eNeuro

Research Article: New Research | Disorders of the Nervous System

Non-cell autonomous regulation of retrograde motoneuronal axonal transport in an SBMA mouse model

Muscle AR regulation of axonal transport in SBMA

Katherine Halievski[1], Michael Q. Kemp[1], S. Marc Breedlove[1], Kyle E. Miller[1,2] and Cynthia L. Jordan[1,3]

[1]*Neuroscience Program, Michigan State University, 108 Giltner Hall, East Lansing, MI 48824-1115.*
[2]*Department of Integrative Biology, Michigan State University, East Lansing, MI 48824-1115*
[3]*Department of Physiology, Michigan State University, 108 Giltner Hall, East Lansing, MI 48824-1115.*

DOI: 10.1523/ENEURO.0062-16.2016

Received: 15 March 2016

Revised: 23 June 2016

Accepted: 14 July 2016

Published: 26 July 2016

Funding: National Institute of Health
NS045195

Funding: Ruth L. Kirchstein National Research Service Award
1F32AR055848-01

Funding: Ruth L. Kirchstein National Institutional Research Service Award
T32-MH070343

Funding: NIH
1R01MH094607-01A1

Funding: NSF
IOS_0951019

Correspondence should be addressed to Cynthia L Jordan, Neuroscience Program and Physiology Department, 108 Giltner Hall, Michigan State University, East Lansing, MI 48824, E-mail: jordancy@msu.edu

Cite as: eNeuro 2016; 10.1523/ENEURO.0062-16.2016

1. **Manuscript title:** Non-cell autonomous regulation of retrograde motoneuronal axonal transport in an SBMA mouse model

2. **Abbreviated title:** Muscle AR regulation of axonal transport in SBMA

3. **List of authors and affiliations:**
Katherine Halievski[1], Michael Q. Kemp[1], S. Marc Breedlove[1] and Kyle E. Miller[1,3], Cynthia L. Jordan[1, 2] *

[1]Neuroscience Program and [2]Physiology Department, 108 Giltner Hall, Michigan State University, East Lansing, MI 48824-1115.

[3]Department of Integrative Biology, Michigan State University, East Lansing, MI 48824-1115

4. **Author contributions:**
MQK, SMB, KEM, and CLJ Designed research; MQK, SMB, KEM, and CLJ Performed research; SMB, KEM, and CLJ Contributed unpublished reagents/analytic tools; KH, MQK, SMB, KEM, and CLJ Analyzed data; KH, MQK, SMB, KEM, and CLJ Wrote the paper.

5. ***Correspondence should be addressed to:**
Cynthia L Jordan
Neuroscience Program and Physiology Department
108 Giltner Hall
Michigan State University
East Lansing, MI 48824
Phone: 517-355-1722
FAX: 517-432-2744
jordancy@msu.edu

6. **Number of figures:** 4

7. **Number of tables:** 1

8. **Number of multimedia:** 0

9. **Number of words for abstract:** 247

10. **Number of words for introduction:** 708

11. **Number of words for discussion:** 2597

12. **Funding sources:** This research was supported National Institute of Health grant NS045195 (to C.L.J.), a Ruth L. Kirchstein National Research Service Award 1F32AR055848-01 (M.Q.K.), a Ruth L. Kirchstein National Institutional Research Service Award T32-MH070343 (M.Q.K.), NIH 1R01MH094607-01A1 (K.E.M.) and NSF IOS_0951019 (K.E.M.).

13. **Conflicts of interest**: The authors declare no competing financial interests.

