THE ROLE OF MUSCLE-DERIVED NEUROTROPHIC FACTORS IN SPINAL BULBAR MUSCULAR ATROPHY

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ABSTRACT

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Muscle-derived neurotrophic factors are critical to the survival and maintenance of a healthy neuromuscular system and their expression is often perturbed in neuromuscular disease, such as in spinal bulbar muscular atrophy (SBMA). SBMA is a disease that occurs only in men, is androgen-dependent, and is characterized by the progressive weakness and atrophy of muscles that are innervated by lower motoneurons of the brain stem and spinal cord. Moreover, a polyglutamine expansion mutation in the *androgen receptor* (AR) gene is linked to SBMA. CHAPTER 1 provides a general introduction on SBMA pathophysiology, and describes the various implicated roles of neurotrophic factors in health and disease, with a particular emphasis on the role of brain-derived neurotrophic factor in the neuromuscular system. In this dissertation, I examine the status and potential contribution of muscle-derived brain-derived neurotrophic factor in SBMA pathogenesis using two mouse models of this disease. I conclude that supplementing diseased muscle with exogenous BDNF may offer therapeutic benefit for treating symptoms of SBMA.

In CHAPTER 2, I describe my discovery that skeletal muscle from diseased SBMA mice were deficient in BDNF. I found this deficit in two different mouse models of SBMA (one that overexpresses wild-type AR in a muscle-specific manner as well as one that globally expresses a polyglutamine-expanded AR). I also found comparable deficits in BDNF in both fast and slow muscles. To explore whether the deficit in muscle BDNF is relevant to SBMA pathology, I asked whether it was androgen-dependent like the disease. Indeed, BDNF mRNA levels were reduced in the presence of androgens, correlating with motor dysfunction, and when androgens were removed, both BDNF expression and motor function were restored to normal. Moreover, I found

that BDNF levels were reduced *prior* to the loss of motor function, thus indicating that impaired BDNF expression in skeletal muscles is an early and possibly precipitating event in the emergence of motor dysfunction in SBMA.

In CHAPTER 3, I sought to examine whether relieving the deficit in muscle BDNF would ameliorate disease symptoms, given the strong correlation found in CHAPTER 2, as well as the substantial evidence linking BDNF to the maintenance of proper neuromuscular function. To do so, I used a transgenic Cre/loxP approach to specifically overexpress BDNF in muscle cells of diseased SBMA mice. I found that overexpression of muscle BDNF slowed disease progression after its onset, slowing the normal rate of decline in hang performance. Moreover, this improvement was associated with an improvement in the expression of genes relevant to muscle contraction in slow-twitch muscle, but not fast.

CHAPTER 4 provides an overview of my findings and describes remaining questions and future work to be done. The approach to delineate which disease mechanisms are improved by muscle BDNF to slow disease progression will be discussed in CHAPTER 4. Candidate mechanisms include improved neuromuscular transmission and muscle contractile force tension in slow-twitch muscles.

This dissertation is dedicated to
my parents for bringing me here.

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PREFACE

At the time of writing my dissertation, CHAPTER 2 has been published in full in *Experimental Neurology.* CHAPTER 3 will be submitted for publication following the completion of several experiments proposed in CHAPTER 4. CHAPTER 1 will be published as a review.

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KEY TO ABBREVIATIONS

SBMA .....spinal bulbar muscular atrophy

AR.....androgen receptor

BDNF .....brain-derived neurotrophic factor

CK.....creatine kinase

IGF-1 .....insulin-like growth factor 1

ASO .....antisense oligonucleotide

N/C .....amino and carboxyl

ARE .....androgen response element

ER.....endoplasmic reticulum

NADH .....nicotinamide adenine dinucleotide

JNK.....cJun N-terminal kinase

EDL.....extensor digitorum longus

LA .....levator ani

RyR.....ryanodine receptor

SERCA .....sarco/endoplasmic reticulum Ca^{2+}-ATPase

NMJ .....neuromuscular junction

CREB.....cAMP response element-binding protein

RMP.....resting membrane potential

AChR .....acetylcholine receptor

DRG.....dorsal root ganglion

NGF .....nerve growth factor

NT-3.....neurotrophin-3

NT-4.....neurotrophin-4

GDNF .....glial cell line-derived neurotrophic factor

Trk .....tropomyosin related kinase receptor

Tg .....transgenic

PLC-γ.....phospholipase C-γ

PI3K.....phosphatidylinositol 3-kinase

Erk .....extracellular signal-regulated kinase

IP_3.....inositol tris-phosphate

DAG.....diacylglycerol

PKC .....protein kinase C

mTOR.....mammalian target of rapamycin

MAPK .....mitogen-activated protein kinase

TNF.....tumor necrosis factor

NF-κB .....nuclear factor kappa-light-chain-enhancer of activated B cells

ALS.....amyotrophic lateral sclerosis

VEGF.....vascular endothelial growth factor

CNTF .....ciliary neurotrophic factor

ACh.....acetylcholine

DHPR .....dihydropyridine receptor

SR.....sarcoplasmic reticulum

EPP .....endplate potential

mEPP .....miniature endplate potential

Pr.....release probability

RRP.....readily releasable pool

qRT-PCR .....quantitative reverse-transcription polymerase chain reaction

TA.....tibialis anterior

PCR.....polymerase chain reaction

SEM.....standard error of the mean

CHAPTER 1: Introduction

I. Overview of SBMA

i. Spinal bulbar muscular atrophy: History and clinical presentation

SBMA is an X-linked, slowly progressive neuromuscular disease that affects 1-2/100,000 men (Katsuno et al., 2012). The first written account of SBMA dates back to 1897 by Kawahara, and was further described in 1968 by Kennedy and colleagues. Since then, SBMA patients have been described from all over the world, with no known racial biases (Finsterer, 2010). SBMA is also recognized by over 200 different names, including spinobulbar muscular atrophy, Kennedy's disease, X-linked spinal and bulbar muscular atrophy, and bulbospinal muscular atrophy (Arvin, 2013). A loss of lower motoneurons was thought for a long time to cause dysfunction in SBMA, as this is a common finding in autopsy studies, and led to the description of SBMA as a "motoneuron disease". New evidence from animal models, and a reassessment of human findings, such as muscle histopathology, has put a new focus on SBMA as a myogenic disease, with motoneuron loss being a late event (Jordan and Lieberman, 2008; discussed below). SBMA is an androgen-dependent disease (also described below), although partial androgen *insensitivity* is common in men, with incidences of gynecomastia, infertility, and testicular atrophy (Katsuno et al., 2012).

Owing to the slowly progressive decline of motor function in SBMA, patients will often first see a physician only after many years following the first appearance of subclinical symptoms, usually being in the 3rd-5th decade of life. Early symptoms may occur as early as in the teenage years, and include cramping, twitching, and fatigue after exertion. Elevated creatine kinase (CK) levels, a marker of muscle injury, can occur in patients a decade before overt symptoms appear, and CK remains elevated after symptom onset (Sorenson and Klein, 2007). As SBMA progresses, later symptoms include atrophy of the limb muscles and eventually of the bulbar and facial muscles. This weakness often results in dysphagia (difficulty swallowing) and

dysarthria (difficulty articulating), and patients may present with a nasal speech as well as severe tongue atrophy. Overall life expectancy is not reduced in humans due to SBMA per se, but because of weak bulbar muscles, choking could lead to aspiration pneumonia, resulting in fatal respiratory infection (Atsuta et al., 2006). Moreover, since limbs become weak, falling is a hazard; men in more progressed states of SBMA will require the aid of a walker or wheelchair. Metabolically, SBMA men have a higher occurrence of diabetes, hyperlipidemia, liver dysfunction, and hypertension (Katsuno et al., 2012).

Electrodiagnostic exams of SBMA patients revealed reduced motor unit recruitment (Suzuki et al., 2010), and chronic denervation-reinnervation (Jokela and Udd, 2016). This apparent reinnervation may mean collateral sprouting which likely leads to the increased fatigue and neurotransmission failure observed in SBMA patients (Noto et al., 2013); compound muscle action potentials were also reduced (Suzuki et al., 2008; Fu et al., 2013). Patients also report sensory disturbances, in particular reduced distal sensations (Lee et al., 2005) and reduced sensory nerve action potentials (Suzuki et al., 2008).

Muscle histopathology in SBMA patients revealed what are classically viewed as “myogenic” versus “neurogenic” changes. Myogenic changes include fiber splitting, hypertrophic fibers, internalized nuclei, necrotic fibers, while neurogenic changes indicative of denervation/reinnervation included fiber type grouping, angulated fibers, and target fibers (Harding et al., 1982; Sobue et al., 1989; Soraru et al., 2008; Chahin and Sorenson, 2009). Interestingly, those patients with more severe myopathic findings had worse motor function scores (Soraru et al., 2008).

SBMA is linked to a CAG/polyglutamine expansion mutation in the amino-terminal coding region of *AR* gene (La Spada et al., 1991). A polymorphic CAG tract is located in the first exon of *AR*, near the activation function-1 transactivation domain. Healthy persons typically carry 9-34 repeats, but when the repeat size increases above 38, SBMA symptoms may appear. Length of the polyglutamine repeat has been inversely correlated with age of symptom onset in SBMA

men (Fratta et al., 2014), and genetic anticipation is moderate (Grewal et al., 1998). A shorter CAG repeat has also been found to be associated with a sensory-dominant phenotype, while longer CAGs were associated with a motor-dominant disease presentation in SBMA patients (Suzuki et al., 2008).

Despite the clear association of an expanded CAG allele with SBMA, it is possible that many cases of neuromuscular disease not diagnosed as SBMA but are due to pathological action from AR, but with a non-expanded-polyglutamine tract. For example, there are cases of muscle weakness in men who exhibit core symptoms of SBMA, such as gynecomastia and elevated CK, but were not considered "SBMA" because of a normal repeat size in the *AR* (Ferlini et al., 1995; Mariotti et al., 2000). Indeed, a mouse model that overexpresses a Wt (22Q) AR exclusively in muscle fibers manifests with symptoms strikingly similar to SBMA in humans (Monks et al., 2007).

ii. Delineating site of toxic AR action in SBMA

Motoneuronal loss has been long considered the central cause of muscle weakness and atrophy in SBMA, but a myogenic view of pathogenesis where primary *muscle dysfunction* leads to muscle weakness, is slowly becoming appreciated (Jordan and Lieberman, 2008; Bricceno et al., 2012). Evidence supporting motoneuron death as causal to SBMA came from autopsy studies; patients who lived with overt disease symptoms for at least 10-20 years before examination had fewer motoneurons in the spinal and bulbar regions (Sobue et al., 1989). Nonetheless, muscle histopathology in SBMA patients revealed neuropathic and myopathic changes (described above), of which the *myopathic* features were better predictors of motor dysfunction (Soraru et al., 2008). Moreover, that asymptomatic, carrier women also exhibited myopathic muscle changes suggested this to be an early event in the pathogenesis of SBMA (Mariotti et al., 2000; Soraru et al., 2008). Also, CK is expressed in over 80% of SBMA patients, often 10-20X higher than normal, which is unexpected for a pure motoneuron disease; only

~20% of amyotrophic lateral sclerosis (ALS) patients showed elevations in CK (Chahin and Sorenson, 2009). Moreover, SBMA patients expressed more of the disease-causing AR protein in muscle than in spinal cord, an indication that muscles may be more exposed to toxicity (Tanaka et al., 1999).

Animal models have been extremely useful in testing the hypothesis that SBMA occurs through a myogenic mechanism. Solid evidence first appeared when an exclusive overexpression of AR in muscle cells resulted in a mouse model that strikingly resembled SBMA (Monks et al., 2007). This model exhibited axonopathy and muscle weakness that occurred only in males and was androgen-dependent (Monks et al., 2007; Johansen et al., 2009; Johansen et al., 2011). Other early evidence came from finding that myopathic features were present before the onset of overt and neurological symptoms in a knock-in mouse model of SBMA (Yu et al., 2006).

Targeting muscles has proven successful in preclinical studies of mouse models. For example, an overexpression of muscle-specific insulin-like growth factor 1 (IGF-1) improved SBMA symptoms in a mouse model that globally expresses a polyglutamine AR (Palazzolo et al., 2009). More recently, evidence supporting the myogenic view (Jordan and Lieberman, 2008) came in the form of a selective polyglutamine AR knock-out mouse model. On the background of an SBMA mouse, polyglutamine AR was removed specifically from muscle cells, completely rescuing survival (Cortes et al., 2014b). An accompanying report described that peripheral blockage of AR expression through the use of antisense oligonucleotides (ASOs) was sufficient to slow disease progression in two mouse models, presumably acting on muscles, suggesting this may be a feasible therapeutic approach (Lieberman et al., 2014).

On the other hand, other mouse models have provided evidence for a neural component in SBMA. For example, Ramzan et al. (2015) used an inducible Cre/loxP system to selectively overexpress polyglutamine AR for a four-week period in adulthood in either muscle cells (MyoAR) or motoneurons (NeuroAR). Interestingly, while NeuroAR mice showed an overt motor

phenotype and MyoAR mice did not, cellular markers of disease were more prominent in the MyoAR mice. Conversely, when we use a constitutively expressing Cre recombinase, MyoAR display a more severe and earlier motor phenotype than NeuroAR mice (Jordan lab, unpublished observations). Muscle gene expression and neurotransmission were also more strongly affected in MyoAR mice (Jordan lab, unpublished observations). Nevertheless, a role for neural AR in producing toxicity is further supported by ASO work in a 97Q mouse model that targeted neural tissue only, which demonstrated a therapeutic effect on disease when AR was reduced centrally (Sahashi et al., 2015). This conflicting data prompts further study of muscle versus neural mechanisms of AR toxicity, starting with exploring whether the benefits to treating either hold true across multiple mouse models of SBMA.

Ultimately, as no animal or cell model perfectly recapitulates the human disease, we will need further evidence from human patients regarding whether potential therapeutic interventions indicated from animal studies are also beneficial in humans. Moreover, perhaps different, yet combined, levels of contribution from muscles and motoneurons are required for disease; other unidentified cell types may also contribute to disease (e.g., perisynaptic Schwann cells). The exciting prospect of a strong role for peripheral muscle involvement is its accessibility for therapeutic intervention.

iii. Androgen dependence of SBMA

The presence of androgens is necessary for disease symptoms in SBMA, a development that came relatively recently. Although the mutation was identified to be in the *AR* gene in 1991 (La Spada et al., 1991), it was over a decade later that the requirement for androgens was established in cell and animal models (Katsuno et al., 2002; Takeyama et al., 2002). In a mouse model that globally overexpresses a polyglutamine AR, disease occurred to a greater extent in males than females, and manipulation of androgen levels controlled disease status. Namely, disease was prevented (Katsuno et al., 2002) or reversed (Katsuno et al., 2006) in male mice

that were castrated, and females that were administered testosterone developed SBMA symptoms (Katsuno et al., 2002). Since then, the requirement of testosterone has become an important criterion to demonstrate that a disease mouse is modeling SBMA (Chevalier-Larsen et al., 2004; Yu et al., 2006; Monks et al., 2007; Johansen et al., 2009). Indeed, any phenomenon thought to be related to SBMA pathogenesis should be tested under androgen positive and negative conditions to be truly considered relevant to SBMA.

Clinical evidence matches the androgen dependence requirement. Women, who have low testosterone levels, show only mild subclinical symptoms (Mariotti et al., 2000). SBMA is also not a typical X-linked disease: Even females carrying two copies of the disease allele will not show clinical-level symptoms (Schmidt et al., 2002). Moreover, when testosterone was administered to an SBMA patient, he experienced worsening neuromuscular symptoms that improved following testosterone removal (Kinirons and Rouleau, 2008). However, in an earlier case study, testosterone did not appear to influence disease in two SBMA patients; but these findings were confounded by a simultaneous exercise regime (Goldenberg and Bradley, 1996), an intervention that may provide benefit to some SBMA patients (Shrader et al., 2015). A recent report of SBMA in a patient that underwent male-to-female sex reassignment suggested that low testosterone did not alleviate symptoms; it is possible the anti-androgen used for the transition, spironolactone, had toxic effects as it stimulates nuclear AR localization and transactivation (Lanman et al., 2016), necessary steps to AR toxicity in SBMA (described below).

Pharmacologically reducing androgen levels and AR function in animal models ameliorates disease (Katsuno et al., 2003; Renier et al., 2014). Strong evidence for the curative effects of anti-androgens in animal models led to several clinical trials (Weydt et al., 2016). Leuprorelin is a luteinizing hormone-releasing hormone agonist that acts to reduce testosterone levels by negative feedback through the hypothalamic-pituitary-gonadal axis. Early studies indicated that SBMA patients respond favorably to leuprorelin treatment (Banno et al., 2006),

and a Phase II trial even demonstrated improvements in swallowing function after a 48-week treatment and improved motor function after 96 weeks (Banno et al., 2009). A larger phase III trial with leuprorelin, however, did not support the reduction of testosterone as beneficial in SBMA after a 48 week follow-up (Katsuno et al., 2010). Nevertheless, a subgroup of patients in earlier disease stages did show an improvement (Katsuno et al., 2010). In another anti-androgen trial, the efficacy of dutasteride, a 5-α reductase inhibitor, was assessed, but no improvements were found. Of note, this trial was designed to more specifically target motoneurons, which express higher levels of 5-α reductase than muscle (Fernandez-Rhodes et al., 2011). However, with muscle being a major site of pathogenesis (Monks et al., 2007; Jordan and Lieberman, 2008), that this clinical trial was unsuccessful is unsurprising. As SBMA is a slowly progressive disease, better trial design with more sensitive measures and longer follow-up are warranted (Weydt et al., 2016). Recently, a new SBMA-specific motor function scale was established that might allow for better tracking of progression (Hashizume et al., 2015). Finally, despite the failed anti-androgen trials, androgen reduction therapy is still the best candidate for treating SBMA and should not be discounted.

iv. Cellular and molecular mechanisms of SBMA

Nuclear localization of AR, DNA binding, and interdomain interactions

The normal unbound AR resides in the cytoplasm, where it is kept in its native conformation by chaperone proteins. Upon androgen (testosterone or dihydrotestosterone) binding and activation of the receptor, the conformation changes that involve interactions of the amino and carboxyl (N/C) interaction ends of the receptor, removal of chaperone proteins, and translocation to the nucleus. Once in the nucleus, AR acting a ligand-dependent transcription factor binds to androgen response elements (AREs) as a dimer and recruits transcriptional coregulators to induce transcriptional activation or repression of genes. These same steps in AR signalizing also seem to be involved in triggering SBMA symptoms in animal models.

Nevertheless, some evidence suggests that loss of normal AR transcriptional function also contributes to pathology (Takeyama et al., 2002; Nedelsky et al., 2010; Chua et al., 2015). The requirement of AR to be localized in the nucleus, DNA binding, and of N/C interdomain interactions (Nedelsky et al., 2010; Orr et al., 2010; Zboray et al., 2015) implicates a toxic gain of AR function within the regular cascade of AR events. Targeting AR toxicity function (e.g., N/C interaction without affecting transcriptional activity, Zboray et al., 2015) while maintaining healthy, endogenous AR function is the goal of future SBMA therapeutics. Additional sources of toxicity include aberrant posttranslational modifications of AR, also potential targets for therapy. For example, cell and animal models reveal that enhancing phosphorylation can reduce ligand binding (Palazzolo et al., 2007) or N/C interaction (Zboray et al., 2015), deacetylation can promote degradation of the toxic AR (Montie et al., 2011), inhibiting SUMOylation can restore normal transcriptional function (Chua et al., 2015), and ubiquitination can help remove the disease-causing AR (Adachi et al., 2007). By narrowing down the precise molecular requirements of AR toxicity as well as its site of action, we may be able to achieve an SBMA treatment with limited or no unwanted side effects.

Protein quality control systems

Heat shock proteins bind to and maintain the proper folding of cytosolic AR, but levels of these chaperone proteins are reduced in SBMA due to sequestration in AR inclusions in a cell model (Stenoien et al., 1999). Moreover, when ligands bind to mutant AR, triggering the loss of bound chaperones, activated AR becomes misfolded and toxic. The protein quality control system within a cell can detect misfolding and turn on proteosomal degradation or autophagy pathways to keep toxic AR at bay. However, these pathways are often dysfunctional in SBMA (Rusmini et al., 2016). Indeed, enhancing the heat shock response can ameliorate SBMA symptoms in mouse models (Adachi et al., 2003; Malik et al., 2013) and enhancing autophagy also reduces toxicity through enhanced degradation of the toxic protein in cultured motoneurons

(Montie et al., 2009). However, autophagy genes are over-activated in SBMA mouse and human muscle (Chua et al., 2014), which may explain why inhibition of autophagy ameliorates SBMA symptoms in a mouse model (Yu et al., 2011). Moreover, endoplasmic reticulum (ER) stress was found in SBMA cells (Montague et al., 2014), and the unfolded protein response, which occurs from ER stress was induced in SBMA mouse and human skeletal muscle (Yu et al., 2011). Thus, there is clear evidence that the protein quality control systems are dysfunctional in SBMA, but which AR-dependent mechanisms lead to this and which tissues are affected requires further study.

AR aggregates and nuclear inclusions are key hallmarks of SBMA in humans (Li et al., 1998), but their role in pathogenesis it is not clear. Indeed, recent evidence indicates that aggregates are protective against AR toxicity in SBMA (Rusmini et al., 2016), and that soluble AR species are toxic in mouse and cell models (Li et al., 2007; Heine et al., 2015).

Mitochondrial defects

Mitochondrial dysfunction could lead to loss of oxidative capacity of the cell, a critical mechanism for generating energy in muscles, both fast-oxidative and slow-oxidative. Evidence in SBMA humans and mice suggests that mitochondria are perturbed. Expression of genes important for mitochondrial function was dysregulated in SBMA mouse muscle (Ranganathan et al., 2009; Mo et al., 2010; Giorgetti et al., 2016). SBMA cell models exhibit mitochondrial membrane depolarization (Ranganathan et al., 2009; Cortes et al., 2014a). Moreover, nicotinamide adenine dinucleotide (NADH) staining, a marker of mitochondrial function, was increased in multiple SBMA mouse models and patients (Sopher et al., 2004; Monks et al., 2007; Johansen et al., 2009; Rocchi et al., 2016), but was reduced in another model of SBMA (Ramzan et al., 2015). Interestingly, although NADH staining was increased in a knock-in model of SBMA (Rocchi et al., 2016), mitochondrial function and mitochondrial DNA copy number was decreased in skeletal muscle (Giorgetti et al., 2016). Androgen-dependent hypertrophy of

mitochondria occurs in a myogenic mouse model of SBMA (Musa et al., 2011; Poort et al., 2016), a finding that was corroborated by evidence of mitochondrial swelling in knock-in SBMA mouse muscle, although these changes were proposed to be secondary to defective glycolytic mechanisms (Giorgetti et al., 2016). Nevertheless, an intervention that rescued disease symptoms in the knock-in SBMA mice (inhibition of AR SUMOylation), also rescued mitochondrial gene expression and type I fibers (Chua et al., 2015). Since mitochondrial function is critical to muscles and motoneurons, any changes in structure (mitochondrial number and size) might underlie the changes in mitochondrial function (described above) and could lead to symptoms mimicking mitochondrial myopathy (Pfeffer and Chinnery, 2013). Thus, targeting oxidative pathways can alleviate disease, regardless of what may have initially instigated it.

Axonal transport

Motoneurons project their axons long distances, which can be energetically challenging for a cell. To sustain healthy neuromuscular function, proteins must be transported from the soma to the axon terminal. Likewise, the motoneuronal targets (i.e., muscles) provide trophic factors that signal retrogradely to the presynaptic terminal, to either locally potentiate neuromuscular transmission or be endocytosed and transported back to the soma to provide long-distance survival signals. In several SBMA mouse models, axonal transport has been found to be perturbed. Apart from one report (Malik et al., 2011), both retrograde (Katsuno et al., 2006; Kemp et al., 2011) and anterograde (Morfini et al., 2006) axonal transport are disrupted in early stages of disease, suggesting that loss of this critical mechanism might contribute to neuromuscular dysfunction and ultimately muscle weakness. Evidence also suggests that transport is perturbed in SBMA patients. Dynactin is a protein complex essential for retrograde transport via dynein as it acts as an adapter for cargo binding and enhances processivity. Staining of the dynactin 1 subunit was found to be reduced in post-mortem SBMA human motoneurons (Katsuno et al., 2006). Moreover, axonal transport appears dysfunctional since

axon terminals accumulated neurofilament in post-mortem motoneurons of an SBMA patient (Katsuno et al., 2006). How this disruption in transport comes about may be related to toxic action of AR in the motoneurons themselves (Piccioni et al., 2002; Szebenyi et al., 2003). For example, mutant AR has been found to disrupt core transport machinery involving the pathological activation of cJun N-terminal kinase (JNK) inhibited kinesin binding to microtubules in squid axoplasm (Morfini et al., 2006). However, given that the pathogenic mechanisms in SBMA likely originate and are triggered by mutant AR in muscle, it is not clear that studying how mutant AR in squid axons disrupts transport helps to understand the mechanisms driving SBMA in humans. On the other hand, published evidence also makes clear that retrograde transport in affected motoneurons can be disrupted via a non-cell-autonomous mechanism of toxicity in mouse models, where primary muscle dysfunction leads to inefficient axonal transport in the motoneuron, perhaps through a loss of muscle-supplied neurotrophic factors (Kemp et al., 2011; Halievski et al., 2016).

Mechanisms of muscle force production

The NMJ is the site where electrical signals from the motoneuron (action potential) get converted into a chemical signal to direct the target skeletal muscle to contract (Figure 1). Once the neurotransmitter ACh is released, it binds to its receptors (AChRs) on the postsynaptic sarcolemma (see “Brief tutorial on synaptic transmission” section below for more details). The local depolarization extends into the post-junctional folds, where sodium channels are activated to trigger a muscle action potential, which triggers a conformational change in the transverse-tubule voltage-sensing dihydropyridine receptor (DHPR), which in turn triggers the opening of the calcium ryanodine receptor (RyR) channel in the SR, allowing calcium to flow out of the SR and into the sarcoplasm. This surge of intracellular calcium binds troponin which then removes a tropomyosin block and allows myosin to mediate muscle contraction. Calcium is then pumped

back into the sarcoplasmic reticulum (SR) via sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) to restore the resting calcium levels and contraction ceases.

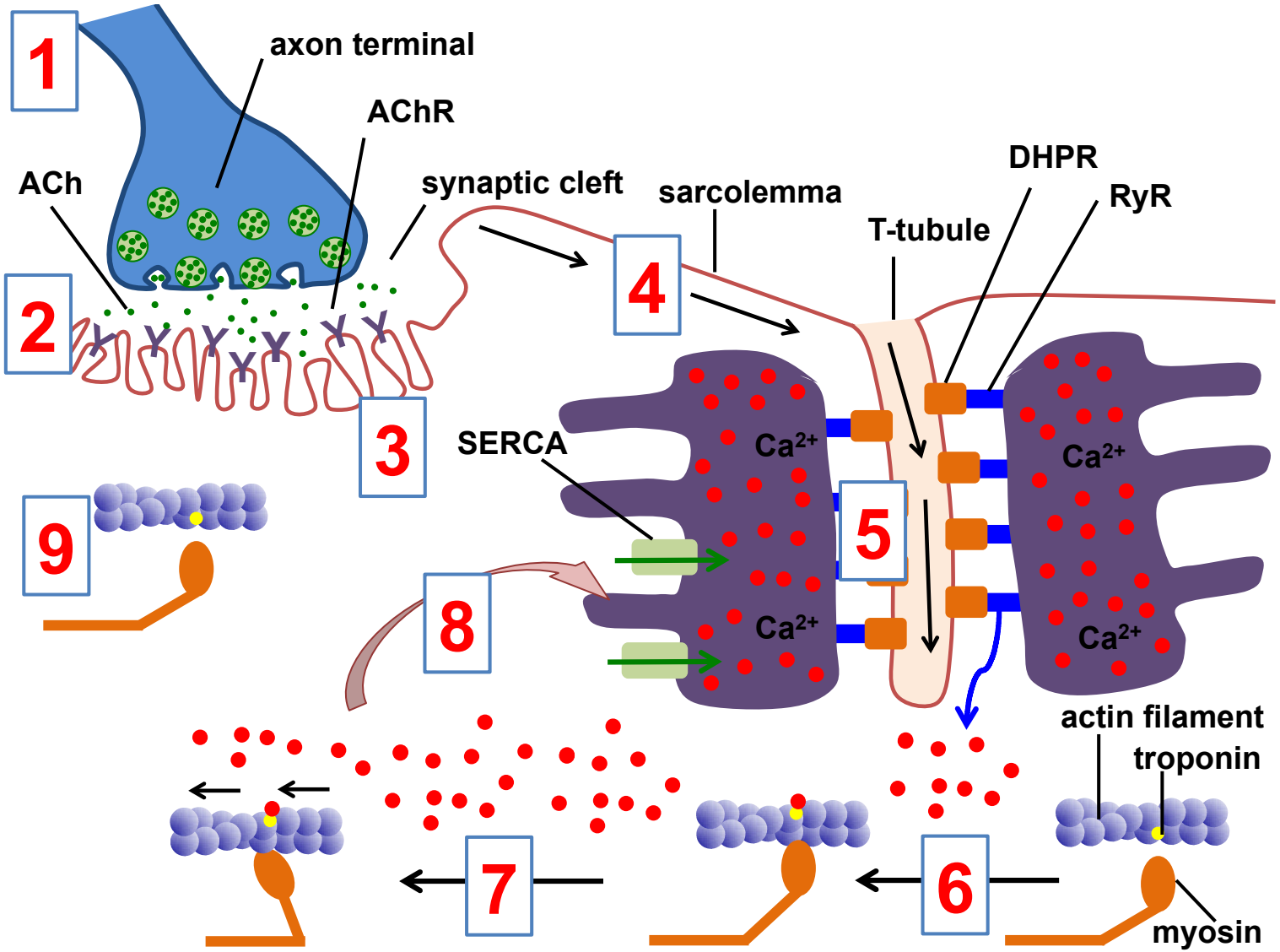

Figure 1. Schematic of events leading up to muscle contraction. 1) An action potential propagates down the motoneuron, reaching the axon terminal where calcium influx occurs and causes the exocytosis of acetylcholine (ACh) containing synaptic vesicles. 2) ACh traverses the synaptic

Figure 1 (cont'd). cleft where it binds to the ACh receptors (AChRs). 3) Voltage changes within the muscle fiber can be recorded intracellularly to detect influx through AChR. Evoked endplate potentials (EPPs) are the muscle's response to the contents of many ACh vesicles that were released due to a presynaptic action potential. Spontaneous exocytosis of single vesicles also occurs and results in a postsynaptic miniature endplate potential response on the muscle fiber. 4) If an EPP is large enough, the sarcolemma will depolarize past threshold and generate an action potential in the muscle that propagates through the transverse (T)-tubules. 5) Voltage sensitive calcium channels called dyhydropyridine receptors (DHPRs) are located on the T-tubules and detect depolarization, at which point they physically interact with and cause opening of ryanodine receptors (RyRs) located on the sarcoplasmic reticulum (SR). The SR stores high concentrations of calcium which is released through open RyRs. 6) Once released, calcium binds to troponin, a regulatory protein controlling actin-myosin interactions. Troponin undergoes a conformational change that allows myosin to bind to actin. 7) Once bound to actin, myosin is in a high energy state and produces a power-stroke that pulls actin filaments to cause contraction. This "cross-bridge" cycle is repeated until the depletion of calcium or ATP. 8) The sarco-endoplasmic reticulum ATPase (SERCA) is responsible for muscle relaxation as it takes up calcium from the sarcoplasm back into the SR. 9) Under low intracellular calcium levels, myosin can no longer bind to actin and contraction ceases.

