as a deficit in neuromuscular endurance when polyQ-AR is selectively expressed in motor neurons. In the older cohort, a repeated measures ANOVA on hang test data did not reveal any significant values.
Figure 11: *Endurance Strength measures.* (A) Time to fall (s) on the rotarod test for Cohort 1 (left) and Cohort 2 (right). (B) Time to fall on the hang test for Cohort 1 (left) and Cohort 2 (right).
3.6.4 Grip Strength

Testing for deficits in peak-force generation required a grip strength test (see Figure 12 on p.84-85 for results). In the younger cohort, there was an effect of Test Session (F=5.195; p=0.007), but Genotype and the interaction between these variables were non-significant. Performance, as measured by the grip strength meter, showed a declining trend over the course of testing for animals of this age group. Adjacent test sessions showing decline in performance on the latter session was found from Post-DOX2 to Post-DOX3 (p=0.005). Other session intervals showing the same general decline over the course of testing were from Pre-DOX1 to Post-DOX2 (p<0.001) and Pre-DOX1 to Post-DOX3 (p=0.016).

Among older adult mice, there was a main effect of Test Session (F=16.512; p<0.001) and the interaction of Test Session X Genotype reached significance (F=2.451; p=0.036). However, post hoc tests revealed no differences based on genotype on any of the test sessions. These mice showed a fairly consistent downward trend in performance with worse performance on latter trials from Pre-DOX1 to Post-DOX1 (p=0.001) and Post-DOX1 to Post-DOX2 (p=0.013). Other comparisons showed the same downward trend from Pre-DOX1 to Post-DOX2 (P<0.001), Pre-DOX1 to Post-DOX3 (p=0.001), and Post-DOX1 to Post-DOX3 (p=0.013).

3.6.5 Gait Analysis

To continue testing for differences between transgenic and WT mice in the predicted pattern, a measure of stride length was performed on young adult mice (see Figure 12 on p.84-85 for results). The repeated measures ANOVA only revealed a main effect of Test Session (F=3.452; p=0.025). Independent samples t-tests of individual sessions revealed a general decline in stride...
length from Pre-DOX1 to Post-DOX3 (p=0.019). Due to the lack of positive results on this measure as well as time constraints, gait analysis data was not evaluated in older adult mice.
Figure 12: *Grip Strength and Gait Analysis.* (A) Grip strength, measured as peak tension (kg) in Cohort 1 (Top) and Cohort 2 (Middle). (B) Stride length (mm) for transgenic and WT mice in Cohort 1.
Chapter 4

Discussion

4.0 Overview

The DLO hypothesis, based upon the work of previous investigators, states that onset of KD involves pathology in motor neurons and perhaps skeletal muscle. This is a departure from the traditional view of KD, whereby motor neurons alone are believed to be primary in disease. In order to test the DLO hypothesis, the general prediction of this investigation was made. The general prediction proposes that measurements taken from muscle cross-sections after four weeks of selective expression of 113Q repeat-AR should indicate abnormalities in both HSA and HB9 transgenic mice.

The general prediction was evaluated based on the outcome of 4 experiments. First, it was proposed that there would be a reduction in cell number for HSA/AR113 and HB9/AR113 mice in the EDL and/or SOL muscles stained using the H&E technique. This aspect of the general prediction was partially confirmed, as HB9 transgenic mice showed a reduction in number in the SOL, while HSA transgenic mice failed to show this histopathological sign.

Next, it was proposed that there would be changes in oxidative metabolism in transgenic mice when compared with their WT littermates. This aspect of the general prediction was roundly confirmed. The fiber-typing method used in this investigation revealed that HSA/AR113 mice showed a switch in fiber-type from FO to FG fibers in the EDL. Despite the fact that a fiber-type switch was found only for HSA transgenic mice, both transgenic mouse groups showed reduced oxidative capacity in the EDL and SOL upon exposure to an NADH stain. An SDH
assay confirmed findings acquired from NADH staining of muscle tissue in transgenic and WT mice. Both transgenic mouse groups showed reduced SDH-driven oxidative metabolism in the EDL, and HB9/AR113 mice also showed this difference in the SOL. In the older cohort of transgenic and WT mice, both HSA/AR113 and HB9/AR113 mice showed reduced NADH-driven oxidative capacity in the SOL and on the SDH assay, HSA/AR113 mice showed reduced oxidative capacity in the EDL.

