Characterization of Myopathy in Mice Overexpressing Androgen Receptor in Skeletal Muscle

By

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Abstract

Although androgens are known to exert anabolic effects in skeletal muscle, overexpression of androgen receptor (AR) selectively in this tissue causes androgen-dependent motor deficits and muscular atrophy. The cellular and subcellular changes underlying this phenotype are unknown. Therefore, this study aimed to elucidate the ultrastructural and histologic changes accompanying myopathy and to determine the importance of androgens and overexpression level for myopathic features. Transmission electron microscopy revealed augmented mitochondrial content and reduced myofibril width in androgen exposed transgenics. Additionally, male transgenics demonstrated increased glycogen content. Histochemical analyses confirmed sex-specific changes in glycogen content and revealed a surprising loss in the proportion of oxidative fibers in symptomatic animals. However, increased mitochondrial content was confirmed by the presence of ragged red fibers. Overexpression of AR in muscle fiber results in mitochondrial pathology and dysregulation of glycogen metabolism, possibly reflecting normal but exaggerated function of androgens in skeletal muscle fibers.

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List of Abbreviations

AR	Androgen Receptor
ARE	Androgen Response Elements
ARKO	Androgen Receptor Knock Out
cAMP	Cyclic Adenosine Monophosphate
CSA	Cross-sectional Area
DBD	DNA-Binding Domain
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic Acid
ER	Estrogen Receptor
ERK	Extracellular Signal Regulated Kinase
F	Female
FG	Fast Glycolytic
FO	Fast Oxidative
FOG	Fast Oxidative Glycolytic
FSH	Follicle Stimulating Hormone
GLUT4	Glucose Transporter 4
Gpx1	glutathione peroxidase
GS	Glycogen Synthase
GTC	Gomori Trichrome Stain
H&E	Hematoxylin and Eosin
HD	Huntington Disease
HSA	Human Skeletal Actin
L141	Animals from Line 141
L78	Animals from Line 78
LBD	Ligand Binding Domain
LH	Luteinizing Hormone
LHRH	Luteinizing Hormone Releasing Hormone
M	Male
MAPK	Mitogen Activated Protein Kinase
MHC	Myosin Heavy Chain
mRNA	Messenger Ribonucleic Acid
NQO1	NAD(P)H:quinine oxidoreductase 1
PAS	Periodic Acid Schiff
PASD	Periodic Acid Schiff Diastase
PKA	Protein Kinase A
PSA	Prostate-Specific Antigen
rtTA	Reverse Tetracycline-controlled Transcriptional Activator
SARM	Selective Androgen Receptor Modulators
SDH	Succinate Dehydrogenase
SHBG	Steroid Hormone Binding Globulin
SOD	Superoxide Dismutase
T	Testosterone

Tg	Transgenic
TRE	Tetracycline Response Element
VEH	Vehicle
Wt	Wildtype

Chapter 1: Introduction

1.1 Overview

Androgens are known to exert masculinizing and anabolic effects in skeletal muscle. These effects are believed to be principally mediated by the androgen receptor (AR). However, it has recently been shown that skeletal muscle-specific overexpression of AR in mice results in an unexpected phenotype marked by androgen-dependent motor deficits and myopathy (Monks, et al., 2007). The principal purpose of this study is to investigate cellular and subcellular features of pathology in these transgenic (Tg) mice.

This introduction begins with an overview of the endocrinology and function of the androgen system with an emphasis on issues relevant to skeletal muscle. An outline of the mechanisms of androgen action follows with an effort to delineate those which are likely or unlikely to occur in skeletal muscle. Next, the Tg mouse model in question is described, followed by a review of its relevance to human health. Finally, the specific objectives and hypotheses of this study are defined.

1.2 The Androgen System

1.2.1 Androgen Pharmacology

Endogenous androgens are steroid hormones and their metabolites that exert masculinizing and often anabolic effects in a number of tissues. They are responsible for

the development and maintenance of male secondary sexual characteristics as well as a variety of sexually dimorphic processes, including development of reproductive organs, myogenesis, gametogenesis, and sexual behaviour (Hines & Kaufman, 1994; Holman & Hutchison, 1991; Pearson, Rait, Nicholls, Yandle, & Evans, 2006). Some of these effects are reversible, others are irreversible and result in important changes in morphogenesis and differentiation (Heinlein & Chang, 2002). In males androgens are synthesized largely in the Leydig cells of the testes and to a lesser extent the adrenal cortex. In females they are synthesized in the adrenal cortex and ovaries.

Testosterone (T) and its reduced form Dihydrotestosterone (DHT) are the primary functional androgens in mammals. T is the predominant circulating androgen while DHT is its most abundant metabolite and the most potent endogenous androgen. Although both hormones bind to AR they have been shown to have different effects on gene transcription. They additionally differ in their biochemistry such that, for example, T can be aromatized to estradiol but DHT cannot. Other androgens such as androsterone, androstenediol, and dehydroepiandrosterone (DHEA), have minor masculinizing effects and have been shown to play roles in cognition and neuroprotection (Nguyen, Yao, & Pike, 2005).

Secretion of T is under endocrine control by the hypothalamus and pituitary. Luteinizing hormone releasing hormone (LHRH) is released by parvocellular cells in specific hypothalamic nuclei into the hypophyseal portal circulation and subsequently delivered to the anterior pituitary. LHRH stimulates the production and secretion of the gonadotropins

follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH targets sertoli cells leading to the formation of sertoli-sertoli tight junctions while LH targets Leydig cells leading to the production and secretion of T (Au, Robertson, & de Kretser, 1985; Dungan, Clifton, & Steiner, 2006). This classical model of the hypothalamic-pituitary-testes axis has been complicated by the discovery of a family of proteins known as kisspeptins. These neuropeptides are ligands of the once orphaned G-protein coupled receptor GPR54 (J. H. Lee, et al., 1996). Kisspeptins of various lengths have been detected, all products of the Kiss1 gene. However the 54 amino acid isoform, henceforth referred to simply as kisspeptin, is believed to be the primary active form (Popa, Clifton, & Steiner, 2008). Neurons that synthesise and secrete kisspeptin reside in the arcuate and anteroventral periventricular nuclei of the hypothalamus and synapse directly on LHRH secreting neurons where they stimulate LHRH release. This triggers the hypothalamic-pituitary-testes cascade mentioned above (Dungan, et al., 2006). As T levels rise, the release of LHRH, LH, FSH, and kisspeptin from the hypothalamus and pituitary declines. In addition to circulating levels of T, kisspeptin neurons are sensitive to leptin, insulin, environmental cues (e.g. light-dark cycle), and endogenous rhythms such as the circadian oscillator in the suprachiasmatic nucleus (Barb, Hausman, & Lents, 2008; Robertson, Clifton, de la Iglesia, Steiner, & Kauffman, 2009; Simonneaux, et al., 2009; J. T. Smith, Acohido, Clifton, & Steiner, 2006). These findings suggest that kisspeptin may act as an important mediator of the neuroendocrine events that trigger puberty (Dungan, et al., 2006).

Like all steroid hormones T can freely traverse the plasma membrane to bind its receptor in the cytosol or the nucleus. Activation of the AR by ligand binding is thought to mediate most androgen actions. The majority of serum T and DHT (approximately 60%) is complexed with a globular protein called Steroid Hormone Binding Globulin (SHBG), with the remainder bound to albumin (30%) or free in the plasma (3%) (Heinlein & Chang, 2002; Siiteri, et al., 1982). Therefore, only a small fraction of T can freely cross cellular membranes. This coupled with the fact that small fluctuations in physiological T have significant effects suggest that the AR is a limiting factor in this pathway (H. J. Lee & Chang, 2003)

1.2.2 Androgen Receptor

AR is a member of the nuclear receptor superfamily and functions as an androgen-dependent transcription factor. Eight exons comprise the AR gene, which resides on the q11-12 locus of the X chromosome. The first exon is the longest and codes for the N-terminal transactivation domain. This exon includes a number of polymorphic regions that affect the transactivational activity of AR as well its sensitivity to androgens. The remaining 7 exons are fairly well conserved both within and between species. These exons code for the DNA and ligand binding domains (DBD and LBD). The former contains a nuclear localization sequence facilitating translocation into the nucleus and DNA binding. Some authors additionally distinguish a hinge domain located between the DBD and LBD allowing conformational changes in response to ligand binding. The hinge domain contains the PEST sequence (a region rich in proline, glutamate, serine, and threonine) which marks proteins for rapid ubiquitin-mediated proteasome

degradation (Sheflin, Keegan, Zhang, & Spaulding, 2000). AR is therefore a protein with a typically brief half-life and its expression is very strictly regulated. A report has been made of an alternately spliced, N-truncated isoform of AR, but this idea has not found general acceptance, probably because numerous partial breakdown products of AR are known to exist (Wilson & McPhaul, 1994, 1996).

T and DHT are known to regulate AR expression but the direction of their effect is tissue-dependent. In prostate cancer LNCaP and breast cancer T47D cells androgens decrease AR mRNA possibly to limit the androgen response in these cells (Krongrad, Wilson, Wilson, Allman, & McPhaul, 1991; Zhou, Lane, Kemppainen, French, & Wilson, 1995). In osteoblast and hepatocellular carcinoma lines androgens have an opposite effect (Wiren, Zhang, Chang, Keenan, & Orwoll, 1997; L. Yu, Nagasue, Makino, & Nakamura, 1995). Of particular interest, androgens upregulate AR expression in skeletal muscle (myofibers and satellite cells) and motor neurons (D. K. Lee, 2002).

Binding of T or DHT to AR protein activates a sequence of events that ultimately triggers the assorted upregulation or downregulation of a set of androgen-responsive genes. Constitutively, AR resides in the cytosol associated with chaperone molecules that maintain the protein in a mature and functional but inactive conformation. Binding of ligand triggers dissociation of AR from its protein complex, dimerization, and translocation into the nucleus (Adachi, et al., 2007). As a homodimer, the AR binds to regions of DNA known as Androgen Response Elements (ARE's) which modulate the expression of target genes. Gene expression may be upregulated or downregulated

depending on which co-transcription factors are recruited by the AR and the transcription machinery. For example, co-activators such as Cyclin E and SRC-1 upregulate expression of target genes, while co-repressors such as Cyclin D1 and F-SRC-1 downregulate expression. AR is expressed in nearly all mammalian tissues. It is found throughout the nervous system, as well as in skeletal muscle, bone, and reproductive organs. Moreover, skeletal muscles differ in their AR expression profiles and androgen sensitivity (Monks, Kopachik, Breedlove, & Jordan, 2006; Thibert, 1986).

1.3 Mechanisms of Androgen Action

1.3.1 Genomic Mechanisms

Androgens exert their effects through genomic and non-genomic mechanisms. Genomic and non-genomic signalling mediate discrete androgen effects. Genomic action is so called because it involves changes in the transcription of androgen responsive genes. Binding of androgens to cytosolic AR leads to translocation of the AR-androgen complex into the nucleus where it binds to specific DNA regions and modifies the transcriptional activity of androgen responsive genes. This mode of action is by far the better characterized of the two and, perhaps because of this fact, is believed to be responsible for the vast majority of androgen effects.

Within genomic action, some authors make the distinction between direct and indirect mechanisms. Direct genomic action involves binding of activated AR to cis-acting regulatory regions in responsive genes. Therefore, the AR binds to a regulatory region within the same gene that is affected. Indirect genomic action can take a number of

forms. Activated AR may regulate the expression of secondary transcription factors which in turn affect gene expression. Alternatively androgens may regulate the expression of hormones that mediate androgenic effects in distal tissue. Similarly, androgens may alter expression of autocrine and paracrine factors which mediate androgen effects in proximal tissue. Each of these products may in turn affect a variety of genes such that the ultimate androgenic effect is caused by a complex cascade of molecular messengers. Since all genomic effects, both direct and indirect, are contingent on AR activation, these mechanisms are often referred to as AR-dependent.

1.3.1.1 Transcriptional Targets of AR

AR exerts its effects primarily by regulating transcription. It does this in concert with a set of transcription cofactors that are essential for normal genomic AR activity and are responsible for the formation of the initiation complex and the recruitment of RNA polymerase II (Shang, Myers, & Brown, 2002). The transcriptional targets of AR vary from tissue to tissue. This selectivity is mediated in part by the expression profiles of transcription cofactors. Different cofactors are expressed in different tissues and these regulate the transcription of different genes. Therefore, in a given tissue, AR will only regulate the expression of genes for which an appropriate cofactor is expressed.

AR transcriptional targets in the prostate are of great interest because of their clinical significance. AR transactivation triggers the onset and progression of prostate cancer (Chen, et al., 2004; Notini, Davey, McManus, Bate, & Zajac, 2005). The primary biomarker for prostate cancer is an androgen regulated gene called prostate-specific antigen (PSA). Elevated levels of PSA indicate greater likelihood of prostate cancer.

However, there is no generally accepted normal or abnormal PSA level (D. S. Smith, Humphrey, & Catalona, 1997; Thompson, et al., 2004). Several additional transcriptional targets have been identified that are involved in protein synthesis, development, secretion, apoptosis and transcription (Massie, et al., 2007).

In motoneurons AR targets several nuclear encoded mitochondrial genes. Several of these seem to be involved in relieving oxidative stress. For instance, AR increases transcription of superoxide dismutase I-II (SODI, II), NAD(P)H:quinine oxidoreductase 1 (NQO1), glutathione peroxidase (Gpx1), and NF-E2-related factor-2 (Ranganathan, et al., 2009). The first three are important antioxidant enzymes while the last is a transcription factor known to bind to an antioxidant response element that activates the expression of several antioxidant enzymes (J. M. Lee, Calkins, Chan, Kan, & Johnson, 2003). There is conflicting evidence as to the effects of T on oxidative stress. Some have found that T diminishes the capacity of cells to resist oxidative stress. Therefore, it is plausible that AR activation of antioxidant genes such as SODI/II, NQO1, and Gpx1 acts as a negative feedback mechanism for T action. Based on these findings it has been proposed that the suppression of antioxidation by T contributes to the honesty of secondary sexual characteristics as markers of male fitness. Males with prominent secondary sexual characteristics, and therefore high peripubertal T, have to endure reduced tolerance to oxidative stress. The endurance of this stress acts as a testament to male fitness (Alonso-Alvarez, Bertrand, Faivre, Chastel, & Sorci, 2007). A similar hypothesis has been proposed based on the immunosuppressive effects of T. However, others have found T to have antioxidant effects mediated by increasing activity of catalase and resisting the

oxidative effects of 3-nitropropionic acid (Chisu, et al., 2006; Tunez, et al., 2007). If T does indeed have an antioxidant effect, this may be mediated by the antioxidant enzymes whose expression is regulated by AR.

In skeletal muscle AR has a wide range of transcriptional targets that span a range of cellular functions. Using serial analysis of gene expression, genes were identified that are involved in muscle contraction, extracellular matrix, transcription, metabolism, proliferation, and cell signalling (Yoshioka, Boivin, Ye, Labrie, & St-Amand, 2006). In particular, several subunits of cytochrome c oxidase and NADH dehydrogenase, critical respiratory chain complexes, are upregulated within 24 hours of DHT treatment suggesting that DHT may result in increased activity of the electron transport chain and increased energy production for anabolism. This form of analysis is instructive with respect to the range of functions regulated by androgen activity. However, it is less informative regarding the importance of T in these pathways and the functional significance of its involvement.

1.3.2 Non-genomic Mechanisms

Non-genomic androgen action is typically classified as that which is independent of direct ligand-AR mediated gene regulation. The role and significance of non-genomic androgen action has only recently garnered recognition. It typically involves the rapid stimulation of second messenger signal transduction cascades including changes in intracellular calcium, and activation of Protein Kinase A (PKA) and MAPK (Heinlein & Chang, 2002). These effects are believed to occur too rapidly (seconds to minutes; to be

caused by changes in gene transcription and are in fact not repressed by transcription and translation inhibitors (Hoffman, Smith, & Verbalis, 1993).

The effects of non-genomic androgen action appear to be receptor mediated. T has been shown to stimulate a G protein coupled membrane receptor in rat skeletal myotubes resulting in ERK1/2 phosphorylation (Estrada, Espinosa, Muller, & Jaimovich, 2003). This effect was not inhibited by AR antagonists suggesting an AR independent effect. Additionally, a T-albumin complex, incapable of traversing cell membranes, exerted the same effect as free T further suggesting that T's effects were mediated through membrane components (Sun, Gao, Tang, Xu, & Wang, 2006). Nonetheless, non-genomic effects are not necessarily AR-independent. In response to DHT, AR has been shown to interact with and activate the membrane-associated tyrosine kinase c-Src within 1 minute (Kousteni, et al., 2001). It had additionally been shown to interact directly with mitochondria via the Cytochrome C Oxidase Vb subunit (Beauchemin, et al., 2001). Finally, there is evidence that membrane-bound SHBG receptors may bind to androgen-SHBG complexes thereby mediating androgen effects (Nakhla & Rosner, 1996). SHBG receptors have not been cloned to date but are believed to be G-protein coupled receptors or functionally linked such receptors and therefore are thought to mediate androgen effects through second messengers such as cAMP and PKA. Although SHBG receptors have been localized in a number of tissues including the prostate, liver, testis, and breast (Fortunati, 1999; Hryb, Khan, & Rosner, 1985; Krupenko, Krupenko, & Danzo, 1994); to date they have not been found in skeletal muscle.

1.3.3 Reduction to DHT

T can exert effects through conversion to DHT and estradiol. The enzymes responsible for these transformations are differentially expressed in mammalian tissue, predominating in some and absent in others. 5-alpha-reductase (5-AR) is the principal isoform of the steroid reductase responsible for converting T to DHT. It is found in various tissues including skin, seminal vesicles, prostate, and epididymis but levels in skeletal muscle are low. The importance of DHT in skeletal muscle remains ambiguous. Likely, the conversion of T to DHT by 5-AR is not critical for androgen action in this tissue since individuals born with congenital 5-AR deficiency do not experience abnormal muscle development during puberty (Marks, 2004). Moreover, 5-AR itself is minimally expressed in skeletal myofibers (Bruchovsky & Wilson, 1968). Nonetheless, DHT does differ from T both in transactivational effects and biochemistry. For example, unlike T, DHT cannot be aromatized to estrogens and is often used to control for aromatase activity. DHT additionally differs with respect to its binding affinity to AR, SHBG (higher in both) and its transcriptional regulation.

1.3.4 Aromatisation to Estradiol

Aromatase is the enzyme responsible for converting androstenedione and T to estrone and estradiol respectively and is critical in the estrogen biosynthetic pathway. It is expressed highly in the testis, brain, adipose tissue, placenta, skin, and bone and to a lesser extent in muscle (Kotula-Balak, Lenartowicz, Kowal, Styrna, & Bilinska, 2007; Larionov, et al., 2003; Sharma, Blache, Roselli, & Martin, 2004). Whether or not aromatase is expressed at significant levels in skeletal muscle is a point of controversy. Aromatase activity in this tissue is lower in men than in women (Bershtein, Larionov,

Kriukova, Kochnev, & Semiglazov, 1996). Larionov et al. (2003) assayed male and female skeletal muscle using RT-PCR and found aromatase gene expression in nearly all subjects. They additionally assayed enzyme activity and found it to range between 2.2 and 6.5 fmol mg^{-1} protein hr^{-1}. These activities are comparable to those found in adipose tissue. They concluded that muscle can be a significant source of estrogen in men particularly considering its bulk. However, others (Sharma, et al., 2004) have shown that these levels are minimal relative to other tissues. For example, AR activity is 400 fmol/mg/hr in the liver, 300 fmol/mg/hr in parts of the brain, and 35 fmol/mg/hr in testes. Moreover, levels of estrogen receptor (ER) are minimal in skeletal muscle (e.g. only 1/1000 of those in uterine tissue) and show low binding activity (Meyer & Rapp, 1985). Others directly assessed the effect of estrogen treatment on skeletal muscle function and development and could not reproduce androgenic effects (McCormick, Burns, Piccone, Gosselin, & Brazeau, 2004; Monks, et al., 2007). This evidence strongly suggests that estradiol is unlikely to be a principal mediator of androgen effects in skeletal muscle

1.3.5 Mechanisms in Skeletal Muscle

Increasingly, the complexity of androgen action and the involvement of a variety of interrelated intracellular and membrane-bound molecular mediators are being recognized. The most thoroughly characterized mode of action involves AR acting as a ligand dependent transcription factor that regulates androgen responsive genes which may include secondary transcription factors, secondary hormones, and secondary autocrine and paracrine factors. Much remains to be learned about the mechanisms and physiological roles of both AR-dependent and AR-independent non-genomic effects. Nonetheless, the principal candidates for mediating AR-independent androgen action

have either failed to be localized to skeletal muscle or have been found to play minor physiological roles with questionable significance. T action in skeletal muscle is therefore believed to be mediated to a large extent by AR-dependent mechanisms stimulated by T itself rather than by DHT or estradiol.