Motor dysfunction is a key trait of SBMA and other neuromuscular diseases. Since skeletal muscle is responsible for the final output of any somatic movement, the muscle's role in healthy motor function is key. Yet, intrinsic muscle force was not studied in SBMA until very recently. Indeed, it was simply assumed that muscle weakness was simply a byproduct of denervation-induced atrophy. Now, strong evidence points to reduced force in fast- and slow-twitch muscles of two mouse models of SBMA which is largely independent of muscle mass. This loss in force occurs early in the disease process, and depends on androgens (Oki et al., 2013), correlating with the androgen-dependent loss of motor function itself. That this loss of force occurs is largely intrinsic to the muscle points to a myogenic role in muscle weakness of SBMA. In a knock-in mouse model of SBMA which exhibits a very mild phenotype, intrinsic muscle force in limb fast extensor digitorum longus (EDL) and slow soleus muscles was unaffected (Oki et al., 2015; Rocchi et al., 2016), perhaps because pathology in this model is most severe in a highly androgen sensitive muscle, the levator ani (LA; for example, see Xu et al., 2016), where force has yet to be assessed. Mechanisms behind this profound weakness in contractile force that occurs independent of mass in the two Tg mouse models may involve defects in calcium handling inside muscle fibers, including possibly defects in the function of RyR, SERCA, and troponin, which critically mediate muscle contraction (also see Figure 1 below). Preliminary data indeed support this possibility, indicating that expression of *RyR1* and *SERCA1* genes are dysregulated in diseased SBMA mouse muscle (Halievski, Haddad, Panek, and Jordan, unpublished observations). Other possible mechanisms involved include disruptions in the sarcomeric structure so that the contractile proteins, actin and myosin, are not properly anchored at the Z line, as seen in a myogenic SBMA mouse model (Musa et al., 2011) or impaired fusion of myotubes in satellite cells derived from human SBMA patients (Malena et al., 2013). Thus, therapies directed at contractile mechanisms in skeletal muscle may improve motor function in SBMA patients.

Neuromuscular transmission

Impaired muscle function can also be due to impaired neural drive. Electrodiagnostic studies in SBMA patients revealed reduced neuromuscular transmission (Noto et al., 2013), and this deficiency was present even in a patient with very mild symptoms (Meriggioli and Rowin, 2003), suggesting neuromuscular transmission failure is an early event in disease. Detailed electrophysiological analysis was carried out recently in three mouse models of SBMA (Xu et al., 2016) and revealed significant deficiencies in quantal content (i.e., the amount of neurotransmitter released in response to a single action potential), in the size of the readily releasable pool, and in release probability. Post-synaptic defects were also apparent that might contribute to the muscle's ability to transduce the signal with normal efficiency. Notably, in these mice, neuromuscular junctions (NMJs) are not denervated (Kemp et al., 2011; Poort et al., 2016); Poort and Jordan, in preparation) but may sometimes fall below threshold when activated repeatedly. Such dysfunction could contribute to motor dysfunction if too little neurotransmitter is released to activate downstream pathways to trigger release of intracellular calcium in the muscle. An assessment of the amount of force produced when contraction is nerve- versus directly-evoked will be necessary to determine whether neurotransmission failure contributes to the apparent loss of muscle strength in diseased mice. While force was reduced in response to nerve-evoked stimulation in SBMA mouse models (Malik et al., 2013; Rocchi et al., 2016), intrinsic muscle deficits cannot be ruled out (see above).

v. Transcriptional dysregulation in SBMA muscle and reverting to a developmental profile of gene expression

AR is a transcription factor and loss of its native function might account for some of the changes observed in gene expression; indeed, partial androgen insensitivity is a symptom in SBMA patients. However, maladaptive gene expression also occurs in SBMA muscle, which cannot be fully accounted for through AR's native action and thus represents some toxic gain of

function (Lieberman et al., 2002). For example, misfolded AR can interfere with cAMP response element-binding protein (CREB)-dependent transcription by sequestering CREB binding proteins into AR-rich aggregates in mouse and cell models, as well as in human scrotal tissue (McCampbell et al., 2000; Sopher et al., 2004). While some defects in mRNA expression occur through faulty RNA splicing via an expanded CAG repeat *RNA* (Mykowska et al., 2011), such defects in SBMA mouse muscle are androgen-dependent, implicating the toxic AR *protein* in perturbing the splicing machinery (Yu et al., 2009). In a more global view using transcriptome analysis, hundreds of genes in SBMA mouse muscle are dysregulated (Mo et al., 2010; Giorgetti et al., 2016) in an androgen-dependent manner (Halievski et al., 2015a). Because expression of the disease is androgen-dependent, changes in gene expression that are also androgen-dependent are likely the most relevant to disease.

In the context of this dissertation, some categories of dysfunctional gene expression in muscle include ion channels, contractile components, and neurotrophic factors. Muscle ion channels are responsible for maintaining a healthy resting membrane potential (RMP) and membrane excitability. For example, *CLCN1* expression is reduced in SBMA mouse muscle (Yu et al., 2006), which may contribute to the reduced RMP in three mouse models of SBMA (Oki et al., 2015). Moreover, the acetylcholine receptor (AChR) is an ion channel concentrated at the postsynapse whose mRNA expression is also defective. Namely, mRNA levels the adult subunit of AChR (ε) are reduced, while mRNA for the neonatal subunit (γ) is increased in SBMA mouse muscle (Xu et al., 2016). This particular subunit controls the kinetics and amount of sodium current through the AChR with the developmental isoform leading a smaller and slower current. Lower overall conductance of the developmental AChR (Mishina et al., 1986) may lead to reduced capacity to reach the threshold needed for generating a muscle action potential. The same is true for the expression of sodium channel in SBMA, where expression of the adult *Scn4a* is reduced with a concomitant increase in developmental *Scn5a* in mouse muscle (Xu et al., 2016). Since the developmental sodium channel becomes inactivated at more negative

potentials (Nuss et al., 1995; Makielski, 1996), it may spend more time in an inactivated state and *not* contributing to action potential generation in the muscle since SBMA mouse muscles are depolarized. Further downstream, we also find reductions of *Ryr1* in skeletal muscle of SBMA mice*,* which encodes the adult RyR responsible for the release of intracellular calcium from stores to produce a contraction (Panek, Halievski, and Jordan, unpublished observations). Developmental isoforms of these channels are generally less efficient than adult isoforms, and the developmental shift might also help explain defects in neuromuscular dysfunction. The same pattern is true even further downstream in the neuromuscular signaling cascade for muscle contractile components. Myosin heavy chain is the critical motor responsible for muscle contraction, and in SBMA mouse muscle, adult isoforms of myosin heavy chain are reduced (Halievski et al., 2015b), with concomitant increases in the embryonic and perinatal isoforms of myosin heavy chain in human and mouse SBMA muscle (Halievski and Jordan, unpublished observations; Palazzolo et al., 2009; Jokela et al., 2016). Also mRNA expression for *SERCA1*, which encodes the protein responsible for calcium uptake back into the SR, is perturbed in SBMA mouse muscle (Halievski, Haddad, and Jordan, unpublished observations). Excess calcium in the fiber could also lead to mitochondria dysfunction as it works to buffer this excess in the sarcoplasmic compartment. Finally, expression of neurotrophic factors in muscle are dysregulated in SBMA patients and mice (described in detail below). Deficits in the availability of muscle-derived neurotrophic factors could lead to overall dysfunction of the neuromuscular system via the loss of long distance support of the motoneuronal cell bodies and/or their localized action to support NMJs or the muscle itself (see below).

II. Overview of neurotrophic factors and the neuromuscular system

i. The neurotrophic theory

Neurotrophic factors as they are known today are involved in many processes throughout the entire body and lifespan of an organism. In the early 1930's Viktor Hamburger and Rita Levi-Montalcini conducted studies that spawned the field of neurotrophic factors and their role in neuronal survival, now referred to as the neurotrophic theory. Hamburger removed a limb bud from a chick embryo and observed fewer neurons that survived in the ventral spinal cord and the dorsal root ganglion (DRG), suggesting that neuronal survival may depend on some target-derived factor (Hamburger, 1993). Later work on DRG sensory neurons suggested that the larger the size of the periphery (i.e., limb bud), the less cell death would occur (Hamburger and Levi-Montalcini, 1949), further implicating trophic support as critical for neuronal survival and the amount of trophic support available calibrated by target size. In a complementary experiment, expanding the periphery by grafting a limb bud resulted in the rescue of excess motoneurons (Hollyday and Hamburger, 1976). Together, this work was instrumental in placing *target* structures as a critical for ensuring the health and survival of neurons. Thus, neurotrophic factor theory states that for a neuron to survive, it must gain access to limiting amounts of trophic factors from the target (Davies, 1996).

The first neurotrophic factor to be isolated came from mouse salivary glands and was identified as nerve growth factor (NGF; Cohen et al., 1954; Levi-Montalcini and Cohen, 1960). Application of this trophic substance induced hypertrophy and neurite outgrowth in explants of the DRG (Levi-Montalcini and Cohen, 1956) and sympathetic ganglia (Levi-Montalcini and Booker, 1960b). Moreover, administering NGF anti-sera decreased in cell survival in the sympathetic ganglia (Levi-Montalcini and Booker, 1960a). Later studies found that NGF was retrogradely transported from its target tissue (Johnson et al., 1978), and that this transport was necessary for the survival of sympathetic neurons (Kuruvilla et al., 2004).

After the discovery of NGF, came the identification of three other factors in the neurotrophin family (BDNF; neurotrophin-3, NT-3; and neurotrophin-4, NT-4), that are also implicated in the survival of various neuronal populations (Barde et al., 1982; Bothwell, 2014). Surprisingly, single mouse knock-out studies of NT-3, NT-4, and BDNF do not influence motoneuronal survival, but when all three are knocked out, significantly fewer facial motoneurons survive (Liu and Jaenisch, 2000). Likewise, knocking out a neurotrophin receptor (TrkB) rather than its ligand (BDNF / NT-4) revealed a reduction in the number of surviving facial motoneurons (Klein et al., 1993), suggesting that both BDNF and NT-4 may be critically involved in regulating the number of motoneurons that survive. Moreover, other target-derived factors (e.g., cardiotrophin-1; glial cell line-derived neurotrophic factor, GDNF) can also promote motoneuron survival (Oppenheim, 1996). More recently, target-supplied neurotrophic factors are proving to be necessary for maintaining functions other than neuronal survival (Davies, 1996). For example, neurotrophins are necessary for proper functioning of the neuromuscular system (Chevrel et al., 2006; effects of BDNF discussed below); effects of BDNF discussed below).

ii. Neurotrophin signaling pathways

Neurotrophins exert their effects through receptors from two families: the tropomyosin related kinase receptor (Trk) and the pan-neurotrophin receptor (p75). Trk receptors are transmembrane receptors with cytosolic kinase activity. NGF preferentially binds TrkA, BDNF and NT-4 bind TrkB, and NT-3 binds TrkC. Upon binding of ligands, Trk receptors dimerize and transphosphorylate each other at tyrosine residues, leading to the activation of the receptor by enhancing tyrosine kinase activity (Segal, 2003). Then, other tyrosine sites are phosphorylated, which recruit effector proteins that lead to downstream signaling cascades, which occur through three major pathways: phospholipase C-γ (PLC-γ), phosphatidylinositol 3-kinase (PI3K)-Akt, and extracellular signal-regulated kinase (Erk) (Deinhardt and Chao, 2014).

PLC-γ activation from Y816 phosphorylation on TrkB leads to production of inositol tris-phosphate (IP_3) that activates the release of intracellular calcium stores, and further leading to activation of Ca^{2+}-calmodulin-regulated protein kinases and calcineurin. Diacylglycerol (DAG) is also produced by PLC-γ and leads to the activation of protein kinase C (PKC), which can stimulate Erk (see below). Activation from this pathway can also leads to CREB activation, which is critical for many of the survival enhancing transcription products.

PI3K-Akt pathway occurs following TrkB phosphorylation at the Y515 site. There, Shc is recruited, which leads to PI3K-Akt-mTOR (mammalian target of rapamycin) activation, leading to increase protein translation. In muscles, Akt signaling promotes hypertrophy and prevents atrophy (Bodine et al., 2001). Akt signaling activation also inhibits pro-apoptotic gene expression.

Erk signaling can be activated through the Shc pathway, or through PKC activation as described above. Erk signaling can also lead to activated CREB transcription of pro-survival genes.

Both motoneurons and muscle express TrkB receptors and significantly, TrkB receptors are concentrated at the NMJ, in both presynaptic motor terminals and postsynaptically at the endplate (Gonzalez et al., 1999; Garcia et al., 2010c). Thus, both pre- and postsynaptic parts of the system are equipped to respond directly to muscle-derived BDNF and NT-4. Local signaling events at the junction can include increases in intracellular calcium or increased trafficking of synaptic vesicle to potentiate neurotransmitter release (Park and Poo, 2013). Not much is known about local postsynaptic events in the muscle; however, one study has demonstrated that full length TrkB is critical for maintaining healthy post-synaptic AChR clustering in the muscle (Gonzalez et al., 1999). Long range signaling of activated TrkB can also occur via the so-called signaling endosome that travels retrogradely to the soma (Koliatsos et al., 1993; Grimes et al., 1996).

The other neurotrophin receptor p75 is part of the tumor necrosis factor (TNF) receptor superfamily. p75 has an intracellular death domain that can lead to pro-apoptotic signaling through a terminal caspase cascade and also through inducing pro-apoptotic gene transcription through activation JNK (Kraemer et al., 2014). However, p75 can also promote survival (Kraemer et al., 2014). For example, one signaling pathway for p75 is activation of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which upregulates expression of survival genes. Notably, pro-neurotrophins, precursors to mature neurotrophins, bind more strongly to p75. Pro-neurotrophins can be cleaved intracellularly by furin or pro-proteases in the ER or Golgi apparatus, or secreted and cleaved extracellularly by plasmin and matrix metalloproteinases (Kraemer et al., 2014).

Finally, Trk and p75 receptors are colocalized and can influence each other's signaling. For example, p75 can act in conjunction with Trk receptors to enhance the binding affinity of BDNF to TrkB (Bibel et al., 1999). Trk isoforms that lack the tyrosine kinase domain also exist. Although originally thought to act for the purpose of sequestering BDNF and NT-4, research is now showing that they can actively signal (Fenner, 2012). Functions of TrkB isoforms are currently being explored.

III. Neurotrophic factors as treatment for neuromuscular disease

Given that neurotrophic factors are critical to the health and survival of neurons, their role in neurodegenerative disease has been explored. BDNF for example, is reduced in Huntington's, Alzheimer's, and Parkinson's diseases, and increasing levels of BDNF in animal models can improve disease symptoms (Weissmiller and Wu, 2012). Preclinical models of ALS have also benefited from exogenous expression of neurotrophic factors (Henriques et al., 2010). Increasingly, researchers are recognizing the potential role of "neurotrophic" factors in non-neural cells, such as muscles, in neurodegenerative disease.

i. Neurotrophic factors in neuromuscular disease and preclinical studies of neurotrophic factors as treatment

In SBMA, mouse models show deficits of several neurotrophic factors. For example, multiple models have reduced vascular endothelial growth factor (VEGF) expression in muscle (Monks et al., 2007; Johansen et al., 2009; Mo et al., 2010). Application of VEGF was beneficial in reversing a disease-related deficit in retrograde axonal transport in a myogenic model of SBMA (Kemp et al., 2011). VEGF has also been implicated in ALS and muscle-supplied VEGF and GDNF improved survival and ameliorated NMJ denervation in a rat model of this disease (Krakora et al., 2013).

In SBMA, several other neurotrophic factors have been implicated. NT-4 is reduced in diseased muscle of a knock-in model of SBMA (Yu et al., 2006); findings from our laboratory also indicate that NT-4 is reduced in two other SBMA models: a myogenic model and a 97Q model of SBMA (Wahl, Halievski, and Jordan, unpublished observations). Also in the knock-in model, GDNF is reduced in diseased muscle, although castration did not rescue its expression in this model (Yu et al., 2006), questioning the relevance to disease. Interestingly, in human SBMA muscle, GDNF is increased (Yamamoto et al., 1999), as it is in ALS muscle (Grundstrom et al., 1999). Even though elevated in ALS muscle, GDNF expressing mesenchymal cells implanted in muscle rescued ALS mice from disease, suggesting that perhaps elevated levels of GDNF represent an attempt to fight disease which falls short. Interestingly, the effect GDNF synergized with VEGF, leading to a far more potent effect than either one alone (Krakora et al., 2013). Expression of IGF-1 is also dysregulated in diseased muscle from SBMA knock-in mice, although in this report, it was not significant (Yu et al., 2006). Nevertheless, IGF-1 supplementation ameliorated disease symptoms quite successfully in an SBMA mouse model (see below, Palazzolo et al., 2009; Rinaldi et al., 2012) as well as in an ALS mouse model (Dobrowolny et al., 2005). Consistent with the possibility that deficits in IGF-1 may be key factor

leading to dysfunction in both SBMA and ALS is finding deficits in IGF-1 levels in human muscle affected by ALS (Lunetta et al., 2012).

In other models of neuromuscular disease, BDNF and ciliary neurotrophic factor (CNTF) have also been studied. For example, in ALS, human studies show that while BDNF is upregulated (something that happens in denervated muscle) and TrkB phosphorylation is reduced (Kust et al., 2002), suggesting a deficit in BDNF and/or NT-4 ability to activate its receptor. In animal models of ALS, expression of BDNF is not affected in limb or extraocular muscles (Harandi et al., 2014). Despite these findings, subcutaneous injection of BDNF and CNTF treatment in the wobbler mouse, a model that mimics ALS, ameliorates disease (Mitsumoto et al., 1994; Ikeda et al., 1995). However, BDNF did not have a beneficial effect in another study on ALS mice that used mesenchymal stem cells engineered to express high levels of BDNF that were implanted into muscle, perhaps because the levels of BDNF produced were not as high as the neurotrophic factors that did slow disease progression in this study (Krakora et al., 2013). Finally, 7,8-dihydroxyflavone, a TrkB agonist had beneficial effects on progression of ALS in a mouse model (Korkmaz et al., 2014). However, it is possible that the beneficial effects were through reduced reactive oxygen species production (Chen et al., 2011). Moreover, Yanpallewar et al. (2012) demonstrated that genetic deletion of the truncated TrkB isoform slowed disease progression in an ALS mouse model, as did phosphorylation of the full length TrkB via an agonist to A_{2A} receptors. A possible explanation is that if the truncated isoform normally sequesters BDNF to reduce signaling through full-length TrkB, reducing truncated TrkB levels will make more BDNF available to activate the catalytic isoform that induces pro-survival signals. Finally, in a *progressive motor neuronopathy* mouse that mimics ALS, BDNF application to the muscle rescued deficient axonal transport (Sagot et al., 1998). Of note, prior to studies described in this dissertation, BDNF has not been studied in SBMA.

BDNF has also been found to exhibit rescuing effects following nerve injury, even though it cannot single-handedly rescue motoneurons from developmental cell death (Ernfors et al.,

1994). Motoneuron death induced by axotomy in neonatal rats was prevented by BDNF administration to the nerve stump (Sendtner et al., 1992; Yan et al., 1992). Adult rats also respond positively to BDNF following axotomy, with an increase in choline acetyltransferase staining (Yan et al., 1994; Wang et al., 1997), an effect not mimicked by either NT-4 or NGF treatment (Wang et al., 1997). Choline acetyltransferase is responsible for producing acetylcholine (ACh), thus a surplus of the neurotransmitter may lead to potentiated transmission, especially at a time when axons are trying to reestablish functional contacts. Moreover, in adult mice, neuromuscular function was improved following sciatic nerve injury when muscles were injected with viral vectors expressing four different neurotrophic factors (BDNF, GDNF, IGF-1, and VEGF; Glat et al., 2016). Finally, combined treatment of CNTF and BDNF rescued fast-glycolytic muscle fibers following sciatic nerve crush in neonatal rats, which would otherwise have led to muscle degeneration (Mousavi et al., 2004); notably, NT-3 or NT-4 treatment with CNTF could not rescue these fibers (Mousavi et al., 2002). This work clearly implicates BDNF as a potential therapeutic but like most other neurotrophic factors, its beneficial action may depend on other neurotrophic factors.

ii. Clinical trials for motoneuron disease using neurotrophic factors

Strong preclinical evidence led to clinical trials starting in the 1990s to test the therapeutic benefits of neurotrophic factors in motoneuron disease. Unfortunately, none of them were deemed successful and the search for therapeutics continues. However, given the continued and compelling preclinical evidence that neurotrophic factors are beneficial in motoneuron disease (see above), it is worth stepping back to reassess these trials and consider why they may have failed (e.g., delivery method or target of delivery). Due to the broad range of beneficial actions of neurotrophic factors on neuromuscular diseases, they still seem like an ideal option to treat diseases that may have many etiopathologies (Henriques et al., 2010).

In 1999, a large Phase III trial was conducted to study the effects of subcutaneous BDNF to ALS patients over a nine-month period (Group, 1999). Although BDNF was well tolerated, overall survival and motor function was not improved in patients treated with BDNF. Notably, a subgroup of “responders” that experienced increased bowel movements or diarrhea due to action of the drug did show a significant improvement in survival. Thus, if BDNF is bioavailable to induce a response, it may exert beneficial effects in disease. It is possible that efficacy could be improved if BDNF were placed in a location where it could act more directly on critical targets before it is degraded. The next clinical trials did exactly this, by intrathecally administering BDNF to get it closer to the motoneurons that it needed to rescue (Ochs et al., 2000). However, using this approach in a small Phase III trial was also unsuccessful in rescuing function (Kalra et al., 2003). Reasons that benefits were not detected include that the treatment period was relatively short (4 weeks), and that the endpoint was a metabolite marker of neuronal integrity detected via magnetic resonance spectroscopic imaging that did not include any measures of overall survival or motor function. Another small Phase II/III trial that did examine motor function, alongside the primary endpoints related to autonomic function, found that intrathecal delivery of BDNF did not slow disease progression (Beck et al., 2005). Since these last two studies were very small, and thus underpowered, it is difficult to draw conclusions. Given BDNF’s benefit when delivered subcutaneously, it is possible that BDNF was producing its beneficial effects peripherally on muscles and NMJs, as those are much more accessible and since BDNF cannot cross the blood brain barrier (Pardridge et al., 1994).

Several other clinical trials with neurotrophic factors have been conducted, but were also unsuccessful. The first ones using CNTF showed no improvements in limb strength or pulmonary function (Group, 1996), and survival was actually decreased when a higher dose of CNTF was administered (Miller et al., 1996). A total of four independent ALS clinical trials have been conducted using IGF-1, but results have been mixed. An earlier clinical trial in North America administered subcutaneous IGF-1 and found that it slowed progression of disease as

measured by a decline in functional impairments over a nine month period (Lai et al., 1997). However, in Europe, a similar trial to the North America one (duration, nine months; dose, 0.1mg/kg/day; and route, subcutaneous) found no slowing of disease progression (Borasio et al., 1998). A trial using intrathecal delivery of IGF-1 produced modest effects on slowing disease progression (Nagano et al., 2005). Most recently, in an extended two-year trial, subcutaneous IGF-1 showed no improvement of function in ALS (Sorenson et al., 2008).

Finally, a recent Phase I/II trial using mesenchymal stem cells that were engineered to express neurotrophic factors (GDNF, BDNF, VEGF, and hepatocyte growth factor) implanted simultaneously in the muscle and intrathecally was found to be safe and well tolerated, and secondary outcomes on disease progression were promising (Petrou et al., 2016). Perhaps applying trophic substances to muscle is key to its therapeutic benefit. Since one of the early events in ALS is degeneration of the NMJ and dying-back axonopathy, early intervention will be critical. This will allow local trophic action from muscle to retain axonal contacts. Moreover, neurotrophic factors might ameliorate disease by acting on the muscles themselves (Boyer et al., 2013).

IV. Brain-derived neurotrophic factor action at muscles and motoneurons

BDNF has not yet been explored in SBMA, yet there are several reasons why it may be involved in this disease considering what we know about the defects caused by SBMA in neurotransmission and muscle contraction. BDNF is expressed in muscle and both motoneurons and muscles have receptors for BDNF. Moreover, BDNF signaling has been shown to facilitate both motoneuron and muscle health, and has been particularly implicated in maintaining healthy NMJs. In this section, I describe some of the known actions of BDNF in the neuromuscular system.

i. BDNF potentiates neuromuscular transmission

Brief tutorial on synaptic transmission

Changes in the membrane potential of muscle fibers in response to presynaptic ACh (Figure 1) can be measured to make inferences about the integrity of pre- and postsynaptic parts of the NMJ. One can measure local synaptic events by blocking skeletal muscle sodium channels with μ-conotoxin to prevent action potential generation. Then, the nerve can be stimulated and voltage changes due to AChR conductance recorded in the paralyzed muscle fiber; these are called evoked endplate potentials (EPPs). The EPP represents the muscle's response to the released ACh contents from synaptic vesicles (quanta). The amount of packets released upon nerve stimulation is called "quantal content" and can be calculated by comparing the amplitude of the EPP to that of the miniature endplate potential (mEPP). mEPPs occur following the spontaneous release of ACh from a single vesicle. The voltage displacement (depolarization) produced by EPPs and mEPPs depends on the RMP of the cell, since the driving force of sodium influx is stronger the more negatively polarized the RMP of the fiber is, and thus needs to be accounted for when calculating EPP and mEPP amplitude, especially if the RMP differs between diseased and healthy groups, as I observed.

Short-term facilitation occurs at the NMJ. If multiple stimulations are given within a short time period, the amount of neurotransmitter released will increase in subsequent stimulations, resulting from a buildup of intracellular calcium in the nerve terminal during the successive stimulations (Zucker and Regehr, 2002). In disease states, such as SBMA, deficits in facilitation occur (i.e., rather than more vesicle release in subsequent stimulations, there is less; Xu et al., 2016). This might be due to lower release probability (Pr) or a reduced size in the readily releasable pool (RRP), two factors that determine quantal content. RRP can be measured by extrapolating the slope of quantal content with repeated stimulations, which is reduced over time under conditions of tetanic stimulation. Pr is simply the value of the initial quantal content (before depression) divided by the calculated RRP. Elucidating the potential effects of disease

on such mechanisms can give us insight into the precise pathogenesis and what might be the appropriate cellular targets for therapeutics.

BDNF effects on neurotransmission revealed by intracellular recording from individual muscle fibers

BDNF was found early on to be factor which could potentiate the function of neuromuscular synapses. Work on frog NMJs in a co-cultured preparation of spinal neurons and myocytes showed that acute application of BDNF enhanced the frequency of spontaneous events and potentiated the release of evoked events (Lohof et al., 1993). Later work in the same preparation suggested that BDNF's potentiating effects were due to enhanced release probability, as the pair-pulse ratio was reduced following BDNF treatment (Stoop and Poo, 1996). In that same study, BDNF was shown to increase presynaptic calcium, and removing extracellular calcium abolished its potentiating effect. These two studies also suggested that BDNF acted locally, acutely, and presynaptically since the amplitude of spontaneous events was unchanged. However, a recent study found that the postsynaptic myocyte can also contribute to BDNF's potentiating action of spontaneous events. Namely, BDNF can act via p75 postsynaptically to activate the calcium transient receptor potential channel 1, increasing the frequency of spontaneous events (McGurk et al., 2011). Notably, a presynaptic response through TrkB was still needed for the full potentiating effects (McGurk et al., 2011). Thus, BDNF acts both pre- and post-synaptically to potentiate neuromuscular transmission.

At mammalian NMJs, BDNF has an effect as well. For example, acute BDNF application increased EPP amplitude in rat diaphragm, but the potentiating effect occurred only following activation of adenosine A_{2A} receptors, suggesting that TrkB needs to communicate with A_{2A} receptors; through pharmacological inhibition experiments, it was found to be likely through a protein kinase A pathway (Pousinha et al., 2006). Moreover, the increased EPP was due to increased calcium levels in the nerve terminal, since inhibitors of PLC-γ abolished the effect

(Pousinha et al., 2006). BDNF also potentiates EPP amplitude in developing (Garcia et al., 2010a) and adult (Garcia et al., 2010b) rat nerve-muscle levator auris longus preparations. BDNF also enhanced spontaneous release in the adult levator auris longus (Garcia et al., 2010b). Inhibiting TrkB or p75 prevented exogenous BDNF from increasing EPP amplitude, and inhibiting TrkB activity (with either K252a or anti-TrkB antibody) reduced EPP amplitude indicating a constitutive role for TrkB signaling at the adult mammalian NMJ (Garcia et al., 2010b). Interestingly, manipulations (e.g., antiserum against BDNF) that prevented endogenous BDNF from acting did not produce any changes in EPP amplitude, suggesting that TrkB activation is possible in the absence of ligand, perhaps through reciprocal actions with muscarinic receptors (Garcia et al., 2010b). Indeed, after a seven-day period of TrkB inhibition through a chemical-genetic approach, quantal content was not different from controls, although mEPP amplitude was elevated in the adult mouse diaphragm (Mantilla et al., 2014). The full mechanisms and sites through which TrkB and BDNF signaling occurs at the NMJ to potentiate synaptic transmission continue to be studied as it is clear they are not yet well understood.

BDNF on neurotransmission failure and muscle contraction

In addition to measuring synaptic transmission via single fiber recording at the NMJ (previous section), the efficacy of neuromuscular transmission can be assessed through a failure analysis using contractile force as the measure. Muscles can be induced to contract by stimulating the nerve. Muscles can also be directly stimulated to induce a contraction. When intense tetanic stimulation is used, both parts of the system fatigue but generally nerve-evoked contractions fail first due to depletion of presynaptic neurotransmitter stores. The deficit in contractile force produced by nerve stimulation compared to direct muscle stimulation is called “neuromuscular transmission failure”, and has been studied in relation to BDNF’s effects.

Acute (30 minutes) BDNF application to a nerve-diaphragm preparation reduced neurotransmission failure (Mantilla et al., 2004). TrkB signaling seemed to be constitutive at this

NMJ since K252a treatment to inhibit TrkB kinase activity increased neurotransmission failure in this paradigm (Mantilla et al., 2004). These findings were replicated more recently using a TrkB agonist, 7,8-dihydroxyflavone, which also reduced failure in a TrkB-dependent manner (Mantilla and Ermilov, 2012). That is, when TrkB was transiently inhibited, 7,8-dihydroxyflavone did not produce its potentiating effect and there was actually more failure than in controls (Mantilla and Ermilov, 2012). These findings were also borne out in a long term model; heterozygous knockout mice carrying only one TrkB allele ($TrkB^{+/-}$) showed an increased failure of neuromuscular transmission in a similar experimental design (Kulakowski et al., 2011).

Although BDNF signaling seems to have a robust influence on preventing neurotransmission failure, evidence suggests muscle contractile properties are not strongly affected. Muscle fatigability, twitch force, and tetanic force were not influenced by BDNF treatment or TrkB inhibition (Mantilla et al., 2004; Mantilla and Ermilov, 2012; Mantilla et al., 2014) although a minor reduction was seen in twitch force following TrkB inhibition in another study (Greising et al., 2015). However, specific force produced by muscle of $TrkB^{+/-}$ mice was reduced (Kulakowski et al., 2011), suggesting that longer-term TrkB may play a role in maintaining healthy contractile properties. Also in this model, expression of the skeletal muscle developmental sodium channel alpha subunit 1.5 was increased (Kulakowski et al., 2011).