The third experiment of this study found a reduction in cell size of FG fibers in HSA/AR113 mice of the younger cohort, partially supporting the idea that abnormalities would be observed in transgenic mice. The fourth experiment of this study partially confirmed the general prediction, as HB9/AR113 mice were alone in showing signs of muscle weakness on the hang test.

As KD is a disorder of middle adulthood, despite expression of polyQ-AR throughout life, a secondary prediction was made towards testing the DLO hypothesis. That is, older adult transgenic mice were predicted to show the same abnormalities as young adult transgenic mice. This prediction was partially confirmed. Older adult HSA/AR113 and HB9/AR113 mice showed the same tendency toward reduced oxidative metabolism. Among older HSA/AR113 mice, deficits in size of FG fibers may be masked by reduced size of fibers as a result of physiological aging. Deficits favoring HB9/AR113 mice (i.e. number and strength on the hang test) were not replicated in older adult transgenic and WT mice, also perhaps due to aging-induced floor effects.
As the bulk of histopathological evidence gathered in this study tends to support the general prediction, the results are consistent with the DLO hypothesis. Contrary to the secondary prediction, the idea that pathological signs exhibited in transgenic mice would be consistent across age, some deficits found in younger transgenic mice were not replicated in the older cohort. Despite this fact, the DLO remains unviolated, as deficits were likely masked in older transgenic mice by the confounding effects of normal aging on animal physiology. Notably, the current investigation was original in finding abnormalities upon selective expression polyQ-AR in motor neurons using the HB9 promoter for transgene activation. The further development of this mouse model has potential to be useful in future hypothesis testing of the muscle weakness of KD. More detailed interpretations of results reported in this thesis document are provided below.

4.1 Interpretations

The most robust finding of this investigation, confirming both the general prediction and the secondary prediction, was the fact that transgene expression in both HSA/AR113 and HB9/AR113 mice resulted in reduced oxidative capacity of skeletal muscle. This finding is consistent with previous work in cell cultures also finding reduced oxidative metabolism when polyQ-AR is expressed (Ranganathan et al., 2009), although the current investigation extends this result to skeletal muscle. Interestingly, reduced oxidative metabolism in skeletal muscle, contradicts previous findings of enhanced oxidative metabolism with over-expression of normal AR in this tissue (Monks et al., 2007). Presumably, this difference is attributable to altered behavior of AR due to the length of the polyQ tract. While WT Q-lengths enhance androgenic interaction with mitochondrial processes, polyQ-AR promotes toxicity in mitochondria-
mediated oxidative metabolism, perhaps by inclusion bodies interacting with mitochondrial biogenesis and enzyme activity. Immunohistochemistry may still be performed on available muscle tissue in order to test this possibility.

A curious aspect of the metabolism changes observed in this investigation is that both HSA/AR113 and HB9/AR113 mice at least showed trends in each statistical analysis toward reduced oxidative capacity in skeletal muscle. This means that polyQ-AR either mediates toxicity to metabolism by a common process when this transgene is expressed in neuronal or muscle tissue, or these similar signs of pathology may do so via separate pathways. The latter idea seems more likely as AR inclusions are known to associate with mitochondrial membrane proteins, suggesting a direct route for polyQ-AR-mediated reductions in oxidative metabolism when this receptor protein is expressed in muscle. By contrast, when polyQ-AR is expressed in motor neurons, for metabolism changes in muscle to occur, intermediate protein interactions are likely necessary at the NMJ. Researchers are encouraged to investigate the synaptic mechanisms responsible for the spread of pathology across the synapse that alter mitochondrial metabolism in skeletal muscle.

This investigation examined whether older adult transgenic mice show the same signs of disease as their young adult counterparts. As the onset of KD strikes at middle age in humans, it was conceived that transgenic mice of corresponding years may exhibit disease in such an age-dependent manner. Measures of oxidative metabolism in the older cohort revealed most of the same trends observed in younger transgenic mice, albeit often in an attenuated fashion. The fact that the effects of transgene expression on oxidative metabolism grew smaller with age may
reflect age-related decline in this kind of energy production (Beal, 1995). Alternatively, the smaller effect on oxidative metabolism in older transgenic mice may be the result of age-related decline of systemically available androgens for activation of disease (Vermeulen, 1991). If the latter idea is correct, it would be fortuitous, as disturbances observed in muscle metabolism may be androgen-dependent. A measure of unbound circulating testosterone contained in blood samples of transgenic mice of both age cohorts may be used as evidence to support the androgen-dependent nature of disturbances in muscle metabolism. If less circulating testosterone is detected in older adult transgenic mice, cellular processes related to reduced oxidative metabolism in skeletal muscle may be due to the toxic-gain of function of polyQ-AR. It is further suggested that this simple measurement may be used to argue that reduced oxidative capacity of skeletal muscle may be a pre-clinical marker of KD.