Abstract

Defects in axonal transport are seen in motoneuronal diseases but how that impairment comes about is not well understood. In spinal bulbar muscular atrophy (SBMA), a disorder linked to a CAG/polyglutamine repeat expansion in the androgen receptor (AR) gene, the disease-causing AR disrupts axonal transport by acting in both a cell autonomous fashion in the motoneurons themselves, and in a non-cell autonomous fashion in muscle. The non-cell autonomous mechanism is suggested by data from a unique "myogenic" transgenic (TG) mouse model in which an AR transgene expressed exclusively in skeletal muscle fibers triggers an androgen-dependent SBMA phenotype, including defects in retrograde transport. However, motoneurons in this TG model retain the endogenous *AR* gene, leaving open the possibility that impairments in transport in this model also depend on AR in the motoneurons themselves. To test whether non-cell autonomous mechanisms *alone* can perturb retrograde transport, we generated male TG mice in which the endogenous AR allele has the *testicular feminization mutation* (*Tfm*), and consequently is nonfunctional. Males carrying the Tfm allele alone show no deficits in motor function or axonal transport, with or without testosterone treatment. However when Tfm males also carrying the myogenic transgene (Tfm/TG) are treated with testosterone, they develop impaired motor function and defects in retrograde transport, having fewer retrogradely-labeled motoneurons, and showing deficits in endosomal flux based on time-lapse video microscopy in living axons. These findings demonstrate that non-cell autonomous disease mechanisms originating in muscle are sufficient to induce defects in retrograde transport in motoneurons.

70 **Significance Statement**

71 Our findings suggest that therapies targeting skeletal muscle could potentially rescue
72 motoneurons from axonal transport dysfunction in neuromuscular disease. Axonal transport is
73 critical for proper motoneuronal functioning and is often impaired in neurodegenerative disease,
74 including spinal bulbar muscular atrophy (SBMA), an androgen-dependent neuromuscular
75 disease linked to a polyglutamine expansion in the androgen receptor (AR). In this study, we
76 show that AR activated by androgens exclusively in skeletal muscle is sufficient to trigger
77 defects in retrograde transport in the motoneurons. Specifically, diseased mice show impaired
78 retrograde labeling of motoneurons *in vivo* and defective endosomal transport in living axons *ex*
79 *vivo.* Thus, one trait of diseased motoneurons, impaired axonal transport, can be conferred by
80 disease processes originating in muscle.
81
82

Introduction

Projection neurons are particularly susceptible to defects in axonal transport due to their long processes (De Vos et al., 2008; Morfini et al., 2009). This has led to an interest in the role of axonal transport in neurodegenerative diseases in which projection neurons are affected, including motoneurons in spinal bulbar muscular atrophy (SBMA), a progressive neuromuscular disease linked to a CAG expansion mutation in the *androgen receptor* (*AR*) gene (La Spada et al., 1991). SBMA occurs only in men and is androgen-dependent (Baniahmad, 2015; Katsuno et al., 2012). With only one exception (Malik et al., 2011), studies of axonal transport in both SBMA models and SBMA patients suggest this key neuronal process is perturbed and may be an early event in disease (Katsuno et al., 2006; Kemp et al., 2011; Morfini et al., 2006; Piccioni et al., 2002; Szebenyi et al., 2003). How AR disrupts axonal transport, however, is not clear. Evidence suggests that the disease-causing AR can act directly in the motoneuron in a cell autonomous fashion to disrupt axonal transport (Szebenyi et al., 2003) and/or indirectly in a non-cell autonomous manner via the target musculature (Kemp et al., 2011). Understanding where and how AR impairs axonal transport and whether perturbed transport contributes to motor dysfunction in SBMA is critical for developing effective treatments for this disease.

That *non*-cell autonomous mechanisms can instigate motoneuronal dysfunction in neuromuscular disease has been the subject of several recent reviews (Ilieva et al., 2009; Jordan & Lieberman, 2008; Sambataro & Pennuto, 2012). Because motoneurons depend on their targets to survive in development (Hamburger & Levi-Montalcini, 1949) and to function normally in adulthood (Navarro et al., 2007), it stands to reason that disease-related dysfunction originating in muscle could trigger dysfunction in the motoneurons. However, whether axonal transport is susceptible to such non-cell autonomous influences has not been firmly established. In these terms, because both motoneurons and muscle function is impaired in disease models of SBMA, they provide an excellent model system for testing whether defects in axonal transport can arise non-cell autonomously.