Interestingly, in a knockout model of the truncated isoform of TrkB ($TrkB.T1^{-/-}$) muscle contraction was enhanced, via increased calcium release from the SR (Dorsey et al., 2012). Since the truncated TrkB isoform does not contain a catalytic domain, it is likely that its deletion led to more ligand available to bind and activate the full length isoform. Indeed, Akt signaling was enhanced in skeletal muscle of $TrkB.T1^{-/-}$ mice (Dorsey et al., 2012). Interestingly, regulation occurs differently in cardiac muscle. While BDNF does enhance calcium signaling, this occurs through the truncated TrkB receptor: $TrkB.T1^{-/-}$ mice exhibit cardiomyopathy and reduced calcium signaling in cardiac muscle in response to BDNF (Fulgenzi et al., 2015). In smooth airway muscle, BDNF increased calcium response and contraction in a TrkB kinase-

dependent manner (Prakash et al., 2006), suggesting that BDNF signaling differs across tissues and even across muscle types.

ii. BDNF influence on neuromuscular integrity

Many of the above studies discussed examined only short-term influences of exogenous BDNF signaling, which are not likely accompanied by substantial reorganization of the neuromuscular system. Nevertheless, BDNF and TrkB signaling can alter structure, gene expression, and development of nerves and muscles, which could also contribute to changes in neuromuscular function. For instance, $TrkB^{+/-}$ mice who experience life-long reductions in TrkB signaling have NMJs that are fragmented and expanded, often taken as a sign of having once been denervated (Kulakowski et al., 2011). Notably, fragmented junctions showed no sign of being denervated as indicated by the precise structural overlap of pre- and post-synaptic components (Kulakowski et al., 2011). These mice also exhibited another marker of denervation—increased staining for the developmental subunit of the muscle sodium channel (NaV1.5). Interestingly, this is similar to what is found in SBMA mice (Poort et al., 2016; Xu et al., 2016). NMJ fragmentation is also induced when TrkB.T1 was overexpressed in skeletal muscles to produce a dominant-negative action, implying that full length TrkB normally has a role in maintaining the clustering of AChR that results in a continuous pretzel-like organization (Gonzalez et al., 1999). Finally, in a conditional TrkB knockout induced in adulthood for seven days, only modest effects were seen on NMJ structure (reduced complexity, increased apposition of pre and post-synaptic sites); paradoxically, these changes were towards what one would expect of healthy NMJs, even though neurotransmission failure was increased (Mantilla et al., 2014). Long-term loss of BDNF signaling could lead to deterioration of NMJ structural integrity, ultimately affecting neuromuscular transmission, while more transient effects (<1 week) could lead to neuromuscular transmission failure through other mechanisms.

In a muscle-specific BDNF knockout model, muscles appear grossly normal, with no differences in fiber number or size (Clow and Jasmin, 2010). On the other hand, muscle fiber size was reduced in heterozygote knockouts for TrkB (Kulakowski et al., 2011), suggesting that some non-muscle or non-BDNF mediated TrkB signaling pathway maintains fiber size. NT-4, the other TrkB ligand may be involved. In the muscle BDNF knockout, however, reductions were found in the number of satellite cells marked by Pax7 and muscle regeneration was delayed following a cardiotoxin injury (Clow and Jasmin, 2010). Thus, BDNF/TrkB also seems important for muscle integrity and becomes evident under challenging circumstances like injury or disease.

V. Overview of dissertation aims

There are currently no disease-modifying therapies that have shown clinical value for SBMA. A common finding is the reduction of muscle-derived neurotrophic factors in SBMA, and give rise to the idea that restoring the production of such factors may prove beneficial to SBMA and other neuromuscular diseases. BDNF is one such neurotrophic factor that plays important roles in the health of both motoneurons and muscles. Nonetheless, BDNF has yet to be examined in the context of SBMA. For this dissertation I have taken on two aims to examine the hypothesis that a loss of muscle-derived BDNF underlies neuromuscular dysfunction in SBMA.

i. Are levels of BDNF reduced in SBMA muscle in androgen-dependent fashion?

To address this aim, I examined BDNF mRNA levels in two SBMA Tg mouse models: a myogenic model that overexpresses a Wt AR exclusively in muscle cells and in a 97Q model that ubiquitously overexpresses a polyglutamine-expanded AR. I also examined two muscles with different metabolic properties to deduce whether any change in BDNF expression was muscle-type specific (i.e., slow or fast). I found that BDNF was reduced in both a slow and fast muscle, and across both models. I then determined whether such deficits were androgen-

dependent, like the disease. I found that the BDNF deficit was androgen-dependent, and expression of BDNF in muscle improved when motor function improved. Finally, to further characterize the BDNF deficit and its relation to disease progression, I performed a time-course experiment, tracking BDNF levels across different disease stages in an acute model of SBMA. I found that BDNF was reduced prior to any overt symptoms, positioning it as an early and possibly proximate event in the course of disease.

ii. Can exogenous muscle BDNF ameliorate symptoms of SBMA?

Findings from Aim 1 were sufficiently encouraging to suggest that a deficit in muscle BDNF was a key aspect of SBMA pathology. Thus, I sought to reverse the deficit in muscle BDNF to determine whether mice could be rescued from disease. To perform such a study, I crossed the SBMA mice with Cre/loxP mice that overexpress BDNF specifically in muscle cells. I found that overexpression muscle BDNF mRNA slowed disease progression by two-fold. To elucidate possible cellular and molecular disease-related changes that BDNF rescued, I also examined neurotransmission using single fiber recording at the NMJ in the EDL and gene expression using quantitative reverse-transcription polymerase chain reaction (qRT-PCR) in the tibialis anterior (TA), both fast-twitch muscles. No improvements were seen in synaptic transmission at the NMJ, nor were there any improvements in gene expression in these muscles. However, I did find that the expression of some genes was rescued by BDNF in a slow-twitch muscle of diseased mice, suggesting that the benefit to motor function may be specific to a given muscle fiber-type.

Findings from this dissertation point to a potential beneficial action of exogenous muscle-supplied BDNF in an SBMA mouse model. Future studies should examine other cellular endpoints to delineate how muscle-supplied BDNF slows disease progression in a SBMA mouse model.

CHAPTER 2: Androgen-dependent loss of muscle BDNF mRNA in two mouse models of SBMA

I. Abstract

Tg expression of neurotrophic factors in skeletal muscle has been found to protect mice from neuromuscular disease, including SBMA, triggering renewed interest in neurotrophic factors as therapeutic agents for treating neuromuscular disease. Because SBMA is an androgen-dependent disease, and BDNF mediates effects of androgens on neuromuscular systems, we asked whether BDNF expression is impaired in two different Tg mouse models of SBMA, the so called “97Q” and “myogenic” SBMA models. The 97Q model globally overexpresses a full length human AR with 97 glutamine repeats whereas the myogenic model of SBMA overexpresses a Wt rat AR only in skeletal muscle fibers. Using qRT-PCR, we find that muscle BDNF mRNA declines in an androgen-dependent manner in both models, paralleling changes in motor function, with robust deficits (6-8 fold) in both fast and slow twitch muscle of impaired Tg males. Castration rescues or reverses disease-related deficits in muscle BDNF mRNA in both models, paralleling its effect on motor function. Moreover, when disease is acutely induced in Tg females, both motor function *and* muscle BDNF mRNA expression plummet, with the deficit in muscle BDNF emerging *before* overt motor dysfunction. That androgen-dependent motor dysfunction is tightly associated with a robust and early down-regulation of muscle BDNF mRNA suggests that BDNF delivered to skeletal muscle may have therapeutic value for SBMA.

II. Introduction

SBMA is an androgen-dependent neuromuscular disorder linked to a polyglutamine expansion mutation in the *AR* gene (La Spada et al., 1991). The disease affects middle-aged men and is characterized by progressive proximal muscle weakness and reduced androgen sensitivity. Muscle-derived neurotrophic factors (e.g., VEGF, IGF-1, GDNF, NT-4) have been

implicated in SBMA (Yamamoto et al., 1999; Sopher et al., 2004; Yu et al., 2006; Monks et al., 2007; Mo et al., 2010); their expression is often reduced by disease and experimentally bolstering their expression in muscle can rescue SBMA mice from disease (Palazzolo et al., 2009). One possible mechanism by which muscle-derived neurotrophic factors combat disease is by correcting disease-related impairments in retrograde axonal transport (Kemp et al., 2011).

BDNF, a member of the neurotrophin family, is also produced by skeletal muscle and implicated in neuromuscular disease (Mitsumoto et al., 1994; Sagot et al., 1998; Kust et al., 2002; Korkmaz et al., 2014) but has not been investigated as a possible player in SBMA. Because BDNF mediates effects of androgens on neuromuscular systems (Ottem et al., 2013; Verhovshek et al., 2013), and SBMA is an androgen-dependent neuromuscular disease, we examined BDNF expression and its androgen-dependence using qRT-PCR in two Tg mouse models of SBMA, the “97Q” and “myogenic” models.

While both models exhibit marked androgen-dependent motor dysfunction that affects only males, recapitulating core features of SBMA (Kennedy et al., 1968; Kinirons and Rouleau, 2008), the genetics behind each model are distinct. Disease in the 97Q model is triggered by global overexpression of a full length human AR containing a 97 polyglutamine tract (Katsuno et al., 2002) whereas disease in the myogenic model is triggered by overexpression of a Wt rat AR exclusively in muscle fibers (Monks et al., 2007). While not fully understood, Wt and polyglutamine expanded AR appear to trigger disease via the same toxic mechanisms (Nedelsky et al., 2010). Thus, the myogenic model offers an opportunity for identifying muscle-specific mechanisms which critically drive SBMA (Cortes et al., 2014b; Lieberman et al., 2014). Studying this model may also shed light on why some men with SBMA symptoms (muscle weakness, elevated CK levels, and gynecomastia) lack the disease-associated CAG expansion in their *AR* gene (Ferlini et al., 1995; Jobsis et al., 1995; Mariotti et al., 2000). Our goal is to identify common pathogenic processes across diverse models of SBMA, reasoning that such processes are also likely to critically mediate SBMA in humans.

The *BDNF* gene undergoes alternative splicing to produce multiple transcript variants. Each variant contains a non-coding exon (I – XIII) and a common coding exon (IX), with non-coding exons differentially expressed across tissue types (Aid et al., 2007). We chose to examine the historically better characterized exons: noncoding exons II, IV, and VI and the coding exon IX. We also examined the expression of BDNF receptors TrkB (full length and truncated) and pan-neurotrophin p75, since complement changes in the level of full length TrkB, for example, could effectively maintain BDNF signaling at a normal level in the face of changes in BDNF expression itself. Both the full length TrkB and p75 receptors signal upon ligand binding (Reichardt, 2006), but the truncated TrkB isoform does not and may serve to concentrate BDNF at critical sites (Huang and Reichardt, 2003).

We find that BDNF mRNA expression in skeletal muscle tightly correlates with motor dysfunction in two SBMA mouse models; levels of muscle BDNF mRNA wax and wane in an androgen-dependent manner, paralleling the effects of androgen on motor function. Furthermore, we find that the deficit in muscle BDNF *precedes* the overt expression of motor symptoms. We do not find that disease affects the expression of TrkB and p75 mRNA in either skeletal muscle or the lumbar spinal cord nor is BDNF mRNA expression affected in the lumbar spinal cord. These data suggest that deficits in muscle BDNF may critically underlie the loss of motor function in SBMA.

III. Materials and methods

i. Animals

Animal colonies were held on a 12h:12h light:dark cycle, group housed, and provided food and water *ad libitum*. All animal procedures were approved and performed in compliance with the Michigan State University Institutional Animal Care and Use Committee in accordance with the standards in the *NIH Guide for the Care and Use of Laboratory Animals.*

97Q model: Tg animals ubiquitously overexpressing a full length human AR with a 97 glutamine repeat and Wt age-matched controls were maintained on a C57Bl/6J genetic background. Mice were genotyped using polymerase chain reaction (PCR) at weaning as previously described (Katsuno et al., 2002).

Myogenic model: Tg animals overexpressing the rat Wt AR exclusively in skeletal muscle fibers and Wt age-matched controls were maintained on a C57Bl/6J genetic background. Mice were genotyped using PCR at weaning as previously described (Monks et al., 2007). Tg and Wt males were exposed prenatally to flutamide, an anti-androgen, to block the effects of prenatal endogenous androgens to enhance survival rate of Tg males neonatally (Johansen et al., 2011). Because survival of Tg females is not affected, females used in our studies were from litters not exposed to flutamide prenatally. In each case, Wt controls used in a given study were treated in the same way as the Tg mice.

ii. Gonadally intact males

We examined BDNF mRNA expression in the fast twitch EDL and slow twitch soleus muscles of adult, gonadally intact age-matched Wt and Tg males (mean age and range in postnatal days: 97Q: 69.1, 59-76; myogenic: 180.4, 171-194). We also assessed BDNF mRNA in lumbar spinal cords of the same Tg and Wt males of the two models. Tissue was harvested from chronically impaired myogenic males with severe motor dysfunction, comparable to what has previously been reported for this Tg model (Monks et al., 2007; Johansen et al., 2011). Tissue was harvested from 97Q males once they reached end-stage, defined as hang time < 30 seconds (see description of test below).

iii. Castrated males

97Q model: Tg and Wt males were castrated (Tg+Castrate, Wt+Castrate) or sham castrated (Tg+Sham, Wt+Sham) on postnatal day 28-32, before symptoms emerged as

previously described (Renier et al., 2014). We monitored motor function twice weekly starting on the day of but prior to surgery, until gonadally intact (Tg+Sham) males reached end-stage (hang time <30 sec), at which point it and its age-matched controls (Tg+Castrate, Wt+Castrate and Wt+Sham) were sacrificed and muscles harvested (mean age and range in postnatal days: 77.8, 55-104).

Myogenic model: Since chronically affected adult myogenic males recover motor function after castration (Monks et al., 2007; Kemp et al., 2011), we castrated a cohort of age-matched adult Tg and Wt brothers to assess whether expression of BDNF mRNA in skeletal muscle of diseased males also recovers. Tg and Wt males were either castrated (Tg+Castrate, Wt+Castrate) or sham castrated (Tg+Sham, Wt+Sham)(mean age and range of postnatal days at castration: 106.3, 87-133). Motor function (described below) was monitored starting on the day of but just before surgery and on days 2, 4, 6, 8, 14, and 21 after surgery. Animals were sacrificed after the last motor test.

iv. Androgen treated females

Tg females offer the experimental advantage in that disease is triggered in this model by exposure to male levels of testosterone (T). Thus, the time of disease onset is known and readily controlled, making this model ideal for examining the time course of change in muscle BDNF mRNA as disease develops. We first examined whether acutely diseased females (age matched to Wt females) exposed to five days of T show the same deficit in muscle BDNF as chronically diseased myogenic males (mean age and range in postnatal days: 117.7, 101-178). Treatment was delivered via Silastic capsules containing either crystalline T (effective release length: 6mm, inner diameter: 1mm, outer diameter: 3mm; soaked in sterile phosphate-buffered saline overnight) or nothing (“Blank”) were implanted subcutaneously just caudal to the scapula under deep isoflurane anesthesia. Such T capsules produce T levels slightly below normal circulating levels for adult male mice (Johansen et al., 2009). Motor function of female mice was

assessed immediately prior to surgery and daily during the five day treatment period, with tissue harvested on the final day of motor testing.

Because acutely disease myogenic (Tg) females also show a deficit in muscle BDNF mRNA after five days of T exposure, we next asked how soon the deficit emerges. We used a separate cohort of age-matched Tg and Wt females to assess BDNF mRNA levels in the soleus muscle after 1, 2, 3, 4, or 5 days of T exposure, monitoring motor function daily based on the hang test (mean age and range in postnatal days: 97.5, 81-130).

v. Motor function tests

Motor function was assessed using three different measures: number of rears in an open field, grip strength, and hang time as previously described (Johansen et al., 2011). The hang test provides a measure of overall limb strength. If the hang time on the first try was less than the maximum time possible, 120 sec, then mice were given two more tries with the highest score recorded for that session on that day.

vi. Tissue collection

The EDL and/or soleus were collected on both sides from deeply anesthetized mice. Lumbar spinal cords were quickly extracted with pressurized air. All tissue was weighed fresh and immediately frozen in RNase-free tubes on dry ice, and held at -80°C until processed. All instruments were sprayed with RNaseZap (Sigma-Aldrich) between animal harvests. Additionally, blood was collected intracardially and plasma isolated to measure circulating plasma T levels as previously described (Johansen et al., 2009).

vii. RNA extraction and quantitative real-time PCR

RNA extraction was performed for the muscles with RNeasy Fibrous Tissue Mini Kit (Qiagen). To improve RNA yield for spinal cords, we used TRIzol reagent (Ambion). Tissue was

mechanically homogenized with a PRO200 homogenizer (Pro Scientific). RNA was purified for each type of tissue according to the respective manufacturer directions. Spinal cord RNA samples were treated with DNase I (Invitrogen). A DNase treatment step is included as part of the Qiagen protocol for muscles. Following extraction, RNA was quantified on a spectrophotometer (Beckman DU 530) by measuring 260 nm absorbance values. RNA for both muscles and lumbar spinal cord was then reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with the following thermocycle: 25°C for 10 min, 37°C for 2 h, 85°C for 5 min. Each sample for quantitative real-time PCR assay included 2.5 ng of cDNA, primers, and Power SYBR Green PCR Master Mix (Applied Biosystems). Thermocycle for the quantitative step on the ABI PRISM 7000 Sequence Detection System was as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. A dissociation curve was determined for each well to confirm that the correct gene was being amplified. Each sample was run in triplicate. Samples without reverse transcriptase during the cDNA conversion were also assessed to ensure that there was no DNA contamination. The reference genes were Cyclophilin A (200nM primers: ccactgtcgcttttcgccgc and cccaagggctcgtcatcggc), GAPDH (200nM primers: ccccagcaaggacactgagcaag and tctgggatggaaattgtgagggaga), β-actin (200nM primers: ttcgttgccggtccacaccc and tttgcacatgccggagccgt), and 18s (100nM primers: either ggaccagagcgaaagcatttg and gccagtcggcatcgtttatg or ttgacggaagggcaccaccag and gcaccaccacccacggaatcg). The reference gene was chosen for each experiment by confirming that levels were equivalent between treatment groups. Transcripts of two non-coding and one coding (i.e., total) BDNF exons were quantified: BDNF IV (200nM primers: ctccgccatgcaatttccac and cgagtctttggtggccgata), BDNF VI (200nM primers: gtgacaacaatgtgactccac and gccttcatgcaaccgaagta), and BDNF IX (200nM primers: gcggcagataaaaagactgc and tcagttggcctttggatacc). We measured an additional non-coding transcript in spinal cord, BDNF II (100nM primers: aacctttttcctcctcctgcg and gagccgaacctcggaaaaga), as this transcript is highly expressed in neural tissue but not in muscle

(Aid et al., 2007). We also measured levels of BDNF's receptors in muscle and spinal cord: full length TrkB (400nM primers: ggcaacttcgggaaaggaga and gtaaacccctcaccgcctac), truncated TrkB (600nM primers: ccattgccctctgctaacca and gagatctgaggtgctctcgc), and p75 (200 nM primers: cgtgaccatctcaggccttt and ggtgcccctgttaccttctc). Optimal concentrations and amplification efficiencies were calculated for each primer set.

viii. Statistical analysis

Relative Expression Software Tool (REST) was used to assess statistical significance and fold change of genes (Pfaffl et al., 2002). Specifically, this software uses the non-parametric Pair-Wise Fixed Reallocation Randomisation Test to account for amplification efficiencies when determining fold change. It measures relative expression of a target gene (BDNF, TrkB, p75) between two experimental groups following the normalization of the target gene to a reference gene (Cyclophilin A, GAPDH, β-actin, or 18s).

We performed a two-factor between subjects analysis of variance to test whether genotype (Tg or Wt) or treatment (sham versus castrate (in males); T versus blank (in females)) influenced motor ability on the day of tissue harvest. Post-hoc pairwise comparisons with a Bonferroni correction were used to determine significant differences between planned comparisons.

IV. Results

i. Diseased 97Q and myogenic male mice show comparable deficits in muscle BDNF mRNA

We find significant deficits in both the coding transcript IX and the noncoding transcripts IV and VI in both the EDL and soleus muscles of symptomatic 97Q and myogenic males (Figure 2). On average, we observe a ~6-7 fold deficit in BDNF transcripts in both the EDL and soleus

of both models compared to these same muscles from their respective Wt controls. However, we find no significant changes in BDNF mRNA levels in the lumbar spinal cord (all $p>.05$ for transcripts II, IV, VI, and IX; 97Q: n=6/group and myogenic: n=5/group) where EDL and soleus motoneurons are located, nor did we detect changes in BDNF receptors TrkB (truncated or full-length) or p75 in muscles or the lumbar spinal cord ($p>0.05$) of either model except for a slight increase (1.8 fold) in truncated TrkB in the EDL of 97Q males ($p=0.026$). We found no differences in circulating T levels between gonadally intact Tg and Wt males for either the 97Q or myogenic models (data not shown), indicating that differences in BDNF expression between Tg and Wt males are not due to differences in circulating T levels.

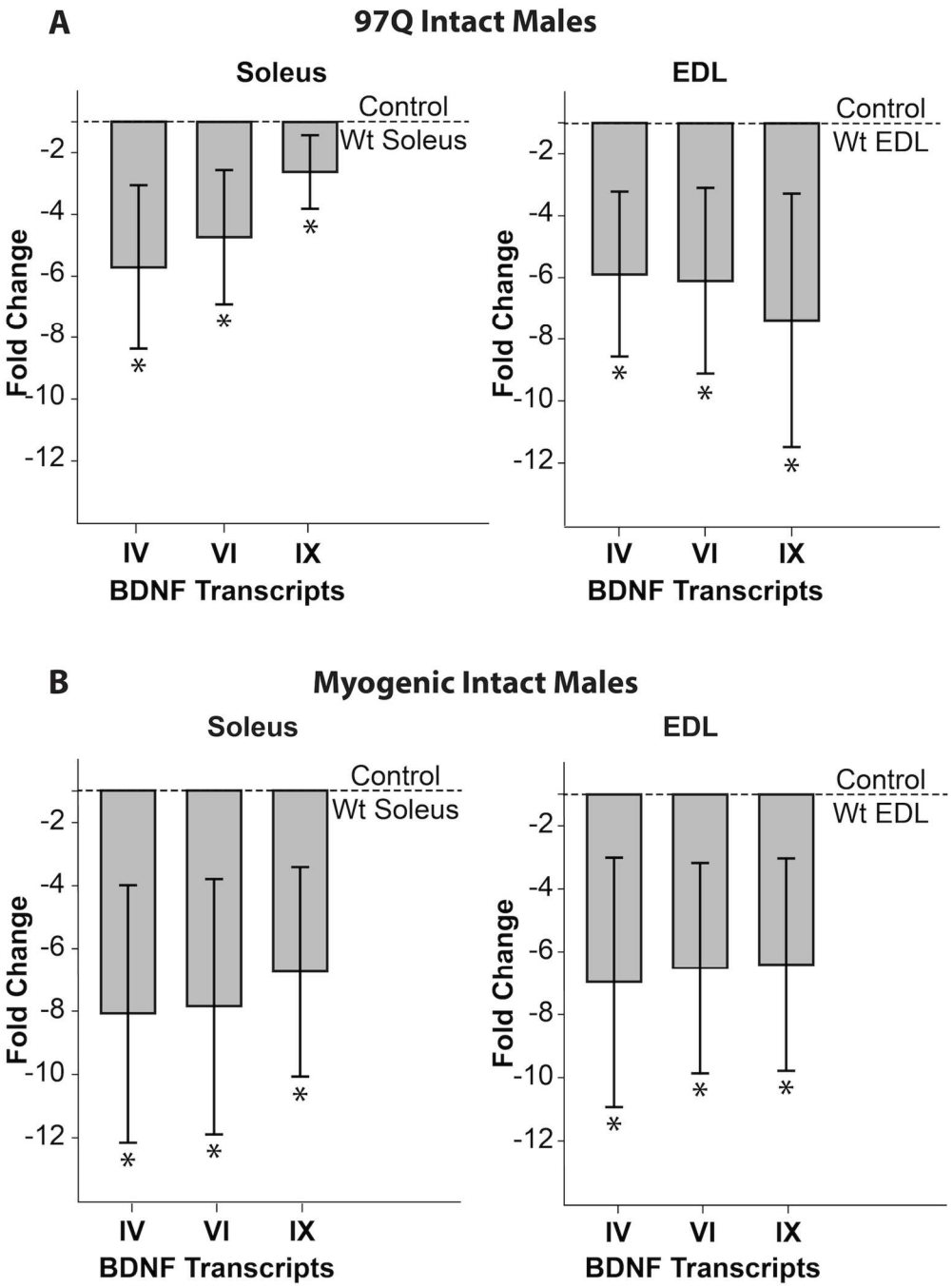

Figure 2. 97Q and myogenic transgenic (Tg) males show comparable disease-related deficits in BDNF transcripts in skeletal muscle. Both slow twitch soleus and fast twitch extensor digitorum longus (EDL) muscles of motor-impaired mice show significant deficits in all three BDNF transcripts (noncoding IV and VI and the coding IX) based on qPCR (A, B). Since the disease-causing AR is expressed only in the muscle fibers of myogenic mice, these data indicate that AR can act directly in the muscle fibers to down-regulate BDNF mRNA (A), possibly triggering dysfunction not only in skeletal muscles, but in the motoneurons too. All plotted values are relative to Wt controls (dashed line). Error bars represent standard error of the mean. n=6-7/group. * $p<0.01$ from Wt control.

ii. Castration rescues BDNF mRNA expression in muscles of Tg 97Q and myogenic males as well as motor function

To examine whether the disease-related loss in muscle BDNF transcripts respects the same rules as motor function itself, we asked whether castration of Tg male mice improves BDNF expression in skeletal muscle, comparable to its effect on motor function. Because motor dysfunction emerges after the onset of puberty in 97Q males, we took advantage of this model to ask whether castration before symptom onset can *protect* BDNF expression from decline, like its protective effect on motor function. In contrast, because myogenic males are chronically impaired from birth, we took advantage of this model to ask whether castration of impaired adult males can *reverse* a disease-related deficit in muscle BDNF mRNA. Given that the effect of disease on BDNF expression did not depend on fiber type composition of the muscle, we examined this question only in the soleus.

We find that castration of asymptomatic 97Q males at the start of puberty protects animals from impairments in BDNF mRNA expression in skeletal muscle, paralleling its effect on motor function, maintaining both at Wt levels (Figure 3). These data show that the transgene alone does not cause the deficit in muscle BDNF mRNA. We note that castration at puberty had no effect on either muscle BDNF expression or motor function in Wt males. We also find that after three weeks, castration of chronically diseased adult myogenic males largely reverses both deficits in motor function and BDNF mRNA (Figure 4), while having no effect on levels of BDNF mRNA in muscle of Wt males. Although neither motor function nor expression of BDNF mRNA fully recovered in castrated myogenic Tg males, both measures approached Wt levels. These data also make it clear that the AR transgene alone does not cause the BDNF deficit. In sum, BDNF mRNA expression in skeletal muscles of 97Q and myogenic SBMA males depends on androgens and is tightly correlated with disease symptoms, suggesting that the loss of muscle-derived BDNF may trigger a loss in function. To further explore this possibility, we next asked whether the loss in muscle BDNF mRNA precedes the loss of motor function using a model in

which disease is acutely induced in adulthood.

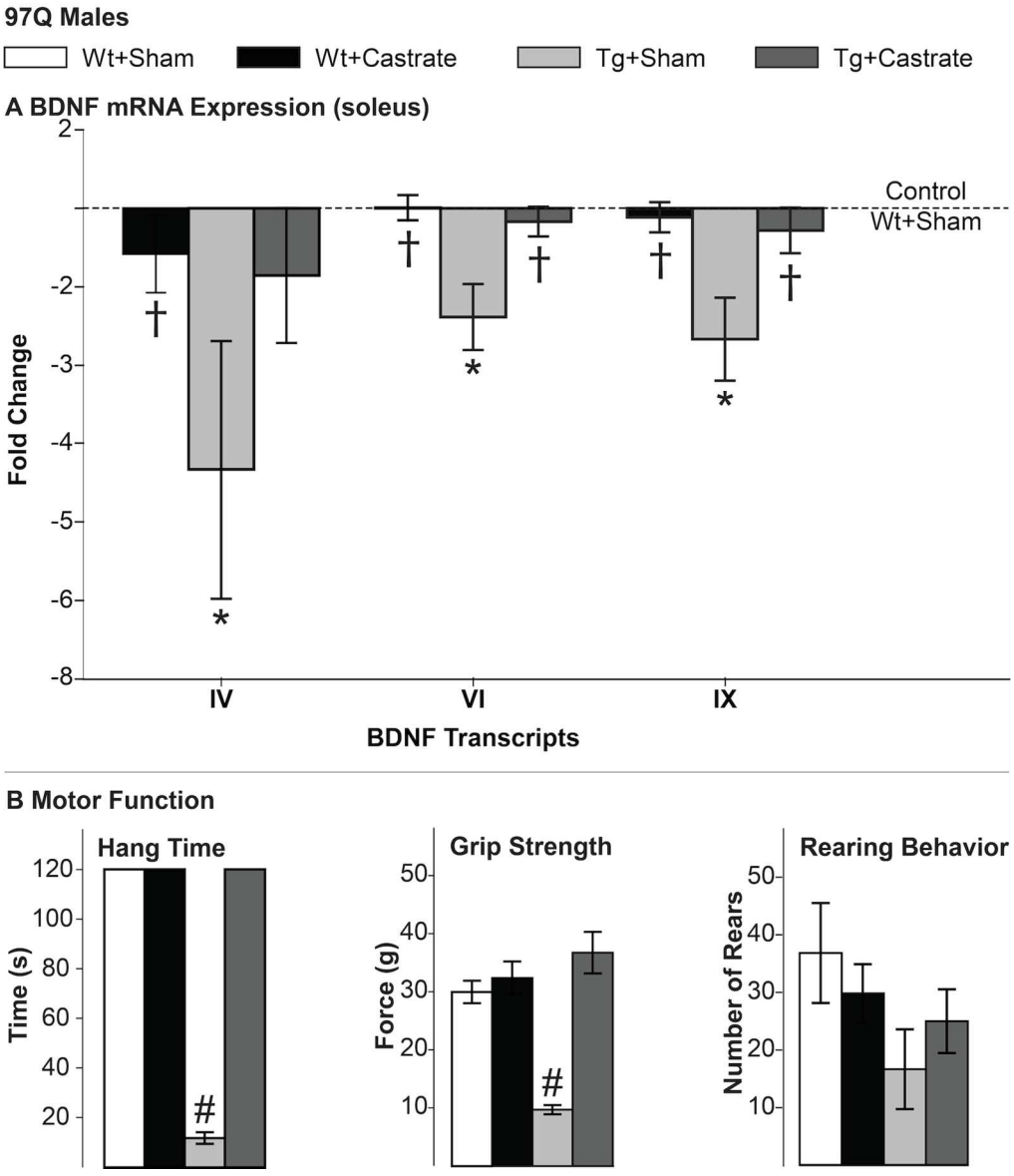

Figure 3. Presymptomatic castration rescues 97Q males from deficits in both muscle BDNF mRNA and motor function. Deficits in muscle BDNF mRNA and motor function of gonadally-intact, end-stage 97Q Tg males are not evident in 97Q males castrated at puberty. Both BDNF expression and motor function of castrated Tg males are comparable to Wt males. Note that we find the same deficit in BDNF mRNA in gonadally-intact 97Q males as in the first experiment (Figure 2A) and that castration of Wt males has no effect on BDNF mRNA (A), nor on motor function. Error bars represent standard error of the mean. n=6/group. A) * $p<0.01$ from Wt+Sham, † $p<0.05$ from Tg+Sham. B) # $p<0.05$ Tg+Castrate versus Tg+Sham.