Besides the observed changes in metabolism, reductions in size of FG fibers in HSA/AR113 mice provided a boost in support for the general prediction. HSA/AR113 mice also showed a greater proportion of this fiber-type in the EDL than WTs, which taken together suggests that FG fibers may be especially involved in the mechanisms of disease onset when polyQ-AR is expressed. Reduced size of fibers may occur with disturbances in muscle that shrink cellular machinery necessary for oxidative metabolism. Musa et al. (2011) reported that HSA-AR mice showed increased mitochondrial content and a corresponding increase in mitochondrial size with expression of normal length AR. Also, hypertrophy of FG fibers was reported for HSA-AR mice and rats, along with increases in oxidative metabolism (Monks et al., 2007; Fernando et al., 2010). Although some of these intracellular measures were not taken in this study (i.e. using electron microscopy), this information suggests that the opposite idea may be valid with
polyQ-AR expression. Muscle fibers may atrophy with longer Q-lengths due to shrinking of mitochondrial size and energy production. This is a potentially important idea to enter into the debate of the mechanisms of onset of KD.

A surprising result of this investigation was that a reduction in number was found for HB9/AR113, but not HSA/AR113 mice. HSA transgenic mice were intact on this measure, an unexpected finding considering the fact that significant expression of polyQ-AR was found in these mice (see Figure 13 on p.92-93 for depiction of this result). Notably, Monks et al. (2007) found reductions in number in the EDL for HSA-AR mice. This discrepancy between the HSA-AR and the HSA/AR113 mice used in this study may reflect the difficulty in initiating sufficient expression of longer Q-length AR, a process that heavily taxes intra-nuclear transcriptional machinery. Also, there was a longer duration of transgene expression in HSA-AR than in HSA/AR113 mice beginning from the prenatal period and extending into adulthood. With greater transgene expression over a longer duration of time, histopathological signs of illness may have been more pronounced in HSA/AR113 mice.
Figure 13: *Relative mutant AR mRNA in AT muscle of Transgenic and WT mice.* HSA/AR113 mice of Cohort 1 show a 10-fold increase in relative mRNA expression of the AR113 transgene 4 weeks after DOX exposure. This is significant expression upon comparison with both HB9/AR113 and WT mice not bearing the transgene (p<0.001 respectively).
Regarding the findings using the behavioral test battery, it was surprising that only HB9/AR113 mice showed behavioral impairments as measured by time to fall on the hang test. This finding suggests the possible relation of reduced oxidative metabolism to behavioral signs of muscle weakness in HB9/AR113 mice. As the hang test may be considered a measure of muscular endurance, impairments shown in HB9/AR113 mice may be related to the reduced oxidative capacity observed in these mice. Unfortunately, this argument suffers upon considering the finding that HSA/AR113 mice showed similar shifts in metabolism despite no evidence of impaired performance on this measure. A direction for further research is to clarify whether reduced oxidative metabolism in muscle is at fault for endurance impairments in animal models of KD.

Of note, the statistical analysis of both the dissection and behavioral strength measures indicate considerations for further work involving HSA and HB9 transgenic mice. Increased seminal vesicle weight in the HSA/AR113 mice of Cohort 2 may reflect an accumulation of compensatory processes for harbouring the polyQ-AR transgene. Whether such compensation involves the androgenic system is possible. Contrarily, increased body weight across all mouse groups of Cohort 1 may reflect lifestyle factors (e.g. physiological maturation, feeding) rather than transgene effects.

The lack of positive results for the disease phenotype in older adult transgenic mice on the hang test may reflect a floor effect, whereby all mice performed poorly as a result of age-related declines in muscular endurance. This may mean that performance on the hang test is not a reliable measure for hypothesis testing using older adult transgenic and WT mice. Regarding
the grip strength and gait tests, all mouse groups of Cohort 1 (and Cohort 2 in the case of grip strength) showed a decline in performance over the course of testing. These results may reflect 

_ fatigue_ endured by repeated use of the behavioral test battery, masking transgene effects on this and other strength measures regardless of age.