Reports indicate that retrograde transport is impaired in an androgen-dependent fashion, paralleling the loss of motor function, in a myogenic model of SBMA (Kemp et al., 2011). This unique myogenic mouse model expresses transgenic (TG) wild-type (WT) AR only in skeletal muscle at high levels. While these data imply that signals originating in muscle can impair retrograde axonal transport in motoneurons, because AR is also expressed throughout the body in TG animals by virtue of the endogenous *AR* gene, it leaves open the possibility that the effect of androgen on motoneuronal retrograde transport may have also depended on AR acting in other cell types, including the motoneurons themselves. To eliminate this possibility, we generated mice in which the only source of functional AR was from the transgene expressed only in muscle. We did this by passing the transgene into males carrying an endogenous allele for AR that is dysfunctional, the *testicular feminization mutation (Tfm*), providing a null background for functional AR. Genetic males carrying the Tfm allele are insensitive to androgens, exhibiting a female phenotype but have normal motor function (Johansen et al., 2011; Zuloaga et al., 2008). Thus, male mice carrying both the *Tfm* allele of the endogenous *AR* gene, and the AR transgene with its expression controlled by the muscle-specific human skeletal α actin promoter (Tfm/TG), have functional AR only in a single cell type—skeletal muscle fibers. This strategy allowed us to ask whether a disease-causing AR allele expressed solely in muscle fibers is sufficient to impair axonal transport in motoneurons via a non-cell autonomous mechanism.

Using cholera toxin (CT) to label retrogradely transporting endosomes, we found fewer *labeled* motoneurons in the lumbar spinal cord of motor-impaired Tfm/TG males than in the lumbar spinal cord of Tfm-only control males with intact motor function, a deficit in Tfm/TG males that is comparable to prior results in myogenic TG males on a background of WT AR (Kemp et al., 2011). Analysis of retrogradely trafficking endosomes in living axons of diseased Tfm/TG male mice also revealed the same deficit in endosomal flux as found previously (Kemp

et al., 2011). The present data confirm that non-cell autonomous mechanisms originating in target muscle fibers of motoneurons can perturb retrograde axonal transport in the motoneurons innervating them.

Materials and Methods

Tfm/TG mouse model.

Tfm/TG mice that express functional AR only in skeletal muscle fibers were generated by *in vitro* fertilization. Sperm was harvested for *in vitro* fertilization from a TG male that overexpresses WT-AR specifically in muscle cells (Monks et al., 2007). Female *Tfm* carriers on a C57Bl/J6 background were superovulated and eggs harvested. The *Tfm* allele renders AR protein nonfunctional and causes androgen-insensitivity uniformly throughout the body (He et al., 1991). After fertilization, embryos were transferred into pseudopregnant recipient B6D2F1/J mice. Four founding females were produced that carried both the transgene and the Tfm allele. Female offspring carrying both the *Tfm* gene and AR transgene were mated to WT C57Bl/6 males to produce the experimental Tfm/TG males.

Many Tfm/TG males die at birth, as do TG males with a WT-AR background (Monks et al., 2007). This perinatal lethality is caused by prenatal exposure to endogenous testicular androgens. To enhance perinatal survival of such Tfm/TG males, pregnant dams were treated with the AR antagonist, flutamide. Pregnant Tfm/TG carrier females were injected subcutaneously with 5 mg flutamide in propylene glycol at the nape of the neck from gestational days 15 – 20 (day of mating designated as day 0). Control males used in this study were littermate males that carried only the Tfm allele (Tfm males) and thus, were exposed to the same prenatal flutamide treatment as Tfm/TG males. Because Tfm male mice develop a deficit in androgen production postnatally (Goldstein & Wilson, 1972; Murphy & O'Shaughnessy, 1991), Tfm/TG males have low, female-like levels of circulating androgens as adults, comparable to Tfm males (Johansen et al., 2011). This means that postnatally Tfm/TG males are asymptomatic (like TG females) and express disease symptoms only when treated with exogenous testosterone (T) to increase T levels to that of normal males (Johansen et al., 2009). All animal procedures were performed in accordance with the [Author University] animal care committee's regulations.

Adult hormone treatment to induce disease symptoms.