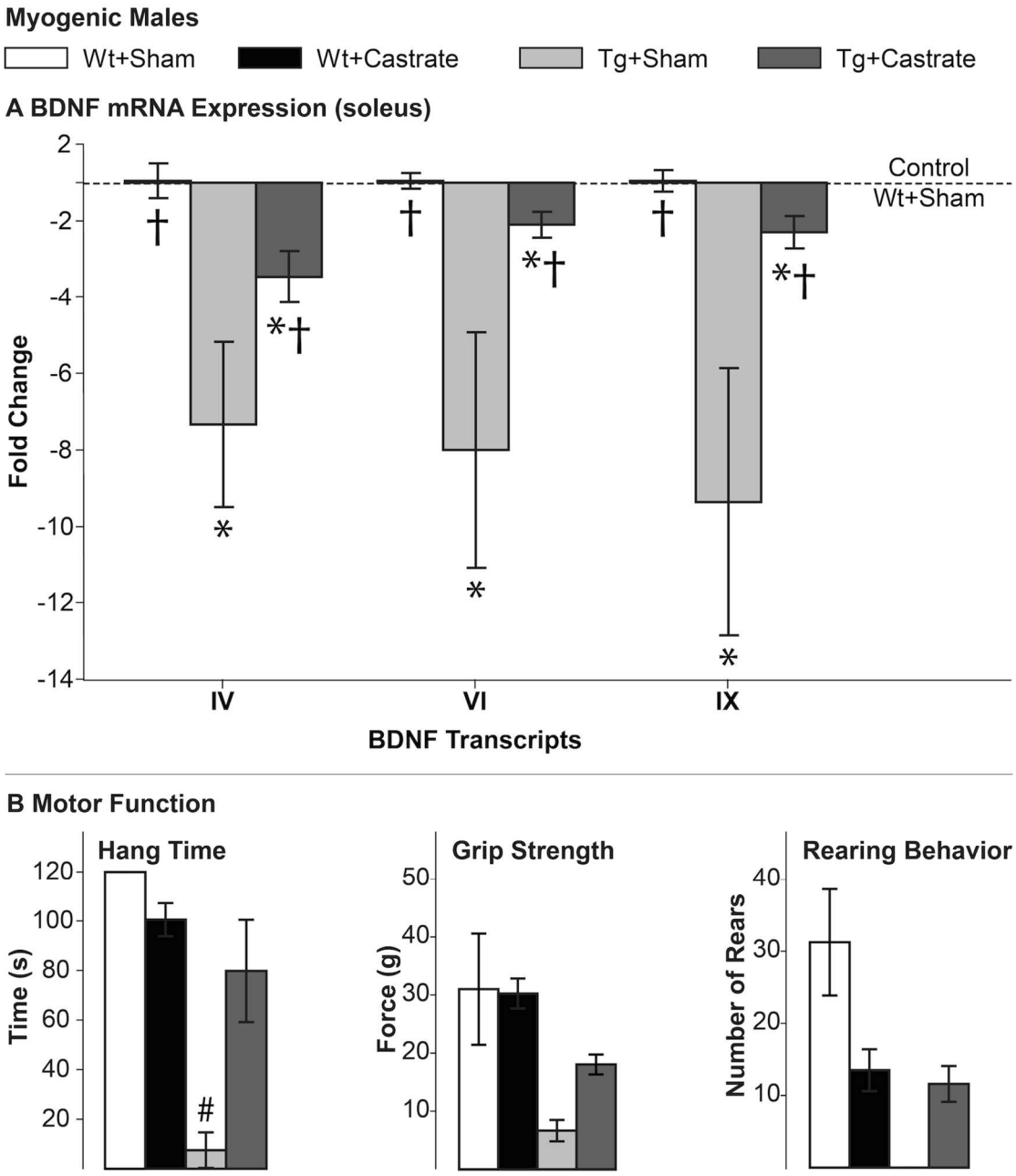

Figure 4. Castration of chronically diseased myogenic males reverses deficits in both muscle BDNF mRNA expression and motor function. Deficits in muscle BDNF (A) and motor function (B) found in gonadally intact Tg males are largely reversed by castration, indicating that defects in both are androgen-dependent. Note that we find comparable BDNF deficits in gonadally-intact Tg males as seen in first experiment (Fig 2B) and that castration of Wt males affects neither BDNF expression nor motor function based on hang times and grip strength but does lower rearing behavior, an androgen-dependent measure of anxiety. That castration did not reverse the rearing deficit in Tg males may reflect increased anxiety-like behavior and not an effect on motor function per se. Error bars represent standard error of the mean. n=4-5/group. A) * $p<0.01$ from Wt+Sham, † $p<0.05$ from Tg+Sham. B) # $p<0.05$ Tg+Castrate versus Tg+Sham.

iii. Deficit in muscle BDNF mRNA levels in acutely diseased myogenic females

Five days of T exposure induced a > 6 fold drop in BDNF mRNA in the soleus of acutely diseased Tg females (Figure 5A), comparable to that seen in chronically diseased myogenic males (Figure 2B). The effect of androgens on expression of BDNF mRNA in muscle again shows a striking correlation with motor function; each are severely compromised after five days of T treatment (Figure 5B). We also found that the level of BDNF mRNA level in the muscle of control Tg females is reduced (<2 fold, Figure 5A) relative to Wt controls, albeit much less than in diseased muscle. While such females exhibit normal motor function (Figure 5B), this somewhat compromised expression of BDNF may account for why motor dysfunction develops so rapidly in Tg females exposed to androgens. These data indicate that the system can tolerate some loss of BDNF expression without deleterious effects on motor function. Treatment with androgens had no influence on BDNF mRNA levels in Wt muscle (p>0.05 for all transcripts), as seen previously in Wt males (Figures 3 and 4).

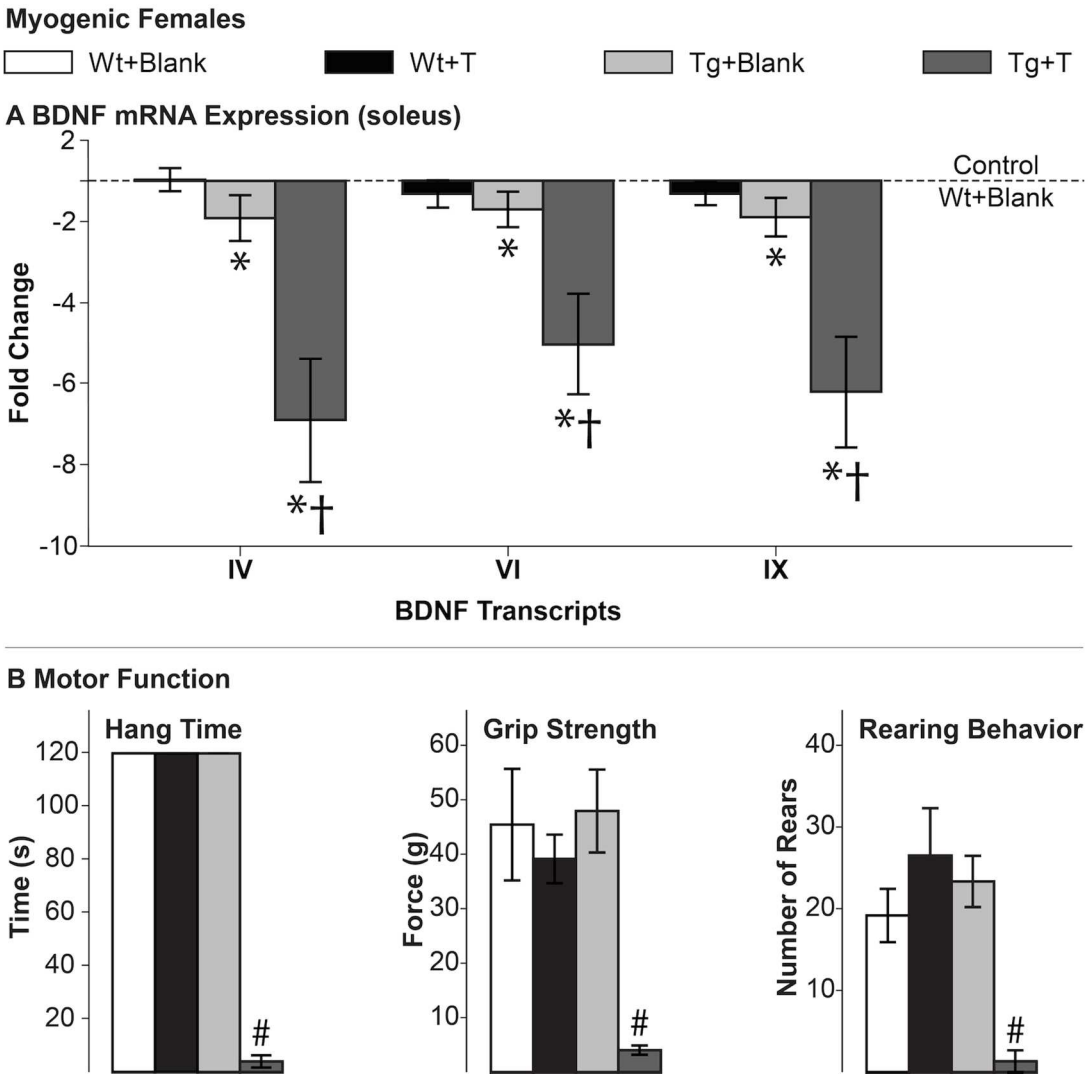

Figure 5. Androgen treatment of myogenic females induces significant losses in both muscle BDNF mRNA and motor function. Exposing asymptomatic myogenic Tg females to testosterone (T) for only 5 days induces a significant, more than 6-fold, decrease in muscle BDNF transcript levels (A) compared to either T-treated Wt controls or asymptomatic control-treated Tg females (Tg+Blank). Androgen's effect on BDNF expression in muscle of Tg females correlates with a robust induction of motor dysfunction (B). Control treated Tg females show a small but significant deficit (< 2 fold) in muscle BDNF mRNA compared to Wt controls (Wt+Blank, dashed line, A) despite having normal motor function, suggesting that a small deficit in BDNF mRNA in asymptomatic Tg females may prime the system for rapid collapse once exposed to male levels of androgens. Error bars represent standard error of the mean. A) * indicates $p<0.05$ from Wt+Blank, † indicates $p<0.05$ from Tg+Blank. B) # $p<0.05$ Tg+Blank versus Tg+T.

Given that motor function declines in a highly predictable manner in this acute model, we next determined *how soon* the >6 fold deficit in muscle BDNF would emerge by examining BDNF mRNA expression in the soleus of Tg females after 1 to 5 days of T treatment. Of note, we included a 5-day treated group in this experiment to determine whether we could replicate the finding of reduced BDNF at this time point from the previous experiment. Because disease had comparable effects on all three transcripts in the first experiment, we measured only the levels of the coding transcript IX in this experiment. Not only did we replicate the same deficit after 5 days of androgen treatment, but we find the same > 6 fold deficit after only 24 hours of T exposure (Figure 6A), a full 48 hours *prior to any overt motor dysfunction*. A deficit in hang time became apparent only after 3 days of T treatment (Figure 6B). The decline in BDNF expression also preceded the decline in open field performance and grip strength (data not shown). Interestingly, we did not find a significant deficit in muscle BDNF mRNA in control treated myogenic females in this cohort of females (p=0.179). This may be because this cohort was slightly younger than in the previous experiment (97.5±1.7 days vs. 117.7 ± 4.2 days).

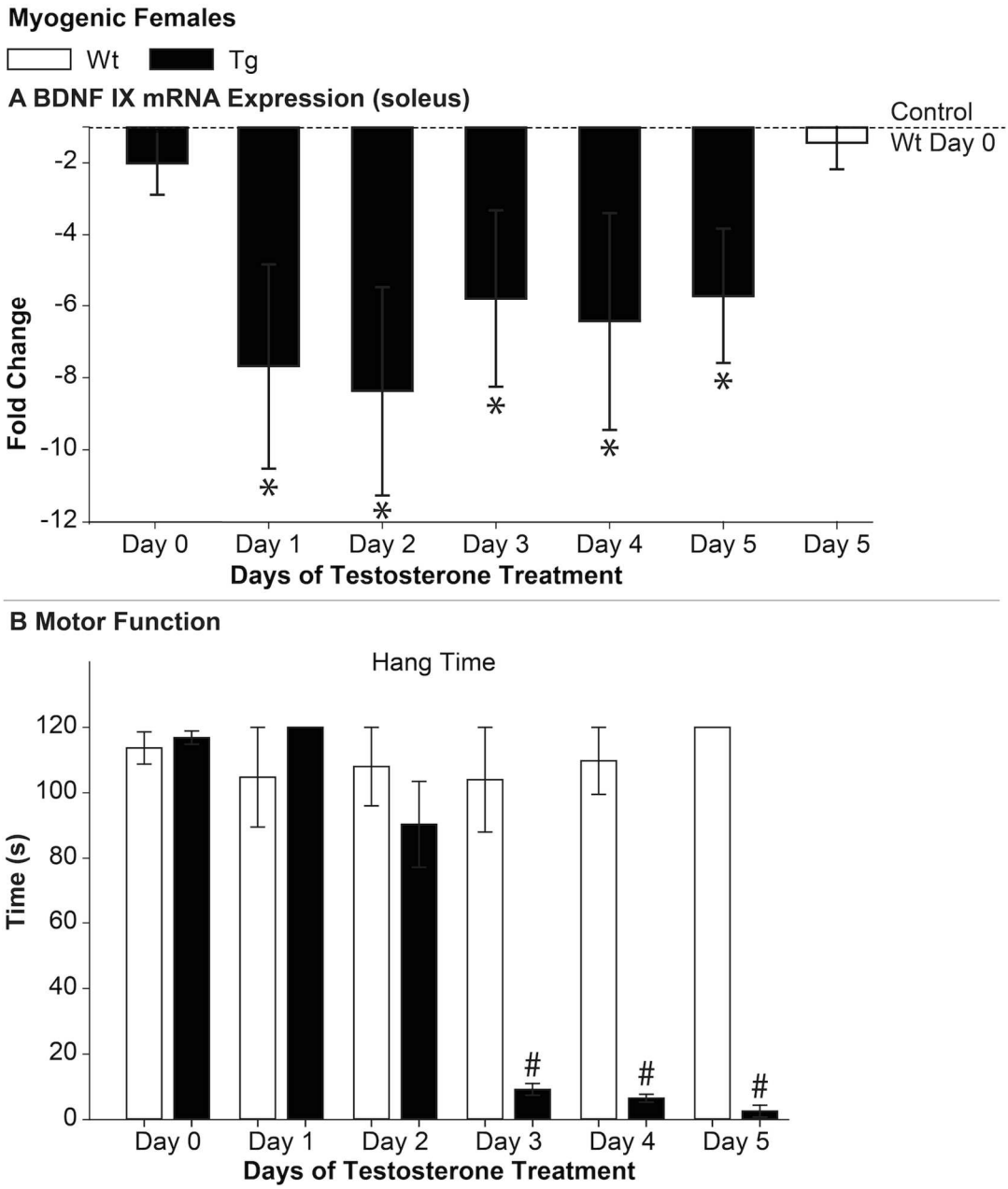

Figure 6. Androgen induces the deficit in muscle BDNF mRNA before detectable motor dysfunction in acutely diseased myogenic females. Muscle BDNF mRNA was significantly reduced after only one day of testosterone (T) treatment (A). Importantly, this deficit preceded the loss in overt motor function; deficits in hang time were evident starting at three days of T treatment but not before. The magnitude of the deficit at 24 hours was not only comparable to that at 5 days of T treatment in Tg females but also comparable to that of chronically affected myogenic and 97Q Tg males (Figure 2), suggesting that a defect in BDNF mRNA expression in skeletal muscle is an early event in disease progression and might trigger the loss in overt motor function. Error bars represent standard error of the mean. n=4-6/group. A) * indicates $p<0.05$ from Wt Day 0. B) # $p<0.05$ Wt versus Tg.

V. Discussion

We find that BDNF mRNA levels are robustly down-regulated in skeletal muscles of motor-impaired male mice in both the 97Q and myogenic models of SBMA. Because motor symptoms in these models are androgen-dependent, we asked whether castration of Tg males could also rescue BDNF mRNA expression, comparable to its beneficial effect on motor function (Katsuno et al., 2002; Monks et al., 2007; Kemp et al., 2011). We also asked the complement question of whether the deficit in BDNF expression in skeletal muscle could be acutely induced by androgens in myogenic females, like motor dysfunction in this model, and if so, whether the deficit emerged before overt motor function. We find that changes in muscle BDNF mRNA parallel changes in motor function in both male Tg models. In the acute female model, we find that the deficit in muscle BDNF mRNA develops within 24 hours of androgen exposure, emerging *before* overt motor dysfunction, supporting the idea that BDNF may critically underlie the loss in motor function.

BDNF expression in skeletal muscle has been shown to be controlled by factors other than disease, including circulating T levels and muscle activity levels (i.e., exercise). Unexpectedly, we find no effect of androgens on the expression of BDNF mRNA in the muscles examined of either male or female Wt mice, contrary to previously published results in rats (Osborne et al., 2007; Ottem et al., 2007; Verhovshek et al., 2010). Nonetheless, the pattern of results in myogenic and 97Q Tg mice underscores the fact that only the combination of T *and* the disease allele leads to impairments in both BDNF mRNA expression and motor function in these SBMA models. Because diseased mice are less active, inactivity may play some role in the loss of BDNF in motor-impaired mice. However, the effect of being less active is likely minor given that complete inactivity of the soleus for one week results in only a 30% decline in the amount of muscle BDNF mRNA (Gomez-Pinilla et al., 2002) whereas disease leads to a deficit in muscle BDNF mRNA that is far greater, a 6-8 fold deficit. Moreover, we find that the deficit in muscle BDNF mRNA is *independent* of activity since T-treated myogenic females show the

deficit well before any loss in motor function. While the *BDNF* gene contains an estrogen response element (Sohrabji et al., 1995), there is no known ARE. One possible mechanism by which *BDNF* expression may be dysregulated in SBMA is through disruption of an AR regulated cyclic AMP-mediated pathway (reviewed in Verhovshek et al., 2013).

There is some dispute over whether the myogenic model is a legitimate mouse model for SBMA since motor dysfunction in this model is triggered by Wt AR rather than polyglutamine-expanded AR (La Spada et al., 1991). There are several compelling reasons to think that the myogenic model has clinical relevance to SBMA. First and foremost, the disease phenotype is expressed only in Tg males and not Tg females, showing the same male-bias as for SBMA in humans and as seen in other mouse models recapitulating this disease (Katsuno et al., 2002; Chevalier-Larsen et al., 2004; Yu et al., 2006; Monks et al., 2007). Disease in the myogenic mice is also androgen-dependent (Monks et al., 2007; Johansen et al., 2009; Kemp et al., 2011; Renier et al., 2014), exhibiting another core trait of SBMA. Moreover, results of two recent studies fully support the conclusions based on the myogenic model that muscle AR critically underlies SBMA pathology in both muscles and the motoneurons (Cortes et al., 2014b; Lieberman et al., 2014). There is also growing precedence linking both mutant and Wt alleles of the same gene to the same neurodegenerative disease. Notable examples in the human population include superoxide dismutase 1 linked to ALS (Bosco et al., 2010) and α-synuclein linked to Parkinson's disease (Singleton et al., 2003). Wt AR has also been shown to exert comparable androgen-dependent toxicity as the polyglutamine-expanded AR in both mouse and fly models of SBMA, and seems to engage common toxic pathways (Mo et al., 2010; Nedelsky et al., 2010). Results of the myogenic model also help to explain the >17% of affected men diagnosed with SBMA that show the expected cluster of symptoms (e.g., slowly progressing motor dysfunction coupled with signs of partial androgen insensitivity (e.g., gynecomastia) and elevated serum CK levels) but *lack* the polyglutamine expansion in their AR gene (Ferlini et al., 1995; Mariotti et al., 2000). Finally, because we find comparable defects in BDNF expression in

a mouse model that expresses a polyglutamine-expanded AR, it is clear that the deficit in muscle BDNF mRNA is not unique to the myogenic model, and thus likely a more general pathogenic mechanism underlying the loss of motor function in SBMA.

As SBMA is an androgen-dependent disease, we asked whether castration of Tg male mice could rescue BDNF expression, akin to its effects on motor function. Castration of presymptomatic 97Q males fully protects the expression of muscle BDNF mRNA, paralleling the protective role of castration on motor function (Figure 3). These data make it clear that nonspecific effects of the transgene are minimal if nonexistent. Note, however, that it is not clear whether castration *prevents* or *reverses* the decline, as prepubertal levels of BDNF were not determined in these animals. Moreover, after three weeks of castration, chronically impaired myogenic males show noticeably improved motor function *and* BDNF mRNA expression, each nearly at Wt levels (Figure 4). While it is possible that the transgene partially impairs BDNF expression independent of androgens in this model, the fact that neither BDNF expression nor motor function *fully* recovers argues against this possibility. Finding that androgen-dependent loss of muscle BDNF mRNA strongly correlates with motor dysfunction in two SBMA models suggests that defects in the expression of muscle BDNF mRNA may trigger neuromuscular dysfunction in SBMA.

To further probe the androgen dependence of the BDNF deficit, we treated myogenic females with T. When adult asymptomatic myogenic females are exposed to male levels of androgens for five days, both motor function and BDNF expression rapidly decline. Androgen-treated Tg females show severe deficits in grip strength, hang time, and rearing ability (Figure 5B), comparable to the severity of deficits seen in chronically impaired (gonadally intact) myogenic males (Figure 4B). Notably, we also saw comparable deficits in BDNF mRNA. The soleus muscle from acutely impaired myogenic females showed ~6 fold deficit in BDNF mRNA after only five days of T exposure, comparable to that found in chronically impaired Tg males, indicating that disease-related deficits in BDNF can be rapidly induced. While control-treated

myogenic females (Tg+Blank) had a small, but significant decrease (<2 fold) in BDNF mRNA, despite normal motor function, we did not replicate this deficit in the second experiment, questioning its significance. We also found that this same deficit in muscle BDNF mRNA emerges within 24 hours of T exposure, a full two days before motor dysfunction emerged. We were surprised to see the full magnitude of the deficit, about an ~8 fold deficit, in muscle BDNF mRNA at the one day mark. These data indicate that significant dysregulation in BDNF expression can occur early in the disease process and may in fact be a proximate event in disease, critically mediating the progressive loss of motor function.

To understand whether BDNF signaling *per se* might be altered, we also examined the expression of BDNF receptors, the high-affinity TrkB receptor (full length and truncated forms) and the pan-neurotrophin p75 receptor. We find no evidence of compensatory expression for these receptors at the mRNA level in either muscle or lumbar spinal cord, apart from a small increase in the truncated form of TrkB in one muscle of one model. While it is possible that other parts of the signaling pathway have compensated for the likely loss of BDNF protein, these results are consistent with the idea that both diseased muscles and motoneurons are deprived of the beneficial effects of BDNF.

The literature provides ample examples of how muscle-derived BDNF maintains key cellular functions including synaptic strength and axonal transport (Koliatsos et al., 1993; Lohof et al., 1993; Stoop and Poo, 1996; Sagot et al., 1998). We previously showed that axonal transport is perturbed in the myogenic SBMA model and can be rescued by exogenous VEGF locally applied to the muscle (Kemp et al., 2011). The results of the current study point to BDNF as another potential neurotrophic factor that may underlie deficits in axonal transport. BDNF has also been shown to overcome retrograde transport dysfunction caused by motoneuron disease (Sagot et al., 1998). Because factors other than BDNF are also perturbed in SBMA, it is always possible that deficits in some combination of neurotrophic factors underlie the overt loss in motor function. Nonetheless, it important to determine what part BDNF might play in the demise

of neuromuscular function in SBMA. For example, because NMJs are much weaker in diseased Tg males of both models (Jordan, unpublished data), the loss of muscle-supplied BDNF may be behind this deficit. Since TrkB receptors are present in all three cell types (motoneuron, Schwann cell, and muscle fiber) of the NMJ (reviewed in Pitts et al., 2006; Garcia et al., 2010c), BDNF may act at any of these locations to enhance synaptic communication. Presynaptically, BDNF might accomplish this through local protein translation, promoting actin polymerization and/or enhancing calcium influx, all known to enhance neurotransmitter release (reviewed in Park and Poo, 2013). Additionally, BDNF acts via the TrkB receptor to promote AChR clustering at the NMJ (Gonzalez et al., 1999; Kulakowski et al., 2011). Interestingly, our models show the same fragmentation of the junction (Jordan, unpublished data) as when TrkB signaling is blocked (Gonzalez et al., 1999; Kulakowski et al., 2011). Moreover, TrkB signaling also appears to be involved in maintaining the expression of the adult isoform of the voltage-gated sodium channel. When TrkB signaling is perturbed, skeletal muscles start expressing the neonatal isoform of the sodium channel (Kulakowski et al., 2011), which leads to slower and less reliable conduction of the action potential along muscle fibers. In sum, muscle-derived BDNF may act presynaptically on motor nerve terminals to mobilize neurotransmitter stores as well as postsynaptically to ensure high fidelity synaptic transmission and efficient excitation of muscle fibers, all of which contribute to high quality motor function. Moreover, because muscle-derived BDNF also promotes myofiber regeneration via the satellite cell population (Clow and Jasmin, 2010), the loss of muscle BDNF in SBMA may also cause fibers to be particularly prone to damage, consistent with the elevated levels of CK in SBMA patients (Chahin and Sorenson, 2009).

iv. Conclusions

Motor dysfunction in SBMA may critically depend on reduced levels of muscle-derived BDNF, suggesting that neuromuscular function can be rescued in disease simply by replenishing the supply of muscle BDNF. Potential sites of BDNF action for promoting

neuromuscular function include motoneurons, muscles, and Schwann cells. Despite a loss of muscle fibers and/or motoneurons, BDNF has the potential to not only promote recovery of function of the remaining motoneurons and muscle fibers, but also to promote the rebuilding of diseased muscle fibers by mobilizing the satellite cell population in the muscle.

CHAPTER 3: Transgenic overexpression of muscle BDNF may benefit slow-twitch muscles to slow disease progression in an SBMA mouse model

I. Abstract

SBMA is an androgen-dependent neuromuscular disease that affects men, resulting in progressive muscle weakness. SBMA mouse models exhibit transcriptional dysregulation in muscle, reduced muscle contractile ability, and defects in neuromuscular transmission. BDNF is a small protein that is produced by muscle and potentiates neuromuscular function; we sought to examine whether enhancing its expression in muscle could ameliorate SBMA symptoms. We previously reported reduced expression of BDNF mRNA levels in diseased muscle of two mouse models of SBMA, a feature that was androgen-dependent and correlated with motor function. In this study, we selectively overexpressed muscle BDNF using a Cre-loxP Tg mouse, which was then crossed to a Tg mouse model of SBMA. We found that overexpressing muscle BDNF slowed SBMA disease progression, doubling the time it took mice to reach endstage after symptom onset. We also found that this intervention rescued mRNA expression of the adult isoform for the slow myosin heavy chain in the slow-twitch soleus, perhaps leading to improved muscle contractile function that may underlie the improved performance on the hang test. Finally, we found that mRNA expression of the developmental isoform of the AChR subunit (gamma) was rescued by Tg BDNF in the soleus, predicting that decay time of synaptic potentials is also restored to normal. Unlike for the soleus, we did not see any beneficial effects of Tg expression of BDNF in a fast-twitch muscle, neither on gene expression, synaptic function, nor muscle function. Our results point to a muscle fiber-specific benefit of BDNF, whereby muscle BDNF appears to protect slow muscles from certain aspects of the disease in SBMA.

II. Introduction

SBMA is a progressive, late-onset neuromuscular disease that affects only men. SBMA symptoms include androgen-dependent muscle atrophy and a loss of motor function; muscle weakness may manifest as difficulty in walking, talking, and swallowing. Partial androgen insensitivity (e.g., gynecomastia, testicular atrophy, infertility) is also characteristic of SBMA (Katsuno et al., 2012), although it does not cause SBMA since androgen insensitivity alone is not accompanied by motor dysfunction (Zuloaga et al., 2008). The underlying genetic mutation linked to SBMA is a polyglutamine expansion in the AR (La Spada et al., 1991).

Recent studies point to muscle as a primary site of disease pathology. Overexpression of the disease allele (AR) exclusively in muscle cells causes SBMA-like symptoms in mice (Monks et al., 2007; Johansen et al., 2009), while removing the disease-causing AR from only skeletal muscle eliminates disease symptoms (Cortes et al., 2014b; Lieberman et al., 2014). Motor dysfunction in SBMA may be caused by weak neuromuscular synapses (Xu et al., 2016) and/or by weakness in the muscle per se (Musa et al., 2011; Oki et al., 2013; Oki et al., 2015). Since healthy neuromuscular function depends on muscle-produced neurotrophic factors, which can act on *both* muscles and their motoneurons to maintain their function, deficits in such factors may explain the loss in strength of muscles and/or their neuromuscular synapses in SBMA.

Expression of several neurotrophic factors, including VEGF, NT-4, GDNF, are low in muscles of diseased SBMA mice (Yu et al., 2006; Monks et al., 2007). Importantly, we previously found that BDNF is reduced in both fast and slow twitch muscles in two SBMA mouse models, and is androgen-dependent, with mRNA levels correlating to motor dysfunction (Halievski et al., 2015c). That is, when motor function was rescued in diseased males by removing testicular androgens, so was expression of BDNF. Because BDNF potentiates neuromuscular transmission (Lohof et al., 1993; Mantilla et al., 2004; Garcia et al., 2010b; McGurk et al., 2011) and promotes muscle development, regeneration, and metabolism

(Mousavi et al., 2004; Matthews et al., 2009; Clow and Jasmin, 2010; Miura et al., 2012; Pedersen, 2013), a defect in muscle BDNF mRNA expression may critically mediate losses in motor function, by failing to maintain normal aspects of synaptic and/or muscle function. Therefore, we tested the hypothesis that deficits in muscle BDNF underlie motor dysfunction in SBMA. Using a Tg approach, we increased the expression of muscle BDNF in muscles of diseased mice and determined whether this increased muscle BDNF ameliorated disease symptoms.

We selectively overexpressed BDNF in skeletal muscle of diseased mice using Cre-loxP mouse models. The *ACTA1* promoter was used to drive the expression of a Cre recombinase specifically in muscle cells under a doxycycline control through the Tet-On system (Rao and Monks, 2009). This mouse was crossed to a Tg BDNF mouse that had a stop cassette placed upstream to prevent transgene transcription in the absence of Cre recombinase-mediated recombination (Chang et al., 2006). Finally, we tested the potential benefits of overexpressing muscle BDNF by crossing the Cre/loxP mouse to the 97Q SBMA mice. The 97Q model of SBMA occurs due to a global overexpression of a polyglutamine AR and exhibits a progressive, androgen-dependent disease phenotype (Katsuno et al., 2002). Mice carrying both the Cre and loxP transgenes on the 97Q SBMA background were then assessed for motor function and disease progression. Cellular and molecular changes were also examined to determine what might underlie any changes in behavior. In particular, synaptic function was measured at the NMJ to examine whether any defects (Xu et al., 2016) were reversed. We also examined whether any rescue of gene expression occurred for select genes important for muscle function that are known to be dysregulated in SBMA (Halievski and Jordan, unpublished observations; Halievski et al., 2015c; Oki et al., 2015; Xu et al., 2016).

Results of this study indicate that BDNF expressed exclusively in muscle can ameliorate disease symptoms in the 97Q mouse model of SBMA. We found that Tg BDNF slowed disease progression, rendering a two-fold extension in survival compared to control disease mice. Our

results point to a fiber type-specific benefit of muscle BDNF: we found that gene expression was partly normalized following BDNF overexpression in a slow- but not fast-twitch muscle. In fast-twitch muscle, we also saw no evidence for improved synaptic transmission. These data suggest that disease-related deficits in muscle BDNF may affect slow-twitch muscles more than fast, consistent with the fact that BDNF is expressed at higher levels in slow muscle compared to fast (Mousavi and Jasmin, 2006). Results identify two molecular targets in muscle that are sensitive to changes in muscle BDNF expression, furthering our understanding of the specific mechanisms that lead to neuromuscular dysfunction in SBMA.

III. Materials and methods

i. Muscle BDNF overexpression in an SBMA mouse model

Animal colonies were held on a 12h:12h light:dark cycle, group housed, and provided food and water ad libitum. All animal procedures were approved and performed in compliance with the Michigan State University Institutional Animal Care and Use Committee in accordance with the standards in the NIH Guide for the Care and Use of Laboratory Animals.

To generate a mouse model that expresses a BDNF transgene specifically in muscle cells, we used a Cre-loxP double Tg mouse, and then crossed it to an SBMA Tg mouse model to explore any potential benefits of muscle BDNF in SBMA. Genotyping of the models was performed with PCR as previously described (Katsuno et al., 2002; Chang et al., 2006; Rao and Monks, 2009). Validation of the Cre-loxP model was based on both male and female mice, and studies involving the disease model used only male mice, as females do not express the disease.