1.4 Functions of the Androgen System

In addition to their role in skeletal muscle, androgens have been shown to play important roles in a variety of tissue types. Best characterized amongst these are the effects of androgens on the development and maintenance of the reproductive organs and central nervous system (CNS). These are briefly reviewed here.

1.4.1 Sexual Development

Androgens are the key hormonal regulators of sexual development throughout the lifespan including the prenatal period. The role of androgens in female sexual development is often as precursor to estrogen synthesis. A discussion of estrogen physiology is beyond the scope of this review and the reader is referred to articles dealing specifically with this topic (Carwile, Wagner, Crago, & Alexander, 2009; Khosla, Melton, & Riggs, 2002; Losordo & Isner, 2001). In order to facilitate a discussion of the role of androgens in sexual development a distinction must be made between the processes of sex determination and sexual differentiation. Sex determination is a binary mechanism in which molecular events lock the embryonic gonads into a fate as testes or ovaries. On the other hand, sexual differentiation is the set of subsequent events required for gonadal development and the formation of secondary sexual characteristics to produce the male or female sexual phenotype (Polanco & Koopman, 2007).

The presence or absence of the SRY gene in a normal developing human or mouse embryo is necessary and sufficient for sex determination. Its expression induces the formation of a male gonad phenotype and the subsequent production of T (Koopman, Gubbay, Vivian, Goodfellow, & Lovell-Badge, 1991). Early T masculinises the embryo both internally and externally. Portions of the male internal reproductive organs develop from the Wolffian duct for which T acts as a survival factor. The Mullerian duct, which would otherwise give rise to portions of the female internal reproductive system, is inhibited by Anti-Mullerian Hormone (AMH) secreted by Sertoli cells during the 6th embryonic week. External virilisation is under more direct control by DHT, the reduced product of T (Asby, Arlt, & Hanley, 2009).

The androgenic effects of T on sexual differentiation are mediated by AR. Male total AR knockout mice (ARKO) exhibit a female-typical phenotype including a vagina with a blind end and a clitoris-like phallus. Since testes are determined by SRY gene expression, these remain present in ARKO males. However, their subsequent development is disrupted as evidenced by their abdominal localization and the failure of germ cell development. These features are similar to a human complete androgen insensitivity syndrome discussed further below.

Spermatogenesis and fertility are likewise dependent on AR signalling. By ablating AR expression specifically in each of the testicular cell-types the role of AR signalling in spermatogenesis has become well understood. In Sertoli cells, AR is important in meiosis

I during spermatogenesis and Sertoli-specific ARKO exhibit an arrest of spermatogenesis at the diplotene primary spermatocyte stage (De Gendt, et al., 2004). In Leydig cells, AR is critical for T synthesis and a Leydig-specific ARKO show spermatogenesis arrest at the spermatid stage (Xu, et al., 2007). AR in the Peritubular Myoid cells, which are contractile cells lining the seminferous tubules, acts to increase sperm output without which males have reduced sperm output but unaffected fertility (Zhang, et al., 2006). Lastly, AR in the gamete progenitor germ cells does not seem to play a role in spermatogenesis and male fertility (Tsai, et al., 2006).

1.4.2 Effects on the CNS

Androgens play a critical role in the development and maintenance of the CNS and therefore of behaviour and cognition. Initially, it was thought that androgens exert purely an activational effect on the CNS, inducing a reversible and temporary change in the probability of a behaviour. However, it was later suggested then demonstrated that the role of androgens in organizing the internal and external genitalia of mammals could be extended to the brain (Cooke, Hegstrom, Villeneuve, & Breedlove, 1998; Phoenix, Goy, Gerall, & Young, 1959).

For instance, astrocytes in the posteriodorsal region of the medial amygdala are more numerous in males than in females. This difference is abolished in ARKO males indicating that this sexual dimorphism is contingent on AR signalling (Johnson, Breedlove, & Jordan, 2008). Similarly, the Sexually Dimorphic Nucleus of the Preoptic Area (SDN-POA) is a nucleus of the hypothalamus in which the sexual dimorphism is so overt as to be visible with the naked eye. This area is 5-6 times greater in volume in

males and is thought to play a role in copulatory behaviour. Blocking androgen action perinatally abolishes this dimorphism.

Since androgens play a role in structuring the CNS it is not surprising that they also play a role in influencing its primary function: behaviour. Several aspects of behaviour and cognition are impacted by circulating levels of T. For instance risk taking behaviour in men correlates positively with salivary T levels and pubertal hormone exposure (Apicella, et al., 2008). Similarly, aggression in several species has been shown to correlate with high levels of T along with low levels of cortisol (Hennessy, Maken, & Graves, 2000; Lynch, Ziegler, & Strier, 2002; Terburg, Morgan, & van Honk, 2009; Y. Z. Yu & Shi, 2009). The behavioural effects of high T levels correlate well with the principal functions of the brain regions whose structure is influenced by T. For instance the morphology of the amygdaloid complex, which projects directly to the periaqueductal gray in the midbrain, a region implicated in aggressive and defensive behaviour, can be altered by T (Johnson, et al., 2008; Manchanda, et al., 1995; Stanton, Wirth, Waugh, & Schultheiss, 2009). It is therefore critical when manipulating elements of the androgen system to control for behavioural changes which may influence the measured outcome.

Much of the sexual differentiation mediated by androgens in the CNS relies on conversion of androgen into estradiol. This single step aromatization reaction is catalyzed by estrogen synthase, also called aromatase. In the brain, the expression of aromatase is induced only by androgens (Tsuruo, 2005). The differentiation of brain regions into the male-typical phenotype is mediated by estradiol which is produced within the CNS from

T. In the absence of circulating androgens, and therefore of CNS estradiol, the female-typical phenotype develops. During fetal development gonadal estradiol binds to α-fetal protein, a glycoprotein present in blood plasma, preventing it from crossing the blood brain barrier and affecting the sexual organization of the brain (MacLusky, Lieberburg, & McEwen, 1979). α-fetal protein levels drop rapidly following birth permitting gonadal estradiol entry into the brain (Tomasi, 1977). However, at that point much of the mammalian brain has been irreversibly organized into a sex-specific phenotype.

Nonetheless, AR does mediate some of the effects of androgens in the CNS. AR is present in several brain regions at varying levels. For instance, all layers and regions of the cerebral cortex express AR but expression levels are particularly elevated in sensory and motor regions in layers II/III and V/VI. By coupling immunocytochemistry with retrograde tract tracing it has been possible to explore the afferent connections of neurons expressing AR. This has identified connections between AR immunoreactive cerebral neurons with local and associational cerebral territories which are involved in sensory processing as well as complex motor and cognitive behaviour (Kritzer, 2004). These findings point to a prevalent role of androgens in organizing and inducing behavioural patterns.

1.5 Skeletal Muscle and the Androgen System

Muscle is one of the most abundant tissues in mammalian bodies. It has long been recognized to be sexually dimorphic and responsive to androgens. Accumulating data has shown that androgens have direct effects on skeletal muscle development, growth, and

maintenance. This section briefly describes muscle at the tissue and cell level and follows with a review of the literature on the roles of androgen in this tissue.

1.5.1 Skeletal Muscle Physiology

Skeletal muscle is a syncytium of myofibers. These cells typically span the length of the muscle tapering at both ends near the tendons. Myofibers comprise a series of longitudinal myofibrils containing a sequence of uninterrupted contractile units called sarcomeres. Myogenesis begins with the maturation of pluripotent mesenchymal-derived stem cells into mononucleated myoblasts that divide repeatedly and fuse with one another to form multinucleated myotubes. These then incorporate additional myoblasts to form long and continuous cells called myofibers. Mature myofibers are post-mitotic and therefore, in adulthood, myofibers are replaced by progenitor cells known as satellite cells. These cells are located on the periphery of myofibers, outside the sarcolemma but within the basal membrane. When muscle is stressed or damaged, quiescent satellite cells are activated into division forming myoblasts prior to differentiating into mature myofibers.

Muscle tissue is one of the most energy demanding tissues in mammalian bodies. Mitochondria play a pivotal role in ATP production and are therefore present in abundance in muscle. Myofibers have two regionally specialized mitochondria populations. The subsarcolemmal mitochondria are found on the periphery of the fiber directly beneath the sarcolemma and are typically large cuboid organelles in close proximity to capillaries. There are also core or intermyofibrillar mitochondria, located

between fibrils within the I-bands of sarcomeres. These mitochondria are believed to provide the bulk of ATP needed for Mysoin ATPase mediated contraction.

1.5.2 Muscle Fiber Types

Whole skeletal muscles differ in their contractile properties. Muscles with high maximal shortening velocity are considered fast while those with low shortening velocity are slow, yet others lie somewhere along this spectrum. These differences in kinetics correspond to differences in cellular composition. Muscles that contract rapidly but not for prolonged periods require transiently high levels of ATP. Therefore, they typically comprise fibers that rely on glycolysis and express high activity myosin ATPase isoforms. In contrast muscles that contract more slowly but for longer periods have fibers that rely on oxidative phosphorylation, have large myoglobin content, and express myosin ATPase isoforms with lower hydrolysis rates.

Skeletal muscle fibers are not homogeneous and can be classified into several different types based on their physiology, morphology, or biochemisty. A number of classification systems have been devised but they do not always agree. One commonly used system differentiates between fibers according to the principal energy producing pathway employed. In this system, Glycolytic (G) cells rely largely on glycolysis, Oxidative (O) cells rely on oxidate phosphorylation, and Oxidative-Glycolytic cells rely on both pathways comparably. This information can be acquired using a biochemical assay for metabolic enzymes such as Succinate Dehydrogenase (SDH). Using a substrate that results in a coloured product (e.g. Nitroblue Tetrazolium) the relative content of

mitochondrial enzymes can be inferred. Ultimately, the fiber type is determined by gene expression profile which in turn is affected by motor unit activation patterns.

A further distinction can be made based on rates of Myosin ATPase hydrolysis. A correlation exists between Myosin ATPase activity and rates of muscle contraction (Scott, Stevens, & Binder-Macleod, 2001). This led to the widespread use of histochemical Myosin ATPase staining to classify fibers. However, these protocols did not directly assess Myosin ATPase hydrolysis rates, but classified fibers based on stain intensity due to differing pH sensitivities of the Myosin ATPases. Scott et al. (2001) claim that since fibers are classified based on the intensity of staining at different pH levels, fibers could be grouped differently by different experimenters. Moreover, rarely does a single study distinguish between all fiber types identified with this method (7 types in humans). Due to these shortcomings more definitive typing strategies are gaining popularity.

Myofibers express different isoforms of Myosin Heavy Chain (MHC) depending on their pattern of activation and ultimately their rate of contraction. The MHC is the polypeptide that serves the ATPase function in myosin protein and therefore there is a correlation between the MHC isoform expressed and the Myosin ATPase staining intensity of a fiber. Myofibers that contract relatively slowly express MHCI (slow MHC) and are classified as Type I fibers by Myosin ATPase staining. Those that contract more rapidly (usually twice the rate of Type I fibers) express MHCII (fast MHC) and are classified as Type II fibers by Myosin ATPase staining. These polypeptide isoforms can be identified

immunohistochemically or by sodium dodecyl sulfate–polyacrylamide gel electrophoretic (SDS-PAGE). In conjunction with enzymatic assays of oxidative metabolism a classification system of a maximum of 6 fiber types is formed:

	Fast	**Slow**
Oxidative	FO	SO
Glycolytic	FG	SG
Oxidative-Glycolytic	FOG	SOG

In practice MHCI is only expressed in oxidative fibers and therefore only SO, FO, FG, and FOG are typically identifiable. Commonly these fibers are referred to as Type I (SO), Type IIA (FO and FOG), and Type IIB (FG).

1.5.3 Role of Androgens in Muscle

1.5.3.1 Anabolic Effects in Skeletal Muscle

Although it had long been controversial, it is now generally established that androgens have anabolic effects on skeletal muscle (Brodsky, Balagopal, & Nair, 1996). Treatment of hypogonadal men with T leads to an increase in fat-free and muscle mass. These changes can in large be attributed to muscle accretion and increases in protein synthesis rates. In particular, rates of MHC and mixed skeletal muscle protein expression are upregulated in hypogonadal men undergoing androgen replacement therapy. These changes in protein synthesis help explain findings of early experiments that demonstrated androgens enhance nitrogen retention in muscle (Kenyon, Knowlton, Sandiford, Koch, &

Lotwin, 1940). Furthermore, T treatment results in a positive linear dose-response curve with respect to fat free mass, muscle volume, muscle strength, and muscle power (Bhasin, et al., 2001). These gains are not limited to hypogonadal individuals. Treatment of eugondal men with supraphysiological doses of androgen leads to further gains in muscle size and strength. In addition to increasing protein synthesis, androgen treatment has been shown to cause individual fiber hypertrophy (Sinha-Hikim, et al., 2002).

1.5.3.2 Reciprocal Effects on Myogenesis and Adipogenesis

Adult stem cells are found within skeletal muscle and have the potential of entering a myogenic pathway through satellite cells or an apidogenic pathway through preadipocytes (Bhasin, Woodhouse, & Storer, 2003). Myogenesis and commitment of stem cells to the myogenic lineage are known to occur when muscle tissue is damaged or in response to hypertrophic factors (Grounds et al., 2002). In particular, androgens have been shown to influence the fate of stem cells by promoting commitment to the myogenic lineage and inhibiting adipogenesis (Singh, Artaza, Taylor, Gonzalez-Cadavid, & Bhasin, 2003). The treatment of C3H 10T1/2 pluripotent stem cells with physiological levels of T or DHT was associated with higher numbers of MyoD and MHC positive myogenic cells. These muscle-specific transcription factors are associated with the commitment of stem cells into the myogenic lineage. Their androgen-linked upregulation suggests that they may play a role in androgen dependent increases in lean muscle mass. Concurrently, peroxisomal proliferator-activated receptor γ 2 (PPARγ2) mRNA and protein levels, both markers of adipocyte differentiation, and number of adipocytes were reduced by T and DHT treatment.

The reciprocal effects of androgens on myogenic and adipogenic differentiation are mediated by AR. Firstly, it has been confirmed by a number of methods that satellite cells express functional levels of AR (Doumit, Cook, & Merkel, 1996). Moreover, the treatment of pluripotent stem cells with bicalutamide (an AR antagonist) precluded the effects of T and DHT on gene expression and cellular differentiation (Singh, et al., 2003). In final support of this mechanism of action, T treatment has been shown to increase the number of myonuclei and satellite progenitor cells in vastus lateralis from eugonadal men and levator ani from rats (Niel, Willemsen, Volante, & Monks, 2008; Sinha-Hikim, Roth, Lee, & Bhasin, 2003). This does not preclude androgen independent contributions to lipid muscle content. For instance, it has been suggested that androgens reduce fat mass by decreasing lipid uptake into and increasing lipid release from abdominal adipose stores (Marin, Krotkiewski, & Bjorntorp, 1992; Marin, Oden, & Bjorntorp, 1995). Regardless of the cellular mechanisms of androgen action, it has now been well established that T reduces whole body and regional fat mass while enhancing muscle volume, mass, and strength.

1.5.3.3 Mitochondria and Oxidative Metabolism

Mitochondrial structure and function are influenced by the androgen system. Mitochondrial profile area has been shown to increase significantly following T treatment in eugonadal young men (Sinha-Hikim, et al., 2003). This increase in size is accompanied by an increase in respiratory chain activity. T levels have been positively correlated with aerobic capacity and the expression of oxidative phosphorylation genes in men (Pitteloud, et al., 2005). In particular, T increases mitochondrial activity by increasing the specific activity of individual mitochondrial enzymes such as cytochrome c oxidase. Additionally,

androgens exert effects on a number of elements in the oxidative metabolic pathway. Both T and DHT have been shown to have recoupling properties in isolated mitochondria treated with protonophores (Starkov, et al., 1997). With respect to fiber type, the most pronounced hypertrophy in response to androgen treatment is observed in Type I oxidative fibers (Ustunel, Akkoyunlu, & Demir, 2003). It has also been found that AR interacts directly with mitochondrial enzyme subunits but the significance of these interactions remains unclear (Beauchemin, et al., 2001). Finally, it has recently been shown that several genes involved in oxidative metabolism (e.g. Carbonyl Reductase 2) are downregulated in males of an ARKO mouse model (MacLean, Chiu, Notini, et al., 2008). Males of this line showed no change in fatigue of a fast glycolytic muscle (EDL) but showed marked reduction in endurance relative to females and Wt males in a slow oxidative muscle (Soleus). Nonetheless, some conflicting findings do exist particularly that ARKO males show reduced MHCI expression, a myosin isoform specific to slow oxidative fibers. In sum, although the issue has not been definitively settled, androgens seem to play a facilitative role in oxidative metabolism by activating transcription of oxidative phosphorylation genes. There is some evidence that this effect is sexually dimorphic (Yoshioka, Boivin, Bolduc, & St-Amand, 2007); this is discussed below.

1.5.3.4 Glucose Metabolism

The androgen system has been heavily implicated in glucose homeostasis, glycogen metabolism, and insulin sensitivity. Moreover, the role of androgens in this capacity seems to be different in males and females (discussed below). Castration of male mice has been shown to dramatically reduce glycogen content in rat skeletal muscle. Subsequent T administration increases glycogen content in intact and castrated animals

(Leonard, 1952). Additionally, glucose Transporter 4 (GLUT4) expression and incorporation into plasma membrane, along with glucose penetration are also enhanced following androgen administration in males (Sato, Iemitsu, Aizawa, & Ajisaka, 2008). These effects are likely mediated by AR induced changes in gene transcription. For one, the time-scale of androgen/castration induced changes in glycogen content (12-24 hours) suggest that changes in gene transcription mediate these effects. Moreover, administration of actinomycin D (a transcription inhibitor) or puromycin (a translation inhibitor) blocks androgen-induced glycogen synthesis (Bergamini, Bombara, & Pellegrino, 1969). These effects may be regulated by androgen mediated changes in the insulin sensitivity of skeletal muscle. Treatment of hypogonadal, hyperglycemic obese men with T for 8 months improves insulin resistance and hyperglycemia (Marin, et al., 1992). Moreover, there was a greater improvement in men with relatively low serum T prior to treatment suggesting that the effect may have therapeutic significance. Androgens therefore play an important role in glucose homeostasis, a role that appears to differ between the sexes.

1.5.3.5 Sexually Dimorphic Effects of Androgens in Muscle

Complex sex differences have been recognized in the effects of androgen on skeletal muscle metabolism and gene transcription. There are well known sex differences in body composition particularly differences in fat-free mass and lean muscle mass. Evidence suggests that these differences are in part regulated by circulating sex hormones including androgens. Moreover, there is growing evidence that adult T treatment can have differential effects in males and females.

For example, T seems to differentially affect insulin sensitivity in males and females. Chronic T administration to ovariectomized female rats results in reduced insulin sensitivity marked by reduced glucose penetration and glycogen synthase expression (Rincon, et al., 1996). These findings are in direct conflict with those obtained in males in whom hypogonadism leads to reduced insulin sensitivity (Pitteloud, et al., 2005). There is also evidence that androgens differentially affect oxidative and glycolytic metabolism in males and females. Men tend to have higher expression of genes encoding mitochondrial proteins, ribosomal proteins, and a few translation initiation factors (Welle, Tawil, & Thornton, 2008). It has been found that DHT treatment of ovariectomized females induced transcription of genes related to fast/glycolytic fiber, glycolysis, and glucose transport (Yoshioka, et al., 2007). DHT treatment of male mice however induced transcripts in oxidative phosphorylation and ATP production. Females showed a repression of these genes with DHT treatment. Therefore, androgens seem to cause disparate effects in males and females with respect to cellular metabolism and therefore fiber type representation.

The mechanisms underlying these sexual differences are not fully understood. Although they may be due to differential gene expression (e.g. aromatase enzyme expression) it is likely that long lasting organizational effects of perinatal androgen play an important role. In neuromuscular systems it has long been recognized that perinatal androgen can permanently alter androgen sensitivity in adulthood. For example, the levator ani muscle, involved in male copulatory behavior, is lost perinatally in females due to the absence of T. In adulthood, T maintains the levator ani in males but does not affect corresponding

myogenesis in females (Sengelaub & Forger, 2008). Therefore, early effects of the androgen system frequently alter adult responsiveness to androgens.