To induce disease symptoms in adult Tfm/TG males, 150 day old Tfm/TG and Tfm male littermates under isoflurane anesthesia received subcutaneous implants at the nape of the neck just below the interscapular fat pad of Silastic capsules containing crystalline T (1.57 mm inner diameter and 3.18 mm outer diameter, effective release length of 6 mm). Such T implants result in serum T levels comparable to that of adult gonadally intact males (Johansen et al., 2009). After five days of treatment, the efficiency of retrograde transport was assessed in two different ways (as detailed below): retrograde labeling of motoneuronal cell bodies with horse radish peroxidase (HRP) injected into muscle, and active transport of fluorescently-labeled endosomes in living axons of the sciatic nerve. In each case, the label was conjugated to cholera toxin (CT). Forelimb grip strength was monitored using a grip strength meter (Columbus Instruments) in Tfm/TG males and their Tfm male brothers during androgen treatment, beginning just prior to implanting T capsules (day 0), and then on days 1, 2, 3, 4, and 5 of treatment. Means and standard errors are calculated and N = number of animals/group are reported in the figure captions.

Retrograde labeling of spinal motoneurons

Spinal motoneurons were retrogradely labeled by injecting CT B conjugated to horse radish peroxidase (CT-HRP; 1µl of 0.2% CT-HRP/muscle; List Biological Labs, USA) into the anterior tibialis (AT) muscles of isoflurane anesthetized mice. Once mice recovered from anesthesia, they were returned to their home cage. After 12 hrs to allow CT-HRP to retrogradely transport to motoneuronal cell bodies, mice were re-anesthetized with an intraperitoneal injection of sodium pentobarbital. Once deeply anesthetized, mice were perfused with 0.9% saline followed by cold 0.1 M phosphate buffer (pH 7.4) containing 0.8% paraformaldehyde and 1.25% glutaraldehyde. Spinal cords were dissected out and placed in the same fixative for 5 h at 4°C, after which they

were transferred to a 10% phosphate-buffered sucrose solution and held overnight at 4°C. The following day, spinal cords were transversely sectioned on a freezing, sliding microtome at 40 µm. Sections were reacted with tetramethylbenzidine (TMB) for histological visualization of HRP (Park et al., 2002). Sections were washed with distilled water and incubated with a mixture of 0.005% TMB and 0.1% sodium cyanide for 20 min. H_2O_2 was added for 20 min, and the reaction was stopped by washing in 0.01 M sodium acetate buffer. Alternate sections were mounted on gelatin-coated slides within 6 h after TMB reaction. The next day, the sections were dehydrated, defatted, and coverslipped. Slides containing TMB reacted sections were stored in opaque boxes at 4°C to prevent degradation of TMB reaction product. All slides were coded and analyzed without knowledge of treatment groups. Estimates of the number of retrogradely labeled AT motoneurons was based on total bilateral counts done on adjacent sections at 100X magnification. Every lumbar section was examined for labeled motoneurons, including several sections beyond the rostral and caudal extent of AT motoneurons. Overall means and standard errors are calculated and N = number of animals/group are reported in the figure captions.

Live imaging of endosomal transport in sciatic nerve axons.

Endosomal trafficking in sciatic nerve axons of diseased (T-treated Tfm/TG) and healthy (T-treated Tfm) male mice was assessed by directly monitoring the retrograde transport of endosomes in living axons. CT B conjugated to Alexa Fluor 488 (CT-AF488; Molecular Probes, USA, 10 µl of 0.2% in 0.9% saline containing 1% DMSO) was injected into the AT and gastrocnemius muscles of isoflurane-anesthetized mice to label endosomes. Retrograde movement of endosomes in living axons of explanted sciatic nerves was monitored four hours after injection. During the 4 hour delay, mice were awake and mobile.