Mouse models

The loxP line contains the human *BDNF* gene downstream from a stop cassette ($BDNF^{stop}$; gift from Dr. Qiang Chang, University of Wisconsin-Madison; (Chang et al., 2006). The stop cassette is flanked by loxP sites thus, upon exposure to a skeletal muscle-specific Cre recombinase, Tg BDNF is expressed in skeletal muscle fibers. The Cre recombinase chosen here specifically drives expression in muscle cells under the control of the *ACTA1* promoter (human skeletal alpha actin 1, HSA-Cre; The Jackson Laboratory, stock no. 012433, B6;C3-Tg(ACTA1-rtTA,tetO-cre)102Monk/J; Rao and Monks, 2009). Moreover, expression of Cre recombinase is doxycycline-inducible in this Cre line through the Tet-On system. Possible genotypes and predicted outcomes based on doxycycline treatment are listed in Table 1. Doxycyline was administered orally for three days in light-proofed water bottles (2mg/ml doxycycline in water containing 5% sucrose).

To study whether restoring muscle BDNF via expression of a BDNF transgene can ameliorate disease in SBMA mice, we used the 97Q model that globally overexpresses a human AR with 97 polyglutamine repeats. Expression of the AR transgene is under the control of the chicken β-actin promoter and CMV enhancer (Katsuno et al., 2002). We chose this model because its slowly progressive phenotype makes it amenable to tracking disease progression, unlike the chronically symptomatic myogenic mouse model (see CHAPTER 2).

Table 1. Possible genotypes, doxycycline treatment, and predicted and actual outcomes for the muscle-BDNF overexpressing genetic cross. Transgenic (Tg).

Genotype and treatment			Predicted BDNF expression	Actual BDNF expression
$BDNF^{stop}$ loxP	HSA-Cre	Doxycycline		
-	-	-	Normal (endogenous)	Normal (endogenous)
+	-	-	Normal (endogenous)	Leaky (endogenous + Tg)
-	+	-	Normal (endogenous)	Normal (endogenous)
+	+	-	Normal (endogenous)	High (endogenous + Tg)
-	-	+	Normal (endogenous)	Normal (endogenous)
+	-	+	Normal (endogenous)	Leaky (endogenous + Tg)
-	+	+	Normal (endogenous)	Normal (endogenous)
+	+	+	High (endogenous + Tg)	High (endogenous + Tg)

Transgene recombination (PCR)

To examine spatial (skeletal muscle) and temporal (doxycycline) specificity of the $BDNF^{stop}$/HSA-Cre model, we used PCR with primers that flank the expected recombined region—i.e., the floxed stop cassette (forward: TGTCCCAAATCTGTGCGGAG, reverse: GCAGCCTTCATGCAACCAAA). The predicted size of the recombination product was 517 bp, while the size of the unrecombined product was predicted to be 3149bp. However, the latter product was unlikely to be synthesized due to its large size under our cycling conditions. Thus, our validation relied on whether the smaller product was present in our samples. The following tissues were examined in both adults and juveniles: skeletal muscle (TA), cardiac muscle (heart), and spinal cord.

Transgenic BDNF mRNA expression (qRT-PCR)

To further confirm and quantify expression of the BDNF transgene, we used qRT-PCR to examine BDNF mRNA. We used primers previously described that specifically detect human (Tg) BDNF mRNA (Chang et al., 2006) and thus, not endogenous mouse BDNF. Since these primers could not amplify endogenous mouse *BDNF* (CT values >35 in our hands), we developed primers that spanned a homologous sequence in the protein-encoding region of

human and mouse *BDNF* mRNA (Table 2). This allowed measurement of endogenous and Tg BDNF levels simultaneously. Tissue used for mRNA quantification was harvested and processed as described in CHAPTER 2 and stored at –80C until use.

Testosterone level analysis

Blood was collected intracardially and plasma isolated to measure circulating plasma testosterone levels as previously described (Johansen et al., 2009).

ii. Motor function testing

Motor function of male mice was assessed weekly starting by PND 40. Genotypes of the animals tested included all combinations of the 97Q transgene ("BDNF overexpressors": $BDNF^{stop}$ / HSA-Cre / 97Q; "Disease controls": $BDNF^{stop}$ / 97Q, HSA-Cre / 97Q, and 97Q) as well as Wt controls for comparison, which do not carry any transgene.

Hang time (up to 120 seconds) was used as the early indicator of disease. When hang times dropped below the full 120 seconds after three tries in a single session, motor function of such mice was then monitored twice weekly. When hang times were below 120 seconds on two consecutive testing days, the first of these two days was considered disease onset.

Experiment 1: Does muscle BDNF overexpression slow disease progression in SBMA mice?

In addition to hang time, grip strength (grams of force), number of vertical rears in an open field chamber, and body weight were recorded as described in CHAPTER 2. Animals were tested until they reached endstage, which was defined as a hang time of <30 seconds. Time to endstage was calculated as the difference in age (days) between endstage and symptom onset.

Experiment 2: Does transgenic BDNF in muscle ameliorate defects in synaptic transmission and muscle gene expression?

For Experiment 2, hang time and body weight were monitored as described above. At 11 ± 2 days post-symptom onset, muscles were harvested for sharp electrode recording of synaptic potentials from single muscle fibers and for gene expression analysis using qRT-PCR. During the 11 days of symptom progression, motor function was assessed 4-5 times distributed evenly across the 11-day window. When the motor function of mice was assessed 5 times, data from the fourth and fifth were averaged.

iii. Experiment 2: Intracellular recording

Neuromuscular transmission was assessed in BDNF overexpressors and disease controls 11 ± 2 days after symptom onset. Age matched, Wt control males were also recorded from for comparison.

Solutions and recording parameters

The EDL and its attached nerve were harvested for electrophysiological recording of muscle fibers *ex vivo* as previously described (Xu et al., 2016). Briefly, mice were made unconscious with CO_2 and rapidly decapitated. During dissection and recording, muscles were kept in oxygenated Ringer's solution (in mM: 135 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 14 HEPES, 11 D-glucose; pH 7.4). Muscles were pinned at approximate resting length on Sylgard-coated dishes and recordings were performed at room temperature with continuously perfusing solution (2-5ml/min). To block muscle contraction, all preparations received 5 μM μ-conotoxin for 30 min, and it was reapplied about every two hours when contraction reappeared.

Evoked EPPs and spontaneous mEPPs were recorded intracellularly with a glass microelectrode (1.0 mm outer diameter; WPI) that had a resistance of 15-20 MΩ when filled with

3 M KCl (Xu et al., 2016). RMP was recorded as the value observed immediately upon electrode insertion.

Synaptic facilitation was evaluated using trains at 0.5 Hz of five pulses at 10, 30, and 100 Hz (100, 30, and 10 ms interpulse intervals, respectively, within the train). Tetanizing stimulation was delivered at 100 Hz for 30 seconds, of which the first 0.5 seconds was used to evaluate the RRP size and release probability (Pr) (Xu et al., 2016).

Electrophysiology data analysis

EPP amplitude, EPP decay time (10-90%), mEPP amplitude, mEPP frequency, and quantal content were analyzed using Clampfit 9.2 software as previously described (Xu et al., 2016). RMP was recorded as the potential observed immediately upon electrode insertion into the muscle fiber. Short-term synaptic facilitation, RRP, and Pr were also analyzed as previously described (Xu et al., 2016).

iv. Experiment 2: qRT-PCR of muscle

Muscle for mRNA quantification was harvested and processed as described in CHAPTER 2 and stored at –80C until use. In some cases, RNA samples of muscle (TA and soleus) came from the contralateral hindlimb that was not used for electrophysiology. In other cases, the soleus muscle was combined from multiple animals of the same genotype to increase RNA yield. N refers to unique samples, and not total number of animals used. Primers used for qRT-PCR are listed in Table 2.

Table 2. List of primers for qRT-PCR are categorized by function.

RNA name	Forward	Reverse
Reference		
Rn18s (18S)	GGACCAGAGCGAAAGCATTTG	GCCAGTCGGCATCGTTTATG
Neurotrophic factor		
Bdnf (Endogenous mouse + transgenic human)	ACCATCCTTTTCCTTACTATGGTT	ATTCACGCTCTCCAGAGTCC
Ngfr (p75)	CGTGACCATCTCAGGCCTTT	GGTGCCCCTGTTACCTTCTC
Ntrk2 (TrkB full length)	GGCAACTTCGGGAAAGGAGA	GTAAACCCCTCACCGCCTAC
Ntf5 (NT-4)	TGAGCTGGCAGTATGCGAC	CAGCGCGTCTCGAAGAAGT
Muscle contractile function		
Myh4	AAGAGCCGAGAGGTTCACAC	TCTCCTGTCACCTCTCAACAG
Myh7	TCTTGTGCTACCCAGCTCCA	GCTTCCACCTAAAGGGCTGTT
Myh8	GTTCACACCAAAATCAGCGCA	CCTCCTGTGCTTTCCTTCAGC
Muscle ion channels		
Chrne (AChR epsilon)	CTCTGCCAGAACCTGGGTG	TGTGCTCTCAGCCACAAAGT
Chrng (AChR gamma)	GGTTGGTGATGGGTATGGTCA	TGACATCAGGAAAGGCAGAGC
Scn4a (NaV1.4)	TGGGGGTCAACTTGTTTGCT	TCGAATCTCTCGGAGGTGGT
Scn5a (NaV1.5)	GTCTCAGCCTTACGCACCTT	TCCCACGATTGTCTTCAGGC
Clcn1	GATTTGCTGCGGGTTCTTGG	TGGCTGAGACACTTGTGCTT

v. Statistical analysis

Mean, standard error of the mean (SEM), and group size (N = animals/samples, n = endplates) are reported, with $p<0.05$ considered to be significant, unless otherwise noted.

qRT-PCR gene expression

Relative Expression Software Tool (REST) was used to determine statistical significance and fold change of genes (Pfaffl et al., 2002). Specifically, this software uses the non-parametric Pair-Wise Fixed Reallocation Randomisation Test to account for amplification efficiencies when determining fold change. It measures relative expression of a target gene between two experimental groups following the normalization of the target gene to a reference gene (18S).

Testosterone analysis

We performed a two-factor between subjects analysis of variance to test whether sex (male or female) or genotype ($BDNF^{stop}$/HSA-Cre, $BDNF^{stop}$, HSA-Cre, or Wt) influenced testosterone

levels.

Motor function

An independent samples t-test was used to measure differences in age of symptom onset and days survived after onset between BDNF overexpressors and disease controls. A Kaplan-Meier log-rank test was used to determine if there were differences in disease progression.

Experiment 1

As disease mice reached endstage, the number of mice per time point decreased over time, precluding use of a repeated measures design. Moreover, hang time data are not normally distributed, given lower and upper boundaries of 0 and 120 sec. Thus, to assess how hang time changed across time in BDNF overexpressors versus disease controls, the non-parametric Mann-Whitney U test was used. Hang time data was grouped into bins, with each bin representing the range of days for the first, second, etc., time that mice were tested. An independent samples t-test was used to assess significant differences within bins between BDNF overexpressors and disease controls on grip strength, number of rears and body weight, with the critical *p*-value adjusted for multiple comparisons using the Bonferroni correction.

Experiment 2

A Mann-Whitney U test was used to determine if there were differences in hang times between the BDNF overexpressors and disease controls on a given post-onset day and in the proportion of hang events that fell under 40 seconds during disease progression. A Friedman test was used to determine whether hang times changed over time across the four symptomatic days examined (0, 4, 7, and 11) within each group (BDNF overexpressors and disease controls). A between-subjects repeated-measures ANOVA was used to assess differences in body weight

across the four symptomatic testing days (0, 4, 7, and 11) between genotypes (BDNF overexpressors and disease controls).

Electrophysiology

A one-way ANOVA with a Bonferroni post-hoc was used to assess differences between BDNF overexpressors, disease controls, and Wt groups on all electrophysiology parameters. n refers to the number of endplates or muscle fibers and was used for statistical analysis; N refers to the number of animals.

IV. Results

i. Skeletal muscle BDNF overexpression: BDNF^{stop}/HSA-Cre model characterization

We first did a number of experiments to validate the model, determining whether Tg BDNF was expressed only in skeletal muscle. We started by screening tissue for gene recombination based on PCR.

Transgene recombination (PCR)

To examine spatial and temporal specificity in the mouse BDNF overexpression model, we examined various tissues for recombination products in adult mice in which recombination in skeletal muscle was expected (BDNF^{stop}/HSA-Cre) and not expected (BDNF^{stop}-only). Tissue was collected from animals that had received doxycycline one month prior to sacrifice (Dox+) or no doxycycline (Dox-). As expected, the recombined product was found only in skeletal muscles of BDNF^{stop}/HSA-Cre mice, indicating good spatial specificity (Figure 7A). Unexpectedly, temporal specificity was not observed since a 517bp recombination product appeared skeletal muscle samples of both the Dox+ and Dox- BDNF^{stop}/HSA-Cre mice (Figure 7A). This may be due to the reported leakiness of this particular Cre mouse line (Rao and Monks, 2009).

We were curious to see whether this leak was also evident in juvenile (PND 28-31) mice since SBMA symptoms develop after this age in the 97Q model and also represent the age at which doxycycline treatment began. Similar to adults, the recombination product was present in skeletal muscle of the BDNF^{stop}/HSA-Cre mice, even in the absence of doxycycline (Figure 7B), suggesting that the BDNF transgene may be expressed well before symptom onset.

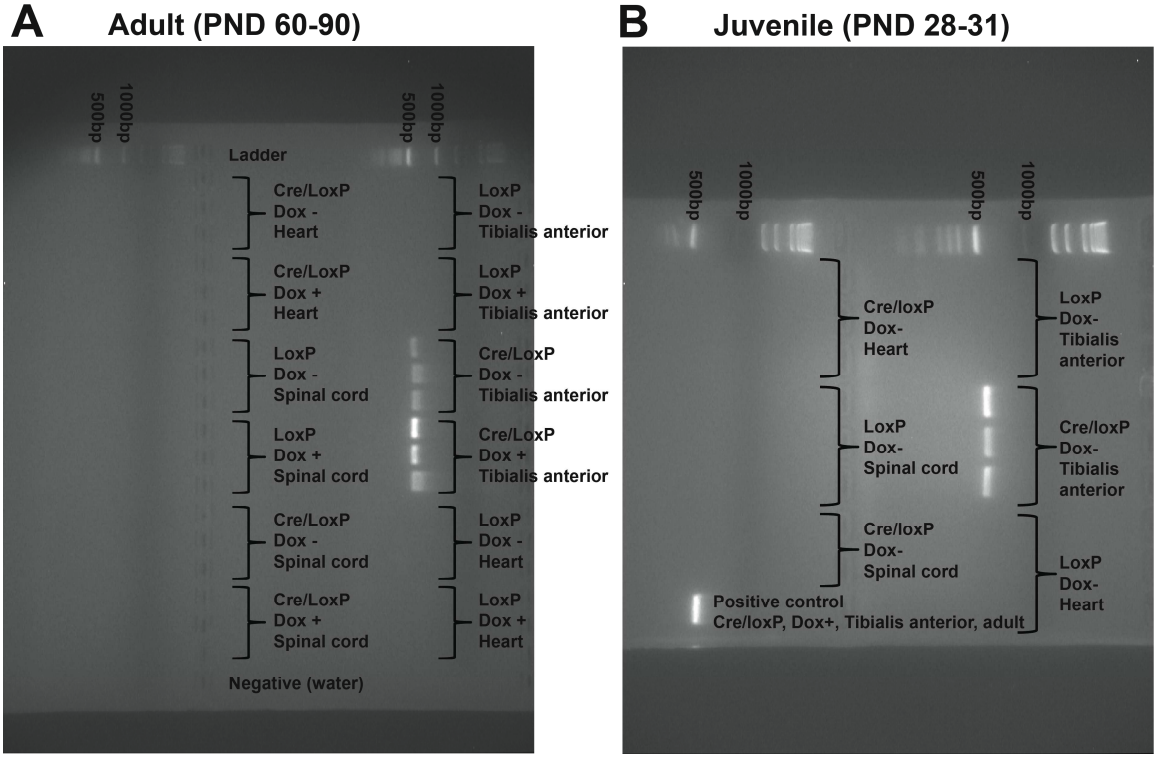

Figure 7. A recombination product is present in a muscle-specific, but not doxycycline sensitive manner in the BDNF^{stop}loxP/HSA-Cre genotype. A) Skeletal muscle (tibialis anterior), cardiac muscle (heart), and spinal cord were examined from adult mice of the BDNF^{stop}loxP/HSA-Cre and BDNF^{stop}loxP-only genotypes. Both Cre and loxP transgenes are necessary for recombination, but doxycycline is not. B) Recombination occurs in the absence of doxycycline as early as PND28-31, since a recombination product is present in juvenile skeletal muscle. Dox+ animals received doxycycline treatment one month prior to tissue collection, and Dox- animals never received doxycycline. Each lane represents a different animal, N=3/group.

Extent of mRNA overexpression (qRT-PCR)

Since this model did not appear to be doxycycline sensitive, with recombination products present regardless of doxycycline treatment, we decided to examine whether this was also the case at the mRNA level. To this end, we used qRT-PCR to examine BDNF mRNA levels in the EDL muscle of age-matched groups that received doxycycline either one week before, one month before, or never. Similar to the recombination results (Figure 7), we found that expression of Tg BDNF mRNA in the BDNF^{stop}/HSA-Cre group did not depend on doxycycline, as levels did not change following doxycycline treatment after either a one week or one month

delay (Figure 8A, $p>0.05$). The other genotypes also did not show changes in BDNF expression (Tg and endogenous) as a result of doxycycline treatment (Figure 8B-D, $p>0.05$). Together (Figures 7 and 8), these data suggest that expression of the human BDNF transgene expression was not doxycycline-inducible when using the HSA-inducible Cre as reports lead one to expect.

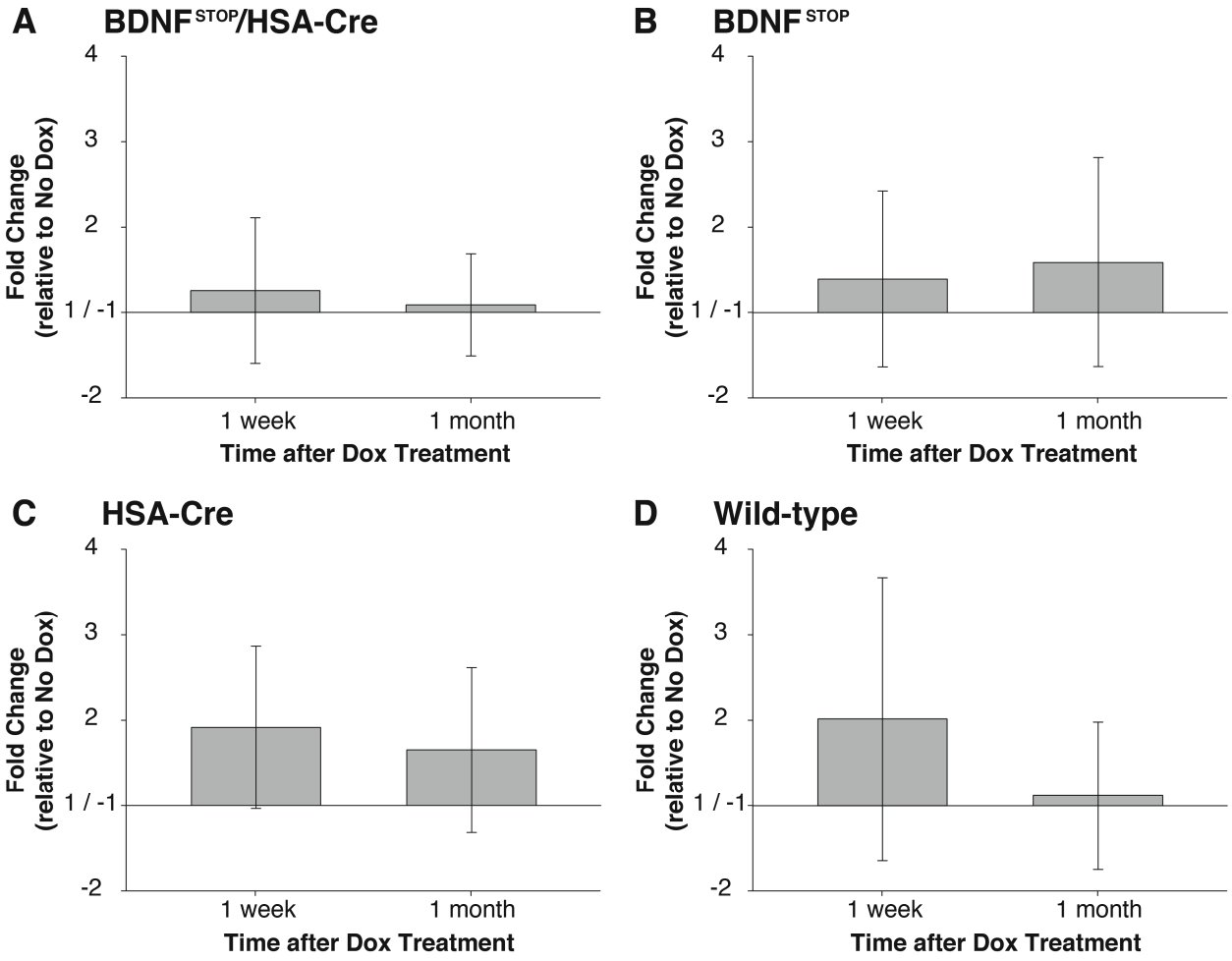

Figure 8. Doxycycline did not alter BDNF mRNA expression in hindlimb skeletal muscle. A) qRT-PCR measurement of BDNF mRNA levels in the extensor digitorum longus of BDNF^{stop}/HSA-Cre group did not differ one week or one month after doxycycline treatment compared to age-matched animals from the same genotype that had never received doxycycline. B-D) Likewise, animals from the BDNF^{stop}-only, HSA-Cre-only, and wild-type groups did not exhibit changes in BDNF mRNA levels following doxycycline treatment. The qRT-PCR primers used can detect both endogenous mouse and transgenic human BDNF mRNA, N=4-5/group. Values are mean fold changes **±** SEMs (standard errors of the mean) based on N/group.

To examine the extent of overexpression between the different genotypes, we collapsed animals from the three doxycycline treatments within each genotype. We found a strong upregulation of BDNF mRNA in the EDL of $BDNF^{stop}$/HSA-Cre mice compared to Wt mice (Figure 9A). We also noted a small, but significant, upregulation of BDNF mRNA in the $BDNF^{stop}$-only group (Figure 9A). No difference in expression was found between the HSA-Cre-only and Wt animals (Figure 9A). Notably, compared to the $BDNF^{stop}$-only group, $BDNF^{stop}$/HSA-Cre animals express ~14-fold more BDNF mRNA (Figure 9A).

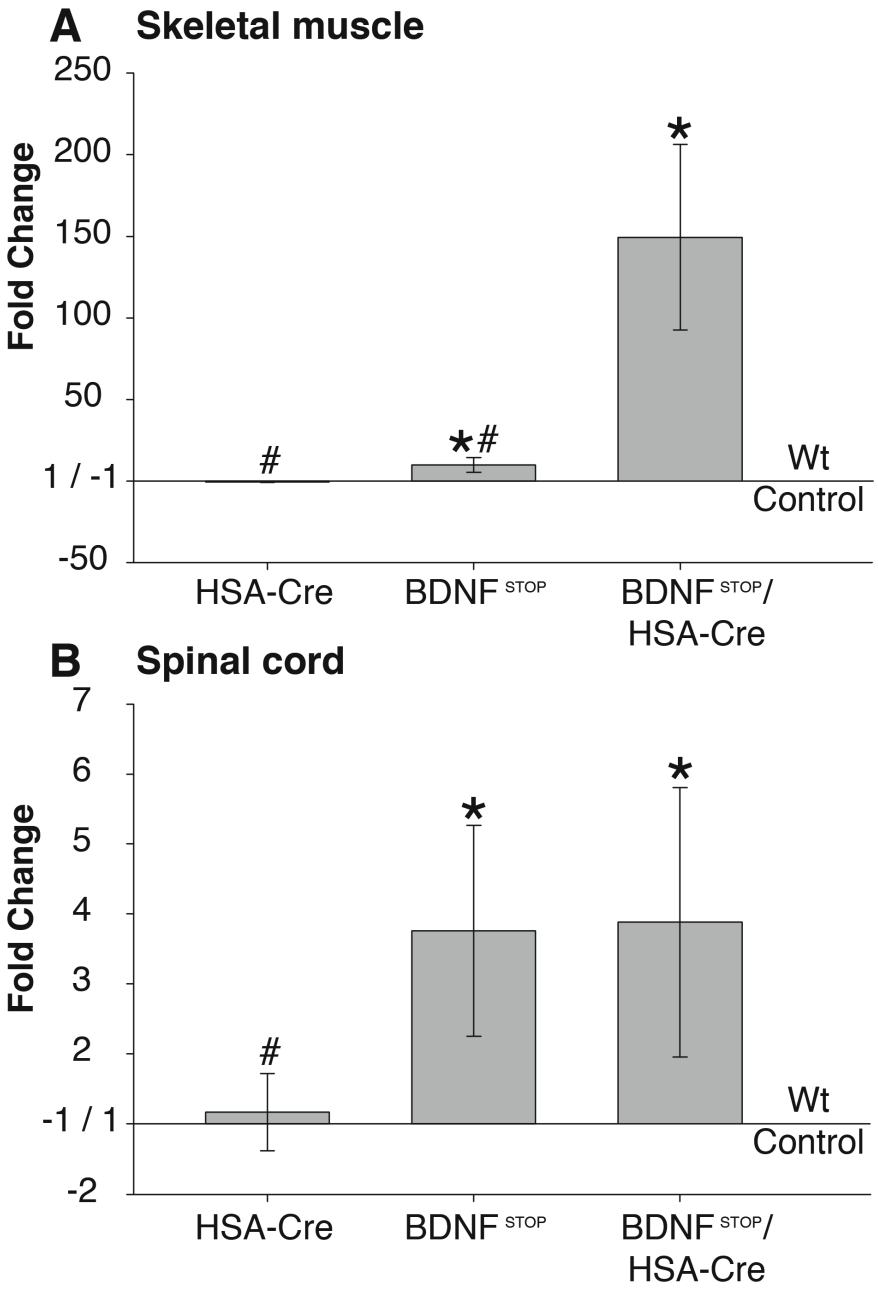

Figure 9. Presence of the BDNF^{stop}/HSA-Cre transgenes together results in strong BDNF mRNA overexpression in skeletal muscle. A) BDNF mRNA levels in extensor digitorum longus skeletal muscle of the BDNF^{stop}/HSA-Cre group are significantly and robustly elevated compared to all other genotypes. Presence of the BDNF^{stop} transgene alone results in moderate BDNF mRNA overexpression in skeletal muscle, but still ~14-fold less than when both BDNF^{stop} and HSA-Cre are present. Animals from all doxycycline treatments were combined, N=15/group. B) BDNF mRNA expression is slightly, but significantly elevated in spinal cord tissue of both BDNF^{stop}/HSA-Cre and BDNF^{stop}-only groups compared to the wild-type group, indicating a likely leak of the BDNF transgene. Spinal cord tissue is from animals treated with doxycycline one month prior, N=5-6/group. *p<0.05 from wild-type, #p<0.05 from BDNF^{stop}/HSA-Cre. Values are mean fold changes **±** SEMs (standard errors of the mean) based on N/group.

Due to the apparently leaky transgene expression in muscle of the BDNF^{stop}-only group, we wanted to determine whether spinal cord tissue also expressed (leaked) Tg BDNF, regardless of the lack of a recombination product (Figure 7). Indeed, lumbar spinal cord from the BDNF^{stop}-only and BDNF^{stop}/HSA-Cre groups showed ~4-fold upregulation of BDNF compared to Wt; this elevation did not differ between the two BDNF^{stop}-containing genotypes (Figure 9B). Together, the recombination PCR and qRT-PCR data suggested the stop cassette was intact (indicated by the much higher level of BDNF mRNA in muscle of BDNF^{stop}/HSA-Cre compared to BDNF^{stop}-only mice) but that the stop codon was not fully efficient (indicated by the increased levels of BDNF mRNA in spinal cord and muscle of BDNF^{stop}-only mice without evidence of recombination). Nevertheless, the HSA-Cre tremendously boosted muscle-specific expression of the transgene when combined with the BDNF^{stop} allele, allowing us to ask whether overexpression of muscle BDNF could rescue mice from SBMA symptoms.

Testosterone

As SBMA is an androgen dependent disease, it was important to rule out any possible effect of the intervening transgene (i.e., BDNF^{stop} and/or HSA-Cre) on testosterone levels, as an apparent rescue by BDNF might simply reflect lowered testosterone levels. Hence, we measured testosterone levels in plasma from the four possible genotypes to determine whether presence of either transgene and/or their combination leading to overexpression of BDNF altered circulating testosterone levels. While testosterone levels differed between males and females (mean (nmol/L) ± SEM: male 13.31 ± 15.81, female 4.87 ± 7.48; main effect of sex $F(1,46)=4.48$, $p=0.04$), there was no effect of genotype ($F(3, 46)=0.438$, $p=0.727$) and no interaction between sex and genotype ($F(3,46)=0.841$, $p=0.478$). Means and SEMs across sex and genotype are listed in Table 3.

Table 3. Testosterone levels (mean (nmol/L) ± SEM) across sex and genotype in a transgenic muscle BDNF overexpression model.

Sex / Genotype	Wild-type	HSA-Cre	BDNF^{stop}	BDNF^{stop}/ HSA-Cre
Male	7.21 ± 1.35	11.10 ± 2.72	20.21 ± 7.79	11.46 ± 2.79
Female	2.74 ± 1.09	7.17 ± 3.50	1.76 ± 0.16	6.71 ± 5.02

ii. Experiment 1: Does muscle BDNF overexpression slow disease progression in SBMA mice?

Having confirmed a 14-fold increase in BDNF mRNA levels in BDNF^{stop}/HSA-Cre mice, we next asked whether this increased expression of muscle BDNF mRNA would benefit diseased SBMA mice who have deficits in muscle BDNF mRNA (CHAPTER 2). Thus, we crossed BDNF$^{stop/}$HSA-Cre Tg mice with 97Q SBMA mice. This cross resulted in eight possible genotypes, five of which were used for the current study (Table 4). All male mice were exposed to doxycycline treatment by PND 25-29 to ensure that the transgene was expressed by this presymptomatic time point.

Table 4. Genotypes used to study the effect of muscle BDNF overexpression in an SBMA mouse model.

Transgenic mouse line			Nomenclature
BDNF^{stop}	HSA-Cre	97Q SBMA	
-	-	-	Wild-type
-	-	+	Disease control, 97Q-only
-	+	+	Disease control, HSA-Cre-only
+	-	+	Disease control, BDNF^{stop}-only
+	+	+	BDNF overexpressor, BDNF^{stop}/HSA-Cre

Motor function and survival

To examine differences in motor function and survival between BDNF overexpressors and disease controls (nomenclature defined in Table 4), mice underwent behavior testing as

described in the methods. While symptom onset did not differ between BDNF overexpressors and disease controls ($t(36)=0.436$, $p=0.666$; Figure 10A), time to reach endstage did (i.e., when hang time <30 seconds). BDNF overexpression in diseased muscles led to a significant slowing of disease progression compared to disease controls ($\chi^2(1)=5.532$, $p=0.019$; Figure 10B). Moreover, the number of days to endstage was doubled for the BDNF overexpressors compared to disease control mice ($t(36)=2.570$, $p=0.014$; Figure 10C).

We also examined whether the $BDNF^{stop}$ allele alone slowed disease progression, since this genotype exhibited a minor increase in BDNF mRNA expression in muscle and spinal cord (Figure 9). Compared to the 97Q-only group, the $BDNF^{stop}$ allele conferred no apparent benefit to 97Q males (days to endstage: $\chi^2(1)=0.007$, $p=0.933$; age at endstage: $\chi^2(1)=0.025$, $p=0.874$; Appendix, Figure 18).