### 4.2 Conclusions

The overall pattern of results of this investigation confirmed the general prediction. Some abnormalities were revealed in dissected muscle of both HSA/AR113 and HB9/AR113 mice. It is proposed then that the DLO hypothesis is consistent with these findings. That is, onset of KD likely involves pathology in two locations, motor neurons and perhaps, skeletal muscle. Despite this support for the DLO hypothesis, some original effects observed in transgenic mice, including the fiber-type switch toward glycolytic metabolism, reduced size of FG fibers in HSA/AR113 mice and reduced number and performance on the hang test in HB9/AR113 mice, were not consistent across age. The DLO survives this problem as deficits on these measures were likely masked by aging-induced floor effects. Of importance, this is the first investigation to find histological and behavioral abnormalities in HB9/AR113 mice.

### 4.3 Future Directions for Research

The results of this investigation are useful for further research of the mechanisms of muscle weakness in KD. Onset of muscle degeneration may be due to intracellular atrophy of machinery devoted to oxidative metabolism (i.e. mitochondrial processes), resulting in FO fibers switching to a weak form of glycolytic metabolism and experiencing reduced size and weight. In order to test this idea, a wide range of measures of muscular pathology is
recommended. HSA/AR113 mice may be used according to the same conditions described in the methods of this study, only EDL and SOL muscles should be weighed prior to assays for both myosin and SDH for fiber-typing (and NADH for a corroborative measurement of metabolism). It is predicted that these mice will have reduced muscular weight relative to WTs, attributable to pathological processes especially within fibers exhibiting the largest reductions in oxidative capacity. In order to suggest this as a possibility, muscle weight would be correlated with proportion of FG fibers. In this analysis, naturally, a negative correlation would be expected in transgenic, but not WT mice.

In the proposed investigation, it would be essential to conduct electron microscopy as was employed by Musa et al. (2011), in order to determine the molecular mechanisms underlying FG fiber pathology contributing to whole muscle atrophy. The same measures (e.g. mitochondrial size and density) would be applied in this proposed follow-up study, whereby FG fibers of HSA/AR113 mice are compared with this fiber-type in WT mice. It is also important to take histological measurements from other fiber-types within HSA transgenic mice in order to determine whether these molecular abnormalities are dependent mainly on FG fiber pathology. In the analysis, FG fibers in transgenic mice would be expected to show more abnormalities in molecular histological measures of oxidative metabolism than these other fiber-types.

If it were possible, electrophysiology performed on isolated HSA transgenic FG fibers would be compared with other fiber-types in order to determine whether subcellular changes in metabolism of FG fibers are related to fiber dysfunction. If this is the case, tetanus amplitude and shortening velocity would be reduced in transgenic FG fibers versus WT fiber-types, while
slow and FO transgenic fibers would show smaller changes on these measures. This analysis is also expected to reveal greater changes in HSA/AR113 FG fibers than in HB9/AR113 FG fibers, with the latter group still showing some impaired mechanical function within this same fiber-type. This idea is based on the fact that reduced size of FG fibers was not found for HB9 mutant mice despite findings of reduced oxidative metabolism in these cells. Thus, the proposal that diminished mitochondria in FG fibers may contribute to whole muscle atrophy in KD mainly reflects the muscular contribution to onset.

In order to further support the argument for molecular involvement in the onset of KD in the pattern already described, immunohistochemistry would be undertaken in the multi-dimensional analysis. Immunoreactive-inclusion bodies may be positive for capture of PGC-1a and/or MAPK proteins necessary for mitochondrial biogenesis and function, especially in FG fibers. Another possible mechanism whereby reduced oxidative capacity and size of muscle fibers can occur is the depletion of neurotrophic factors (e.g. VEGF) promoting growth and maintenance of mitochondria (Annex et al., 1998). As CBP is instrumental for transcriptional activation of VEGF, it is expected that FG fibers in HSA/AR113 mice would show a relatively high proportion of nuclear inclusions positive for CBP. This information would suggest that mitochondrial dysfunction especially in the FG fiber-type is compatible with the VEGF hypothesis of the onset of muscle weakness.