Mice were re-anesthetized with isoflurane to harvest the sciatic nerve. Explants (~10 mm in length) were placed on coverglass, glued in place (VetBond 3M, USA) and covered with 37°C oxygenated bicarbonate Ringer's solution (in mM: 135 NaCl, 5 KCl, 1 $MgCl_2$, 1.5 $NaHCO_3$, 1

213 Na_2PO_4, 2 $CaCl_2$, and 1.1 glucose, pH 7.2). The right sciatic nerve was harvested first and
214 recorded from while the left nerve remained *in situ* in the anesthetized mouse. There is no
215 evidence that transport in left and right nerves systematically differs nor that transport declines
216 over time (data not shown), consistent with previous findings (Kemp et al., 2011). Body
217 temperature was maintained using a heating pad for the 30 minutes between nerve harvests.
218 Once both nerves were harvested, the mouse was killed.
219
220 ***Live image acquisition and processing.***
221 Time-lapse movies of moving endosomes were made using an inverted LiveScan swept
222 field confocal microscope (Nikon Eclipse TE2000-E, Japan) equipped with a 60x PlanApo oil
223 immersion objective (1.4 NA), illuminated with the 488 nm line of a 150 mW argon laser (Melles
224 Griot), and recorded with a Photometrics CoolSNAP HQ2 camera (USA) using the NIS
225 elements software. Temperature of the sciatic nerve explant was maintained at 37°C by a ring
226 incubator. Less than 10 minutes after nerve harvest, recordings began, with two time-lapse
227 movies taken from the same explant within 30 minutes of harvest. Each movie was seven
228 minutes in length, comprising 211 images, with images captured every 2 sec at a 1 sec
229 exposure. Laser power and aperture settings were identical for all captured images. Scanning
230 for trafficking started at the center of the sciatic nerve segment and proceeded toward the distal
231 end of the nerve to reduce variability. Because of concerns about photobleaching, different
232 axons in the same explants were used for the two movies and the second movie was taken from
233 a region distal to the first. To minimize experimenter bias, video capture began when the first
234 moving endosome could be kept in focus.
235
236 ***Trafficking measures from kymographs.***
237 Time-lapse movies were converted into kymographs, single 2-dimensional images
238 showing the movement of endosomes (referred to as “endosomal traces”) along the length of an

axon as a function of time. To make kymographs, the resulting movies were opened in NIH ImageJ as a 16 bit stack of images then rotated 90° counterclockwise. A 60 pixel wide length of axon was then cropped, and using the 're-slice' option, sliced into 1 pixel width sections and z-projected using the SUM option, which resulted in 32 bit 'raw' kymographs. The raw kymographs were converted to 16 bits, and saved as tiff files. To facilitate visibility of transport events, raw kymographs were opened in Adobe Photoshop (7.0 or CS2; USA), color inverted and resampled to increase size by 500%, thus producing the kymographs used for analysis, using ImageJ. We measured flux, net and instantaneous velocity, run length, and frequency of trafficking perturbations (stalls, reversals and velocity changes) for retrogradely transporting endosomes.

Endosomal flux is the number of retrogradely transporting endosomes in an axon per unit of time and was determined by drawing a line through the center of the kymograph parallel to the time axis and counting the number of endosomal traces that cross that line. The total number of endosomes per kymograph was then divided by seven minutes (total time represented on the kymograph) to estimate endosomal flux in number of endosomes/minute. Such estimates were averaged across individual axons within a single animal to obtain a single estimate of flux per mouse. Net velocity of retrogradely transported endosomes is the net distance traveled by each endosome in the distal to proximal direction over time and included stalls, reversals in direction and/or changes in velocity that might have occurred. Measures of net velocity were obtained from kymographs by drawing a box around each endosomal trace such that the far ends of the trace pass through opposite corners of the box. Height and width of the box (in pixels) corresponding to length and time are used to calculate velocity (microns/second) based on the following formula: [Distance (pixels) X 0.10526 (microns/pixel)/ Time (pixels) X seconds per frame]. Net velocities of individual endosomal traces in a single kymograph were then averaged to provide the mean estimate of net velocity/axon. These

264 estimates were then averaged across individual axons sampled within a single animal to obtain
265 a single estimate of net velocity per mouse.