Other behavioral measurements included grip strength, open field (rearing behavior), and body weight. Across the symptomatic phase, average behavioral measures between BDNF overexpressors and disease controls were the same (Figure 11; Table 5), but sample size dropped more drastically for disease controls (Table 5), corroborating the delay to endstage observed for BDNF overexpressors (Figure 10B-C).

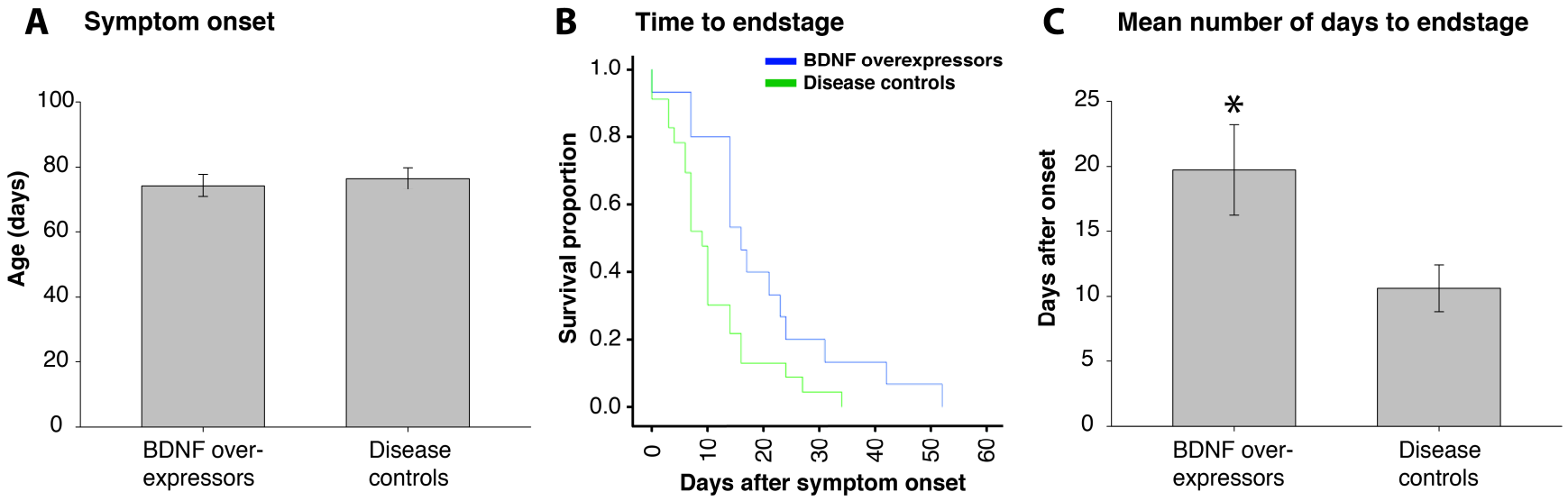

Figure 10. BDNF overexpression in muscle slows disease progression in an SBMA mouse model. A) Symptom onset does not differ between BDNF overexpressors and disease controls. B) However, BDNF overexpressors survive for longer after disease onset compared to disease controls, suggesting that increased muscle BDNF delayed the time it takes to reach endstage ($p<0.05$). C) BDNF overexpressors live for an average of 19.7 days after disease onset while disease controls live only an average of 10.6 days; *$p<0.05$ from disease controls. BDNF overexpressors, N=15; Disease controls, N=23. A, C) Values are mean ± SEMs (standard errors of the mean) based on N/group. B) Values are proportion of mice surviving to age on x-axis.

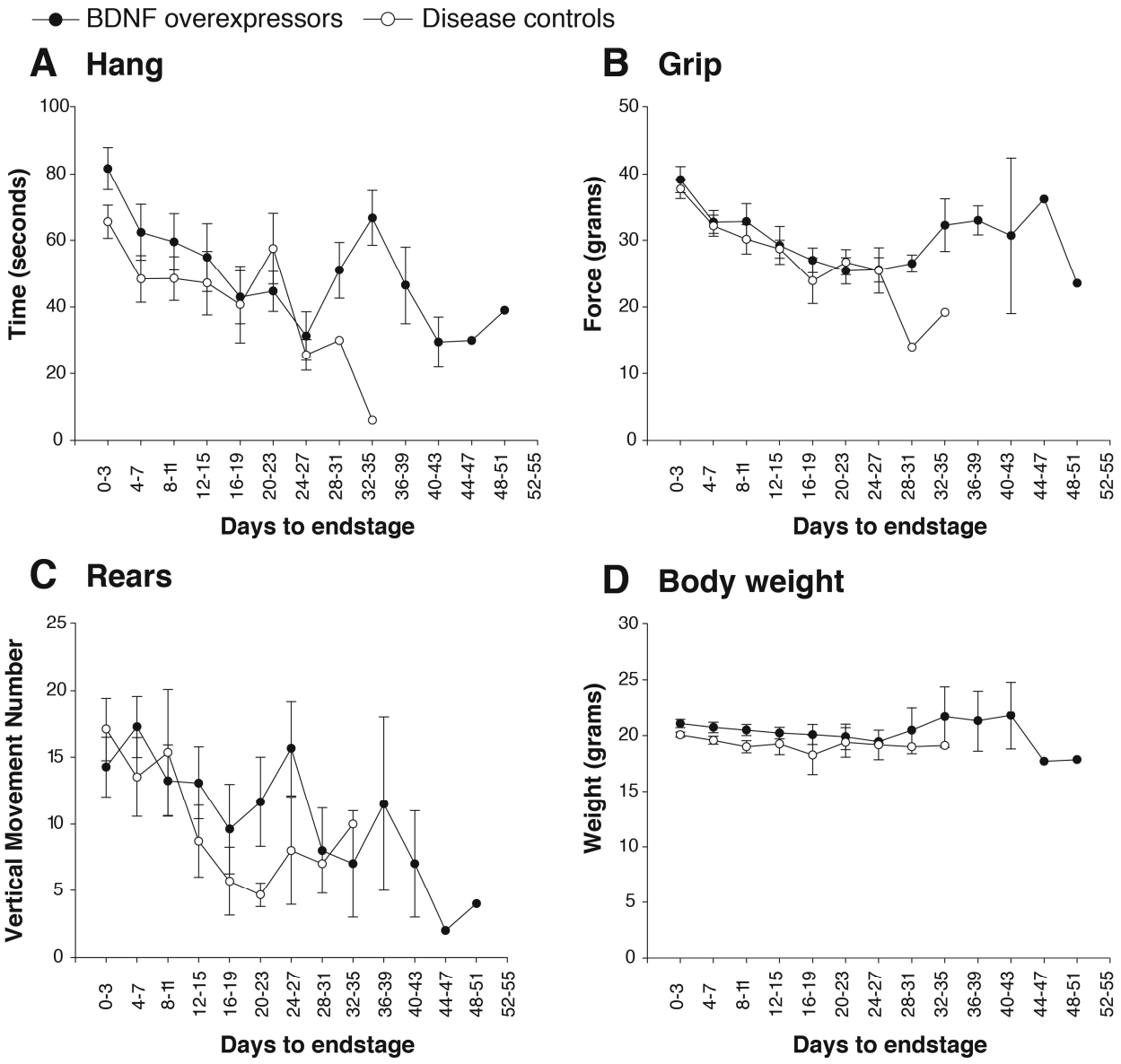

Figure 11. Motor function appears grossly similar between BDNF overexpressors and disease controls during the symptomatic phase, but BDNF overexpressors take longer to reach endstage (see also Table 5). A-D) Behavior (hang time, grip strength, number of rears) and body weight measures reported for BDNF overexpressors and disease controls in bins of days post-disease onset. N per group differs across bin, see Table 5. Values are mean ± SEMs (standard errors of the mean) based on N/ group.

Table 5. Number of animals remaining in the Experiment 1 study as disease progresses from disease-onset. Disease control mice reached endstage appreciably sooner, leading to appreciably lower Ns starting 12 days after the onset of overt expression of disease symptoms. Statistics and p-values reported for behavior measures, where a $p<0.007$ considered significant based on a Bonferroni correction for multiple comparisons.

Binned days after disease onset	**0-3**	**4-7**	**8-11**	**12-15**	**16-19**	**20-23**	**24-27**	**28-31**	**32-35**	**36-39**	**40-43**	**44-47**	**48-51**
BDNF overexpressors (N)	15	13	12	12	8	6	3	3	2	2	2	1	1
Disease controls (N)	22	19	12	7	5	3	3	1	1	0	0	0	0
Summary statistics													
Hang (U, *p*)	106, 0.068	90.5, 0.205	56.5, 0.371	37.5, 0.703	19.5, 0.941	5.0, 0.300	3.5, 0.658	n/a	n/a	n/a	n/a	n/a	n/a
Grip (t, *p*)	0.579, 0.566	0.238, 0.813	0.764, 0.453	0.140, 0.890	0.869, 0.403	0.414, 0.691	0.768, 0.477	n/a	n/a	n/a	n/a	n/a	n/a
Rears (t, *p*)	0.819, 0.419	0.967, 0.342	0.398, 0.695	1.065, 0.302	0.826, 0.426	1.425, 0.197	0.581, 0.586	n/a	n/a	n/a	n/a	n/a	n/a
Body weight (t, *p*)	2.440, 0.020	1.974, 0.058	2.022, 0.056	0.996, 0.333	1.033, 0.324	0.268, 0.796	0.785, 0.468	n/a	n/a	n/a	n/a	n/a	n/a

iii. Experiment 2: Does transgenic BDNF in muscle ameliorate defects in synaptic transmission and muscle gene expression?

Given that muscle BDNF appears to slow disease progression (Figure 10B-C), we next asked how BDNF might be combatting symptoms of disease by examining cellular and molecular underpinnings that may have led to this improvement. For Experiment 2, all animals were sacrificed at 11 ± 2 days post-symptom onset (a time when BDNF overexpressors showed the biggest difference in hang times compared to disease controls). We examined neuromuscular transmission and mRNA expression of several genes known to be affected by disease and clearly linked to neuromuscular function.

Motor function

Behavior testing was conducted as described in the methods. After symptom onset, hang time and body weight were recorded over the course of four trials over eleven days. We replicated the original finding, with BDNF ovexpressors showing improved hang times compared to disease controls (Figure 12A-B). Disease controls exhibited a greater proportion of hang times below 40 seconds at two of the four days examined post-onset (4 days: U=270.0, p=0.045; 7 days: U=243.0, p=0.037; Figure 12A), suggesting that disease controls were more likely to reach endstage criteria sooner, like in Experiment 1. Notably, disease controls showed hang deficits across time ($\chi^2(3)=11.35$, p=0.010), while hang times in BDNF overexpressors did not decline significantly ($\chi^2(3)=2.45$, p=0.485), indicating that BDNF overexpressors are able to maintain sufficient motor function during this phase of disease (Figure 12B). As in Experiment 1, age at symptom onset did not differ between BDNF overexpressors and disease controls ($t(51)=0.045$, p=0.964; Figure 12C). While body weight dropped over time, it did not differ between BDNF overexpressors and disease controls (main effect of weight: $F(1.83, 91.35)=14.877$, $p<0.001$; weight by genotype interaction: $F(1.83, 91.35)=0.315$, p=0.711; Figure 12D).

In sum, overexpression of BDNF in skeletal muscle conferred some protection of disease on motor function, as in Experiment 1, indicating that muscle BDNF impedes disease progression. We next examined neurotransmission and gene expression to see whether the benefits of BDNF on motor function was reflected in these measures.

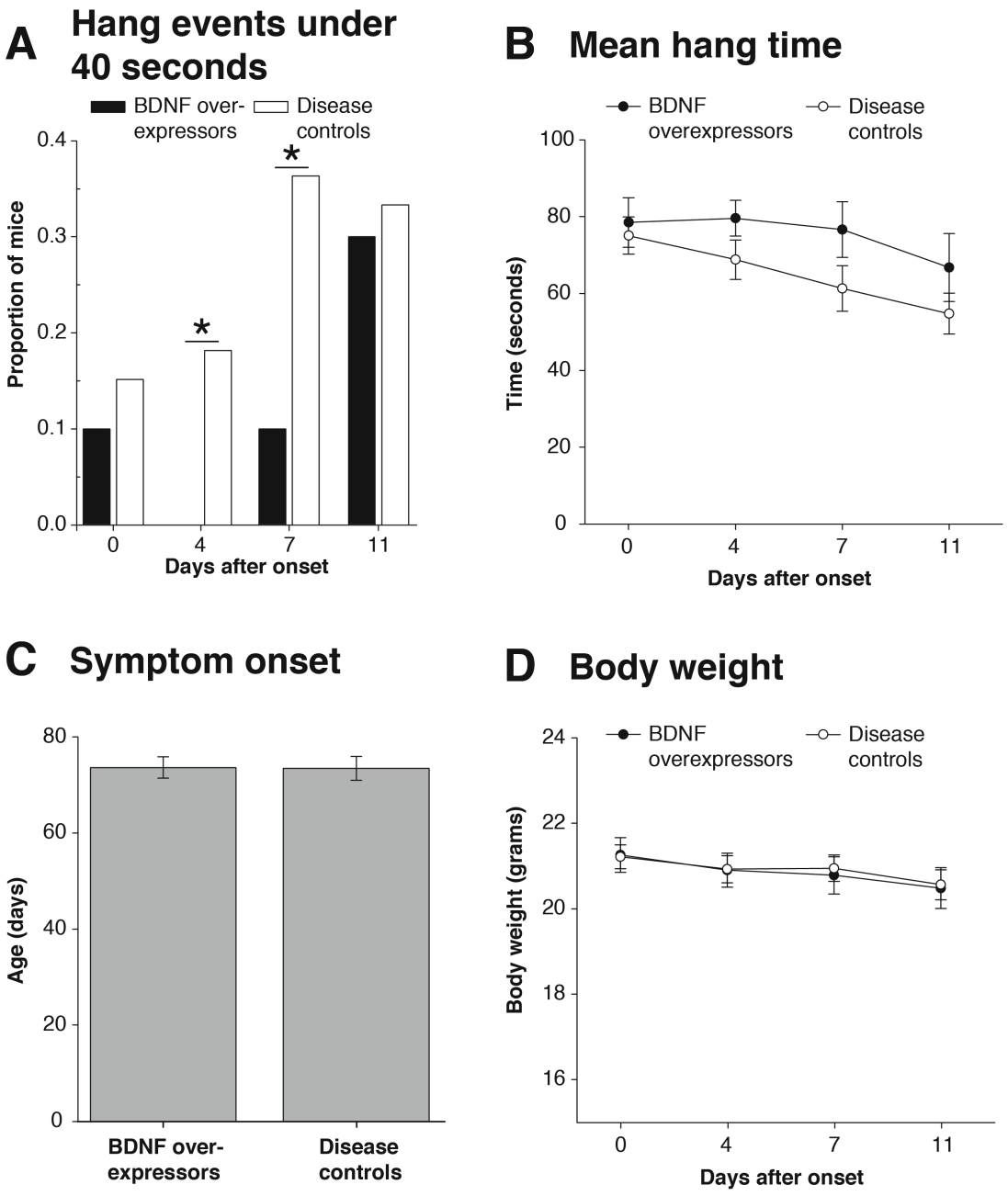

Figure 12. Muscle BDNF overexpression strengthened hang test performance in 97Q SBMA mice. A) Disease controls obtained hang times of less than 40 seconds more often than BDNF overexpressors, suggesting that muscle BDNF improved ability to maintain a functional, healthier score. B) Average hang time declined over time for disease controls, but did not for BDNF overexpressors, indicating that muscle BDNF maintained motor function after symptom onset. C) Age at symptom onset did not differ between groups, suggesting that the improvement caused by increased muscle BDNF acts by decelerating disease progression after it has begun. D) Body weight also does not differ between BDNF overexpressors and disease controls; thus, muscle BDNF may ameliorate disease through a functional, rather than mass related, target. *p<0.05 Mann Whitney U. BDNF overexpressors, N=20; Disease controls, N=32. A) Values are proportion of mice that obtained hang scores less than 40 seconds of total mice (N). B-C) Values are mean ± SEMs (standard errors of the mean) based on N/group.

Basic electrophysiological parameters at the NMJ

Many aspects of neurotransmission at the NMJ are defective in the EDL of the 97Q SBMA mouse model (Xu et al., 2016). Hence, we examined whether there were any neurotransmission improvements in BDNF overexpressors that may explain the slowed disease progression. We measured several parameters in the EDL at 11 ± 2 days post-disease onset. In all cases, BDNF overexpressors and disease controls did not differ from each other, and were comparably different from Wt controls. We replicated the previously reported deficiency in quantal content for mice carrying the 97Q transgene, in BDNF overexpressors and disease controls, each compared to Wt controls (main effect of genotype: $F(2,395)=168.22$, $p<0.001$; post-hoc Bonferroni reported in Figure 13A). Also like before, mEPP amplitude was increased in 97Q animals compared to Wt controls (main effect of genotype: $F(2,395)=75.69$, $p<0.001$; post-hoc Bonferroni reported in Figure 13B). Interestingly, we detected a small, but statistically significant, increase in EPP amplitude in the 97Q groups compared to Wt that was not apparent previously (main effect of genotype: $F(2,395)=6.11$, $p=0.002$; post-hoc Bonferroni reported in Figure 13C). Moreover, EPP decay time was elongated in the disease groups, as previously reported (main effect of genotype: $F(2,395)=9.68$, $p<0.001$; post-hoc Bonferroni reported in Figure 13D); on the other hand, mEPP frequency was not increased in 97Q groups, as previously shown (main effect of genotype: $F(2,395)=0.23$, $p=0.797$, post-hoc Bonferroni reported in Figure 13E). Finally, RMP was not improved in BDNF overexpressors, with both 97Q groups showing partially depolarized RMPs compared to Wt (main effect of genotype: $F(2,351)=286.14$, $p<0.001$; post-hoc Bonferroni reported in Figure 13F). In sum, basic neurotransmission in the fast-twitch EDL did not differ between BDNF overexpressors and disease controls, finding largely comparable defects as previously reported (Xu et al., 2016).

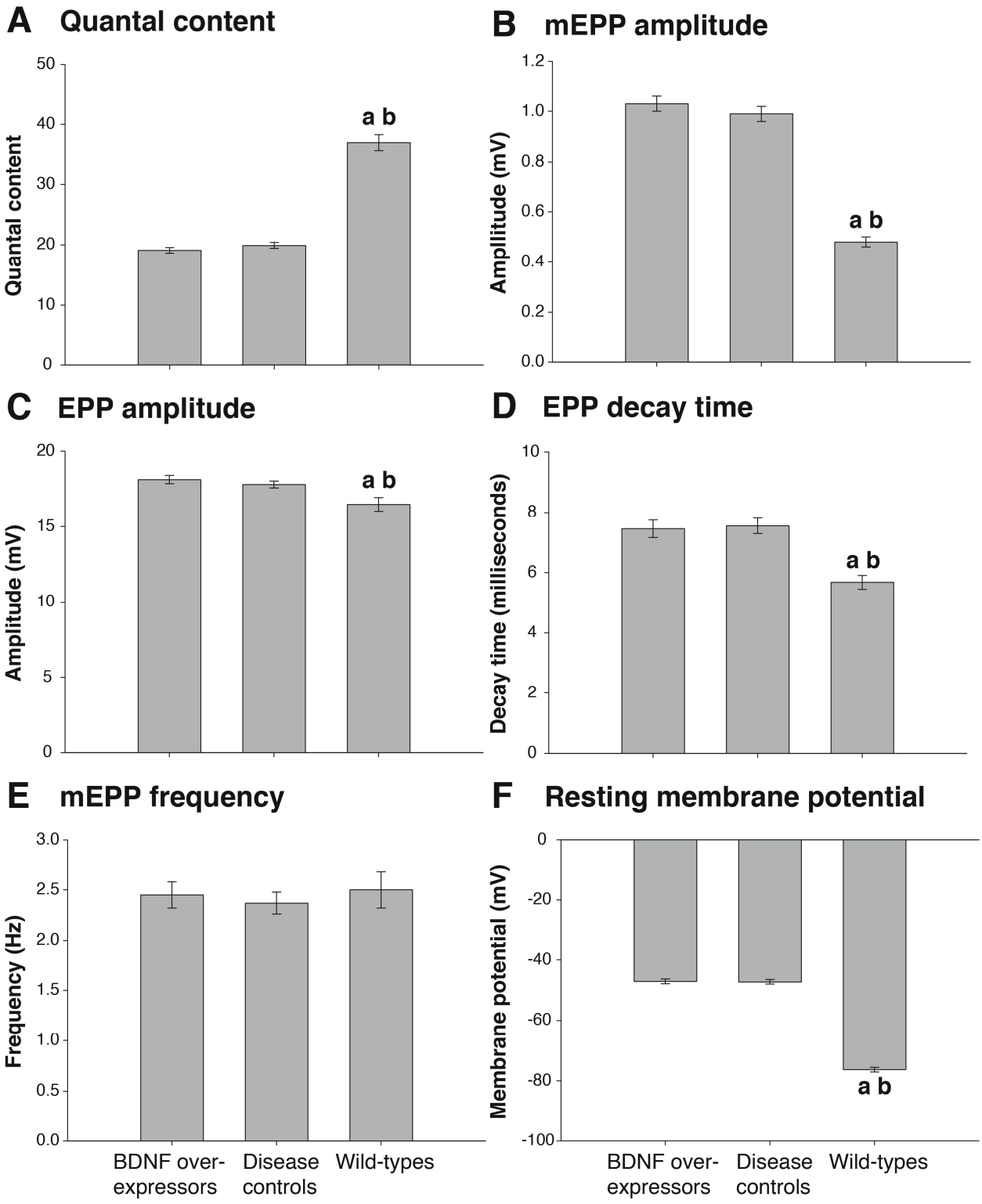

Figure 13. Muscle BDNF overexpression did not improve neuromuscular transmission despite improvements in motor function in a fast-twitch muscle at 11 ± 2 days post-disease-onset. A) Quantal content is reduced, while B) mEPP amplitude and C) EPP amplitude are increased in 97Q males compared to age-matched, healthy wild-types. D) Diseased animals also showed a prolonged EPP decay

Figure 13 (cont'd). time, corroborating the similarly increased AChR gamma subunit mRNA expression in the fast-twitch tibialis anterior of BDNF overexpressors and disease controls. Interestingly, the soleus in BDNF overexpressors does not show increased AChR gamma mRNA expression, suggesting that perhaps decay time is brought back to wild-type levels by muscle BDNF overexpression in slow-twitch muscles. Finally, E) mEPP frequency is unchanged between all groups examined and F) the depolarized resting membrane potential is not rescued in BDNF overexpressors. In sum, defects at the neuromuscular junction in the EDL persist and likely do not underlie improvements in motor function. Recordings were taken from the extensor digitorum longus muscle. Bonferroni post-hoc: $^{a}p<0.05$ compared to BDNF overexpressors, $^{b}p<0.05$ compared to disease controls. BDNF overexpressor, n/N=144/12; Disease controls, n/N=179/16; wild-type, n/N=75/8. Values are mean ± SEMs (standard errors of the mean) based on n/group.

Short-term plasticity at the NMJ

Since muscle BDNF overexpression did not rescue any basic parameters of neuromuscular transmission (Figure 13), we sought to examine whether there was an improvement when the system was challenged. Short-term synaptic facilitation is reduced in diseased 97Q males (Xu et al., 2016) and we also found the same pattern in the current experiment in 97Q animals 11 ± 2 days post-onset (Figure 14, Table 6). However, BDNF overexpressors did not differ from disease controls, suggesting that this endpoint measure in the EDL also did not contribute to improvement in motor phenotype. Interestingly, the SBMA animals from this study also showed a reduced facilitation at 10Hz and 30Hz stimulation parameters compared to Wt controls (Figure 14A-B), which was not detected previously (Xu et al., 2016).

Table 6. BDNF overexpressors and disease controls differ from Wild-type mice at all stimulation frequencies (10, 30 and 100 Hz) used to assess short-term facilitation. Main effect of genotype reported here, Bonferroni post-hoc comparisons reported in Figure 14.

Frequency	Pulse Number	One-way ANOVA
10Hz	2	$F(2,224)=7.94$, $p<0.001$
	3	$F(2,224)=9.12$, $p<0.001$
	4	$F(2,224)=11.19$, $p<0.001$
	5	$F(2,224)=5.78$, $p=0.004$
30Hz	2	$F(2,222)=21.20$, $p<0.001$
	3	$F(2,222)=22.36$, $p<0.001$
	4	$F(2,222)=18.24$, $p<0.001$
	5	$F(2,222)=18.69$, $p<0.001$
100Hz	2	$F(2,217)=9.34$, $p<0.001$
	3	$F(2,217)=13.61$, $p<0.001$
	4	$F(2,217)=12.57$, $p<0.001$
	5	$F(2,217)=11.82$, $p<0.001$

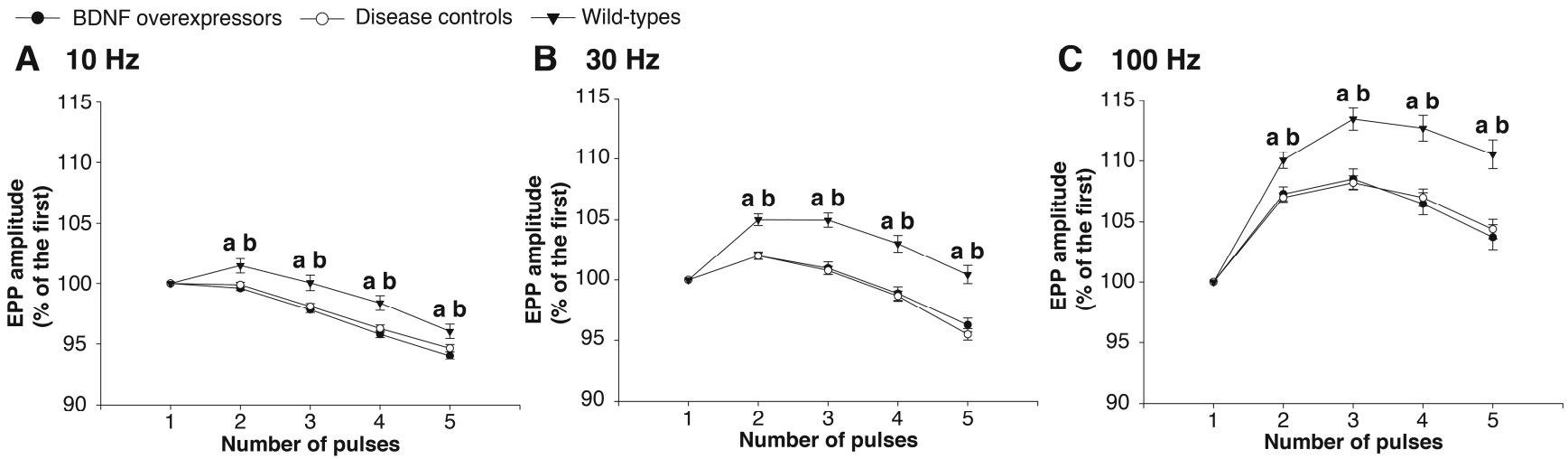

Figure 14. Short-term synaptic facilitation was reduced in a fast-twitch muscle of 97Q males 11 ± 2 days post-onset, and was not improved by muscle BDNF overexpression. A) 10Hz, B) 30Hz, and C) 100Hz stimulation frequencies of 5 pulses were used to examine synaptic facilitation. In all cases, BDNF overexpressors and disease controls did not facilitate as extensively as wild-types. Recordings were taken from the extensor digitorum longus muscle. Bonferroni post-hoc [a]$p<0.05$ Wild-type different from BDNF overexpressors, [b]$p<0.05$ Wild-type different from disease controls. BDNF overexpressors, n/N=64/7; Disease controls, n/N=104/11; Wild-type, n/N=52/7. Values are mean ± SEMs (standard errors of the mean) based on n/group.

Readily releasable pool and release probability at the NMJ

Finally, we also examined the size of the RRP and Pr to determine if one or both of these measures of synaptic function were improved by overexpression of muscle BDNF, since these parameters are known to respond to BDNF (Stoop and Poo, 1996; Tyler et al., 2006). Using quantal content data from the initial depression phase of a tetanizing stimulation (100Hz), we analyzed RRP and Pr. As shown previously, both RRP and Pr are reduced in diseased 97Q animals compared to Wt controls (Xu et al., 2016), but we found no difference between BDNF overexpressors and disease control mice on these two measures (main effect of genotype: RRP: $F(2,116)=13.85$, $p<0.001$, Figure 15A; Pr: $F(2,116)=7.174$, $p=0.001$, Figure 15B; post-hoc comparisons reported in figure captions).

In sum, we did not find that overexpression of BDNF in skeletal muscle ameliorated synaptic defects at the NMJ in the fast-twitch EDL muscle, raising the question of whether BDNF improves motor function by improving neuromuscular function in slow-twitch muscles.

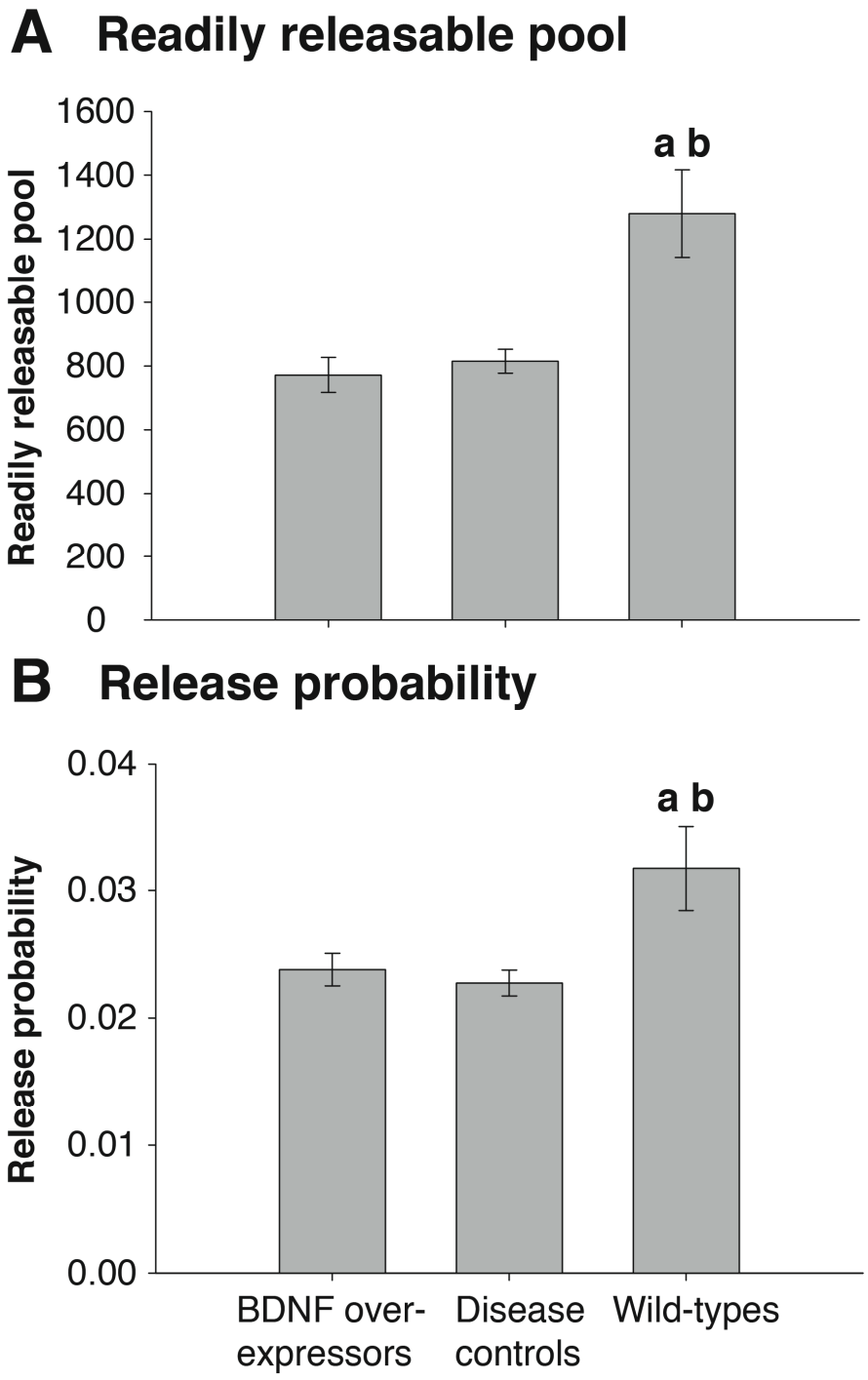

Figure 15. Readily releasable pool and release probability are reduced in 97Q SBMA fast-twitch muscle 11 ± 2 days post-onset, even in the face of increased muscle BDNF. A) Readily releasable pool and B) release probability are equally reduced in BDNF overexpressors and disease controls compared to Wild-types, suggesting that these synaptic parameters in the extensor digitorum longus are not the source of improved motor function in BDNF overexpressors. Bonferroni post-hoc [a]$p<0.05$ Wild-type different from BDNF overexpressors, [b]$p<0.05$ Wild-type different from disease controls. BDNF overexpressors, n/N=35/7; Disease controls, n/N=61/11; Wild-type, n/N=23/7. Values are mean ± SEMs (standard errors of the mean) based on n/group.