1.5.3.7 Motoneuron Regulation

Skeletal muscle is innervated by motor neurons originating in the spinal cord. Many of these neurons express AR and are responsive to androgens. Neuromuscular systems differ in the extent to which they are androgen responsive and sexually dimorphic. The Spinal Bulbocavernosus motoneurons for example are sexually dimorphic neurons that innervate the bulbocavernosus and levator ani muscles in males. A perinatal surge in T is thought to masculinize the neuromuscular system in rodents and maintain it throughout life. The absence of early androgen in females leads to the regression of both muscle and motoneuron, a phenomenon that can be reversed by early T treatment. Present evidence suggests that motoneuron AR and muscle AR play dissociable roles in the development of neuromuscular systems (Freeman, Watson, & Breedlove, 1996; Monks, Vanston, & Watson, 1999). It has long been thought that T acted on AR in bulbocavernosus and levator ani muscle resulting in rescue of these muscles and subsequent survival of bulbocavernosus motoneurons (Johansen, Jordan, & Breedlove, 2004). However, it has recently been reported that AR in skeletal muscle is not sufficient for the masculinisation of the spinal nucleus of the bulbocavernosus system and that AR in other yet undetermined cell types is necessary (Niel, et al., 2009). By crossing AR deficient mice with mice expressing functional AR exclusively in skeletal myofibers, it was possible to generate mice that express functional AR only in myofibers. Males from this line showed feminine motoneuron and muscle phenotypes indicating that AR in other tissue types, possibly is necessary for the sexual differentiation of this system. The close association

of muscles and the motoneurons which innervate them make motoneurons an important target for androgen mediated effects in muscle.

1.6 Androgens and Disease

The androgen system has been implicated in a number of clinical conditions. These conditions either involve changes in androgen production or dysfunctional responses to androgens as mediated by AR. The fact that the AR gene is X-linked makes it particularly susceptible to the adverse effects of constitutional mutations. Several such mutations have been identified including large gene deletions, single nucleotide mutations, and trinucleotide repeat expansions. Many have been implicated in disease.

1.6.1 Androgen Deficiency

Sarcopenia is the loss of muscle mass and function associated with aging. It is frequent in older men and is often accompanied by neuromuscular impairment which can lead to falls and fractures. Since androgens are known to increase muscle volume and strength and since androgen levels decline with age, it is possible that sarcopenia is caused by reduced androgen action on skeletal muscle. Additionally, reduced serum T in men has been repeatedly linked to insulin resistance and obesity (Naharci, Pinar, Bolu, & Olgun, 2007; Pitteloud, et al., 2005). Symptoms of these putative androgen deficiency conditions support the notion that the general effect of androgens in muscle is anabolic and further supports the relationship between androgens and diabetes.

1.6.2 Androgen Insensitivity

A number of large deletions in the AR gene have been shown to cause complete or mild Androgen Insenstivity (AIS), (Jeske, et al., 2007). In AIS, mutations in the AR gene sequence including the loss of entire exons coding for the LBD, render the AR irresponsive to androgens. This leads to a variety of clinical manifestations in male patients ranging from complete external female phenotype to milder symptoms of androgen insensitivity which may include gynecomastia and reduced infertility (Hiort, Sinnecker, Holterhus, Nitsche, & Kruse, 1996). Complete AIS is effectively modeled by AR Knock-out (ARKO) animal models. These loss of function AR mutant animals do not typically display muscular wasting or other fatal phenotypes. However, males from ARKO lines show intersex external genitalia, impaired brain masculinization, late-onset obesity, osteopenia, reduced serum T, and reduction in male-typical behaviours (Notini, et al., 2005; Yanase, et al., 2008). As the AR gene is X-linked and ARKO males are infertile, it is not possible to generate female ARKO mice using traditional knock-out strategies. Therefore, several labs (Hu, et al., 2004; MacLean, Chiu, Notini, et al., 2008; Yeh, et al., 2002) used a Cre-lox system to generate ARKO mice under the control of Beta-Actin resulting in gene knock-out during embryogenesis. Male mice from this line show feminine anogenital distance, diminished testes, reduced serum T, reduced male sexual behavior and halted spermatogenesis. Females on the other hand seemed largely unaffected by the knock-out: sexual behavior, bone density, and body weight remained within the Wt range. However, it has been repeatedly reported (Hu, et al., 2004; Matsumoto, Takeyama, Sato, & Kato, 2003) ARKO females present with premature ovarian failure such that females bear progressively smaller litters and eventually (at 40 weeks) become infertile. As a caveat, a recent study (MacLean, Chiu, Ma, et al., 2008)

found that mice expressing a floxed AR gene in isolation of the Cre recombinase transgene show evidence of hyperandrogenism. This finding suggests that the loxP system may have an effect on gene expression independent of Cre induced excision. In summary, studies of ARKO mice continue to shed light on complete and incomplete AIS and have revealed a differential role of the androgen system in males and females particularly with respect to fertility and reproduction.

1.6.3 Prostate Cancer

Prostate cancer is the leading diagnosed cancer amongst men in several countries including Canada (Muir, Nectoux, & Staszewski, 1991). It is typically diagnosed in men over 50 and incidence increases with age. A family history of breast or prostate cancer is a significant risk factor. Additional factors associated with incidence rate include race, geographic location, and nutrition. In particular this cancer is very frequent in North America and in certain European countries but very rare in much of Asia. It is also more common in black than white Americans and seems to be associated with a high-fat low-fiber diet (Rowley & Mason, 1997). Interestingly, the rate of prostate cancer is higher in asian men living in North America than those living in Asia heavily implicating environmental factors. A subclinical form of prostate cancer known as ‘latent cancer of the prostate’ is far more frequent and far less variable than clinical prostate cancer. It is believed that the latent form is a precursor to full blown prostate cancer which is triggered in only a subset of the patient population (Muir, et al., 1991).

The etiology of prostate cancer is unclear but appears to involve increased AR activity that is initially under the control of androgens. Accordingly, androgen ablation is a

common and effective treatment for advanced prostate cancer (Scher, et al., 1997). This may involve androgen depletion with a Luteinizing Hormone Releasing Hormone analogue, treatment with an anti-androgen such as flutamide, or treatment with a 5α-reductase inhibitor such as finasteride (Wang, Mencher, McCarron, & Ferrari, 2004). However, recurrence is common and is frequently associated with androgen independence (i.e. androgen ablation ceases to be an effective therapy). Androgen independent prostate cancer is thought to arise as a result of increased AR signalling pathways (Mimeault & Batra, 2006). In this form of prostate cancer AR is transcriptionally active suggesting that uncurbed transactivation may play a role in pathogenesis (van der Kwast, et al., 1991). Moreover, down regulation of AR using small intereference RNA inhibits cell growth in androgen-independent prostate cancer (Haag, Bektic, Bartsch, Klocker, & Eder, 2005). Therefore, AR is involved in both androgen dependent and independent forms of prostate cancer. Several of the biomarkers currently used for early detection of prostate cancer are androgen regulated genes such as PSA. These are inconclusive in isolation and must be coupled with familial history of cancer, age, and other factors in order to accurately assess risk.

1.6.4 Androgen Abuse

Due to their anabolic effects in skeletal muscle, androgens and their anabolic analogues are frequently abused. Side effects of excessive doses of anabolic steroids include fluid retention, premature atherosclerosis, liver disease, hypercalcaemia, and avascular necrosis of bone (Laseter & Russell, 1991). These changes may lead to secondary conditions such as quadricep ruptures due to bone resorption and cardiac sudden death caused by electrolyte imbalance (David, Green, Grant, & Wilson, 1995; Fineschi,

Baroldi, Monciotti, Paglicci Reattelli, & Turillazzi, 2001). More recently, the cosmetic use of anabolic steroids amongst men sparked the classification of a novel psychiatric condition known as "muscle dysmorphia" (Pope, Gruber, Choi, Olivardia, & Phillips, 1997). This disorder is a subtype of body dysmorphic disorder and affects the individual physically, psychologically and socially (Dawes & Mankin, 2004). Patients are obsessed with a muscular appearance and many believe they are weak and thin despite being very strong and large relative to their peers (Rohman, 2009). Individuals with muscular dysmorphia develop unhealthy dieting and weight control practices that frequently interfere with work, relationships, and family (Rohman, 2009). The unregulated use of androgens in non-clinical contexts raises numerous ethical and health concerns of which clinicians must be aware. The development of selective androgen receptor modulators (discussed next) is likely to raise additional novel concerns about the non-clinical use of anabolic drugs.

1.6.5 Selective Androgen Receptor Modulators

There is intense interest in artificial non-steroidal compounds that can modulate AR function in a tissue-selective manner. These so-called selective androgen receptor modulators, or SARM, are being developed for therapeutic purposes. The ubiquity of AR and the steroidal backbone of androgens limits the therapeutic use of natural androgens (Narayanan, Mohler, Bohl, Miller, & Dalton, 2008). Selective ER modulators, such as tamoxifen, have been in clinical use for several decades as estradiol agonists in postmenopausal women (Ward, 1973). The first SARM were described simultaneously by two labs in 1998 and were synthesised from tricyclic quinolinones and the anti-

androgen R-Bicalutamide (Dalton, Mukherjee, Zhu, Kirkovsky, & Miller, 1998; Edwards, et al., 1998). Since then, SARM have been developed with the intent to selectively exert anabolic rather than androgenic effects and to act in certain tissue and not others. The anabolic effects of SARM are targeted largely at bone and skeletal muscle for the therapeutic benefit of osteoporotic, sarcopenic, and cachexic patients (Frisoli, Chaves, Pinheiro, & Szejnfeld, 2005). Simultaneously, these compounds must exert minimal androgenic side effects such as virilization in women prostate growth in men (Narayanan, et al., 2008). To date, a compound with purely anabolic properties has not been developed. However, several SARM with high ratios of anabolic to androgenic effects have been developed and some are presently under clinical trials (Gao & Dalton, 2007a; Hanada, et al., 2003; Ostrowski, et al., 2007).

1.6.5.1 Mechanisms of SARM Action

Although the development of novel SARM has progressed rapidly, an understanding of tissue selectivity mechanisms has lagged behind. How do SARM exert tissue specific effects and how do they promote anabolism over androgynism? Several plausible mechanisms have been suggested thus far. Firstly, all SARM developed to date are resistant to 5α-reductase and aromatase, the enzymes responsible for converting T into DHT and estradiol respectively (Gao & Dalton, 2007b). The tissue-specific expression of these enzymes confers tissue selectivity to SARM. For instance, DHT is the most active androgen in prostate and accordingly this tissue has high levels of 5α-reductase (Wright, Douglas, Thomas, Lazier, & Rittmaster, 1999). SARM that are immune to reduction by this enzyme exert minimal effect on the prostate. Similarly, since 5α-reductase levels are low in skeletal muscle and bone, several SARM have potent effects in these tissues. The

tissue-specific expression of aromatase confers similar selectivity (Matzkin & Soloway, 1992).

Another line of evidence implicates transcriptional cofactors. AR regulates transcriptional activity by recruiting numerous coactivators and corepressors. Different sets of these factors are recruited in different tissue types. Ligand binding to AR induces a conformational change which determines what type of cofactors may be recruited cofactors. Different ligands result in subtly different conformational changes in AR and therefore in the recruitment of different cofactor (C. Chang, et al., 1999). By inducing certain conformational changes, SARM lead to recruitment of different cofactors and not others leading to effects restricted to particular tissue types (C. Y. Chang & McDonnell, 2002).

A final possible mechanism for SARM selectivity invokes a distinction between genomic and non-genomic androgen action. As described above, androgens can exert non-genomic effects, which may or may not be AR-independent. Non-genomic and genomic signalling mediate discrete androgen effects. For instance, non-genomic signalling is necessary for the bone protective effects of androgen but not for the development of sexual organs (Kousteni, et al., 2001). By selectively targeting one of these two pathways SARM may exert tissue-selective effects (e.g. bone protection but not prostatic hyperplasia). It is also possible that SARM act on AR-independent pathways. These effects are mediated by intracellular signalling cascades that vary in different tissues. For instance, T acts by inhibiting p38 MAPK in macrophages but is independent of this pathway in bone. This

finding suggests that some SARM may not act by modulating AR at all, which may prompt a reconsideration of the name or a new classification of drugs.

1.6.6 Polyglutamine Disease

There are a set of diseases whose etiology has been linked to polymorphisms in the first exon of the AR gene. This region of the AR gene contains a stretch of CAG trinucleotide repeats that translate into a polyglutamine region in the transactivation domain of AR protein. The length of this repeat is polymorphic and in healthy individuals can range between 9 and 36 trinucleotides and amino acids. The length of this repeat has additionally been shown to directly impact the transactivation function of the AR as well as androgen sensitivity (Mhatre, et al., 1993). Longer repeats are associated with reduced transactivation and reduced androgen sensitivity, while shorter repeats with greater transactivation and greater androgen sensitivity (Rocha, et al., 2008). Expansions of the CAG repeat above the normal range, or contractions below it have been linked to a variety of clinical conditions. These include male infertility, female breast cancer, and endometrial cancer when the CAG region is expanded, and prostate cancer, esophageal cancer, and colon cancer when the region is contracted (Lieberman & Robins, 2008; MacLean, Brown, Beilin, Warne, & Zajac, 2004).

Spinal and Bulbar Muscular Atrophy (SBMA), or Kennedy Disease (KD), is presently the only condition that has been directly linked to an expansion of the CAG repeat in the AR gene. It is an X-linked progressive neuromuscular disorder that afflicts men typically in middle to late adulthood. It is characterized by loss of lower and bulbar motoneurons in the spinal cord and brainstem respectively. The symptoms include symmetrical

proximal muscle weakness, atrophy and fasciculation and may involve bulbar dysfunctions such as dysarthria and dysphagia. Patients additionally present with signs of androgen insensitivity such as gynecomastia, reduced sperm count, and testicular atrophy.

Repeat length is inversely correlated with age at onset and directly correlated with symptom severity. Additionally, anticipation has been observed in SBMA patients, wherein consecutive generations carrying the SBMA genotype have longer CAG repeats and earlier age of onset. SBMA is a member of a class of neurodegenerative disorders known as polyglutamine diseases. All diseases within this class (total of 9) are caused by an expanded CAG tract in culpable genes. These include Huntington disease (HD), several Spinocerebllar Ataxias, and Haw River syndrome. Polyglutamine diseases are themselves members of a broader class called the trinucleotide repeat disorders.

Tg animal models of SBMA have focused on replicating the CAG trinucleotide expansion. However, it has been recently reported that overexpression of Wt AR in mouse skeletal muscle leads to an SBMA-like phenotype (Monks, et al., 2007). These animals, in which AR overexpression is driven using the Human Skeletal Actin (HSA) promoter, have been named HSA-AR mice.

1.7 HSA-AR Tg Mice

1.7.1 Tg Construct and Phenotype

Monks et al. (2007) created Tg mice to examine the role of skeletal muscle AR in the differentiation of a sexually dimorphic neuromuscular system. Using the Human Skeletal Actin (HSA) promoter, an endogenous promoter of an alpha-actin isoform specific to skeletal muscle, myofiber-specific overexpression of widltype rat AR cDNA was achieved. Overexpression models lend themselves well to the study of systems that naturally vary in an age and sex dependent manner such as the androgen system. An unexpected phenotype was observed in one line (L141) marked by motor deficits and severe wasting in males, whereas females appeared normal and healthy. T administration to females of this line resulted in a dramatic pathology marked by motor deficits, loss of body weight, and ultimately death. Male but not female viability at birth was reduced and this was ameliorated by flutamide (an anti-androgen) administration to pregnant dams. Finally, surgical castration of surviving males leads to a striking improvement in motor function. These characteristics suggest that pathology associated with AR overexpression is androgen dependent.

A second founding line (L78) that differs from the first in transgene copy number (L78<L141) was also characterized. Tg males from this line were significantly smaller than their Wt littermates. Female L78 mice did not show any signs of pathology and T treatment did not significantly alter motor function or body weight. These findings suggested that severity of pathology was associated to level of AR expression.

1.7.2 Preliminary Characterization

A preliminary characterization of sketelal muscle revealed loss of myofibers in L141 and L78 males but not T treated females of either line (Monks, et al., 2007). In L141 males, fiber loss was accompanied by loss of motor neuron axons but not motoneuron somas whereas L78 males and T treated L78 and L141 females showed no signs of axonopathy. Collectively these findings suggest that primary pathology in these mice lies within myofibers and neuronopathy is a consequence of this pathology.

A histochemical assay of oxidative metabolism (NADH) revealed that Tg males of both lines had augmented but disorganized oxidative metabolism. Dark NADH staining suggested that mitochondrial content or function was augmented and that changes in oxidative metabolism were involved either as a cause or an effect of pathology. An Hematoxylin and Eosin (H&E) stain revealed grouped atrophic fibers, split fibers, and internal nuclei all suggestive of denervation. Finally, in all these histochemical assessments symptomatic L141 males showed more dramatic changes than L78 males suggesting once more that AR expression levels are linked to the severity of pathology.

1.7.3 Explaining the Paradox

It might be argued that the HSA-AR phenotype is merely a manifestation of non-specific toxicity caused by overexpressed protein. This possibility is unlikely given that the phenotype is androgen dependent which suggests a functional role of AR specifically. Moreover, overexpression of other proteins under the control of HSA (e.g. the LacZ reporter gene) has not been shown to result in myopathy. Finally, AR mRNA levels are

actually reduced during pathology in males and T treated females suggesting secondary pathologic pathways.

That the level of overexpression plays a critical role in inducing pathology is supported by several observations. As described above, L78 animals show a far more moderate set of symptoms relative to L141 animals. Moreover, Tg rats that also overexpress AR specifically in skeletal muscle but to a far lower extent do not show evidence of myopathy but rather reveal an anabolic effect of AR overexpression (Monks lab, unpublished observations). Therefore, there appears to be a critical level of AR expression beyond which adverse cellular events occur. The nature of these events and the value of the critical level remain unknown. Furthermore, the striking similarities between HSA-AR mice and SBMA models point to a possible common mechanism of myopathy. Given these facts, a thorough investigation of the cellular alterations and mechanisms mediating pathology in HSA-AR mice is warranted.

1.8 Objectives and Hypotheses

The implication of the androgen system in muscle health and pathology warrants a thorough characterization of the effects of AR in this tissue. In particular, the pathologic effects of AR overexpression in muscle merit exploration. This study aimed to identify cellular and subcellular features of myopathy in order to begin elucidating possible mechanisms of pathology. Particular focus was placed on mitochondria and sarcomere ultrastructure to identify effects on metabolism and muscle contraction. The study further aimed to identify the role of T in inducing any observed subcellular changes. All

experiments were conducted on both L78 and L141 animals to determine the significance of AR overexpression. Accordingly, our hypotheses were:

1. Muscular atrophy in Tg males has cellular origins that will be revealed by an ultrastructural analysis
2. Tg males will reflect enhanced NADH staining with increases in mitochondrial number, size, or activity
3. Shifts in fiber type composition will reflect enhanced oxidative metabolism such that there will be an increase in oxidative and oxidative-glycolytic cells.
4. All the aforementioned changes will be androgen dependent and can be induced by T treatment of Tg females.
5. Level of overexpression will be related to severity of pathologic changes such that L141 mice will have more pronounced ultrastructural and histological changes than L78.

Given the small number of L141 males in this study, the final hypothesis will be tested using data collected on L78 and L141 females only.

Chapter 2: Materials and Methods

2.1 Overview

This study consisted of 4 experiments each, save for 1, comprising electron and light microscopic components (experiment 4 did not include light microscopy). In each experiment, Extensor Digitorum Longus (EDL) and Anterior Tibialis (AT) muscles were dissected from hind limbs of adult mice. The EDL was used for transmission electron microscopy while the AT was used for light microscopy. These muscles were selected because they are easily extracted and because they are commonly used in electron and light microscopy respectively. The EDL is narrow enough to allow for efficient infiltration of epoxy resin in preparation for electron microscopy and the AT is broad enough to facilitate histological analysis and to encompass a variety of fiber types.