If the results of these experiments conform to expectations, patient analysis would next be performed to further the argument suggested in this discussion. It is yet to be determined whether a fiber-type switch occurs in human patients with polyQ-AR, but the results reported in
this investigation suggest that this may be the case. Assuming that appropriate patient samples are available, post-mortem histology of early stage KD may reveal a fiber-type switch toward glycolytic metabolism along with reduced size of these fibers. Then using the same techniques already proposed for transgenic mice, similar findings in post-mortem patients would support the idea that mitochondrial dysfunction of FG fibers is especially related to whole muscle atrophy during the early stages of disease.

It is suggested that existing patients may be useful as participants in determining whether the location of polyQ-AR expression produces separate sets of symptoms via effects on oxidative metabolism in muscle. For example, expression in motor neurons may be the culprit in the presentation of neuromuscular jitter due to downstream effects on muscle metabolism. If polyQ-AR expression in skeletal muscle is benign with regards to this symptom, long-term physical activity may be affected by pathology originating in muscle by other, as yet, undetermined means. This aspect of KD may be tested by including motor conduction studies in the suggested multi-dimensional approach.

Treatments for KD have, on the whole, been unsuccessful in restoring proper motor function to patients with this illness (Merry, 2005). However, a recent discovery suggests a possible avenue for effective treatment. Cell culture studies reveal that phosphorylation of polyQ-AR by Akt protein in muscle blocks the activation of this disease causing receptor protein (Palazzolo, 2009). Thus, insulin-like growth factor 1 (IGF-1), which activates Akt, may serve as a protective factor for muscle, involved in clearing accumulated mutant AR prior to inclusion formation (Palazzolo, 2009). The evidence described in this investigation, that is, that KD may
involve a fiber-type switch toward reduced oxidative metabolism, provides an opportunity to further test the therapeutic efficacy of IGF-1. It is expected that i.p. injections of IGF-1 in HSA/AR113 mice may reverse the magnitude of the fiber-type switch observed in afflicted animals. This technique would also involve re-measurement of mitochondrial characteristics, expecting return of size and density estimates in muscle cells to within the normal range.

It would be useful to infuse the IGF-1 chemical into activated HB9/AR113 mice, showing that this treatment may be effective for disease originating in motor neurons as well as muscle. As the route to muscle fiber abnormalities in these mice may be different than that suspected in HSA/AR113 mice, with disease transmission at the synapse playing a role, IGF-1 may act as a “stop-gap” on pathology in these mice, reducing changes in muscle despite the site of origin of many aspects of disease lying within the pre-synaptic neuron.

Despite some gains made by this investigation, a particularly troubling finding is that older adult transgenic mice displayed fewer and attenuated differences in contrast with age-matched WT mice. As KD is an adult-onset disorder, it was speculated that polyQ-AR expression in these mice would have at least as strong effects as those observed in their young adult counterparts. Comparisons based on fiber-typing, cell size, number and performance on the hang test were null due to aging-induced floor effects. Possible reasons for null and reduced effects on these and other measures in the older cohort (e.g. for oxidative capacity), besides reductions in systemically available androgens or fatigue induced by old age, include the levels of transgene expression. It may be that transgene expression in 250-350 day old mice is reduced compared with 90-100 day old mice such as those used in this investigation. This idea
may reflect age-related changes in the efficiency of gene transcription and/or translation (Lee, Klopp, Weindruch, & Prolla, 1999). It is convenient to investigate this possibility due to the availability of muscle and spinal cord samples taken from older adult transgenic and WT mice for comparison with the younger cohort. If reduced expression levels of mutant AR are found in older transgenic mice using Q-PCR, reduced effects observed in the older cohort may be explained by the altered behavior of transgene methods with age. Although there are difficulties in testing mice of older age status, other researchers are encouraged to engage in similar age-based designs. Such experimental designs may hold potential in explaining some of the unknown aspects of genetics as well as the pathogenesis of adult-onset disease.

While there is still much work to be performed towards a complete representation of KD in mutant mice, it is reported here for the first time that HB9/AR113 mice show histopathological and behavioral strength deficits after a short duration of transgene expression. The missing link in explaining the onset of KD lies in the mutual interaction of diseased motor neurons and skeletal muscle made possible at the NMJ. Such an investigation is a difficult task as candidate proteins for the spread of KD across the synapse are often fatal in KO models. For example, mice homozygous for deletions on the agrin encoding gene die prior to birth, preventing observation of the illness phenotype (Gautam et al., 1996). Future work must attempt to address such problems while building on the findings of this investigation in order to provide a more holistic picture of early mechanisms of neuromuscular disease.
References