Expression of muscle genes for ion channels, muscle contractile machinery, and neurotrophic factors

As we did not identify any benefit of overexpressing muscle BDNF on basic synaptic transmission parameters, short-term facilitation, RRP size, or Pr in the EDL, we looked to other potential mechanisms by which increased muscle BDNF might ameliorate disease. Expression levels of many genes involved in muscle contraction, neurotransmission, and general muscle health are perturbed at the mRNA level in SBMA muscle (e.g., Halievski and Jordan, unpublished observations; Halievski et al., 2015c; Oki et al., 2015; Xu et al., 2016). To begin exploring whether overexpression of BDNF in muscle rescued the expression of critical genes in muscle, we examined the expression of several genes falling into distinct categories (Table 2) in both a fast-twitch (TA) and slow-twitch (soleus) muscle of BDNF overexpressors and disease controls, 11 ± 2 days post-onset, and comparing gene expression in these two groups to that of the TA and soleus of Wt mice. To confirm that BDNF was indeed overexpressed in mice carrying all three transgenes (BDNF^{stop}/HSA-Cre/97Q), we measured BDNF mRNA levels. In both the TA and the soleus, BDNF mRNA in mice carrying all three transgenes was significantly increased compared to Wt controls. Interestingly, the extent of upregulation was stronger in the TA (~144-fold compared to Wt) than in the soleus (~45-fold compared to Wt; Figure 16A and Figure 17A). As expected (see Figure 9), we also noted a small but significant increase in BDNF mRNA in BDNF^{stop}-97Q muscle: ~13-fold in the TA and ~3-fold in the soleus compared to Wt controls. We also detected a deficit in BDNF mRNA in both the TA and soleus of 97Q control mice *not* containing the BDNF^{stop} allele (i.e., 97Q-only and HSA-Cre-97Q; Figure 16A and Figure 17A), as expected based on CHAPTER 2. Apparently at least a 45-fold upregulation of BDNF mRNA is required for any benefit on motor function, since the ~3 to ~13-fold increase in 97Q mice having the BDNF^{stop} allele but not the HSA-Cre did not slow disease progression (Appendix, Figure 17).

To determine whether the beneficial effects of increased muscle BDNF might be mediated via increased expression of its receptors, we examined mRNA levels of p75 and full length TrkB, as well as expression of another ligand for these receptors (NT-4). In the fast-twitch TA, we did not find any differences due either to disease or BDNF overexpression in receptor expression levels (Figure 16B), as previously described (CHAPTER 2). However, we did find decreased levels of both p75 and TrkB mRNA in the *soleus* muscle of diseased 97Q animals compared to Wt controls (Figure 17B), which was not found previously (CHAPTER 2). Nevertheless, in either the TA or soleus, BDNF overexpressors and disease controls did not differ from each other, with each comparably different from Wt controls (Figure 16B and Figure 17B). Thus, muscle BDNF overexpression does not appear to influence motor function by changing expression levels of genes involved in neurotrophic factor signaling.

An emerging theme in SBMA pathophysiology is the reappearance of developmental mRNA isoforms in muscle—adult isoforms decrease, while neonatal isoforms increase. Hence, we examined mRNA levels of adult and perinatal isoforms of myosin heavy chain, a protein critical for muscle contraction, in 97Q animals (BDNF overexpressors and disease controls) and Wt. In line with previous reports, we detected a decrease in the adult isoform and an increase in the perinatal isoform of myosin heavy chain compared to Wt in both the TA and soleus (Figure 16C and Figure 17C). Notably, BDNF overexpression in muscle seemed to confer some level of protection in the soleus muscle, with BDNF overexpressors expressing higher mRNA levels than disease controls of the adult *Myh7*, bringing them closer to healthy, Wt levels. These data suggest that BDNF overexpression in muscle may ameliorate the detrimental loss of the adult isoform for myosin heavy chain in *slow*-twitch muscle. This could contribute to the loss of inherent muscle force in disease muscle.

Our electrophysiological data on neuromuscular transmission from the fast-twitch EDL predicted that we would not see improvements in the fast-twitch TA in the expression of any ion channels contributing to EPP and mEPP decay times and RMP. Indeed, this was the case for

the TA. 97Q animals (BDNF overexpressors and disease controls) showed comparable and significant defects compared to Wt in mRNA levels of sodium channel subunits, AChR subunits, as well as the chloride channel (Figure 16D); adult isoforms were downregulated, while neonatal isoforms were upregulated compared to Wt and there were no differences between BDNF overexpressors and disease controls. However, for the soleus, we again saw that BDNF reversed the effect of disease on mRNA expression for AChR gamma, lowering levels to that of normal adult muscle. Moreover, BDNF overexpression also appeared to provide a low level benefit to virtually every gene examined for both the slow-twitch soleus and the fast-twitch TA, being less different from Wt than control disease muscle was. This is likely *not random* and suggests there may be broad but subtle benefits to the expression of many genes critical to normal motor function.

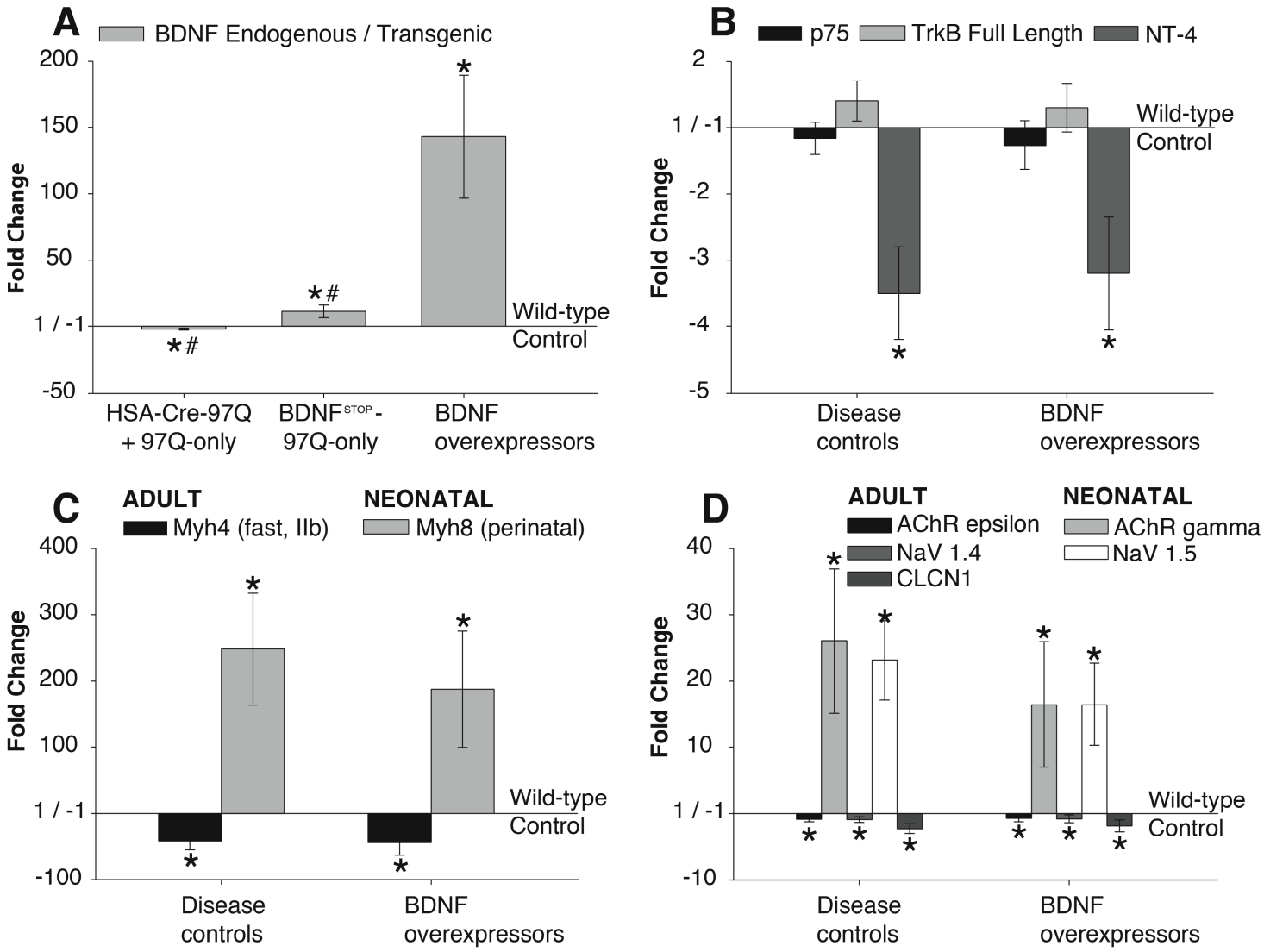

Figure 16. Muscle BDNF overexpression does not improve pathological gene expression (mRNA levels) in the primarily fast-twitch tibialis anterior (TA) muscle. A) Cre-loxP mediated overexpression of muscle BDNF is strong in the TA of BDNF overexpressing males (~10-

Figure 16 (cont'd). fold higher than the BDNF^{stop}-97Q group and ~410-fold higher than the HSA-Cre-97Q + 97Q-only group). B) Expression levels of receptors for BDNF and NT-4 are unaltered compared to wild-type, regardless of BDNF overexpression. NT-4 levels remain reduced in BDNF overexpressors compared to wild-type, as they did for disease controls. C) Expression levels of myosin heavy chain mRNA isoforms did not improve in TA as they did in slow-twitch soleus in response to muscle BDNF overexpression (Figure 17C). D) While fold changes of all ion channel mRNAs appear subtly closer to wild-type levels in the BDNF overexpressors, expression levels do not differ from the disease control group, unlike in the soleus (Figure 17D). *p<0.05 from Wild-type, # p<0.05 from BDNF overexpressors. Disease controls: BDNF^{stop}-97Q, N=4 and 97Q-only + HSA-Cre-97Q, N=11. BDNF overexpressors, N=9. Wild-type, N=6. Values are mean fold changes ± SEMs (standard errors of the mean) based on N/group.

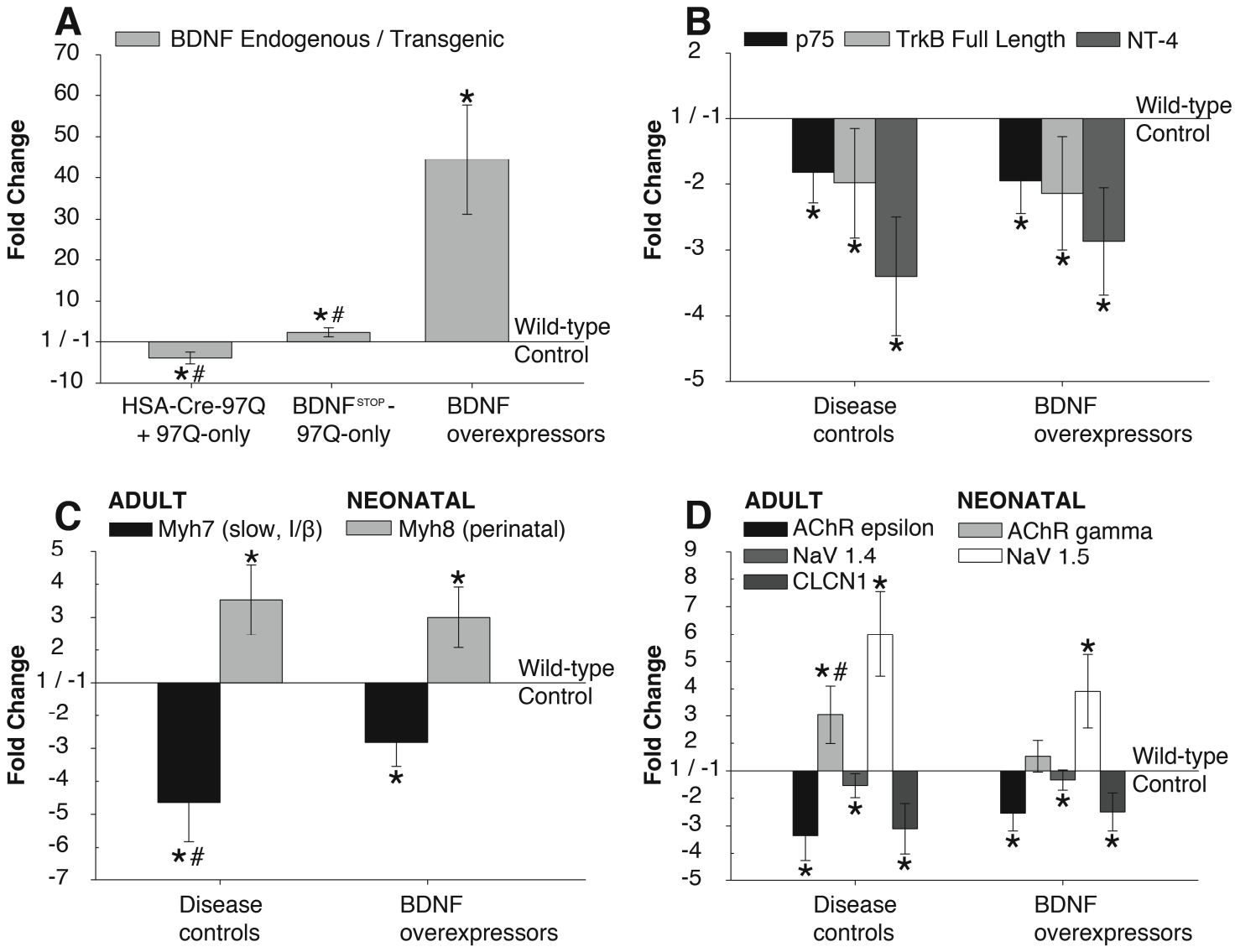

Figure 17. Supplemental muscle BDNF ameliorates an SBMA gene expression phenotype in the slow-twitch soleus muscle. A) Cre-loxP mediated overexpression is very efficient in the soleus muscle of BDNF overexpressing transgenic males (~14-fold higher than the BDNF^{stop}-97Q

Figure 17 (cont'd). group and ~222-fold more than HSA-Cre-97Q + 97Q-only group). B) BDNF receptors p75 and TrkB are downregulated in soleus muscle of both BDNF overexpressors and disease controls, a finding previously undetected in the soleus. NT-4 mRNA levels also remain low in SBMA males, regardless of muscle BDNF status. C) Although myosin heavy chain isoforms are expressed in the predicted disease pattern for BDNF overexpressors and disease controls, the adult isoform (Myh7) is upregulated towards the healthy, wild-type levels in the BDNF overexpressing group compared to controls. This suggests that muscle BDNF may ameliorate contraction dysfunction associated with SBMA in a muscle-type specific manner, since the same was not found in TA (Figure 16C). D) Ion channels critical for synaptic transmission at the neuromuscular junction are perturbed in disease controls as previously described. BDNF overexpressors show a milder phenotype, with no pathological upregulation of the developmental AChR gamma subunit mRNA compared to wild-types. These gene expression data suggest that overexpression of muscle BDNF ameliorates soleus gene expression, and the slowing of SBMA disease progression in the BDNF overexpressors (Figure 4B-C) is likely a result from benefit to slow-twitch muscles. *p<0.05 from Wild-type, $^{\#}$ p<0.05 from BDNF overexpressors. Disease controls: BDNF^{stop}-97Q, N=5 and 97Q-only + HSA-Cre-97Q, N=8. BDNF overexpressors, N=8. Wild-type, N=5. Values are mean fold changes ± SEMs (standard errors of the mean) based on N/group.

V. Discussion

The goal of the current study was to examine whether reversing a deficit in muscle BDNF in diseased SBMA mice could ameliorate disease. We overexpressed BDNF specifically in skeletal muscle using a Cre-loxP Tg mouse model that drove expression of human BDNF selectively in skeletal muscle, crossed with a 97Q SBMA mouse model. We found that overexpression of BDNF in skeletal muscle slowed disease progression nearly two-fold: SBMA mice that overexpressed muscle BDNF ("BDNF overexpressors") lived 19.7 days after symptom onset, while those with low BDNF levels ("disease controls") lived only 10.6 days. We confirmed these findings in a second cohort of animals, where BDNF overexpressors exhibited longer hang times than disease controls during a symptomatic phase. Furthermore, motor function in BDNF overexpressors did not drop during the 11-day symptomatic period like it did for disease controls (Figure 17B), confirming in a second cohort that supplemental muscle BDNF slowed the rate of disease. To determine what mechanisms might underlie the slowing of disease by muscle-produced BDNF, we examined synaptic and muscle function based on intracellular recording of skeletal muscle fibers and the expression of multiple genes implicated in neuromuscular dysfunction in SBMA based on qRT-PCR. Our results point to a fiber type-specific action of BDNF. Namely, we found that defects in the expression of two genes, myosin heavy chain and AChR, were reversed in a slow- but not a fast-twitch muscle. Likewise, neurotransmission in a fast-twitch muscle was not improved but amelioration of select genes predict improvement in some specific aspects of neurotransmission and muscle contractile function in slow-twitch muscle. Hence, supplementing muscle BDNF slowed disease progression, perhaps through action on slow-twitch muscles.

i. Gene expression – Myosin heavy chain

Myosin heavy chain is a motor protein that plays an integral role in force production of skeletal muscle (Schiaffino and Reggiani, 2011). Muscle weakness is a key trait of SBMA in humans (Katsuno et al., 2012), and animal work points to reduced contractile ability (i.e., specific force) in SBMA muscle, which is independent of mass and nerve contributions (Oki et al., 2013; Oki et al., 2015). Several isoforms of myosin heavy chain exist, which are differentially expressed across developmental stages and muscle types, and have distinct contractile properties (Schiaffino and Reggiani, 2011). The neonatal isoforms of myosin heavy chain produce less force and exhibit slower kinetics than the adult isoforms for both slow and fast muscles (Johnson et al., 1994; Racca et al., 2013), reminiscent of muscle contractile properties in diseased muscle of SBMA mice (Oki et al., 2013; Oki et al., 2015). We and others have previously shown an increase in the expression of the developmental isoforms of myosin heavy chain in SBMA muscle (Palazzolo et al., 2009; Halievski et al., 2015b; Jokela et al., 2016). While developmental isoforms are often upregulated during denervation, SBMA muscles are *not* structurally nor apparently functionally denervated despite a loss of motor function (Poort et al., 2016). Our lab has also shown reduced *adult* myosin heavy chain, with slow- and fast- isoforms decreasing in their respective muscle groups (Halievski and Jordan, unpublished observations). These changes are androgen-dependent, as testosterone-treated females, with motor function at its nadir, show reduced levels of adult slow isoform *Myh7* mRNA in slow-twitch muscle (Halievski and Jordan, unpublished observations). However, myosin heavy chain expression was not improved in the TA, even though overall disease progression was slowed. Thus, fast-twitch muscles may *not* be involved in the motor improvement seen in BDNF overexpressors in the current study. That SBMA and control mice express equal levels of adult myosin heavy chain mRNA levels in the quadriceps, a primarily fast-twitch muscle, further supports the idea that in fast myosin heavy chain may not be involved in motor loss (Palazzolo et al., 2009). As our data revealed healthier mRNA levels of the slow/β myosin heavy chain isoform in the soleus

of BDNF overexpressors, it will be interesting to examine the contractile force of slow twitch muscles, which we expect to also be improved.

ii. Gene expression – Muscle ion channels

Similar to myosin heavy chain, mRNA expression of ion channels in diseased SBMA mouse muscle is dysregulated, with developmental isoforms upregulated and adult isoforms down-regulated. Such changes affect the AChR, and the sodium and chloride channels. In the TA muscle, we confirmed these previously reported differences between Wt and diseased control mice (who do not overexpress muscle BDNF), as previously reported for the fast-twitch EDL muscle of 97Q SBMA male mice (Oki et al., 2015; Xu et al., 2016). Interestingly, dysregulation of ion channel expression has also been detected in heart muscle of human SBMA patients, finding a reduction in the adult sodium channel subunit (SCN5A) (Araki et al., 2014). We now extend these findings, by not only showing that expression of the AChR is dysregulated, with the neonatal gamma subunit upregulated in the slow-twitch soleus of symptomatic 97Q SBMA mice, but that this dysregulation can be reversed by muscle-derived BDNF. While we did not detect any improvement in decay time of EPPs in the EDL of BDNF overexpressors, a parameter dependent on the balance of gamma and epsilon subunits of the AChR, it seems likely that kinetics of synaptic potentials will be improved in the *soleus*.

iii. Synaptic transmission at the NMJ

Synaptic transmission at the EDL NMJ is markedly defective in 97Q animals (Xu et al., 2016) which were largely replicated in the present study. While BDNF seemed to slow the course of disease, it apparently does not achieve this by increasing the effectiveness of neurotransmission in the EDL. Evidently, defects in neurotransmission in the EDL do not underlie the defects reversed by overexpression of muscle BDNF to improve motor function. This observation also indicates that defects in EDL neurotransmission do not fully explain motor

dysfunction in the 97Q SBMA model. Moreover, asymptomatic juvenile 97Q males exhibit most of the same neurotransmission defects with comparable severity in the EDL as adults (Xu and Jordan, unpublished observations), further indicating that defects in neurotransmission in the EDL are not necessarily a reliable proxy for the quality of motor function as whole. Clearly, more muscles and more aspects of neurotransmission need to be considered. Data thus far suggest that neuromuscular function in slow twitch muscles such as the soleus may critically mediate losses in motor function, at least in the early stages, and that muscle-derived BDNF is able to reverse some of this dysfunction by correcting the expression of at least two genes with well understood functions.

Notably, innervation is required for mRNA expression of slow (I/β) myosin heavy chain (Jerkovic et al., 1997). Given that BDNF increased mRNA levels for the slow adult isoform suggests that the soleus is more functionally innervated, and thus, BDNF may have rescued quantal content or aspects of synaptic plasticity that keep the synapse above threshold under prolonged periods of activity. Finally, neurotransmission in the soleus of asymptomatic juvenile 97Q male mice was not as perturbed as it was in diseased adult mice, suggesting that dysfunction in the soleus may be a better predictor of motor dysfunction in this model (Xu and Jordan, unpublished observations).

In the current study we did not fully replicate differences in neurotransmission between 97Q animals and Wt. Namely, here we detected an increase in EPP amplitude of both groups of mice carrying the disease allele compared to Wt, while previously, EPP amplitude was normal in diseased mice. This difference may have not been detected earlier due to relatively lower statistical power in the previous study (current study N=8-11, previous study N=4); indeed, mean EPP amplitude in the EDL is consistently higher in both juvenile and adult 97Qs compared to Wt (Xu and Jordan, unpublished observations; Xu et al., 2016). Moreover, EPP amplitude was increased at the NMJ in a mouse model of motoneuron disease (ALS) and in the polyglutamine Huntington's disease (Rozas et al., 2011; Arbour et al., 2015), suggesting that

increased EPP amplitude is common in diseased junctions and may simply reflect the reduced size of diseased EDL muscle fibers (Oki et al., 2015). In the current study, we also detected reduced facilitation at all stimulation frequencies for the short-term plasticity studies. Discordant with the previous study, we detected differences between disease and Wt groups at the 10Hz and 30Hz stimulation parameters. These discrepancies may again be due to greater power to detect smaller differences in the current study, differences in the mouse genetic background between the two studies, or differences in the severity of motor dysfunction shown. Such possibilities may also explain the discrepancy in mEPP frequency in the two studies.

iv. Neurotrophin-related gene expression

Expression of the BDNF transgene was apparently quite efficient in SBMA muscle—on the scale of 45- to 145-fold higher than normal Wt levels depending on the muscle. For this reason, we also examined the expression of genes related to BDNF signaling to determine whether any regulatory feedback occurred. This information is valuable if BDNF given to muscle is used therapeutically in the future. Moreover, it also helps us to understand what factors might have limited BDNF efficacy. For example, if high BDNF levels lead to a downregulation in its receptors, this could explain why quite high levels of BDNF expression in muscle were not more effective in rescuing motor function. Thus, we would need to consider preventative measures or adjust BDNF dose accordingly. It is also important to consider whether BDNF overexpression also affects levels of NT-4 expression, a neurotrophin that also signals via the same receptors as BDNF. In CHAPTER 2, we reported that diseased 97Q mice did not exhibit differences in TrkB or p75 compared to Wt in the EDL and soleus. We replicated these findings in the TA of diseased mice of the current study. However, discordant with findings reported in CHAPTER 2, the soleus of diseased 97Q animals (BDNF overexpressors and disease controls) showed a small, but significant, reduction of both TrkB and p75 mRNA levels compared to Wt, a deficit that BDNF overexpression did not change in either the TA or soleus. Despite this

downregulation in receptor mRNA, BDNF was still capable of producing beneficial effects in the soleus. Perhaps overexpressing muscle BDNF compensates for a deficit in receptors to combat disease progression by helping to maintain some aspects of neuromuscular function. It is important to note that BDNF was able to improve function even in the face of reduced NT-4 levels (Wahl, Halievski, and Jordan, 2015, unpublished observations; Yu et al., 2006) and presumably reduced levels of other neurotrophic factors, including VEGF and GDNF, also known to be perturbed in SBMA muscle (Yu et al., 2006; Monks et al., 2007). It seems likely that reversing the deficit of other key neurotrophic factors would provide additional benefits to motor function (Fan et al., 2000).

v. Fiber-type effect of muscle BDNF

That muscle BDNF overexpression may have selectively improved slow-twitch muscle function in an SBMA mouse model is consistent with what is known about BDNF and TrkB signaling. First, in healthy adult mice, BDNF is more highly expressed in the soleus than the EDL (Mousavi et al., 2004), suggesting that perhaps slow-twitch muscle is more dependent on TrkB signaling. Indeed, the soleus but not the EDL experiences greater fatigue in response to tetanic muscle stimulation when the other ligand for TrkB, NT-4 is knocked out (Belluardo et al., 2001). Likewise, a muscle-specific TrkB knockout showed increased failure of neuromuscular transmission, more NMJ fragmentation, and decreased muscle strength in the slow-twitch soleus compared to Wt controls (Kulakowski et al., 2011). NT-4 signaling is also critical for the normal expression of type I slow myosin heavy chain in the soleus (Carrasco and English, 2003). Moreover, NT-4 and to a lesser extent BDNF treatment to the nerve stump after sciatic nerve injury preferentially rescued soleus mass and the size of slow fibers (Simon et al., 2003). Finally, after three months of BDNF treatment to extraocular muscles in non-human primates, muscle fiber and NMJ size was increased in slow, but not fast, fibers; the overall proportion of slow-staining fibers was also higher in BDNF treated muscle (Willoughby et al., 2015). In the

current study, we saw that overexpressing BDNF in skeletal muscle normalized the expression of some muscle genes, but only in the slow-twitch soleus and not the fast-twitch EDL. Thus, the selective beneficial effect of Tg expression of BDNF on the soleus may underlie the improved hang times seen in diseased mice. One could argue that the hang test is a measure of slow-twitch muscle function as it requires the animal to grasp the cage lid for an extended period of time, recruiting more slow fibers. Nevertheless, BDNF and NT-4 signaling also contribute to proper fast-twitch muscle function and thus effects there cannot be ruled out (Sakuma et al., 2001; Mousavi et al., 2004; Clow and Jasmin, 2010). Although our gene expression data in the TA did not reveal significant improvements, the changes observed were in the direction of healthy, Wt levels. Thus, BDNF may have improved both fast and slow muscle function, but with a stronger influence on slow-twitch muscles.

As muscle BDNF may produce its beneficial effects through fiber type-specific mechanisms, how different fiber types might contribute to SBMA bears a brief discussion. In SBMA humans, fast-fibers seem preferentially affected: highly atrophic type IIa (fast-oxidative) fibers were frequent in the TA (Jokela et al., 2016); fewer type IIb (fast-glycolytic) fibers, with a concomitant increase in type I (slow-oxidative) fibers, were observed in the vastus medialis and triceps (Harding et al., 1982); and number of type IIX (fast-intermediate) fibers was reduced in the biceps (Yamada, Halievski, Jordan, and Sobue, unpublished observations). Similarly, in the 97Q mouse model, contractile function of the fast-twitch EDL was more greatly affected than the slow-twitch soleus; however, the opposite was true in a myogenic model of SBMA (Oki et al., 2015). Additionally, in the diseased 97Q quadriceps, there was a loss of a healthy mosaic pattern of NADH staining, indicating that oxidative capacity was perturbed in this fast muscle (Rinaldi et al., 2012). In two other SBMA mouse models, an oxidative-to-glycolytic shift in fiber type occurred only in the fast EDL, with no fiber-type switching in the slow soleus; oxidative capacity (NADH stain) was reduced in both EDL and soleus (Ramzan et al., 2015). Yet in a fifth, knock-in model of SBMA, slow muscles were grossly unaffected, but fast muscles exhibited a

glycolytic-to-oxidative switch, with proportion of fibers shifted from IIb (fast-glycolytic) to IIa (fast-oxidative), and NADH staining increased compared to controls (Rocchi et al., 2016). Interestingly in this same knock-in model, transcriptional restoration through a genetic disruption of AR SUMOylation rescued specifically type I (slow-oxidative) fiber atrophy, which correlated with survival and improved motor ability on an endurance test that required slow muscle function (Chua et al., 2015). In sum, these data suggest that fast-fibers are primarily affected, but improving function of *slow* fibers is enough to rescue motor function suggesting that the loss of motor function caused by SBMA is partly related to dysfunction in slow twitch muscles. Thus, a beneficial effect of BDNF on slow-twitch fibers may support the organism when function in fast fibers is lost and/or enhance the function in fibers that have recently converted to slow.

vi. BDNF^{stop} / HSA-Cre Model validation

We were surprised to find that BDNF mRNA was expressed in animals containing only the BDNF^{stop} allele. We believe that the strong promoter (CAGGSS) combined with the location of the transgene (downstream of the efficiently expressed collagen 1a1 locus) allow the stop cassette to be bypassed by some possibly cell-specific factors since we detected BDNF mRNA levels in loxP-only mice without the benefit of the Cre recombinase transgene (McCreath et al., 2000; Chang et al., 2006). This seems plausible, as leakiness in muscle is more so than in spinal cord (~14-fold versus 2-fold, respectively) and fibroblasts, a cell type that strongly expresses collagen 1a1 (McCreath et al., 2000), are abundant in muscle tissue (Gillies and Lieber, 2011). We also did not detect doxycycline sensitivity in this model as previously reported, and found that recombination had already occurred in juvenile HSA-Cre / BDNF^{stop} animals. While this Cre model is known to exhibit some leakiness (Rao and Monks, 2009), we found that doxycycline did not enhance Tg BDNF expression whatsoever. This was unexpected, as others have required a longer doxycycline treatment to fully reach the desired expression (Li et al., 2015). It is possible that the BDNF^{stop} allele, which is downstream of the strong collagen

1a1 locus, combined with the leaky HSA-rTTA resulted in maximum transgene expression early on.

vii. Conclusions

BDNF overexpression in muscle slowed disease progression in a mouse model of SBMA. Notably, this intervention doubled the time to end-stage of diseased animals from the time of symptom onset. During this period, mice with BDNF maintained a higher level of motor function. At the molecular level, we saw significant improvements in gene expression in the slow-twitch soleus muscle, although there was an overall tendency towards healthy, Wt mRNA levels in both fast and slow twitch muscles of mice that overexpressed BDNF mRNA in their muscles. Future studies should focus on the slow-twitch muscle as a target for beneficial effects of BDNF to ameliorate symptoms of SBMA.