In Experiment 1, unmanipulated adult Wt and Tg L78 males were examined. After noting several overt ultrastructural changes in animals overexpressing AR in skeletal muscle, the role of T was investigated. Experiment 2 therefore examined the role of T using T or vehicle (VEH) treated Wt and Tg L78 females. Next, the significance of level of AR overexpression was investigated. Experiment 3 included T or VEH treated widltype and Tg L141 females.

Males from the L141 line are extremely difficult to acquire. The transgene has a negative impact on male but not female viability. In the year encompassing this study 3 Tg males were born in our colony. For the purpose of other studies in our laboratory one of these animals was gonadectomized. Nonetheless, this animal was included in the analysis and

identified as such where applicable. Moreover, the scarcity of L141 males and the use of their tissue in other experiments prohibited histological analysis of their AT muscle. Therefore, in Experiment 4 only ultrastructural analyses were conducted on unmanipulated (INTACT) or gonadectomized (GDX) L141 males. All animals were housed in controlled laboratory conditions with ad libitum access to food and water. Ambient temperature was 24^{o}C, and the light-dark cycle was set at 12 hours. All morphometric analysis was carried out using ImageJ software from NIH.

2.2 Subjects

In all experiments littermates were used in sibling pairs in treatment and control conditions. In *Experiment 1* 10 unmanipulated L78 males (5 Wt, 5 Tg) were used (ages 144-148 days, mean 146 days). *Experiment 2* consisted of 32 L78 females divided equally into four treatment conditions: (i) Wt VEH treated (Wt-VEH; 8) (ii) Wt T treated (Wt-T; 8) (iii) Tg VEH treated (Tg-VEH; 8) and (iv) Tg T treated (Tg-T; 8) animals (ages 97-202 days, mean 133 days). *Experiment 3* consisted of 16 L141 females divided equally into four treatment conditions: (i) Wt VEH treated (Wt-VEH; 4) (ii) Wt T treated (Wt-T; 4) (iii) Tg VEH treated (Tg-VEH; 4) and (iv) Tg T treated (Tg-T; 4) animals (ages 100-264 days, mean 143 days). Finally, *Experiment 4* consisted of 6 L141 males (3 Wt, 3 Tg). One pair was castrated prior to dissection (ages 96-116 days, mean 105 days). Unless otherwise stated, L141 male data was pooled regardless of manipulation. Since only one pair of L141 males was castrated, the statistical significance of this manipulation could not be assessed.

2.3 Surgery and Dissections

T and VEH implants were prepared with silastic surgical tubing. T was packed to a 6mm release length while VEH implants were vacant save for silicone caps at each end. Typical serum T concentrations in males and female mice are approximately 2-4ng/ml and 0.3ng/ml respectively (Sengstake & Chambers, 1991). An implant of release length 60mm has been shown to produce serum T concentrations of around 3.5ng/ml. Animals were placed under light isofluorine anesthesia while the capsules were implanted dorsally at the nape of the neck. Hibitane cream was applied to inhibit infection and the animals were left to recover on clean bedding. After 1 week the animals were euthanized with 0.1ml Sodium Pentobarbital and EDL and AT muscles were rapidly dissected. Dissected EDL were fixed in 1% glutaraldehyde in 0.1M phosphate buffer for 15 minutes then cut at the belly with a scalpel into 3 or 4 discs each approximately 1mm thick. These discs were stored in glutaraldehyde fix for subsequent electron microscopy. Dissected AT were oriented in cryomolds containing Tissue-tek, flash frozen in liquid nitrogen, and subsequently stored at -80^{o}C until used for histochemical analysis.

2.4 Transmission Electron Microscopy

2.4.1 Sample Preparation

Sections were removed from glutaraldehyde and fixed in 1% sodium osmium tetroxide in 0.1M phosphate buffer for 1 hour. The sections were then washed repeatedly with 0.1M phosphate buffer and dehydrated in graded alcohol (70%, 95%, 95%, 100%, 100%, 100%). Samples were gradually infiltrated with epoxy resin by diluting in diminishing volumes of propylene oxide (75%, 50%, 0%) and were rotated in 100% epoxy resin

overnight. The resin mix was degassed using a vacuum pump until frothing ceased. Mold wells were labeled, filled with 100% resin, and the samples were then placed into their corresponding wells. The tissue was oriented such that the cutting edge lay parallel to the long axis of the muscle fascicle. The mold wells were then baked at 60^{o}C for 48 hours to permit polymerization.

Ultra thin longitudinal sections (90nm) were prepared using an ultramicrotome and mounted on 300 mesh copper grids. Each grid was occupied by two sections and four grids were prepared per animal. Finally, sections were stained with uranyl acetate and lead citrate. Sections were viewed on a Hitachi-7000 Transmission Electron Microscope at 75kV acceleration.

2.4.2 Sampling Strategy

Myofibers were sampled pseudorandomly by photographing myofibers in alternate EM grids. Images for sarcomere and mitochondrial analysis were taken at x20,000 and x40,000 magnification respectively. Glycogen was analyzed using the latter magnification. Core, intermyofibrillar mitochondria rather than subsarcolemmal mitochondria were photographed. Subsarcolemmal mitochondria have a distinct quantity, morphology, distribution, and functional role. Fibers narrower in diameter than 15 μm were excluded from analysis to avoid inclusion of subsarcolemmal mitochondria.

2.4.3 Measurements

Sarcomere width (short axis) and height (long axis) were measured as well as mitochondria number, profile area, and volume density. Volume density was calculated

using the point-counting method (Weibel, 1979) on a 12x12 (144 point) test grid. The grid was generated using the Grid plug-in for ImageJ by Wayne Rasband. In an attempt to quantify differences in glycogen content, the proportion of micrographs that displayed obvious glycogen granule presence was calculated. If ten distinct myofibers could not be identified in a given grid, another grid was used. Each micrograph was of an independent fiber that had visibly defined sarcolemma; this in effect generated fields of ten distinct fibers within the EDL per animal.

2.4.4 Volume Density Estimates

A 144 point grid was superimposed on each micrograph using ImageJ v1.38x and the Grid plug-in by Wayne Rasband (http://rsb.info.nih.gov/ij/plugins/grid.html). The choice of a Total Point (P_T) value of 144 was derived as follows (adapted from Weibel, 1979):

The smallest measurable mitochondrial profile area must be smaller than the area of a single square on the point grid. The number of squares that can be fit into a single micrograph field established the number of points on the field and therefore the grid's dimensions. For example, if the smallest mitochondrion is 10000nm^2, then the smallest square must be 10000nm^2. The number of squares that can fit into a micrograph of fieldsize 1440000nm^2 is 144. One hundred and forty four squares translate into 144 points and therefore the grid is at least 12x12.

2.5 Light Microscopy

Anterior Tibialis muscle was sectioned at 10um thick with a cryostat at -22^{o}C. Serial sections were used to identify fiber type using a SDH stain and an immunohistochemical assay of fast Myosin Heavy Chain. Additionally, histochemical stains were used to

examine lipid content (Sudan Black B), glycogen content (Periodic Acid Schiff/Diastase), and mitochondria (Gomori Trichrome).

2.5.1 Fiber Typing

In order to distinguish between oxidative (O), glycolytic (G), and oxidative-glycolytic (OG) fibers, sections were incubated for 60 minutes at 37^{o}C in Nitroblue Tetrazolium (Nitro BT). Dark, light, and medium staining fibers were identified as O, G, and OG respectively. A monoclonal anti-skeletal myosin antibody (MY-32, Sigma M4276) was used to distinguish fast and slow fibers. Sections were treated with 3% H_2O_2 to block endogenous peroxidase activity and with avidin-biotin blocking solution to reduce nonspecific staining. Further blocking with MOM IgG blocking reagent was employed (Vector, MKB-2213). Biotinylated horse anti-mouse 2^{o} antibody and avidin-biotin-peroxidase complex (ABC) were used and visualized with diaminobenzidine (DAB) and Nickel Chloride. This protocol stains cells that express the fast isoform of MHC only. Secondary-free sections were used as negative controls. Micrographs of SDH and MHC stained sections were overlayed when necessary to identify fiber type.

2.5.2 Histochemical Assays

2.5.2.1 Periodic Acid Schiff (Diastase)

We followed previously described protocols for the semiquantification of glycogen content in muscle fibers using Periodic Acid Schiff stain (PAS; (Fairchild & Fournier, 2004; van der Laarse, van Noort, & Diegenbach, 1992). This two step stain involves oxidation of a 1,2-glycol group to a dialdehyde that then condenses with Schiff's reagent to give a bright red stain. PAS staining is not specific to glycogen but to any molecule containing a 1,2-glycol group. Therefore, sections digested by amylase (enzyme that

more specifically catalyzes glycogen lysis in myofibers) were used as a background control. Sections were fixed in Carnoy's Fix (chloroform and acetic acid in anhydrous alcohol) for 10 minutes, oxidized with periodic acid for 10 minutes and finally stained in Schiff's Reagent for 5 minutes. High glycogen content was marked by darker pink/red staining. Diastase digested sections were stained and used as a negative control (PASD). Photomicrographs were taken on an Olympus BX-51 compound light microscope with a mounted Olympus D70 CCD camera attached to a computer. The microscope was allowed to stabilize for 15 minutes with the bulb set at 11V prior to photography. Three regions of one PAS and one PASD section were photographed at 40x. All images were converted to 8-bit grayscale and mean gray values (in pixels) were measured. To convert from pixels to optical density units (O.D.) a calibrated Kodak No.3 Step-tablet was used along with Rodbard calibration function on ImageJ software. All measures were corrected by subtracting corresponding PASD O.D. values. Micrographs of PAS stained tissue were used for morphometric analysis. Myofiber cross-sectional areas (CSA) were calculated using the minor axes of elipses fitted on individual fibers. This minor axis was used as an unbiased estimate of fiber diameter to correct for imperfect sectioning planes.

2.5.2.2 Sudan Black B and Gomori Trichrome Stain

Muscle lipid content was qualitatively assayed using Sudan Black B. Sections were fixed for 5 minutes in 10% formalin stained in Sudan Black B in propylene glycol, rinsed and cover-slipped with DPX. Changes in mitochondrial content were assayed with one-step Gomori Trichrome (GTC) stain. Slides were stained in GTC for 30 seconds, differentiated in glacial acetic acid, dehydrated, and cover-slipped with permount.

2.5 Statistical Analyses

Ultrastructural differences between Wt and Tg males in experiments 1 and 4 were tested using two-way independent samples t-tests. Ultrastructural differences in the females of experiments 2 and 3 were tested using two-way multivariate ANOVA and significant differences were evaluated with a Tukey's post-hoc test. Differences in fiber type parameters were tested with repeated measure ANOVA's with genotype and treatment (where applicable) as between subject variables. Paired samples t-tests were used to identify disparate groups. Since proportion values are discontinuous, glycogen percentage and volume density values were transformed using an arcsine function prior to statistical analysis.

Chapter 3: Results

3.1 Overview

Changes in muscle ultrastructure and histology were evident in androgen exposed Tg animals (males and T treated females). Males overexpressing AR in skeletal muscle showed an increase in mitochondria number, profile area, and volume density. Females overexpressing AR did not show these changes unless treated with T. Wt females did not show these features regardless of T treatment. A similar pattern was observed with respect to myofibril width, which was narrower in Tg males and only in Tg-T females. Males but not females showed an increase in intermyofibrillar glycogen content. The two Tg lines differed in the extent rather than in the quality of ultrastructural alterations.

Histological analysis revealed differences in fiber type representation and morphology. Tg males showed a reduction in the proportion of oxidative fibers but an increase in that of glycolytic fibers. These findings were mirrored in Tg-T L141 females but not in L141 females of other conditions. L78 females did not show any significant change in fiber type proportion. Tg males additionally exhibited increased overall fiber CSA. Specifically Tg males had larger FO and FOG fibers but smaller FG fibers. No changes in fiber CSA were observed in L78 females regardless of genotype and treatment. However, Tg-T L141 females showed an enlargement of oxidative fibers relative to other groups of L141 females. Augmented glycogen content in males but not females was confirmed histologically using PAS stain. Tg males had greater PAS optical density than Wt males but females did not differ significantly from one another. Ragged red fibers (RRF) were

clearly evident in Tg males whereas lipid content did not appear to be affected by transgene expression or T treatment.

3.2 General Subject Characteristics

General physical characteristics of subjects are summarized in table 1. Tg males from the 78 line had reduced mean AT weights relative to Wt littermates ($t_{2,8}$=2.65; p=0.042). They also had lower body weights although the difference was not significant ($t_{2,8}$=1.75; p=0.12). Seminal vesicle weight (an estimate of serum T level) did not differ significantly. Data is unavailable for L141 males. The physical characteristics of L78 females were largely unaffected by genotype or treatment. In contrast, an ANOVA followed by a Tukey post-hoc revealed that Tg-T L141 females showed a significant reduction in body weight relative to Wt-VEH L141 females (p=0.046).

3.3 Ultrastructural Features

3.3.1 Increased Mitochondria Number, Profile Area, and Volume Density in Tg Males

Mitochondrial ultratructure was examined to investigate the effects of skeletal muscle-specific AR overexpression on metrics of oxidative metabolism. Tg L78 males had almost twice the number of mitochondria found in Wt littermates ($t_{2,8}$=8.48; p<0.001; figure 1). Mean profile area of mitochondria tended to be greater in transgenics but not significantly so ($t_{2,8}$=1.92; p=0.09). Correspondingly, the volume density of mitochondria (i.e. the proportion of cell volume occupied by mitochondria) was greater in Tg relative to Wt ($t_{2,8}$=3.45; p<0.01). L141 males similarly showed greater mitochondrial profile area

($t_{2,4}$=2.80; p=0.049; Figure 2) and number, although the latter was not statistically significant ($t_{2,4}$=1.07; p>0.10). Once more, these changes were reflected in a greater mitochondrial volume density in Tg relative to Wt ($t_{2,4}$=2.86; p=0.046).

3.3.2 Reduction in Myofibril Width in Tg Males

Since symptomatic HSA-AR mice show motor deficits and reduced muscle strength, changes in sarcomere dimensions were examined. No differences were found in sarcomere length (long-axis), but Tg L78 males had narrower and visibly disorganized myofibrils ($t_{2,8}$=9.46;p<0.001; figure 3). Myofibrils were similarly reduced in width in Tg L141 males relative to Wt ($t_{2,4}$=3.59;p<0.05).

3.3.3 Only T Treated Tg Females Recapitulate Changes in Mitochondria and Fibril Ultrastructure

To investigate the importance of T in the observed ultrastructural changes, T and VEH treated L78 females were examined. When treated with T, Tg females recapitulated male changes in mitochondrial and fibril morphology (figure 4). Multivariate ANOVA revealed a main effect of Genotype (Tg or Wt) on myofibril width mitochondria number, and volume density. An additional main effect of Treatment (T or VEH) on mitochondrial volume density and an interaction on all measures were identified. However, a Tukey post-hoc test revealed that Tg-T animals were actually the only group to significantly vary from all others such that mitochondria were most numerous (p<0.05 for all comparisons) and occupied the greatest volume in this group (p<0.01 for all

comparisons). Mean myofibril width was additionally narrower in Tg-T L78 animals relative to all other groups ($p<0.05$ for all comparisons; figures 5 and 6).

L141 females similarly showed transgene and androgen dependent changes in mitochondrial and fibril ultrastructure. There were main effects of genotype and treatment on mitochondrial profile area and volume density as well as fibril width. There was an additional interaction in mitochondrial area, number, volume density, and fibril width. A Tukey post-hoc revealed that Tg-T L141 females significantly differed from each other group in all measures of mitochondria and fibril width.

3.3.4 Increased Granular Glycogen Content in Males but Not Females

Glycogen content in L78 and L141 Tg males was augmented as evidenced by large collections of granules within the sarcoplasm (figure 1). These accumulations of intermyofibrillar glycogen granules were evident in Tg but not Wt males. In an attempt to quantify these changes the proportion of electronmicrographs presenting with granular glycogen was calculated. A much greater proportion of L78 Tg male fields showed glycogen presence relative to Wt ($t_{2,8}=0.93$; $p=0.001$; figure 3). Similarly, the proportion of micrographs that evidenced glycogen was greater in L141 Tg males than Wt ($t_{2,4}=4.07$;$p=0.015$). In contrast to these findings in males, females of both lines showed no significant evidence of glycogen accumulation regardless of treatment (figure 6).

3.3.5 Level of Overexpression Associated with Extent of Ultrastructural Change

We assessed whether level of transgene expression affected the extent of ultrastructural changes using a series of independent samples t-tests. Relative to L78 Tg males, L141 Tg males had narrower myofibrils although this difference was not significant ($t_{2,6}$=1.96; p=0.09). And although L141 Tg had larger mitochondria, greater volume density, and more micrographs presenting with glycogen granules, none of these differences were statistically significant.

A comparison of Tg-T L141 and L78 females was also conducted. As expected, L141 females had a lower body weight relative to L78's ($t_{2,10}$=6.69; p<0.001). Additionally, L141's had significantly narrower myofibrils ($t_{2,10}$=2.91; p=0.016) and greater mitochondrial profile area ($t_{2,10}$=2.73; p=0.021). Mitochondria volume density showed a trend to be larger in L141's relative to L78's ($t_{2,10}$=1.88; p=0.089). No differences were observed in the number of mitochondria. Ultrastructural features in all experiments features are summarized in table 2.

3.4 Histological Features

3.4.1 Loss of Distinction in Fiber Type Representation and Morphology in Tg L78 Males

To examine how fiber type representation and fiber morphology were affected by transgene activity, a histologic examination was performed. Immunohistochemistry revealed that the Anterior Tibialis in mice is an entirely fast muscle (figure 11). The validity of this finding was confirmed by repeating the procedure on sections of soleus

muscle (a hind limb muscle known for its slow fiber content). Fibers were therefore classified as Fast Oxidate (FO), Fast Glycolytic (FG), and Fast Oxidative/Glycolytic (FOG).

Amongst L78 males, a repeated measure ANOVA revealed that fiber type proportions varied by genotype ($F=8.48$; $p=0.02$). An independent samples t-test demonstrated that Tg L78 males had roughly half the proportion of FO ($t_{2,8}=3.30$; $p=0.011$) and displayed an increase in the proportion of FOG fibers ($t_{2,8}=6.53$; $p<0.001$; figure 7). In Wt animals AT muscle is composed of distinct proportions of fiber-types such that the majority of cells are FO and the remainder are FG or FOG. This distribution is absent in Tg animals wherein all fiber-types are approximately equally represented (Figure 3). This was demonstrated statistically with a series of paired samples t-tests. Whereas Wt animals showed significant differences between the proportions of FG and FO fibers ($t_{2,4}=3.37$; $p=0.028$) and FO and FOG fibers ($t_{2,4}=4.25$; $p=0.013$), Tg showed no such distinctions (figure 8).

A repeated measure ANOVA revealed that fiber types differed in CSA and that there was a significant interaction with genotype ($F=31.11$; $p=0.001$; figure 7). A comparison between genotypes revealed a trend towards greater overall CSA in Tg ($t_{2,8}=2.34$; $p=0.055$). More specifically, FO ($t_{2,8}=5.95$; $p<0.001$) and FOG ($t_{2,8}=2.62$; $p=0.03$) were larger in Tg, whereas FG were smaller in Tg ($t_{2,8}=2.61$; $p=0.03$). Once again the normal distribution of fiber size by type was lost in Tg. Typically, glycolytic fibers have the greatest CSA while FOG and FG have smaller CSA. Whereas, Wt L78 males showed this

distribution (FG>FOG>FO), Tg showed no distinction in size by fiber type. FG fibers were not significantly larger than FOG ($t_{2,4}$=2.24; p>0.05) or FO ($t_{2,4}$=0.78; p>0.1) fibers.

3.4.2 L141 but not L78 Females Recapitulate Loss of Fiber Type Distinctions

To investigate whether these effects were contingent on T activation of transgene AR, muscle histology was examined in T and VEH treated L78 and L141 females. The changes in fiber type proportions seen in L78 males were not recapitulated exactly in Tg-T L78 females (figure 9). Nonetheless, some differences in fiber type proportions were observed. An ANOVA followed by a Tukey post-hoc revealed that Wt-VEH females had a greater proportion of FOG fibers than all other groups (p<0.01 for all groups). Additionally, treatment of Wt females with T resulted in an increase in the proportion of FO fibers ($t_{2,6}$=2.58; p=0.042). As can be seen in figure 10, no loss of fiber type distinction was observed. Apart from a trend towards larger FO fibers in Tg-T females (p=0.065) no differences were observed in fiber CSA amongst the conditions in L78 females (Figure 6).