266 Instantaneous velocity was measured by drawing a one pixel wide line parallel to the
267 time axis, bisecting the kymograph with respect to distance. For each endosomal trace crossing
268 the center line, a 20 pixel box was drawn around it, centering it with respect to the bisecting line,
269 with width and height of the box representing distance and time, respectively. The width of the
270 box (20 pixels) accounted for 6.6% (1.67 mm) of the total distance traveled and any continuous
271 velocity within it was considered instantaneous. The time it took to travel this short distance was
272 calculated based on the height of the box, which was adjusted so that its corners intersected the
273 most distal and proximal aspects of the trace. On rare occasions, perturbations in endosomal
274 velocity, including changes in speed, stalls and/or reversals, appeared within the box. In such
275 cases, the box was moved immediately distal to the trafficking perturbation or until the box
276 included a part of the endosomal trace that contained no trafficking perturbations. Instantaneous
277 velocity (microns/second) was calculated based on the same formula used for net velocity.
278 Measures of instantaneous velocity for each endosome were averaged within a single
279 kymograph and such estimates from two to four axons of the same mouse were averaged to
280 obtain a single estimate of instantaneous velocity per mouse.

281 We also assessed the frequency of trafficking perturbation events, which reflects the
282 efficiency by which endosomes are transported retrogradely. We counted the number of
283 trafficking perturbation events per trace, which included changes in velocity, stalls, and/or
284 reversals that lasted for $\geq$ two seconds (Figure 1). Our measure of velocity changes was based
285 on visible changes in velocity in the kymograph without directly measuring changes in the slope
286 of the endosomal trace, indicating that subtle changes in velocity may have not been detected.
287 Stalls were defined as an endosome that was stationary for ≥ 2 s. Stalling prevalence was
288 assessed by determining the number of stalls per endosomal trace and the proportion of traces

that had stalls. We also measured average run length, which was estimated by adding together the total distance of endosomal transport observed in a kymograph and dividing this by the number of stalls. As above, measures were averaged across multiple kymographs for each animal, with N = number of animals per group for all statistical analyses. Data for reversals, stalls, velocity changes, and overall trafficking perturbations are presented as a percentage of total endosomes. Mean estimates of trafficking measures were based on 15-30 endosomal traces per kymograph, with 2-4 kymographs per mouse and N = number of animals/group. Analyses of kymographs were done by an experimenter blind to animal ID and genotype. Refer to statistical table for description of data structure, statistical test used, and 95% confidence intervals of data (Table 1).

Results

Activation of transgenic WT-AR in skeletal muscle fibers is sufficient for androgen-dependent deficits in both motor function and retrograde transport.

We find that motor function based on forelimb grip strength of adult Tfm/TG males rapidly declines during the five days of androgen treatment while motor function in Tfm-only males remains stable and is unaffected by androgen (Figure 2A). After only 24 hours of androgen exposure, motor function in Tfm/TG males is significantly reduced compared to Tfm-only males (day 1 p=0.002) and continues to drop progressively during the next four days (day 2 p=0.0004, day 3 p=0.0003, day 4 p=0.00001, day 5 p=0.00006)[a], exhibiting the same time course of demise as seen in T-treated myogenic TG females (Johansen et al., 2009). These data also replicate previous results for Tfm/TG males (Johansen et al., 2011). Both Tfm and Tfm/TG males without T treatment have T levels about 10 times lower than do adult WT males (Johansen et al., 2011), suggesting that the low circulating level of T is why adult Tfm/TG males do *not* develop motor dysfunction on their own.

313 Muscles of Tfm/TG mice and Tfm controls were injected with CT-HRP at the end of
314 treatment (5 days) to assess the efficiency of transport based on the number of retrogradely
315 labeled motoneurons. We find that motor-impaired Tfm/TG males have a significant deficit in the
316 number of retrogradely labeled motoneurons compared to Tfm controls (Figure 2B, p=0.0027)[b].
317 Interestingly, the number of labeled motoneurons for Tfm control males is comparable to that
318 previously reported for *gonadectomized* WT males who lack testicular androgens (Kemp et al.,
319 2011), with the number of retrogradely labeled motoneurons in both gonadally intact Tfm males
320 and gonadectomized WT males somewhat reduced compared to those of gonadally intact WT
321 males. It is possible that one of the normal roles of WT AR when activated by endogenous
322 androgens is to enhance the retrograde transport of cargo to motoneuronal cell bodies.