CHAPTER 4: General discussion

I. Overview of findings

The experiments presented in this dissertation propose a novel therapeutic direction to ameliorate disease in SBMA by using the muscle-supplied neurotrophic factor, BDNF. We discovered that BDNF mRNA levels were reduced in androgen-dependent manner in muscles of diseased SBMA mice, and subsequently found that transgenically replacing muscle BDNF slowed disease progression and rescued gene expression in a slow-twitch muscle.

We found a 6-8 fold deficiency in muscle BDNF in two SBMA mouse models: 1) a myogenic model that overexpresses a disease-causing Wt AR exclusively in muscle cells, and 2) a 97Q model that ubiquitously overexpresses a polyglutamine-expanded AR. The loss of BDNF mRNA was androgen dependent, paralleling the androgen-dependent nature of SBMA. Moreover, when chronically diseased myogenic male mice were castrated to remove endogenous androgens, motor function *and* BDNF mRNA expression was restored. In the 97Q model, male mice were castrated presymptomatically, which prevented the expression of an overt motor phenotype, and also prevented the loss of muscle BDNF levels. As a final experiment to link the loss of muscle BDNF mRNA to disease in SBMA, we conducted a time course study in the myogenic model. Tg females that are asymptomatic rapidly show SBMA symptoms when exposed to male-typical androgen levels, reaching baseline motor function after 5 days of treatment (Monks et al., 2007). Thus, we administered testosterone to females and examined BDNF mRNA expression in muscle on days 1 through 5 of treatment. After the first day of treatment (prior to overt motor dysfunction), females already showed a robust downregulation of BDNF mRNA, but levels before testosterone treatment were normal. Motor dysfunction manifested only on the third day of treatment. Together, these findings strongly linked the expression of muscle BDNF mRNA to disease progression, perhaps even being causal in disease since the drop in BDNF levels occurred *before* the drop in motor function.

Given this information, as well as previous work linking BDNF signaling to healthy neuromuscular function (Clow and Jasmin, 2010; Kulakowski et al., 2011; Mantilla et al., 2014), we sought to test whether providing exogenous BDNF to BDNF-depleted SBMA muscles would ameliorate defects in motor function, synaptic transmission, and muscle gene expression.

To this end, we used a Tg approach to overexpress BDNF specifically in muscle cells using Cre/loxP mice crossed to 97Q SBMA mice. This allowed us to compare SBMA mice that overexpressed muscle BDNF to disease controls that had deficits in muscle BDNF. We found that muscle BDNF overexpression benefited SBMA mice by slowing disease progression by two-fold after disease onset. To determine the underlying mechanisms that might account for improved motor function, we assessed synaptic function at the NMJ using single fiber recording in the EDL, a fast-twitch muscle that is known to exhibit defects in neurotransmission (Xu et al., 2016). We found that BDNF overexpression did not ameliorate disease in this measure in the EDL, as all SBMA animals showed defects in synaptic transmission compared to healthy Wt controls, regardless of whether they overexpressed BDNF or not. Another route through which BDNF overexpressors might exhibit improved motor function is through the rescue of muscle gene expression, known to be greatly perturbed in SBMA (Mo et al., 2010). We examined the expression of genes in muscle that are critical for proper neuromuscular function and known to be dysregulated in SBMA (Halievski et al., 2015c; Oki et al., 2015; Xu et al., 2016). We found that myosin heavy chain expression was rescued in the slow-twitch soleus muscle but not in the fast twitch TA. This rescue effect might account for improved motor function, perhaps through improved contractile properties, a finding that needs further exploration. Finally, AChR gamma subunit expression was also restored in the soleus, being expressed at healthy Wt levels, unlike the pathological upregulation in non-BDNF overexpressing disease controls. That gene expression is rescued primarily in the soleus, a slow twitch muscle, suggests that BDNF might preferentially act on slow-twitch muscle fibers to improve neuromuscular function in the face of disease.

While the experiments in this dissertation provide solid groundwork to move forward with exploring muscle BDNF as therapeutic for SBMA, more work needs to be done to understand which mechanisms are improved that lead to a rescue in overall motor function.

II. Future Directions

Muscle BDNF overexpression slowed disease progression, but the underlying cellular mechanisms responsible for the improvement remain largely elusive. Fortunately, the findings from this dissertation call for specific predictions proceeding forward.

i. Does BDNF improve neurotransmission in slow-twitch muscle?

Findings from CHAPTER 3 suggest that BDNF preferentially rescues slow-twitch muscles, since improved motor function was seen on an endurance (hang) test and the expression of some genes was rescued by Tg BDNF in the slow-twitch soleus. Although neurotransmission was not rescued in the fast EDL, the situation may be different in the slow soleus. Thus, intracellular recording experiments in the soleus will need to be done to determine whether quantal content, short-term facilitation, RRP, and/or release probability are improved. Gene expression findings hint that a rescue *will* be seen in synaptic transmission in this slow-twitch muscle. In particular, mRNA of the developmental AChR subunit gamma was not pathologically upregulated and was expressed at healthy, Wt levels in the soleus of BDNF overexpressing SBMA mice. Thus, the postsynaptic response to neurotransmitter may be normalized; the specific prediction is a rescue of EPP and mEPP decay times, which were elongated in SBMA mouse models (Xu et al., 2016). Indeed the epsilon isoform confers a greater conductance to the AChR (Mishina et al., 1986), more likely resulting in action potential generation in the muscle fiber.

ii. Does BDNF overexpression improve muscle contraction and increase the reliability of neurotransmission?

To determine whether muscle contraction and neurotransmission is improved in SBMA mice overexpressing BDNF, muscle tension could be assessed in response to direct-muscle and indirect-nerve stimulation. Rescued gene expression in the soleus of BDNF overexpressors predicts that muscle contraction would be improved for both muscle- and nerve-evoked contraction. Specifically, since expression of the gamma subunit of AChR was rescued, nerve-evoked transmission might be improved due to higher conductance of the adult epsilon subunit. It is also possible that soleus muscle will exhibit improved presynaptic properties, perhaps with an enhanced release of neurotransmitter (to be determined). Muscle-evoked contraction (i.e., intrinsic muscle force) may also be improved since the adult myosin heavy chain expression was rescued in the soleus. Indeed, adult muscles produce more specific force than fetal muscles (Racca et al., 2013). It would also be interesting to examine whether expression of myosin heavy chain IIa, the other predominant fiber-type (fast-oxidative) in the soleus (Totsuka et al., 2003), is affected in the 97Q SBMA model and rescued by BDNF overexpression.

Examining neurotransmission failure by comparing contraction produced by challenging muscle-evoked and nerve-evoked stimulation could further glean insight into how neuromuscular transmission is affected in SBMA, and whether any components are improved by BDNF overexpression. Indeed BDNF reduces failure of neuromuscular transmission as detected by muscle contraction, although it does not affect intrinsic contractile function (Mantilla et al., 2004). Nerve-evoked (Malik et al., 2013; Rocchi et al., 2016) and muscle-evoked (Oki et al., 2013; Oki et al., 2015) contraction are reduced in SBMA. What is unclear is whether defects in neurotransmission produce defects in muscle contraction that are over and above what is seen in muscle-evoked contraction. That motoneurons release less neurotransmitter (Xu et al., 2016) predicts an even greater dysfunction in contraction in response to nerve stimulation. Examining failure of neurotransmission by comparing nerve- and muscle- evoked contraction in

SBMA muscle could reveal perturbations in presynaptic release or postsynaptic response that contribute to reduced muscle force production, separate from the downstream steps of force generation. Moreover, neuromuscular function would be examined at a broader, motor unit population level in this nerve- versus muscle-evoked paradigm. Improvement is predicted in slow muscles based on gene expression and an improvement may also be found in fast muscles since there was a general tendency for expression levels to be closer to that of Wt in EDL of BDNF overexpressors (Figure 16), suggesting that subtle differences may exist, which may be brought out in this paradigm.

Overall, assessing muscle contraction as a readout of neuromuscular function will provide useful information about the underlying changes in the BDNF overexpressors that allow them to perform better at the behavioral level (hang test).

iii. Might the benefits of BDNF overexpression be better detected at an earlier disease stage?

An alternative explanation for the modest effects of muscle BDNF on improving synaptic function and gene expression SBMA may be that our chosen time-point of examination was too far into disease to detect substantial differences between BDNF overexpressors and disease controls. Nevertheless, that the slowed disease progression was replicated in two cohorts suggests that the effect is reliable although subtle. Perhaps exogenous BDNF is providing a more global enhancement of neuromuscular function via small benefits to a number of different mechanisms as our data suggest is the case for the fast twitch TA. Alternatively, the underlying improvements might only be detected at an earlier age, prior to motor dysfunction, which the system later succumbs to as disease progresses. Muscle gene expression is perturbed very early in disease (Appendix, Figure 19; Table 7). In the TA of juvenile, presymptomatic (29-32 day old) 97Q male mice, gene expression was already significantly perturbed compared to Wt controls (Appendix, Figure 19) for most of the same genes that were perturbed in the

symptomatic TA of 97Q disease controls (Figure 16). The only exception in presymptomatic muscle was that no increases were yet detected in the levels of the developmental isoforms of the AChR (gamma) and sodium channel subunit 1.5 (Appendix, Figure 19; Table 7), which could mean that this upregulation is a late stage occurrence. Nevertheless, although the gene expression changes were significant in presymptomatic mice, they were much milder than that at symptomatic stages. For example, the perinatal myosin heavy chain was upregulated ~4-fold in juveniles, while the upregulation was ~250-fold in adults (see Appendix, Table 7 for full list). Thus, the severity of gene expression dysregulation correlates with severity of motor symptoms, suggesting that these changes may be relevant to disease progression.

There are at least three possible explanations for the early defects in gene expression. The first is that many cellular processes become slowly defective over time under the stresses of toxic AR in SBMA, and thus an overt motor dysfunction is preceded by weeks of neuromuscular dysfunction that is not detected with the behavioral motor tests used. Gene expression defects could be a part of this early pathology. Another explanation could be that the SBMA muscle never reached healthy, adult-level expression. Finally, these defects could be due to the transgene alone, and thus not relevant to the SBMA phenotype. However, this last explanation is unlikely as the fold-changes become stronger with motor dysfunction (Appendix, Table 7). Moreover, in the myogenic model of SBMA, these gene expression changes are androgen-dependent, thus not occurring solely due to transgene expression (Henley and Jordan, unpublished observations). Therefore, disease seems to start early in the 97Q model.

To detect an improvement due to muscle BDNF overexpression, examining at an earlier age may be warranted. Indeed, the animals examined here were collected *after* disease onset, indicating that the integrity of the neuromuscular system has already declined to a detectable threshold. However, cellular (synaptic function; Xu and Jordan unpublished observations) and molecular (gene expression, Appendix, Figure 19) defects are already apparent at young age, much earlier than motor dysfunction. Thus, there is substrate in 97Q males at a young age to

examine differences between BDNF overexpressors and disease controls. At this earlier age, a more ubiquitous rescuing of gene expression may be revealed, and possibly improvements in synaptic transmission at the EDL. These predicted outcomes would be in congruence with the slowing of disease progression, since disease apparently begins much earlier than previously thought.

A final consideration of this in the translational sense would be whether BDNF administration *after* disease onset is sufficient to ameliorate disease. If not, supplementation would need to happen presymptomatically and this will be difficult in SBMA patients unless an early genetic diagnosis is received.

iv. Does mRNA expression predict protein expression?

Another consideration of why muscle BDNF overexpression provided such subtle effects is whether BDNF protein was overexpressed to the extent that the mRNA was based on qRT-PCR. BDNF mRNA levels were robustly elevated (45-144 fold) in muscle as a result of Cre/loxP mediated expression. However, it is unclear whether BDNF *protein* was also enhanced and there are a couple of reasons to think that may not have been. First, mice carrying the disease 97Q gene and the $BDNF^{stop}$-loxP transgene without the HSA-Cre did not exhibit a slowing of disease progression (Figure 18), even though they did overexpress BDNF mRNA to some extent (3-13 fold), essentially reversing the disease-related deficit in BDNF mRNA. Nevertheless, no benefit was seen. Thus, it is possible that the level of mRNA expression in $BDNF^{stop}$-loxP controls did not lead to a comparable increase in protein. Alternatively, if it did, perhaps a threshold of BDNF overexpression is required to produce beneficial effects in SBMA. Another reason to believe that mRNA levels are not a perfect readout of protein, especially in SBMA, is that SBMA cells are inherently unhealthy, thus unlikely to efficiently translate all of the available mRNA. Indeed, the unfolded protein response, which occurs during ER stress, is

upregulated in muscle of a mouse model of SBMA (Yu et al., 2011). Thus, the observed benefit of muscle BDNF on motor function might be subtle due to limited production of BDNF protein.

Unfortunately, measuring BDNF protein is notoriously difficult, especially in muscle (personal observation; personal communication with C.B. Mantilla, N. Garcia; also see Greising et al., 2015). Thus, an accurate estimation of BDNF in muscle due to Tg expression is not feasible. One possibility to measure Tg BDNF protein would be to detect the hemagglutinin tag in the $BDNF^{stop}$ loxP allele (Chang et al., 2006). Although this would not tell us relative amounts of BDNF overexpression compared to Wt, it may provide a hint as to whether any BDNF protein is produced in $BDNF^{stop}$-loxP mice. If so, then the relative BDNF expression could be compared between $BDNF^{stop}$-loxP and HSA-Cre/$BDNF^{stop}$ groups.

v. Does increasing muscle BDNF mRNA globally only benefit some fiber types affected by SBMA?

Based on the data presented in this dissertation, BDNF appears to preferentially act on slow-twitch muscles to rescue disease in the 97Q mouse model of SBMA. This was interesting to note since the extent of transgene upregulation (as detected via qRT-PCR) was only ~45-fold in the slow soleus, while it was ~144-fold in the fast TA, even though improvements that achieved statistical significance were detected only in the soleus. It could be that slow-twitch muscles are less pathological in SBMA from the outset, lowering the threshold required to rescue them. Alternatively, normal function of slow twitch motor units may depend more on TrkB signaling than fast twitch motor units (see CHAPTER 3 discussion). Whatever the case, it will be important to further study slow muscles to determine the exact benefits that BDNF is imparting. Finally, if BDNF improves only slow fibers, an intervention for fast-twitch muscles will need to be established.

That BDNF preferentially improves slow-twitch muscles also has to be considered in light of SBMA in general. The muscles affected by disease may be different across different

SBMA models and in humans. Many models of SBMA exist, each with their own unique mode of toxicity (Wt or polyglutamine AR; muscle-specific, motoneuron-specific, or global expression), disease progression (chronic, slow, sporadic death), and model organism (mouse, fly, cell culture). An apt example is in two Tg SBMA models studied here, slow twitch muscles are much more affected in the myogenic model while in the 97Q model fast twitch muscles are more affected (Oki et al., 2015). Thus, if slow-twitch muscles are largely affected in patients with SBMA, BDNF could offer significant benefit. Alternatively, if fast-twitch fibers are more affected in SBMA, the benefits of BDNF could be limited. However, given that a switch from fast to slow is part of the disease process, BDNF may also have therapeutic potential for muscles that were originally fast. Thus, although a BDNF intervention was examined in only one model of SBMA, an improvement of some degree would be expected in other models regardless of muscle-type pathology, and also hopefully in SBMA patients.

III. Questions remaining

If muscle BDNF treatment is to be considered as a therapeutic for SBMA, there are several items to consider before moving forward to clinical trials. This includes 1) assessing whether a more potent benefit could be produced with the combination of multiple neurotrophic factors, 2) whether there are any deleterious effects of BDNF treatment, and 3) how BDNF could be administered to humans since a Tg approach is not possible.

i. Could the effect of BDNF be synergized with other neurotrophic factors to rescue the neuromuscular system in trying times?

The neuromuscular system depends on the action of more neurotrophic factors than just BDNF for proper function (Chevrel et al., 2006). Hence, it is not surprising that BDNF alone may not be enough to produce full rescue of a pathological state. Starting early on, many muscle-

derived neurotrophic factors are needed to rescue motoneurons from developmental cell death (Oppenheim, 1996; Henderson et al., 1998). Indeed, there is ample evidence for neurotrophic factors to act in a synergistic or additive manner to promote neuromuscular function. For example, BDNF and CNTF treatment to *Xenopus* nerve-muscle co-cultures produced a synergistic potentiating effect on spontaneous synaptic transmission since the two factors enhanced neurotransmitter release through different mechanisms (Stoop and Poo, 1996). In disease and injury, combining neurotrophic factors also produces greater effects than treatment with a single factor. In neonatal nerve injury, neurotrophic treatment can rescue motoneurons and muscle fibers from death. When both BDNF and GDNF were used after a sciatic nerve transection, a greater survival of motoneurons was seen compared to when either factor was used alone (Vejsada et al., 1998). In a similar nerve injury model, type IIb (fast-glycolytic) muscle fibers were rescued when BDNF and CNTF were co-administered, while CNTF and BDNF alone did not rescue any type IIb fibers (Mousavi et al., 2004); notably, treatment with NT-3 or NT-4 in combination with CNTF did not enhance rescue (Mousavi et al., 2002), suggesting BDNF is necessary for this effect.

In motoneuron disease models, treatment with multiple neurotrophic factors also produces greater effects than single neurotrophic factors. For example, in Wobbler mouse model, subcutaneous treatment of BDNF and CNTF improved motor function and disease progression to a much greater extent than either factor did alone (Mitsumoto et al., 1994). More recently, in a rat model of ALS, synergistic effects on survival were seen following implantation in skeletal muscle of human mesenchymal stem cell transplants expressing both GDNF and VEGF (Krakora et al., 2013). However, BDNF or IGF-1 treatment alone did not produce any benefit, which may have been due to the lower expression of these factors in the stem cells than that of VEGF and GDNF in that study (Krakora et al., 2013). Perhaps a synergistic approach with BDNF and VEGF/GDNF would produce a greater benefit as well, given the above evidence of BDNF to act synergistically in rescuing the neuromuscular system. Finally, concerning the ALS

clinical trials with neurotrophic factors, hints of benefits on patients were seen in early pilot work, similar to hints observed in animal work following administration of single neurotrophic factors. That the outcomes of the clinical trials were unsuccessful is not surprising since those trials were conducted with single treatment of either BDNF, CNTF, or IGF-1. Future clinical trials should take advantage of synergistic and additive effects of combined neurotrophic factor treatment in neuromuscular disease.

ii. Could there be deleterious effects of BDNF overexpression in skeletal muscle? The yin and yang hypothesis of BDNF.

The experiments in CHAPTER 3 were based on the findings of reduced BDNF mRNA in muscle of SBMA mice. However, whether BDNF protein was reduced, or what the balance between proBDNF and mature BDNF was, remains unknown. By overexpressing muscle BDNF, we hoped to remedy this loss. As mentioned above, it is quite difficult to measure BDNF protein, let alone differentiate between the pro- and mature forms. Perhaps examining expression of enzymes involved in BDNF processing (e.g., sortilin) and cleavage (e.g., furin or plasmin) would provide insight into whether the balance of the isoforms perturbed. Why does this matter? Since proBDNF and mature BDNF preferentially bind different receptors to activate different downstream signaling pathways, with proBDNF activating p75 leading to “death signaling”, benefits are only likely when the mature form of BDNF is favored.

Signaling through the p75 receptor is often associated with pro-apoptotic or synaptic depression-like events, while TrkB signaling is associated with survival and potentiating effects on synaptic transmission (Lu et al., 2005). Hence, a Tg overexpression of muscle BDNF might inadvertently produce excess proBDNF that worsens the neuromuscular state in SBMA. On the post-synaptic side, this is unlikely to produce detrimental effects. For example, p75 signaling promotes myogenic differentiation and muscle repair (Deponti et al., 2009), which are necessary functions in damaged muscles. Since SBMA patients exhibit high levels of CK, a marker of

muscle damage, improved regeneration could benefit SBMA patients (Chahin and Sorenson, 2009). Moreover, muscle strength is reduced in p75 deficient mice (Reddypalli et al., 2005), indicating that p75 signaling is important for muscle function. p75 signaling on the muscle is also important for communication with the innervating motoneuron: enhancing post-synaptic calcium through a p75-mediated route provides a retrograde signal to potentiate neuromuscular transmission (McGurk et al., 2011). Finally, blocking p75 inhibits the potentiating effect of BDNF on EPP amplitude, suggesting that p75 signaling can also enhance neurotransmission (Garcia et al., 2010b).

Although it seems that BDNF overexpression is unlikely to be deleterious to muscle, it could potentially have deleterious retrograde effects on the motoneuron in other ways. For example, if the balance is tipped toward more proBDNF production, negative signals could be sent to destabilize the NMJ. For example, during development, proBDNF inhibits NMJ formation by causing retraction of the axon terminal, while mature BDNF stabilizes it (Je et al., 2013). Also in development, blocking p75 activity with antiserum against p75 resulted in a delay of neuromuscular synapse elimination (Garcia et al., 2011), suggesting signaling through p75 functions to promote synapse elimination. Moreover, p75 seems to mediate axonal degeneration and motoneuron death following nerve injury, since p75 deficient mice exposed to nerve crush exhibited better recovery than mice capable of expressing p75 (Ferri et al., 1998). Endogenous BDNF has also been shown to enhance excitotoxic sensitivity of motoneurons through TrkB signaling, since treating with BDNF antiserum or blocking TrkB activity by overexpressing a dominant negative TrkB protected motoneurons from excitotoxic insult (Hu and Kalb, 2003). We have hopefully avoided this latter possibility by driving BDNF overexpression only in muscle cells, but it remains possible that muscle-supplied BDNF that is taken up and retrogradely transported by motoneurons could increase the level of toxicity there rather than reduce it.

In sum, given the potential for negative effects of supraphysiological levels of BDNF, more attention should be given to the approach of combining neurotrophic factors. Combined expression could provide synergistic benefits and reduce the need to express any one neurotrophic factor at a high (perhaps toxic) level.

iii. How might BDNF treatment be translated for use in SBMA patients?

Obviously a Tg approach will not be feasible in SBMA patients, thus the findings here will need to be first replicated using alternative treatment methods. One straightforward option is injections, but the short half-life (10 minutes) of BDNF (Sakane and Pardridge, 1997) does not make this a favorable approach. Instead, BDNF mimetics, such as 7,8-dihydroxyflavone could be used (Jang et al., 2010). Apart from the poor pharmocokinetic profile of BDNF, a problem with straight injections could be that the drug does not reach the necessary location to produce its action, potentially reducing the efficacy of mimetics. Indeed, BDNF has important functions in muscles, at the NMJ, as well as for the motoneuron itself. Moreover, since motoneurons can retrograde transport BDNF-TrkB, administration in the muscle could provide supportive effects to all of these dysfunctional sites. However, injecting mature BDNF or TrkB agonists might reduce the benefits produced through p75, although there are also risks through stimulating p75 (see above). To bypass these potential roadblocks, one could use mesenchymal stem cell implants that have been induced to express BDNF and perhaps other neurotrophic factors. These cells can then be implanted in the muscle where they can continuously secrete neurotrophic factors. This sort of approach has been recently tested and deemed safe in humans with ALS, with some indications of efficacy when intramuscular implantation was combined with intrathecal implantation (Petrou et al., 2016). That muscle BDNF overexpression produces beneficial effects in an SBMA mouse model suggests that intramuscular implants alone may be sufficient in SBMA. The questions that remain would be whether other neurotrophic factors can provide additional or synergistic benefits.

IV. Conclusions and final remarks

Supplementation of Tg BDNF to muscle in an SBMA mouse model slowed disease progression. Improvement in slow-twitch muscle appears to be the route through which muscle BDNF is producing a beneficial effect. At this time, the supporting data include rescued gene expression of myosin heavy chain and AChR subunits in the slow-twitch soleus. Thus, more studies are required to determine whether BDNF also rescues neuromuscular function, specifically, synaptic transmission and muscle contractile force. Regardless of how muscle BDNF produces its beneficial effect at the cellular and molecular level, it clearly has an impact on overall motor function and thus may have therapeutic value in slowing the loss of motor function in SBMA patients.

APPENDIX

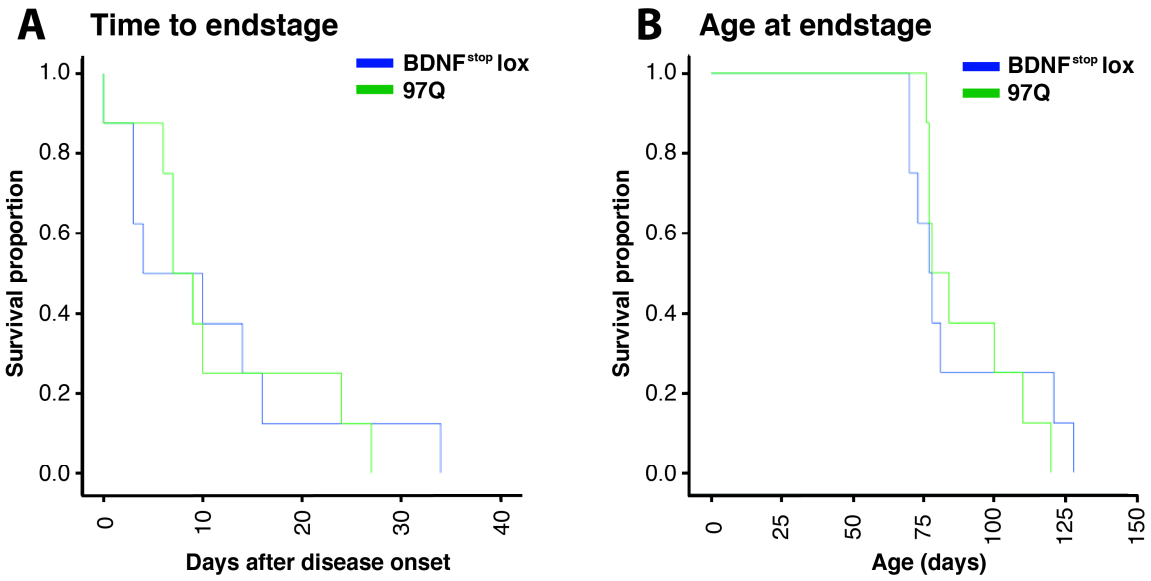

Figure 18. Minor muscle and spinal cord BDNF mRNA expression in the BDNF^{stop} genotype is not sufficient to improve disease phenotype in an SBMA mouse model. Neither A) time to endstage nor B) age an endstage are improved in mice expressing the BDNF^{stop} allele, in whom we find leaky transgenic human BDNF mRNA expression. At least a 45-fold upregulation of BDNF mRNA in muscle is required for any benefit. 97Q, N=7 BDNF^{stop}, N=8. Values are proportion of mice surviving to age on x-axis.

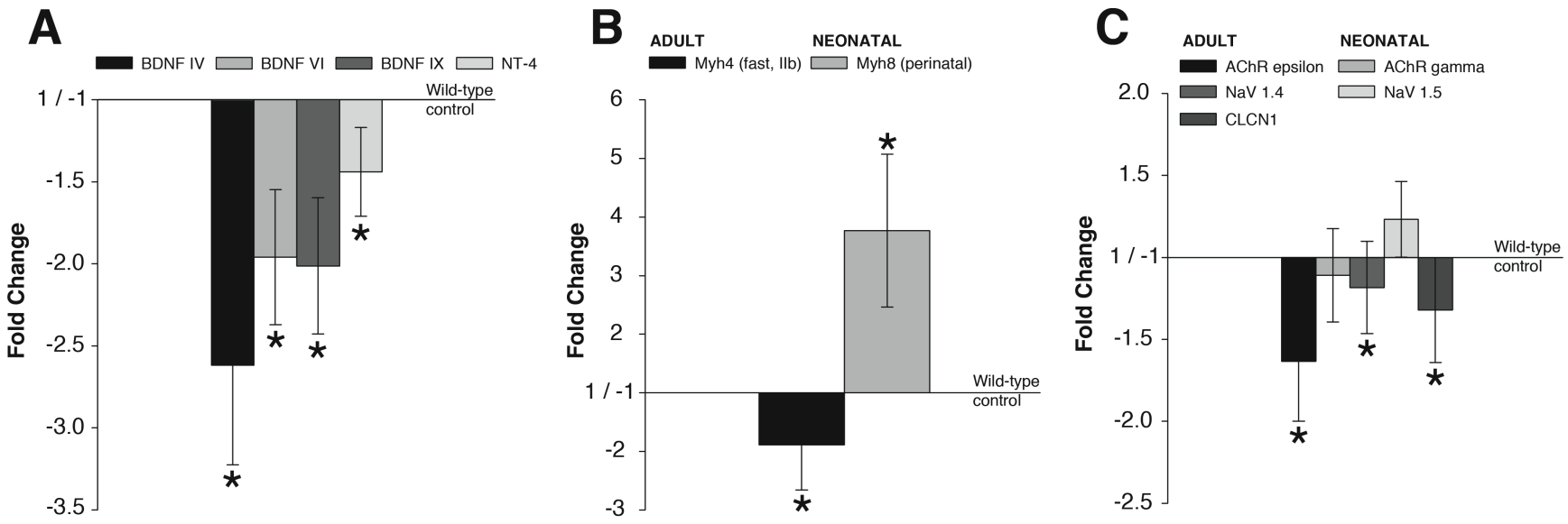

Figure 19. Gene expression is perturbed in juvenile (postnatal days 29-32) 97Q tibialis anterior muscle, despite lack of any motor dysfunction. A) Total BDNF mRNA (exon IX) as well as transcripts containing exon IV and VI are downregulated early on. NT-4 mRNA levels are also downregulated at the juvenile stage. B) Myosin heavy chain mRNA expression is also perturbed at this early stage, with adult transcripts lower and neonatal transcripts higher than wild-type controls. However, note that the fold changes in muscle of symptomatic mice are more severe (Table 7), suggesting that these changes correlate with motor dysfunction. C) mRNA expression of muscle ion channels is also perturbed in presymptomatic 97Q muscle. However, while adult isoforms are downregulated, the upregulation in neonatal isoforms has not yet occurred. *p<0.05 from Wild-type. 97Q, N=6. Wild-type, N=8. Values are mean fold changes ± SEMs (standard errors of the mean) based on N/group.

Table 7. Comparison of qRT-PCR fold changes in presymptomatic and adult 97Q mice in tibialis anterior muscle. Fold changes are relative to cohort-matched wild-types. *p<0.05.

Gene	Juvenile, presymptomatic (from Figure 19—Halievski and Jordan, unpublished)	Adult, symptomatic
Neurotrophins		
BDNF IV	-2.6*	-2.3* (Wahl, Halievski, Jordan, unpublished)
BDNF VI	-2.0*	-1.9 (Wahl, Halievski, Jordan, unpublished)
BDNF IX (total)	-2.0*	-1.7 (Wahl, Halievski, Jordan, unpublished) -3.0* (from CHAPTER 3)
Neurotrophin 4	-1.4*	-3.0* (from CHAPTER 3)
Myosin heavy chain		
Myh4 (fast, IIb)	-1.9*	-42.0* (from CHAPTER 3)
Myh8 (perinatal)	+3.8*	+248.9* (from CHAPTER 3)
Ion channels		
AChR epsilon	-1.6*	-1.8* (from CHAPTER 3)
AChR gamma	-1.1	+27.0* (from CHAPTER 3)
NaV1.4	-1.2*	-1.9* (from CHAPTER 3)
NaV1.5	+1.2	+24.2* (from CHAPTER 3)
CLCN1	-1.3*	-3.3* (from CHAPTER 3)

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