Unlike L78 females, Tg-T L141 females did recapitulate some of the histological changes observed in males (figure 9). Repeated measure ANOVA revealed that fibers differ in their proportions by a three-way interaction of fiber type, genotype, and treatment. Paired samples t-tests revealed that the distinction between FG and FO fibers found in Wt-VEH, Wt-T, and Tg-VEH groups is lost in the Tg-T group (Figure 10). In particular, the proportion of FO fibers is reduced in Tg-T females such that it is significantly lower than

Wt-T females ($t_{2,6}$=3.01; p=0.024; Figure 7). An ANOVA revealed a main effect of genotype and treatment on FO fiber CSA. A Tukey post-hoc revealed that Tg-T L141 females had overall larger fibers and specifically had larger FO fibers than all other groups ($p<0.05$ for all comparisons; figure 10). Additionally, independent t-tests revealed that relative to Wt-VEH, Tg-T females had greater CSA of FOG fibers ($t_{2,6}$=3.21; p=0.018).

3.4.3 Augmented Glycogen Content Confirmed by Microdensitometry in Males but not Females

To confirm and further investigate increased glycogen content in L78 males, Periodic Acid Schiff (PAS) stain was used. Relative to Wt, Tg L78 males showed greater glycogen content as evidenced by more intense PAS staining ($t_{2,8}$=5.61; p=0.001; figure 7). Specifically, FO ($t_{2,8}$=3.40; p=0.009) and FOG ($t_{2,8}$=4.22; p=0.003) fibers showed greater PAS optical density in Tg relative to Wt (figure 8). In accordance with ultrastructural data, females did not evidence accumulation of glycogen regardless of genotype or treatment (figure 10).

3.4.4 RRF Identified but not Alterations in Fiber Lipid Content

Qualitative histochemical stains were used to confirm alterations in mitochondrial content and to investigate changes in lipid content. Gomori Trichrome stain was used to search for RRF which are indicative of fibers with proliferative mitochondria. RRF's were identifiable in Tg L78 males and Tg-T L141 but not L78 females (figure 11). Sudan

Black B staining did not reveal any changes in lipid storage within any of the experimental groups.

3.5 Summary

Study findings are summarized in tables 2 and 3. In brief, overexpression of AR in male skeletal muscle leads to increased mitochondrial and glycogen content and diminished fibril width as assessed by electron microscopy. In Tg females, increased mitochondria are observed only after T treatment. Females did not demonstrate increased glycogen content regardless of treatment or genotype. SDH staining did not reflect increased mitochondrial content. Rather, the proportion of FO fibers estimated by SDH staining declined in Tg males and Tg-T L141 females. However, that mitochondrial content was augmented by T and Tg AR were confirmed by the identification of RRF's. Fibers were additionally found to be hypertrophic in Tg males and Tg-T females. Lastly, lipid content appeared to be unaffected by transgene or treatment.

Chapter 4: Discussion

4.1 Overview

Overexpression of rat AR in mouse skeletal muscle caused dramatic androgen-dependent changes in mitochondria, glycogen storage, and fibril organization. Androgen exposure and transgene expression were both necessary to elicit changes in mitochondrial morphology and number. Mitochondria were distended and proliferated in Tg males and seven days of T treatment sufficed to distend and proliferate mitochondria in Tg females. In L141 animals, where AR overexpression is greatest, mitochondrial distention was most dramatic. Tg males and T treated females additionally evidenced narrow and disorganized myofibrils. Some of the effects of activating overexpressed AR were sexually dimorphic. Glycogen accumulation in male muscle was evident at the light and electron microscope level but at neither level in females. Greater overexpression of AR in L141 females did not alter this sexual dimorphism. Suprisingly, histological findings based on SDH staining did not corroborate EM changes in mitochondrial content. There was a significant shift in Tg L78 males and Tg-T L141 females from oxidative to glycolytic fibers as assessed by SDH staining such that FO and FG fibers were approximately equally represented. However, that mitochondrial content was augmented in these animals was established histochemically by the presence of RRF's as revealed by Gomori Trichrome stain. In contrast, Tg-T L78 females showed the predicted enhanced SDH staining (and therefore proportion of FO fibers) rather than a diminution. Lipid content as assessed by Sudan Black B stain was found to be normal in all animals.

4.2 Mitochondrial Distention as a Normal or Pathologic Feature

Mitochondrial enlargement was observed only in animals that both overexpressed AR and were exposed to T. That androgens can influence mitochondrial ultrastructure has previously been demonstrated in humans, mice, and fish (Brantley, Marchaterre, & Bass, 1993; Satoh, Gotoh, & Yamashita, 2000; Sinha-Hikim, et al., 2003). Both subsarcolemmal and intermyofibrillar mitochondria increase in size in response to androgens. These changes are reversible as the removal of T implants reverts mitochondria to their pretreatment size. Moreover, mitochondrial content in androgen sensitive muscle is greater in males relative to females (Bass & Marchaterre, 1989). Enlargement in response to physiological levels of T are typically on the order of 1.3x the size of control animal mitochondria. In this study, males overexpressing AR had enlarged mitochondria relative to those with normal receptor levels suggesting that changes in mitochondrial ultrastructure in response to androgens are mediated by AR. Therefore, it may be argued that supraphysiological increases in mitochondrial size are a reasonable response to supraphysiological AR expression.

However, there is evidence suggesting that this inadequately explains the case in HSA-AR animals. Although acute androgen administration can lead to mitochondrial enlargement, it has been found that this effect is transient. Mitochondria gradually (by 8 weeks) return to their pretreatment size even if androgen administration persists (Satoh, et al., 2000). In this study, male HSA-AR mice were exposed to high levels of AR throughout life and were examined in adulthood (22 weeks minimum). Therefore, one would expect any mitochondrial enlargement driven by androgen action to have

dissipated by the time of this study; this was not the case. Of course, it remains possible that the results of previous studies were driven by a compensatory downregulation of AR expression in response to persistently high levels of T. Since this downregulation could not occur in HSA-AR animals, mitochondrial enlargement may have been mediated by androgen action.

It is possible that mitochondrial enlargement and proliferation is a secondary response to negative changes in cellular energetics. These changes may represent an attempt by the cell to salvage its aerobic capacity and to rescue itself from energy deficiency. Mitochondrial myopathies are myogenic disorders of mitochondrial origin, typically caused by mutations in mitochondrial or nuclear DNA leading to deficiencies in respiratory chain complexes (Fayet, Aure´, & Lombe`s, 2007). Such myopathies typically involve an increase in mitochondrial content as revealed by the presence of RRFs and RBFs. Wredenberg et al. (2002) found that mice with a respiratory chain deficiency had increased mitochondrial mass and that this was sufficient to compensate for the deficiency and sustain ATP production in muscle. This idea is supported by the finding that increases in mitochondrial size beyond the normal physiological range are inversely correlated with mitochondrial metabolic competence (Bertoni-Freddari, et al., 2003). Uncurbed increases in mitochondrial content may additionally trigger apoptotic cascades particularly if outer-membrane permeabilization occurs. For instance, unrestrained mitochondrial proliferation has been found to be accompanied by membrane dysfunction and changes in membrane potential (Mancini, et al., 1997). Moreover, it has been demonstrated *in situ* that apoptosis, as marked by Bax overexpression and caspase 3

activation, is tightly linked to mitochondrial proliferation (Fayet, et al., 2007) assessed with SDH stain. That mitochondrial changes in our Tg animals may be a prelude to apoptosis is supported by the fact that ruptured mitochondria were observed in some of our Tg males (personal observation) and that these animals have fewer myofibers than controls (Monks, et al., 2007).

4.3 Compression of Myofibrils in T Exposed Tg

It is uncertain whether mitochondrial distention leads to sarcomere compression in HSA-AR animals or whether secondary changes, such as dysregulation of contractile protein expression, are responsible. Regardless, sarcomere CSA, and therefore width, is definitive of maximum contractile force and narrowed fibrils therefore undoubtedly impact motor strength and function. As described below, fiber hypertrophy frequently involves an increase in fibril splitting resulting in more numerous but narrower fibrils. Although such an explanation may account for reduced fibril width it does not explain the motor deficits observed. A more likely explanation is that disruption in fibril organization is a feature of this myopathy and that distention of mitochondria has contributed to fibril compression.

4.4 Glycogen Accumulation in Tg Males

We also identified an accumulation of glycogen in Tg male but not Tg-T female mice. It is not surprising that the manipulation of AR expression impacted glucose homeostasis. The androgen and insulin systems are interactive and the effects of androgens on insulin sensitivity are well documented. Castration of males leads to a decline in skeletal muscle glycogen and T treatment causes glycogen accumulation (Bergamini, et al., 1969). This is

thought to occur by increased glycogen synthase activity and glucose penetration as mediated in part by enhanced GLUT4 expression (Barros, Machado, Warner, & Gustafsson, 2006; Bergamini, 1972). These transactivation effects are likely mediated by AR. Therefore, overexpression of AR may amplify these normal responses to androgen leading to exaggerated accumulation of glycogen in muscle. We also identified sex differences in glycogen accumulation.

4.4.1 Sexual Dimorphisms in Glycogen Accumulation

Although Tg males of both lines accumulated intermyofibrillar glycogen, Tg-T females did not. This observation is perhaps partially explained by the fact that there are sexual differences in the effects of androgens on gene transcription (Yoshioka, et al., 2006). Rincon et al. (1996) showed in rat skeletal muscle that T treatment of oopherctomized females leads to a decline in insulin-mediated glucose uptake and that insulin is able to stimulate GLUT4 insertion into the plasma membrane of control but not T treated females. Moreover, glycogen synthase expression in T treated females is reduced relative to controls; an opposite effect, it will be recalled, occurs in males. On the other hand, glucose penetration, glucose phosphorylation, and glycogen synthase activity have all been found to increase with T treatment in castrated males. It is possible that these sex differences are mediated by differential responses of the insulin system to T (Rincon, et al., 1996). In hypogonadic men there is increased insulin insensitivity and incidence of the metabolic syndrome (Pitteloud, et al., 2005), whereas the opposite is true in women, for whom hyperandrogenism (e.g in polcystic ovarian syndrome) has been associated with reduced insulin sensitivity and non-insulin dependent diabetes (Nestler & Strauss, 1991). It is also possible that disruptions in oxidative metabolism (discussed further

below) that lead to accumulated metabolites (e.g. pyruvate) can stimulate the cell into glycogen synthesis. Pyruvate can be used to synthesise glycogen in liver and muscle when glucose levels are low (Mahler, 1966). Therefore, if HSA-AR mice experience a rise in intracellular pyruvate glycogenesis may be stimulated which may account for the findings in this study. However, this explanation is unlikely correct since in females disruption of oxidative metabolism occurs without glycogen accumulation. Therefore, it is likely that this finding is a result of normal AR mediated androgen action on glucose homeostasis through an insulin related mechanism.

The process by which differential responses to androgens may develop is not fully understood. One possibility is that perinatal androgen exposure has long lasting organizational effects on gene expression and its regulation. Such a mechanism has been found to mediate expression of arginine vasopressin in the bed nucleus of the stria terminalis (Han & De Vries, 2003). Another possibility is that genetic differences between males and females are involved in androgen regulation of gene transcription. It is also possible that transactivation patterns differ in response to chronic versus acute androgen treatment. Further study is necessary to distinguish between these and other possibilities.

4.5 Fiber Hypertrophy

In this study we observed an increase in fiber CSA in response to AR overexpression coupled with T exposure. The origin of this increase is difficult to determine particularly because androgens and skeletal myopathy both often have similar effects on fiber

morphology. Therefore, the most likely interpretations of fiber enlargement are androgen induced anabolic hypertrophy, myopathy induced compensatory hypertrophy, fiber type transitions, and any combination of these mechanisms.

4.5.1 Anabolic Hypertrophy

That androgens have a dose-dependent hypertrophic effect in muscle fibers is well documented. Administration of androgens to eugonadal young men, hypogonadic HIV patients, and healthy women has been shown to increase myofiber CSA accompanied with gains in strength. The cellular changes that underlie this anabolic hypertrophy have not been fully elucidated but changes in gene transcription and protein synthesis, particularly contractile proteins, are implicated. For instance, mixed muscle protein and MHC synthesis were found to increase with CSA in individuals treated with T. These proteins are incorporated into the sarcomere protein complex/contractile filaments leading to a measurable increase in myofibril width. Enlargement of myofibrils typically occurs at the periphery of the muscle and is a limited means of hypertrophy. Once this limit is reached, progressive myofibrillar splitting occurs near the tendon-junction resulting in new fibrils of shorter widths (Goldspink, 1970).

This complex array of morphological changes accompanying hypertrophy makes it difficult to assess whether the ultrastructural changes observed in HSA-AR mice is due to normal androgen induced mechanisms. It is possible that androgen induced widening of myofibrils ultimately resulted in frequent fibrillar splitting resulting in more numerous fibrils but of narrower widths. This would account for both the reduced mean fibril width as well as the increased CSA. Additionally, since fibril splitting is accompanied by

intermyofibrillar mitogenesis this mechanism may account for the increased mitochondria number observed. Unfortunately, the accuracy of this hypothesis is difficult to determine without knowledge of the average number of fibrils per fiber. In addition to changes in myofibril morphology, increases in mitochondria size and volume, myonuclei number, satellite cell number, and fibril density have all been shown to accompany anabolic fiber hypertrophy (Beck & James, 1978; Kadi, Eriksson, Holmner, & Thornell, 1999; MacDougall, Sale, Elder, & Sutton, 1982). Since HSA-AR mice show several of these features it is likely that androgen mediated hypertrophy occurs in these animals.

4.5.2 Fiber type transformation

Fiber type transitions are known to occur in response to changing functional demands, myopathy, and certain hormones (Mendler, Pinter, Kiricsi, Baka, & Dux, 2008). These changes can include alterations in metabolic activity, protein isoform expression levels, and fiber morphology. Fibers of different types differ in their morphology. In particular the CSA of fast twitch fibers (type II) is typically greater than slow twitch fibers (type I). More nuanced variations in fiber size exist within the fiber subtypes (Scott, et al., 2001). A reduction in the proportion of oxidative fibers and an increase in the proportion of glycolytic fibers as assessed by SDH staining was recognized in this study. However, the accuracy of this conclusion is doubtful as the mitochondrial content of fibers in T exposed Tg was found to be increased by a variety of approaches. This directly contradicts the likelihood that the SDH stain reflects accurately on the metabolic type of the cell. Moreover, fibers of all types were seen to be hypertrophic suggesting a type-independent mechanism of fiber enlargement. Lastly, L78 males showed a significant

(17.2%) reduction in glycolytic cells despite overall hypertrophy. Therefore, it is unlikely that fiber type transitions are principally responsible for the observed hypertrophy.

4.5.3 Compensatory Hypertrophy

When fibers are required to support a greater load they undergo compensatory hypertrophy. This effect has been demonstrated with a variety of paradigms. For example, disabling synergistic hindlimb muscles by surgical severance (tenectomy) leads to a reduction in force diffusion and an increase in load on individual muscles . This leads to whole muscle and myofiber compensatory hypertrophy (Roy & Edgerton, 1995) . Graded load-training regiments in rodents have shown similar results (Goldberg, Etlinger, Goldspink, & Jablecki, 1975). Such fiber hypertrophy is thought to occur through a number of mechanisms independent of androgen action. Firstly, increased strain on whole muscle may lead to greater functional activity as mediated through stretch receptors and reflex pathways (Schiaffino & Margreth, 1969). Secondly, greater passive strain on muscle has been shown to increase metabolic activity and stimulate angiogenesis. Therefore, fiber atrophy leading to increased strain on residual fibers may lead to fiber hypertrophy independent of androgen action. However, this does not preclude contributions of androgens to fiber hypertrophy. In fact, the hypertrophic effect of load is enhanced by androgen treatment (Celotti & Negri Cesi, 1992; Hickson, Kurowski, Galassi, Daniels, & Chatterton, 1985; Taylor, Brooks, & Ryan, 1999). Therefore, it is likely that both increased strain on residual muscle in conjunction with anabolic androgen action is responsible for the observed fiber hypertrophy in HSA-AR mice.

4.5.4 Summary

Both strain induced and androgen induced fiber hypertrophy is thought to be mediated by androgens and AR. Passive and active muscle strain have an enhancing effect on AR protein expression and are known to increase serum T. Likewise, T treatment of muscle leads to an increase in AR mRNA in this tissue. Once activated, AR function includes DNA accretion, satellite cell division and differentiation, and reduced myostatin expression in myotubes (Haddad & Adams, 2006). Myostatin is a secreted protein that limits the number of myofibers formed during embryogenesis and inhibits muscle growth in adulthood (S. J. Lee, 2004). Moreover, androgens can antagonize catabolic pathways such as those mediated by glucocorticoids in order to reduce protein break down (Matlock, Sheffield-Moore, & Urban, 2005). These cellular changes concertedly lead to increased fiber hypertrophy. In this study it is likely that compensatory hypertrphy as a result of increased load on residual fibers in conjunction with augmented androgen signalling lead to the observed hypertrophy. This possibility is all the more likely as only symptomatic subjects (Tg and androgen exposed L78 males and L141 females) demonstrated myofiber hypertrophy.

4.6 Unexpected Shifts in Fiber Type Representation in L78 Males and L141 Females

Although ultrastructural analysis revealed mitochondrial proliferation and distention, histochemical analysis showed a reduction in oxidative fibers and in overall fiber-type distinction. This additionally contradicts the presence of RRF in the present study and the dark NADH staining previously reported (Monks, et al., 2007).

4.6.1 Deficits in Respiratory Chain Complex

NADH Dehydrogenase is the first and largest member of the respiratory chain complexes. It is localized in the inner mitochondrial membrane and removes electrons from NADH transferring them to Cytochrome C Reductase (Complex III) via coenzyme Q10. SDH is the second complex in the electron transport chain and is additionally active in the tricarboxylic acid cycle in oxidizing succinate to fumarate. It too transfers electrons from reduced FAD to Complex III through coenzyme Q10 such that it and Complex I work in parallel to oxidize electron carriers.

The conspicuous absence of intense SDH staining in androgen exposed Tg animals suggests a distinct deficiency in the activity of this enzyme. This possibility becomes all the more likely when considering that distinctions between fiber-types in size and proportion are lost when classified using this stain. Additionally, a loss in SDH activity might explain why Tg males and androgen exposed Tg L141 females are symptomatic while androgen exposed L78 females are asymptomatic. Since L78 females have intact SDH activity as revealed by normal stain intensity they do not appear to suffer from respiratory chain dysfunction. (Vladutiu & Heffner, 2000) report that SDH deficiency typically presents as pale SDH staining coupled with the presence of RRF. They also reported that SDH deficiency can usually be detected histochemically and that pale SDH staining is correlated with reduced enzyme activity.

4.7 Model of AR Overexpression Action

Thus, we have shown that overexpression of AR in skeletal muscle leads to androgen dependent mitochondrial distention and proliferation, male-specific glycogen accumulation, and SDH deficiency. These changes may reflect normal responses to T that are amplified by the presence of excessive AR. They may also reflect cellular compensation strategies to reduced respiratory chain activity. We present a model, summarized in figure 12, to explain the observed phenomena .

4.7.1 Normal but Excessive AR Action is Pathogenic

The simplest and most direct explanation for HSA-AR myopathy is that normal, but excessive, AR function in skeletal muscle is pathogenic. Several of the myopathic features observed are known to occur, albeit more moderately, in response to androgens. Firstly, T levels have previously been correlated with aerobic capacity and the expression of oxidative phosphorylation genes (Pitteloud, et al., 2005). Mitochondrial profile area has been shown to increase with T treatment. Therefore, it is possible that the exaggerated changes in mitochondrial morphology observed in our animals is merely an amplification of normal responses to T mediated by AR. However, since we observed a number of additional pathological features, this is unlikely a complete picture. Excessive AR action may mediate an exaggerated distention of mitochondria leading to compromised aerobic respiration. Mitochondrial distention and proliferation in our animals may therefore reflect a combination of exaggerated androgen-AR mediated changes to mitochondria as well as a response to metabolic deficiency. Moreover, fiber atrophy may occur if the structural integrity of mitochondria or the cellular energy balance is disrupted.

Similarly, glycogen storage is stimulated in response to androgens in males but inhibited in females. Accordingly, overactivity of AR may have contributed to the accumulation of glycogen in males but not female HSA-AR mice. Such an effect may secondarily impact the glycolytic pathway, insulin sensitivity, and oxidative metabolism. This model is summarised graphically in Figure 10.