323 We also find that endosomal transport in axons of diseased Tfm/TG males was affected
324 when assessed directly in living axons *ex vivo* using video microscopy. Specifically, endosomal
325 flux (p=0.0079)[c], but not net velocity (p=0.411)[d], is significantly impaired in sciatic nerve axons
326 (Figure 2C, D), suggesting that deficits in flux may account for the reduced number of
327 retrogradely labeled motoneurons in diseased Tfm/TG mice. This pattern of results is also seen
328 in both chronically diseased myogenic TG males and acutely diseased myogenic TG females
329 (Kemp et al., 2011).

330 We also measured instantaneous velocity and trafficking perturbations and unexpectedly
331 detected a deficit in instantaneous velocity (Figure 3A, p=0.0178)[e], an outcome that was *not*
332 apparent in myogenic TG males (Kemp et al., 2011). These data suggest that endogenous WT-
333 AR may have protected against the effects of a toxic muscle AR on instantaneous velocity.
334 Altered instantaneous velocity in Tfm/TG males suggests defects in dynein function, a notion
335 not without precedence as another SBMA mouse model showed reduced dynein heavy chain
336 levels in the ventral spinal root (Katsuno et al., 2006). We also find a greater percentage of
337 endosomes show trafficking perturbations in diseased Tfm/Tg males (Figure 3B, p=0.0387)[f],

caused by more endosomes in diseased Tfm/TG males showing changes in velocity (Figure 3C, p=0.0435)[g] and reversals (Figure 3D, p=0.0314)[h] compared to endosomes in axons of healthy Tfm males. Surprisingly, although more endosomes tend to stall in diseased axons than in healthy axons, this difference was not significant (Figure 3E, p=0.0955)[i]. Likewise, while the mean run length of endosomes in Tfm/TG is shorter, it too was not significantly decreased compared to that of healthy Tfm males (Figure 3F, p=0.2784)[j]. Together these data suggest that endogenous WT-AR may have protected against some of the effects of a toxic muscle AR on transport and converge on the conclusion that AR acting *only* in muscle fibers is sufficient to impair retrograde transport/trafficking of endosomes in motoneurons.

Table 1. Statistical table. Structure of data, statistical test used, and 95% confidence intervals are listed for each variable measured.

	Data Structure	Type of test	95% Confidence Interval for the mean Tfm (Lower bound, Upper bound); Tfm/TG (Lower bound, Upper bound)
[a]Grip strength	Normal	Repeated measures ANOVA	Day 0: n/a
			Day 1: Tfm (84.72, 136.78); Tfm/TG (37.74, 79.06)
			Day 2: Tfm (69.78, 124.22); Tfm/TG (15.43, 49.37)
			Day 3: Tfm (60.88, 142.62); Tfm/TG (10.06, 30.34)
			Day 4: Tfm (82.57, 118.43); Tfm/TG (-6.59, 25.39)

			Day 5: Tfm (74.99, 113.01); Tfm/TG (-8.81, 31.61)
[b]HRP filled motoneurons	Normal	Independent t-test	Tfm (93.81, 195.19); Tfm/TG (51.44, 90.96)
[c]Flux	Normal	Independent t-test	Tfm (3.49, 8.16); Tfm/TG (2.22, 4.52)
[d]Net velocity	Normal	Independent t-test	Tfm (0.25, 0.53); Tfm/TG (0.30, 0.45)
[e]Instantaneous velocity	Normal	Independent t-test	Tfm (0.41, 0.86); Tfm/TG (0.34, 0.58)
[f]Overall trafficking perturbations	Normal	Independent t-test	Tfm (3.46, 18.79); TfmTG (10.52, 34.11)
[g]Velocity changes	Normal	Independent t-test	Tfm (1.12, 5.61); Tfm/TG (2.86, 11.61)
[h]Reversals	Normal	Independent t-test	Tfm (3.10, 18.79); Tfm/TG (11.20, 34.10)
[i]Stalls	Normal	Independent t-test	Tfm (0.75, 8.92); Tfm/TG (4.53, 16.11)
[j]Run length	Tfm group positively skewed (skewness: 2.198)	Independent t-test	Tfm (-444.43, 1881.98); Tfm/TG (41.77, 499.35)