4.7.2 Relationship of Overexpression to polyQ

Despite similarities between AR overexpression and polyQ AR phenotypes, it remains unclear what may underly this relationship. To address this issue it is imperative to form an appreciation for the molecular function of the polyQ region. A region of repeated glutamines is common to several transcription factors and is thought to play a role in binding to functionally complementary proteins. In the case of AR, the domain aids in the formation of a homodimer and the binding to transcription cofactors. This so-called polar zipper is comprised of antiparallel β-pleated sheets of poly(L-glutamine) bound together by hydrogen bonds between their main and side chain amides (Perutz, Staden, Moens, & De Baere, 1993). This secondary structure is insoluble in water. The polar zipper is akin to the well studied leucine zipper which is a common dimerization domain found in transcription factors (Boysen, Jong, Wilce, King, & Hearn, 2002). It has been suggested that expansion of the glutamine repeat region may cause disease by increasing non-specific affinity between factors containing this domain (Perutz, Johnson, Suzuki, & Finch, 1994). It is plausible that high concentrations of Wt AR may likewise favour excessive or non-specific binding of AR to other proteins. It has also been suggested that the gradual precipitation of proteins bound by polar zippers leads to dysfunction (Perutz,

et al., 1994). In support of this notion, several polyQ disorders present with insoluble aggregates of the expanded protein (Ross, et al., 1999). In particular these aggregates are formed by polyQ regions in isolation of the remainder of the protein (Cooper, et al., 1998). Although distinct, such a mechanism may not be mutually exclusive to non-specific AR binding and both may occur in SBMA. Here we have not searched specifically for aggregates in HSA-AR mice nor were any overtly present. However, non-specific AR binding as a result of excess AR may explain the similarity to polyQ disease in the absence of aggregates.

The glutamine repeat region appears to influence AR association with mitochondria. Longer repeats lead to greater association which is likely based on increased polar zipper formation. The precise target of this association remains unclear but likely involves respiratory chain complexes. This association occurs in the absence of androgens and is not disruptive. However, and most remarkably, in the presence of androgens polyQ AR causes mitochondrial depolarization, an increase in reactive oxygen species, and an increase in caspase 9 activity which then leads to an increase in caspase 3 activity (Ranganathan, et al., 2009). Therefore, a mitochondria mediated apoptotic pathway appears to be triggered when excessive AR is associated with mitochondria and is then activated by androgen. Hence, it is very plausible that a similar mechanism operates in HSA-AR mice. High levels of AR are likely to increase the amount of AR associated with mitochondria. Upon activation by T, this AR may trigger a mitochondrial apoptotic pathway similar to that observed with polyQ AR. And in final support for a relationship between overexpression and glutamine repeat expansion is the finding that in a Tg model

of SCA1, a related polyQ disease, overexpression of Wt Ataxin 1 is sufficient for polyQ-like neurodegeneration (Fernandez-Funez, et al., 2000; Tsuda, et al., 2005).

4.8 Future Directions

4.8.1 Biochemical Assays of Respiratory Chain Complexes

Respiratory chain dysfunction has been implicated in the pathogenesis of a number of neurodegenerative disorders such as Parkinson disease, HD, Friedreich ataxia, hereditary spastic paraplegia, Alzheimer disease and amyotrophic lateral sclerosis (Bonilla, et al., 1999; Heales, et al., 1999; Schapira, 1999). Such dysfunction leads to impaired energy production, increased intracellular calcium, generation of free radicals, opening of the mitochondrial transition pore and activation of the apoptotic cascade (Beal, 1998).

This study comprises only a primary characterization of myopathy in response to AR overexpression in skeletal muscle. A number of cellular and subcellular features have been implicated in pathology but the precise causes and effects of these features remain undetermined. Particularly, although mitochondrial changes have been identified, the precise significance of these changes is unknown. For instance, whether or not the numerous and enlarged mitochondria are metabolically functional has not been assessed. We have identified that at least one of the respiratory chain enzymes is deficient but this does not preclude the involvement of other respiratory chain complexes or co-enzymes. Based on previous evidence (Monks, et al., 2007) we can exclude complex I deficiency, but the activities of other respiratory chain complexes remain unknown. Moreover, a histological determination of enzymatic activity is neither definitive nor quantitative. To

quantitatively determine the condition of the respiratory chain complexes a biochemical assay of Complex I-V must be conducted. Moreover, to determine the involvement of either of the coenzymes Q10 and cytochrome C, combined biochemical assays of Complex I+III/II+III and Complex III+IV should be conducted.

Although mitogenesis may be a response to energy deficiency, it may also be a pathologic effect of transgene AR activation. If the latter case is true then two further possibilities exist: (i) that the mitochondria are hypermetabolic leading to energy deficiency or (ii) that the mitochondria are nonfunctional or dysfunctional. Therefore, apart from a mitochondrial enzyme assay, an investigation of energy state (e.g. ATP content) should be made.

Both outcomes, increased or decreased respiratory complex activity, are plausible. Based on the model proposed here that pathology is a product of normal yet amplified AR activity, mitochondrial complex activity can be expected to be greater in diseased animals. Greater activity might lead to depletion of energy resources. This would be compounded by a simultaneous increase in glycogen synthesis stimulated by AR. Starved of energy, mice may undergo muscular atrophy (Stevenson, Koncarevic, Giresi, Jackman, & Kandarian, 2005). On the other hand, decreased respiratory complex activity would be congruent with present findings of SDH deficiency. Deficiencies in other complexes or their coenzymes would suggest impaired energy production, leading to energy starvation, and muscular atrophy.

4.8.2 Subcellular Localization of AR

Determining the location of AR in Tg myofibers may shed light on the mechanism of AR action in HSA-AR mice. In the absence of T, AR is normally located in the cytosol bound to chaperone heat shock proteins. Ligand binding dissociates AR from its cytosolic complex allowing it to translocate into the nucleus. In SBMA, polyQ AR form insoluble nuclear aggregates in the presence of T. These are thought to disrupt transcriptional activity leading to pathology. Therefore, it is plausible that an accumulation of transgene AR in HSA-AR myonuclei could act through similar mechanisms to result in myopathy.

Recent evidence suggests that AR may have androgen mediated effects independent of the nucleus. Notably, AR may interact directly with the nuclear encoded mitochondrial enzyme cytochrome c oxidase (Beauchemin, et al., 2001). Interestingly, cytochrome c oxidase is a transmembrane protein located in the inner mitochondrial membrane (Wikstrom, 2004). Therefore, in vivo AR interaction with this protein would require the transport of AR across the outer mitochondrial membrane into the inter-membranal space. However, the outer membrane, although relatively permissive, restricts passive diffusion to species approximately 5 kDa or smaller (Waterhouse, Ricci, & Green, 2002). And since full length AR is 110 kDa, its delivery into the intermembranal space must be mediated by selective mitochondrial membrane transport proteins. Moreover, it was found that in AR-cytochrome c oxidase interactions heat shock proteins are facilitative rather than prohibitive (Beauchemin, et al., 2001). Such an elaborate mechanism for the regulation and delivery of AR to respiratory chain complexes would suggest a specific role of AR in mitochondria beyond modulation of nuclear gene transcription.

Subcellular localization of AR may be used to investigate the potential for direct transgene AR action on mitochondria. One possible approach is using immunoelectron microscopy. By labelling AR primary antibody with gold particles and applying them to ultrathin sections, AR can be visualized on a transmission electron microscope. In fact, by using gold particles of different diameters, it is possible to co-localize multiple proteins, such as cytochrome c oxidase, with AR (Renno, 2001). Excessive localization of AR within the inter-membranal space or elsewhere in mitochondria would implicate a direct mechanism of transgene AR action independent of gene transcription. Whether such localization is androgen dependent would also be of interest. It has recently been shown that although polyQ AR localizes to mitochondria in the absence of androgens, it does not result in membrane depolarization or caspase 9 activation unless androgen is present (Ranganathan, et al., 2009). Similarly, in HSA-AR mice, transgene AR may localize to mitochondria in the absence of T, but induce mitochondrial dysfunction only in its presence. The localization of high levels of AR in mitochondria of HSA-AR mice would not be surprising. The presence of steroid receptors, including ERα and ERβ, in mitochondria of mammalian cells has been well documented (Solakidi, Psarra, Nikolaropoulos, & Sekeris, 2005; Yang, et al., 2004).

4.8.3 Apoptotic or Necrotic Pathway

Whether atrophic fibers in HSA-AR mice are lost by an apoptotic or necrotic pathway remains to be determined. Although it had once been thought that differentiated skeletal muscle does not undergo apoptosis, it has now been shown in androgen sensitive muscle that apoptosis does take place (Boissonneault, 2001). Gonadectomized male rats lose a

significant number of myofibers in the androgen responsive levator ani muscle. This loss is accompanied by the appearance of DNA fragments in the sarcoplasm along with the overexpression of the apoptotic marker gene trpm-2/clusterin (Boissonneault, 2001). Moreover, it has already been described above that polyQ AR can trigger mitochondrial apoptotic cascades by activating Bax and caspase 9 and 3 in motoneurons. Whether this occurs in skeletal myofibers has yet to be determined. Considering the cellular and sub-cellular similarities between polyQ AR and AR overexpression phenotypes it is plausible that a similar apoptotic cascade is triggered in HSA-AR mice. Increased mitochondrial fission and membrane permeabilization may release pro-apoptotic factors into the sarcoplasm. These may result from excessive mitochondrial growth as was observed in this study. In fact, several ruptured mitochondria were observed in Tg males but their prevalence was not quantified. Therefore, markers of apoptosis and necrosis should be investigated in HSA-AR muscle. A definitive assay would examine factors such as caspase 3, cytochrome c, and Bax. A finding that these proteins are elevated in Tg animals would support an apoptotic mechanism of fiber loss and motor deficit. However, the possibility remains that necrotic pathways mediate adult fiber death. This possibility is supported by the fact that necrotic cascades typically involve enlargement of mitochondria. An assessment of serum myoglobin levels would indicate whether or not necrosis is taking place (Almog, Isakov, Ayalon, Burke, & Shapira, 1987). Such a finding would be interesting in that it would imply damage to myofibers secondary to transgene AR activation.

Yet another possible finding is that neither of these pathways is active. Since female T treated Tg mice do not seem to undergo myofiber loss (Monks, et al., 2007) it may be that myofiber loss in males is due to impaired myogenesis and not muscular atrophy. Such a developmental effect may be observed in males but not females because the former are exposed to high levels of T throughout prenatal and postnatal life whereas the latter are exposed to high T for a single week during experimentation. This mechanism would be supported by the absence of apoptotic and necrotic markers in Tg males. To determine whether myogenesis is impaired during development, embryos should be removed by c-section at various points of development and a histological assay of myofiber number may be conducted. A consistently reduced myofiber number would suggest dysfunctional myogenesis. This in turn would suggest that transgene AR interferes with normal molecular processes involved in myogenesis during development. It would then be of great interest to identify the transcriptional and cellular targets of AR that leads to this dysfunction. In females, low levels of prenatal T may permit the normal development of skeletal muscle. T administration during adulthood may result in a distinct pathway leading to myofiber dysfunction and motor deficits. The existence of a distinct mechanisms active in adulthood is supported by the finding that Tg males recover considerable motor strength following gonadectomy (Monks, et al., 2007). However, this does not preclude the existence of a developmental effect of transgene AR on muscle development in general and myogenesis in particular.

4.8.4 Spatiotemporally Selective Overexpression of AR

Our lab has developed a Tg mouse line that has the potential to conditionally activate or inactivate in myofibers any appropriately engineered gene (Rao & Monks, 2009). In

particular, using these mice AR can be overexpressed selectively in skeletal muscle at any stage of development or adulthood. The newly developed line relies on two transgenes. The first utilizes the HSA promoter to drive the skeletal muscle-specific expression of reverse tetracycline-controlled transcriptional activator (rtTA). This protein is an artificial transcription factor that binds to promoter sequences known as tetracycline response elements (TRE) and promotes the expression of adjacent genes. However, rtTA only binds TRE in the presence of tetracycline allowing for specific regulation of TRE-associated gene expression using tetracycline. The second transgene consists of TRE promoter linked to Cre recombinase. Combined, these transgenes allow for selective tetracycline induced Cre expression in skeletal muscle. Cre catalyzes the recombination of DNA sequences between loxP sites and can be used to excise any such pieces of floxed DNA. Therefore, by crossing this new line with an appropriately floxed AR Tg line one could create Tg mice that express or overexpress AR selectively in skeletal muscle under the control of tetracycline. Although a similar Tg line has been created for the conditional expression of genes in myofibers, its reliance on tamoxifen, a poorly understood ER ligand, makes it unsuitable for studies of androgen and AR function (Schuler, Ali, Metzger, Chambon, & Metzger, 2005).

This new line may be used to determine the particular effects of AR overexpression at one point in development without being confounded by early transgene effects. In particular, the lethality of the HSA-AR transgene in males can be overcome if gene activation is delayed until adulthood. Similarly by activating AR expression during different points in the animal's life, the temporal properties of AR action can be explored.

For instance, whether the reduced number of myofibers observed in Tg males is due to muscular atrophy or impaired myogenesis during development can be detetrmined by activating the transgene in adulthood and measuring myofiber number. If transgene activation in adulthood does not affect myofiber number, it may be inferred that myofiber loss is due to disruption of myogenesis during development. However, if myofiber loss is observed, this would suggest that muscular atrophy does occur in adult males. This would further suggest that distinct mechanisms of pathology operate in males and females, because adult Tg females do not show myofiber loss when treated with T.

Adapting this novel Tg line to selectively express genes in other tissues is quite feasible, requiring mostly the replacement of the HSA promoter with another tissue-specific promoter.

In sum, future work must be directed at understanding cellular and molecular mediators of androgen induced muscular atrophy. This will shed light on the subcellular interactions of AR within myofibers and may be helpful in understanding related human disease.

4.9 Conclusions

This study identified the cellular and subcellular changes that underly myopathy in HSA-AR mice. It has specifically shown that overexpression of AR in HSA-AR mouse myofibers leads to androgen dependent changes in mitochondrial content and function, glycogen storage, and myofibril width. Particularly, androgen activation of excessive AR leads to enlargement and increase in number of mitochondria as well as an increase in glycogen content (in males) and a reduction in myofibril width. Pathology also involves a deficiency in Complex II activity and does not appear to involve aberrations in lipid storage. It is likely that normal but exaggerated AR function in conjunction with cellular responses to metabolic insufficiency induce the changes observed. This study has additionally affirmed that sexual differences exist in AR-mediated effects of T. The male-specific accumulation of glycogen highlights the importance of sex differences in androgen action. That the androgen system is a critical regulator of a variety of cellular activities in muscle has been well established. Therefore, a more comprehensive understanding of AR action in healthy and pathologic muscle is critical for targeted therapeutic intervention.

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Tables and Figures

Table 1: Summary of subject physical characteristics in experiments 1, 2, and 3. Physical characteristic data was unavailable for experiment 4. Tg males from the 78 line were smaller than their Wt littermates and their AT muscles weighed significantly less. Seminal vesicle weight was used as a relative estimate of serum T levels and was not found to vary by genotype. Females from the 78 line did not differ significantly in any of the physical characteristics whereas 141 females had significantly reduced body weights relative to Wt-VEH control of the same line. (Independent samples t-tests used for male comparisons and ANOVA with Tukey post-hoc for female comparisons. Significance set at 0.05, a and * refer to $p < 0.05$)

Experiment	Line-Sex	Genotype	Treatment	Mean Age (days)	Mean Body Weight (g)	Mean AT Weight (g)	Mean Seminal Vesicle Weight (g)
1	78-M	Wt	none	146.4±1.0	28.8±2.1	0.076 ± 0.007	0.31±0.02
	78-M	Tg	none	145.6±1.0	24.6±1.2	0.057 ± 0.003*	0.29±0.02
2	78-F	Wt	VEH	115.3±5.7	22.5±1.1	0.074±0.002	NA
	78-F	Wt	T	130.1±15.5	22.5±0.4	0.073±0.003	NA
	78-F	Tg	VEH	119.9±15.0	25.0±0.9	0.079±0.003	NA
	78-F	Tg	T	166.9±11.8	25.5±0.6	0.077±0.004	NA
3	141-F	Wt	VEH	146.0±24.8	23.7±1.8[a]	0.062±0.006	NA
	141-F	Wt	T	142.5±27.3	22.5±0.6	0.077±0.002	NA
	141-F	Tg	VEH	150.5±20.2	23.4±0.2	0.074±0.003	NA
	141-F	Tg	T	134.8±28.1	19.3±0.7[a]	0.074±0.003	NA

Table 2: Summary of ultrastructural changes in experiments 1, 2, 3 and 4. Tg males of both lines evidenced augmented mitochondrial content. T but not VEH treated Tg females evidenced similar changes. All experimental but not control animals evidenced narrowed sarcomeres. Increased granular glycogen content occurred only in Tg males and not females regardless of treatment. '+' signifies a significant ($p<0.05$) change in measure relative to the listed control; '-' signifies no change relative to control.

Tg Animals	Enlarged Mitochondria	More Numerous Mitochondria	Increased Mitochondrial Volume Density	Narrowed Myofibrils	Increased Glycogen content
78M	+	-	+	+	+
141M	-	+	+	+	+
VEH + 78F	-	-	-	-	-
T + 78F	-	+	+	+	-
VEH + 141F	-	-	-	-	-
T + 141F	+	+	+	+	-

Table 3: Summary of histological changes in Experiments 1, 2, and 3. Histological analysis was not conducted on subjects of experiment 4 (L141 males). Unexpected loss of oxidative fibers was observed in Tg males and T but not VEH treated L141 females. Fiber hypertrophy was evident in all T treated animals as well as unmanipulated males overexpressing AR. RRF and intense PAS staining were also evident in L78 males but not Tg-T females. '+' signifies a significant ($p<0.05$) change in measure relative to the listed control; '-' signifies no change relative to control.

Tg Animals	Loss of Oxidative Fibers	Fiber Hypertrophy	RRF	Increased PAS Staining
78M	+	+	+	+
141M	N/A	N/A	N/A	N/A
VEH + 78F	-	-	-	-
T + 78F	-	+	-	-
VEH + 141F	-	-	-	-
T + 141F	+	+	+	-

Figure 1: Ultrastructural myofiber features of Tg and Wt L78 and L141 males. Males that overexpress AR in skeletal muscle (Tg) show enlarged and more numerous interfibrillar mitochondria (M). Additionally, these animals show increased interfibrillar granular glycogen (G). Sarcomeres appear narrowed in Tg animals.

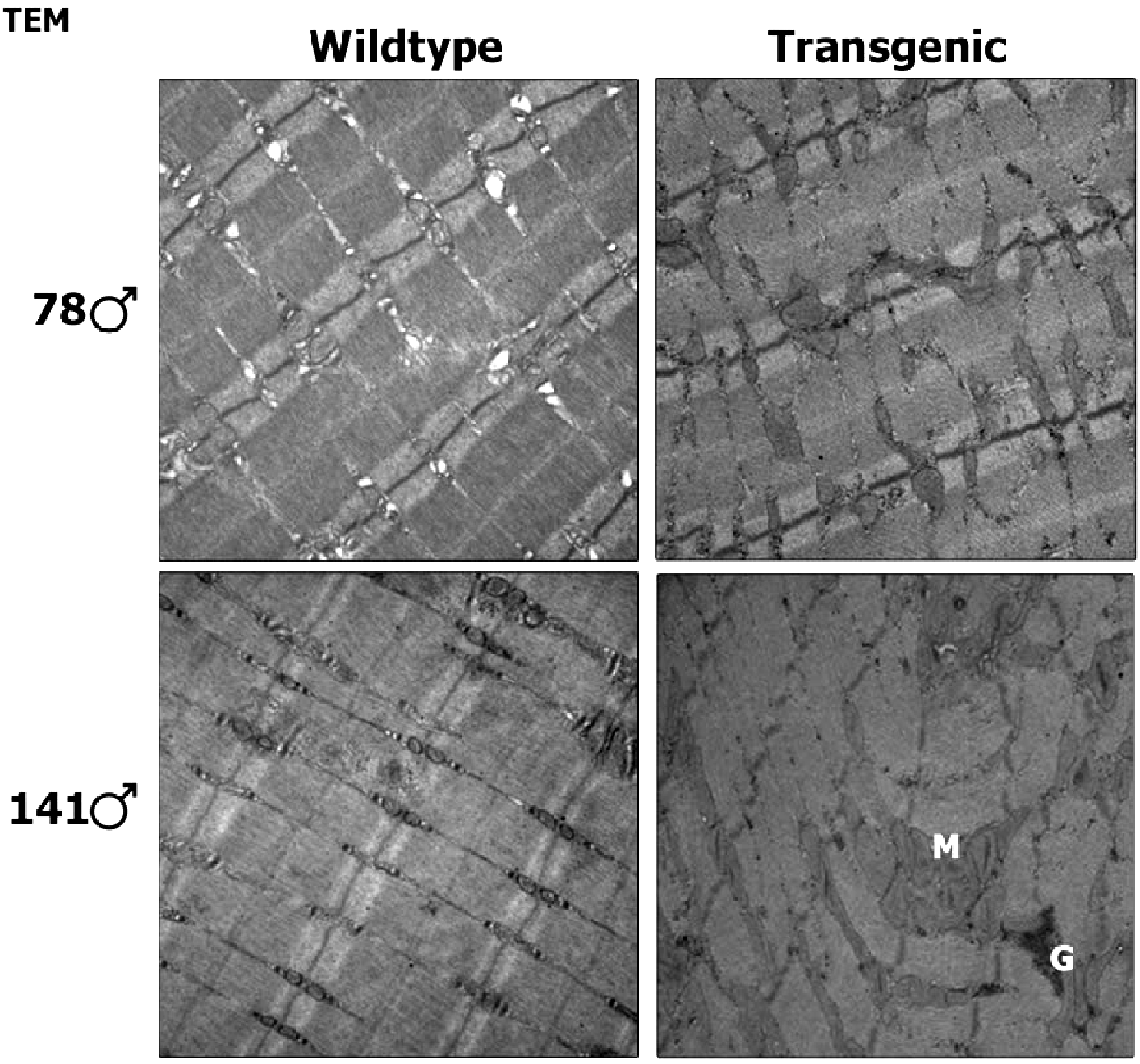

Figure 2: Mitochondrial volume density (A), size (B), and number (C) in Tg and Wt L78 and L141 males. Increases in mitochondrial size and number in males that overexpress AR in skeletal myofibers is reflected as augmented mitochondrial volume density (Vv). Myofibril width is narrower in transgenic males possibly as a result of mitochondrial enlargement. (* $p < 0.05$; ** $p < 0.01$; † number of mitochondria per 8.92 μm^2)

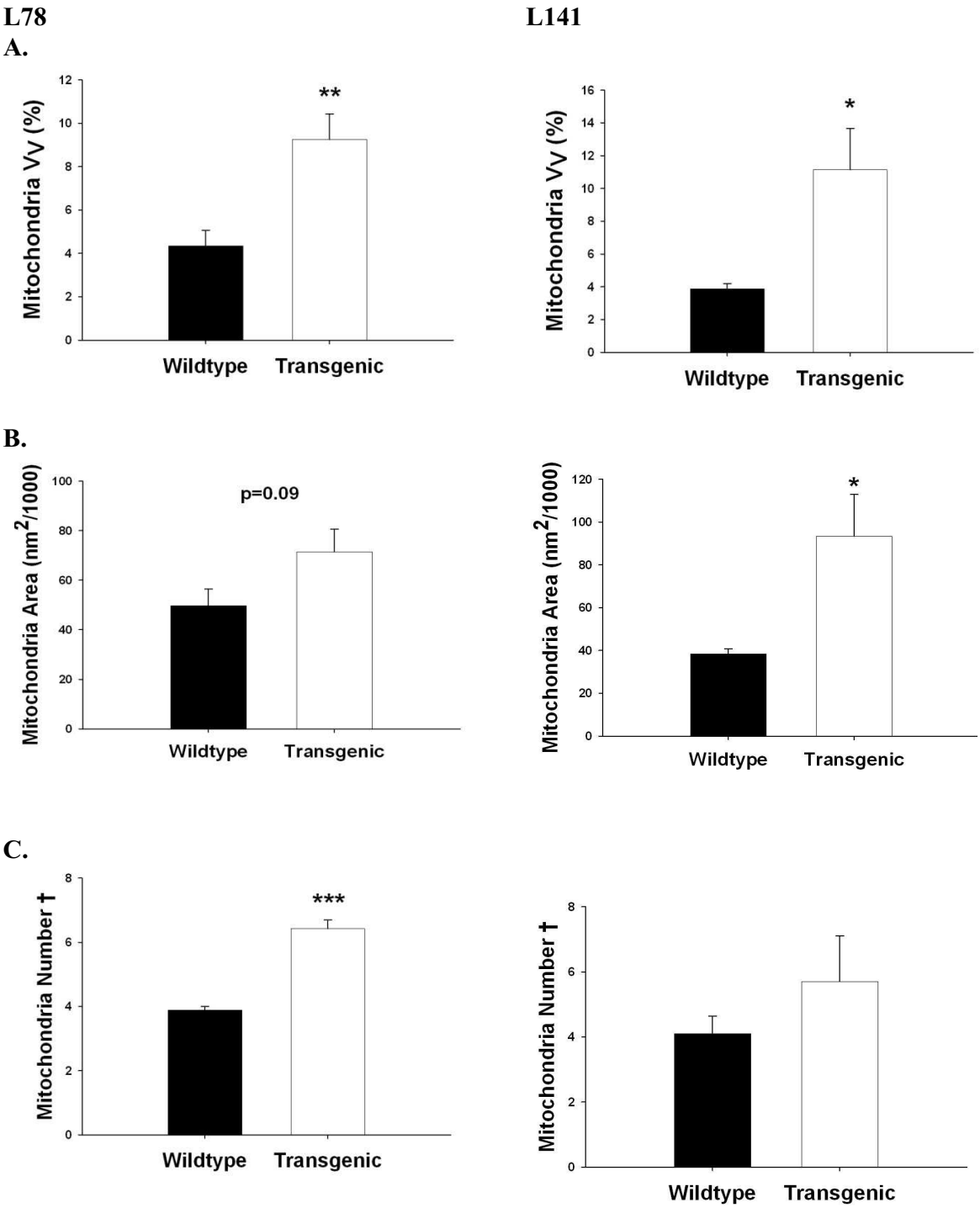

Figure 3: Myofibril width (A) and granular glycogen content (B) in Tg and Wt males. Fibrils are significantly narrower in Tg males of both lines relative to Wt littermates. Note that the mean fibril width in Tg L141 males is narrower than that of the L78 line. The proportion of micrographs presenting with granular glycogen is greater in Tg relative to Wt. Once more L141 males appear to have greater glycogen content relative to L78. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

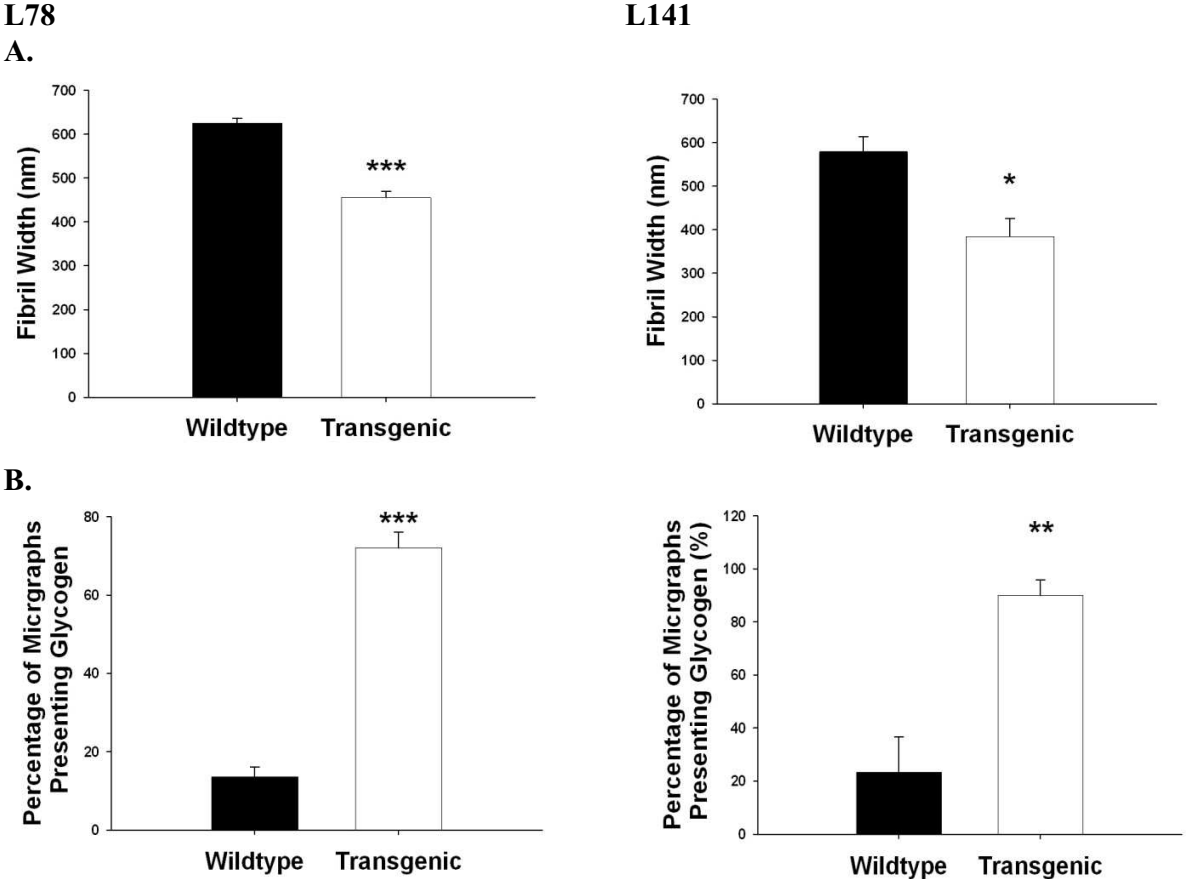

Figure 4: Ultrastructural features of EDL muscle in mice overexpressing AR and their Wt littermates. T treatment is necessary to induce changes in mitochondrial size and number in Tg animals. Wt and vehicle controls are largely unaffected by their treatment.

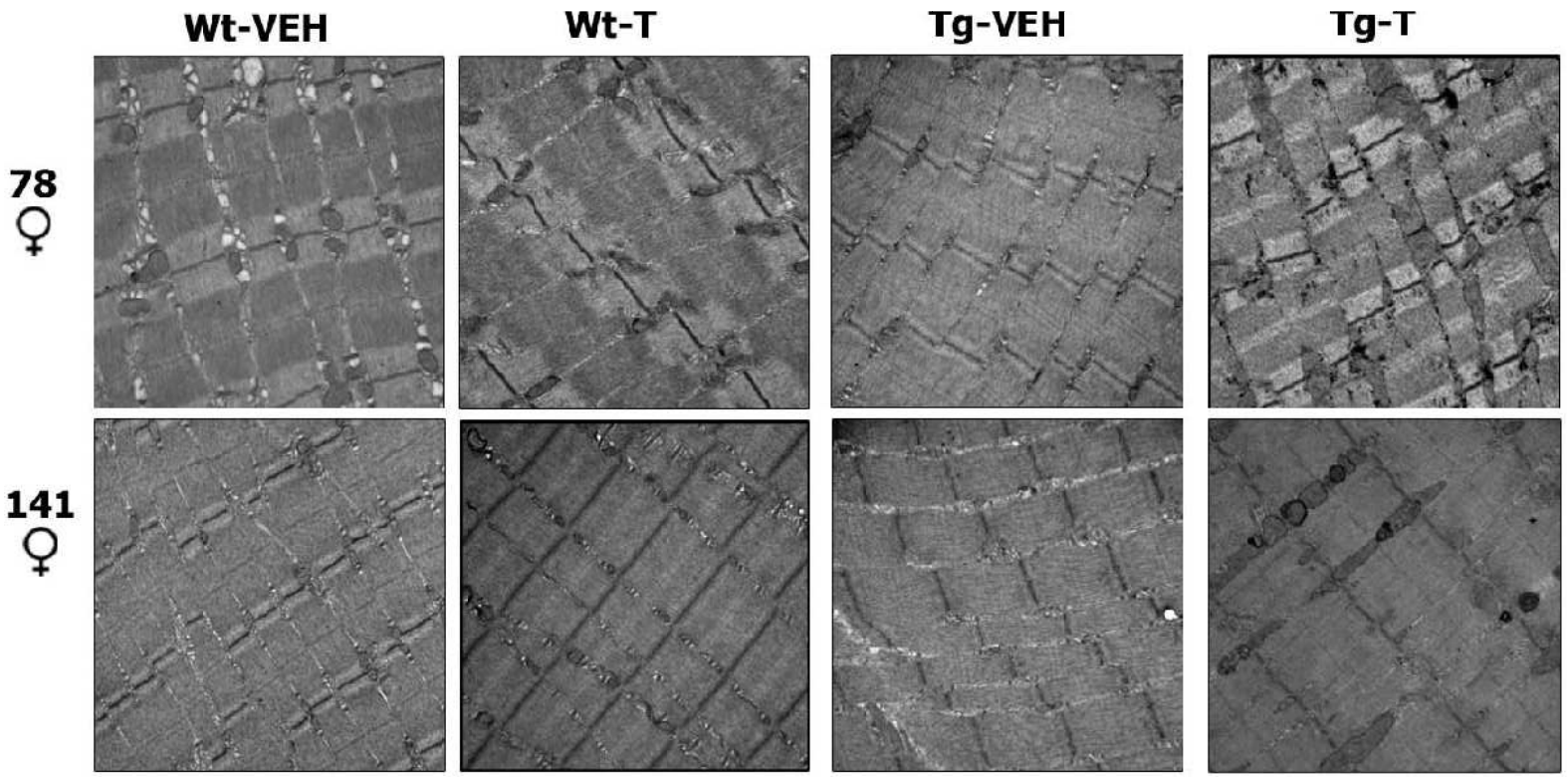

Figure 5: Mitochondrial volume density (A), size (B), and number (C) in Tg and Wt L78 and L141 females. An increase in the number and size of mitochondria in Tg animals treated with T is reflected as greater mitochondrial volume density (Vv). Myofibril width is narrower in Tg-T possibly due to enlargement of mitochondria. (* $p < 0.05$; ** $p < 0.01$; † number of mitochondria per 8.92 μm^2).

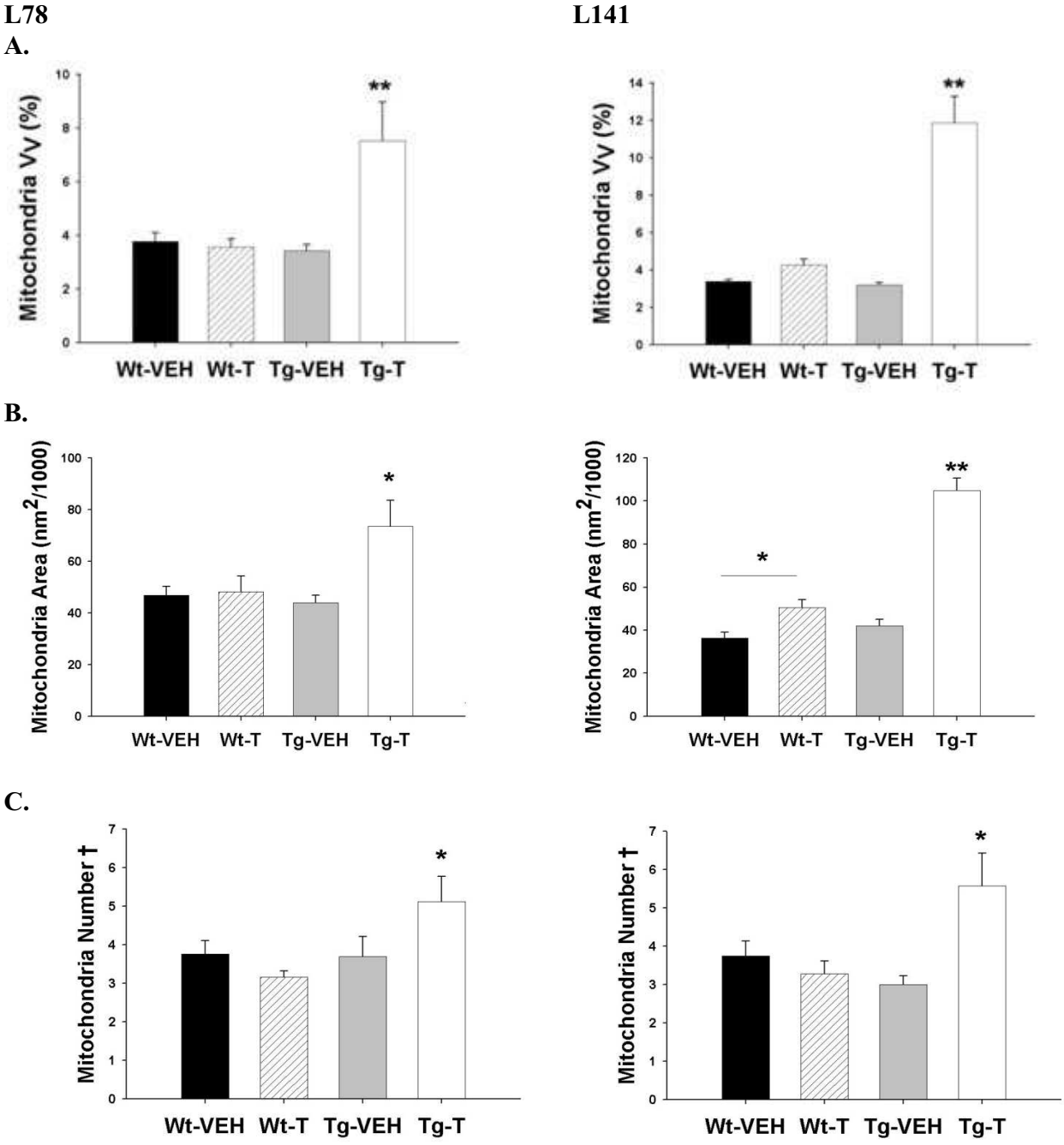

Figure 6: Myofibril width (A) and granular glycogen (B) content in L78 and L141 females. Fibrils are significantly narrowed in Tg-T females of both lines relative to genotype and treatment controls. Note that fibrils in Tg-T L141 females are narrower than those of the L78 line. The presence of granular glycogen was inconsistent in females of both lines. None of the groups differed significantly from one another. (* $p < 0.05$).

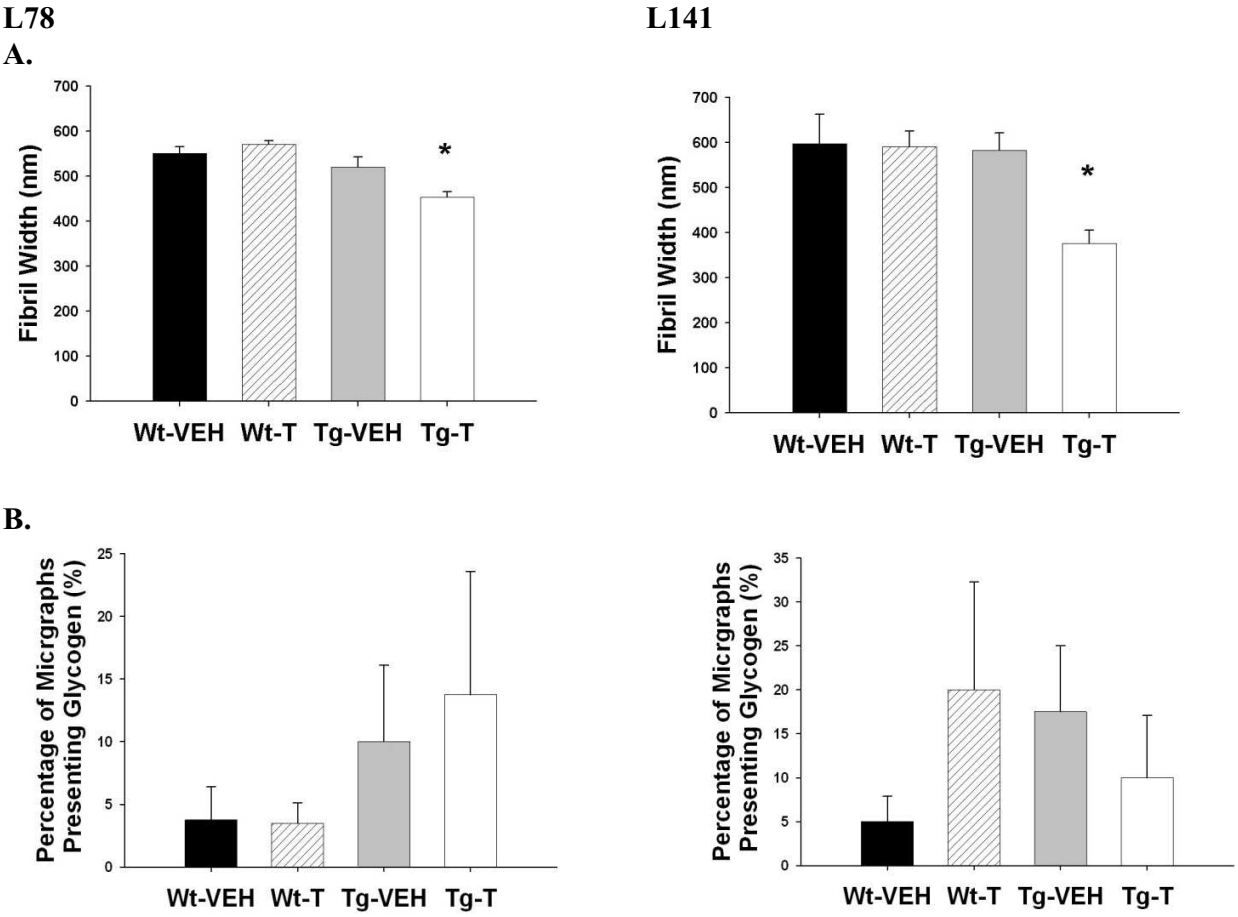

Figure 7: Periodic Acid Schiff (PAS) and SDH staining of L78 males. Mice that overexpress AR in skeletal muscle (Tg) demonstrate accumulation of glycogen in fibers in addition to fiber hypertrophy. There is additionally a reduction in the overall intensity of SDH stain in Tg and a loss of oxidative fibers. Amylase digested negative control is included for comparison.

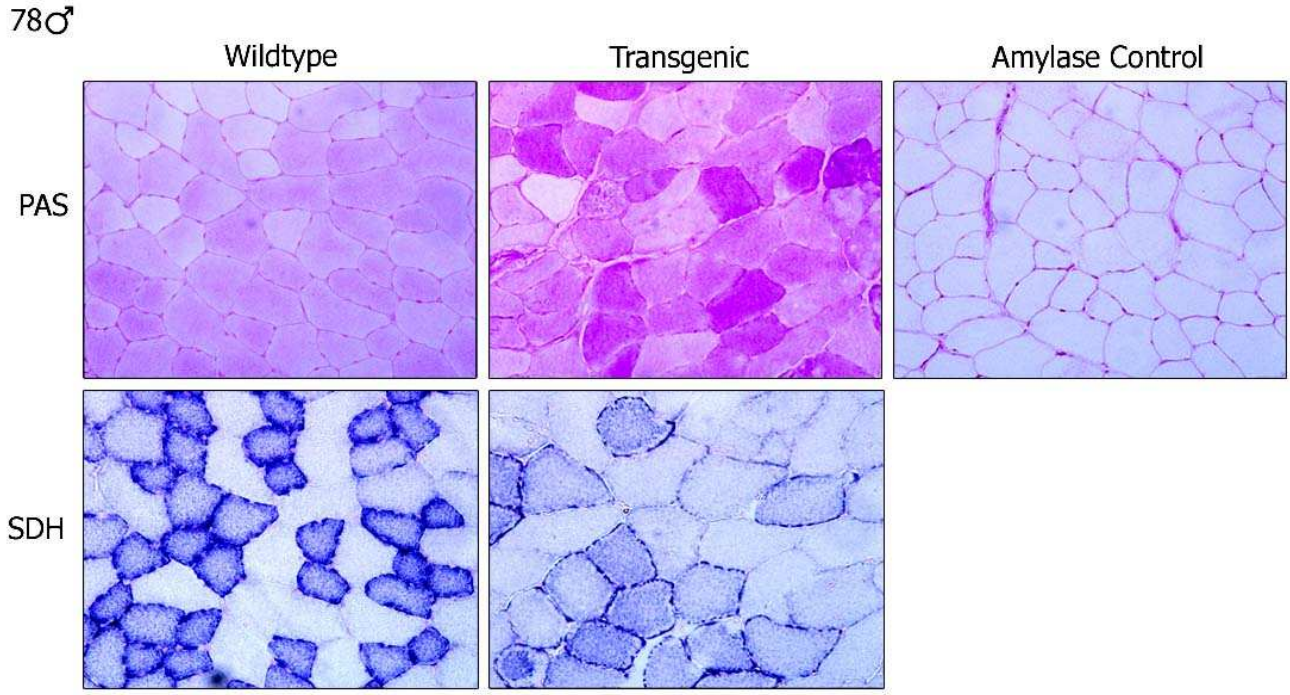

Figure 8: Fiber type proportions (A), calculated CSA (B), and PAS stain optical densities (C) in L78 males. Tg males lose distinctions between fiber types with respect to proportion and size. Whereas Wt have a high proportion of FO and lower proportions of FG and FOG, Tg males have approximately equal proportions of each fiber type. A similar loss of fiber CSA distinction by type is observed. Glycogen content in Tg males is augmented in all fiber types as evidenced by more intense PAS staining. (* $p < 0.05$).

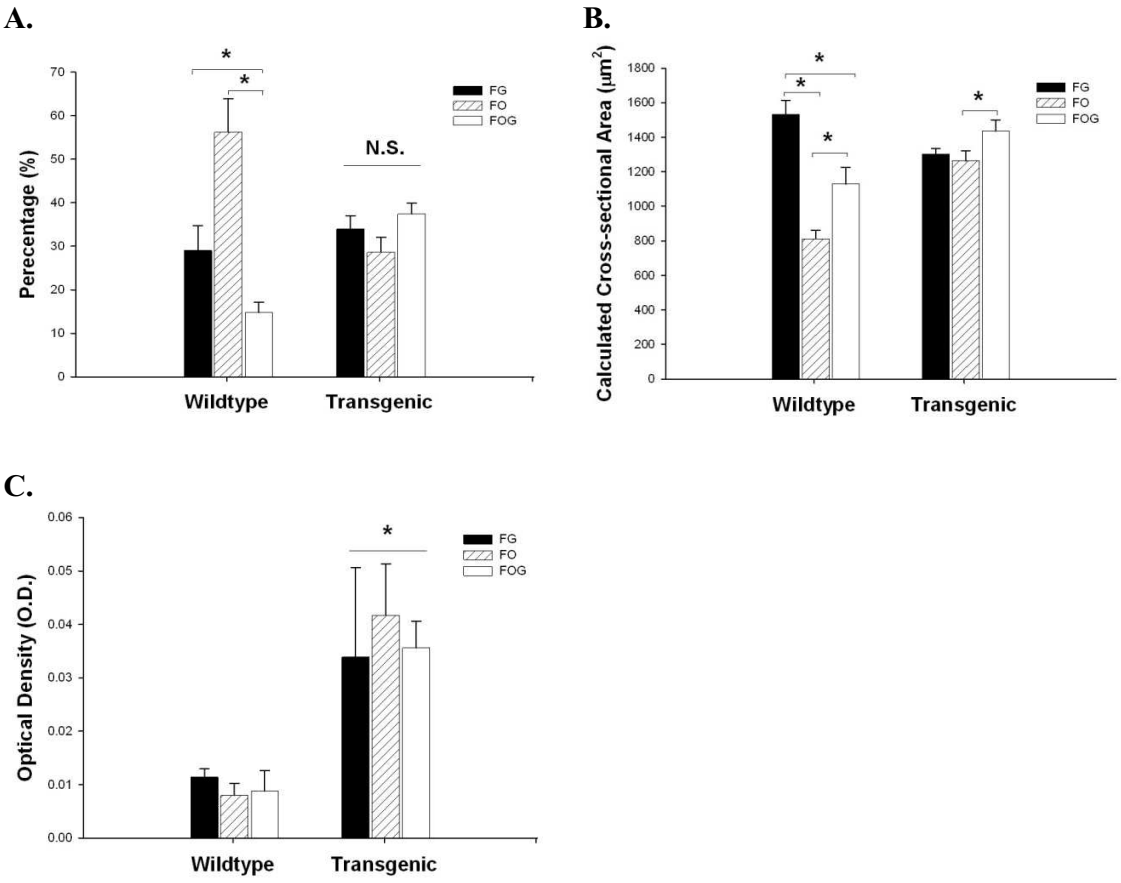

Figure 9: SDH Staining in L78 and L141 females. A reduction in SDH stain intensity is noticeable with T treatment in Tg animals. This occurs only in mice that overexpress AR maximally (L141) relative to those that overexpress it to a lesser extent (L78 Tg).

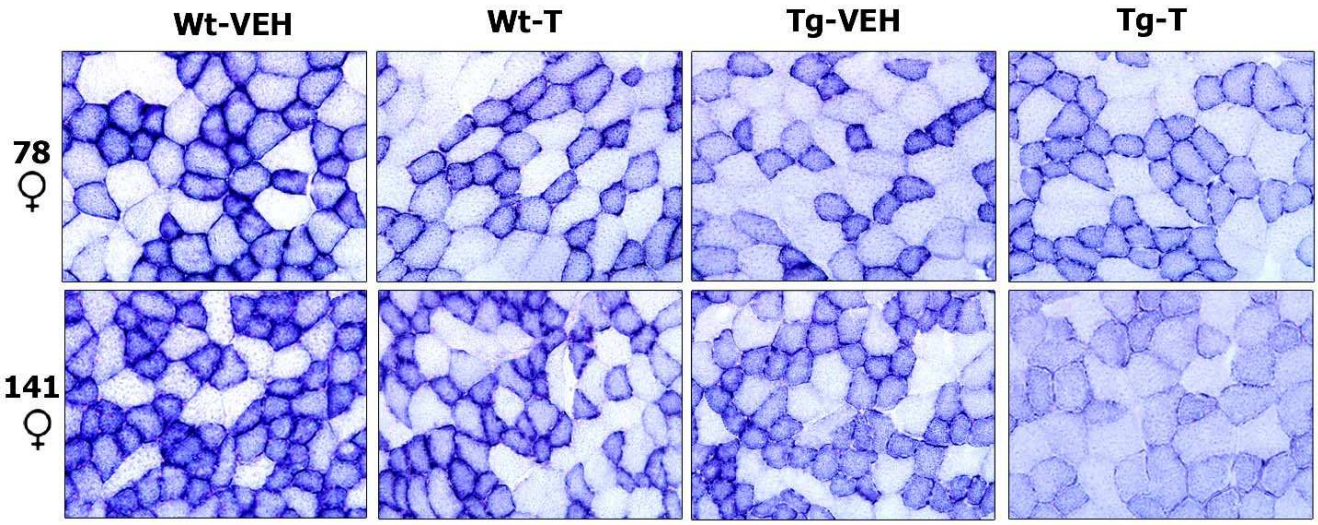

Figure 10: Fiber type proportions (A), calculated CSA (B), and PAS stain optical densities (C) in L78 and L141 females. Fiber type proportions ad CSA are unaffected by transgene expression or androgen treatment in L78 females. These animals maintain distinctions between FO and FG fibers. L141 females however show a reduction in the proportion of FO fibers such that they are approximately equal to that of FG fibers. Hypertrophy in L141 females is limited to FO fibers in the Tg-T group. Although fibers of different types appear to differ marginally in their glycogen content no significant differences between groups were found in either line. (* $p < 0.05$).

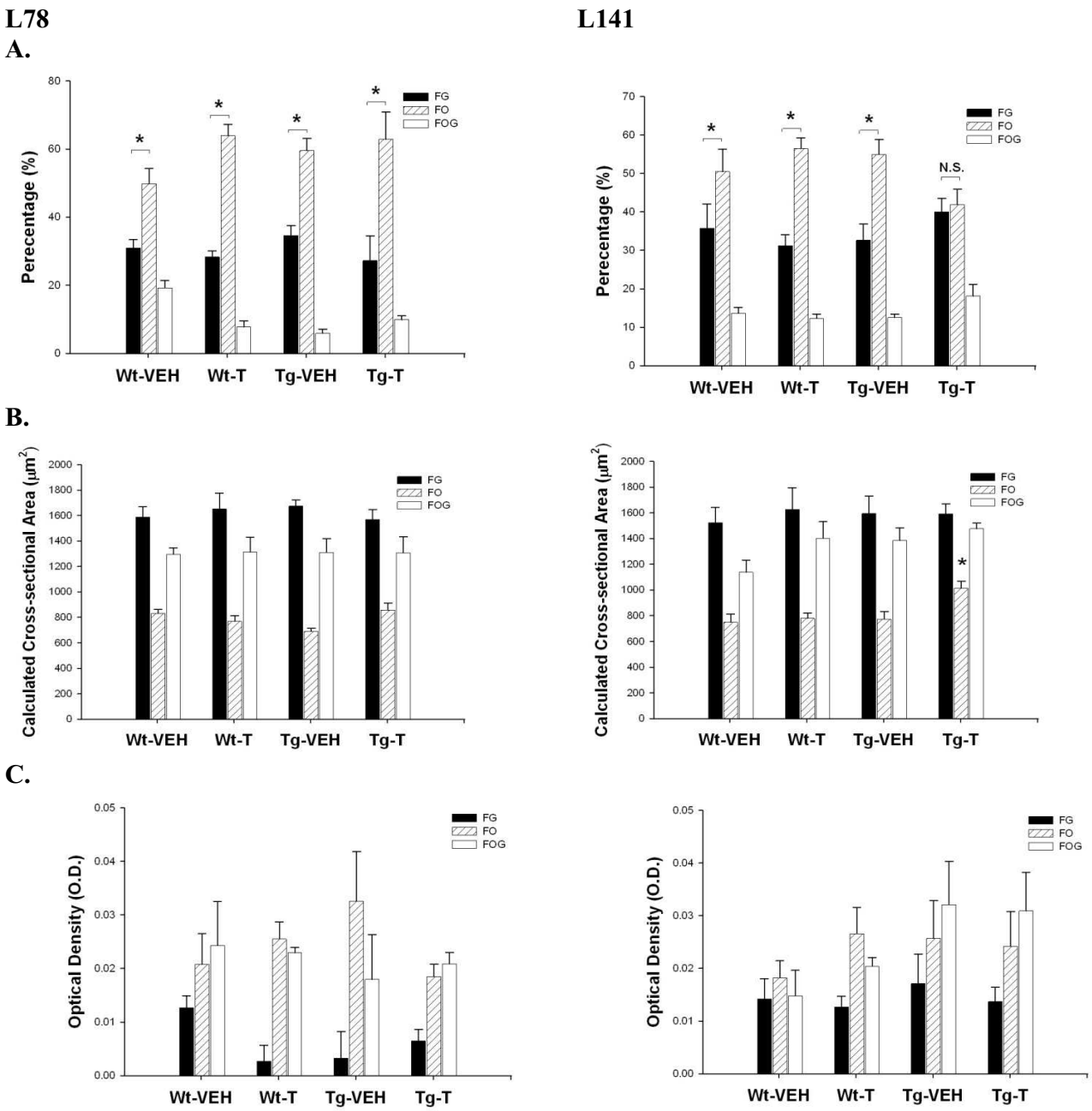

Figure 11: Gomori Trichrome (A), Sudan Black B (B), and MHC II (C) staining in L78 males. The top panel shows Wt (left) and Tg (right) male AT muscle stained with Gomori Trichrome stain. RRF are evident in Tg but not Wt males. This micrograph additionally reveals fiber hypertrophy in Tg. The middle panel shows Wt (left) and Tg (right) AT stained with Sudan Black to examine lipid storage. Lipid storage appears unaffected by transgene expression. The bottom panel shows representative AT (right) and Soleus (Sol; left) muscle sections stained by DAB reaction using My32 primary antibody against MHCII. The antibody reveals both fast (dark) and slow (white) fibers in the largely slow soleus but does not distinguish any slow fibers in the AT.

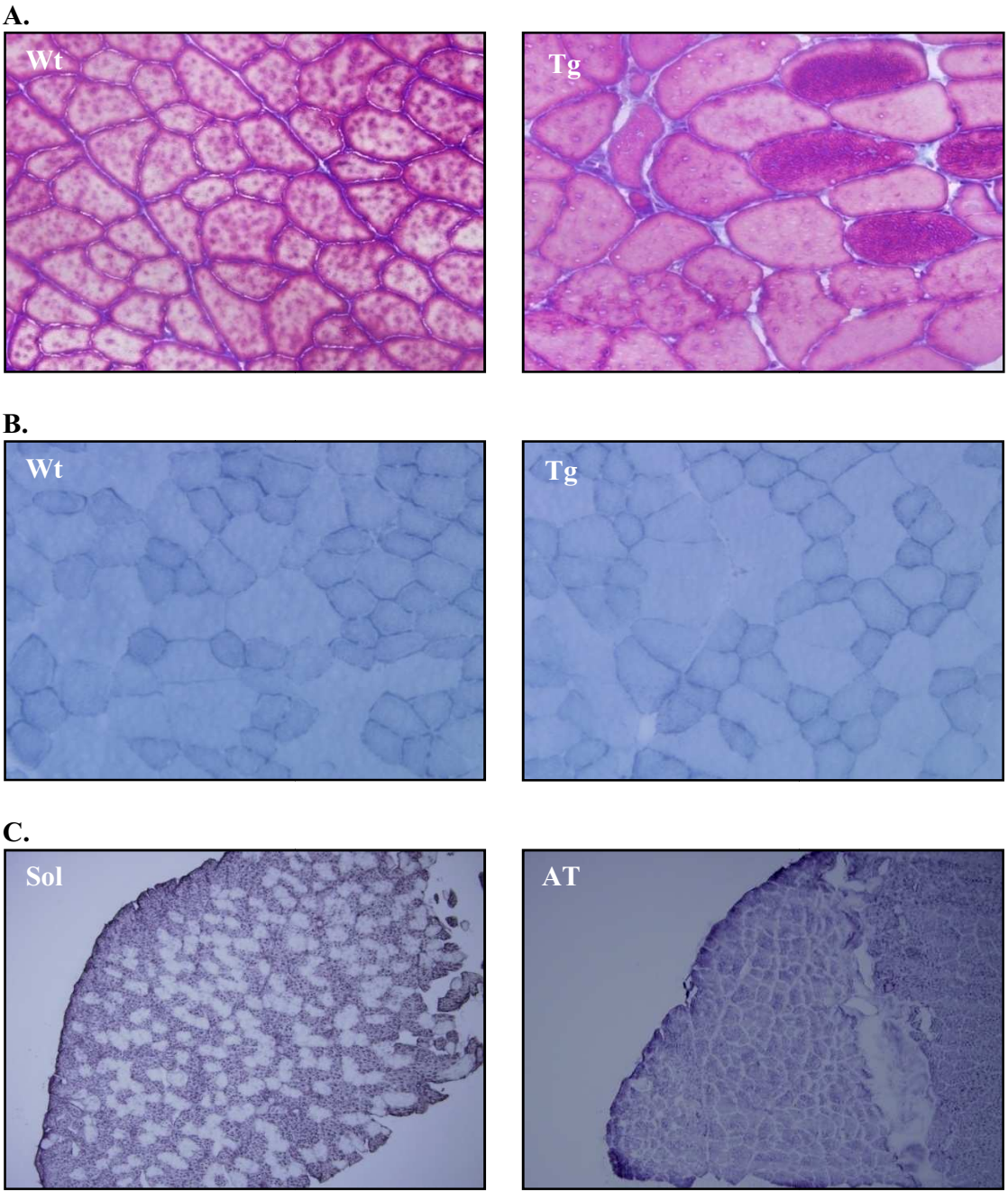

Figure 12: A model of pathology in Tg HSA-AR mice induced by amplified androgen signaling. Blue arrows indicate normal response to androgen, red arrows indicate amplified response associated with AR overexpression. Normally, AR and T regulate glucose and oxidative metabolism partly by affecting glycogen synthesis and mitochondrial morphology. Androgens have dimorphic effects on glycogen synthesis in males and females. Overexpression of AR leads to an amplified androgen signal marked by excessive glycogen synthesis in males and reduced synthesis in females. Mitochondria number and size are likewise augmented excessively in response to AR overexpression. These changes in mitochondrial and glycogen content may negatively impact energy balance leading to apoptosis. Respiratory chain deficiency may accompany changes in mitochondrial morphology further aggravating energy imbalance.