350

351

Discussion

Here we demonstrate that retrograde axonal transport in motoneurons is impaired via *non-cell autonomous* AR action in muscle. Myogenic contributions to SBMA were first recognized in two different mouse models (Monks et al., 2007; Yu et al., 2006), where myopathic changes were discovered in a knock-in model well before neuropathy and transgenically overexpressed WT-AR *specifically* in muscle cells triggered SBMA-like symptoms only in myogenic TG males, including a loss of ventral root axons. These unexpected findings suggested that non-cell autonomous mechanisms originating in muscle could drive pathogenesis in motoneurons in SBMA mice. We now find that AR in muscle is sufficient to disrupt retrograde transport in the motoneurons which innervate diseased muscle. Previous findings indicated that myogenic TG mice exhibit perturbed axonal transport compared to WT littermates (Kemp et al., 2011). However, such TG mice also had AR in other cell types due to expression of the endogenous gene, leaving open the possibility that endogenous AR in the motoneurons and/or in other cell types was responsible for the transport deficits found in this model. Thus, we asked whether the effects of TG AR in muscle fibers depend on endogenous AR in other cell types by assessing axonal transport in myogenic TG males that also have the *Tfm* allele of the endogenous *AR* gene (Tfm/TG males), thus eliminating all functional AR from the endogenous gene. Consequently, such Tfm/TG males had functional AR *only* in skeletal muscle fibers due to expression of the AR transgene. We found that diseased Tfm/TG males showed comparable deficits in the number of retrogradely labeled motoneurons and in endosomal flux as previously reported for diseased TG males on a WT AR background (Kemp et al., 2011), indicating that AR acts solely in muscle fibers to perturb retrograde transport in motoneurons.

As expected, Tfm/TG males exhibited androgen-dependent motor dysfunction, replicating a previous finding from this same mouse model showing that such dysfunction is fully

reversible when androgen treatment ceases (Johansen et al., 2011; Johansen et al., 2009; Monks et al., 2007; Figure 2A) and consistent with the phenotype of SBMA mouse models expressing a CAG expanded AR allele (Chevalier-Larsen et al., 2004; Katsuno et al., 2002; Renier et al., 2014; Sopher et al., 2004; Yu et al., 2006). Such mice also showed a striking deficit in the number of retrogradely labeled motoneurons compared to non-diseased Tfm male controls (Figure 2B), correlating with the deficit in motor function (Figure 2A). Interestingly, the magnitude (~50%) of the labeling deficit in Tfm/TG males is comparable to that previously reported for both chronically diseased myogenic TG males and acutely diseased myogenic TG females (Kemp et al., 2011), indicating that TG AR in muscle, when activated by androgens, rapidly perturbs the uptake/retrograde transport of CT-HRP by motoneuronal axons independent of AR expressed by the native gene, including that in motoneurons and/or other cell types.

Strong evidence argues that the deficit in axonal transport exhibited by androgen-treated Tfm/TG males is largely comparable to that previously observed in TG-only males and females. Notably, deficits in CT-HRP staining and grip strength are apparent after only 24h of T-treatment in TG-only females (Kemp et al., 2011), the time-point at which Tfm/TG males, given the same T-treatment, show deficits in grip strength. Moreover, CT-HRP transport recovers fully in chronically symptomatic TG males that have been castrated, as does motor function in symptomatic Tfm/TG males following T removal (Johansen et al., 2011; Kemp et al., 2011). It is clear that the transgene alone without androgen stimulation is not sufficient to induce transport dysfunction, in parallel with the effect of the transgene on motor function, since castrated TG males and untreated TG females show deficits in neither motor function nor axonal transport (Kemp et al., 2011). Unfortunately, any direct comparison between TG and Tfm/TG males in disease traits would be confounded by long term versus short term effects of disease. TG-only males are exposed throughout life to endogenous male levels of androgens, causing secondary effects of disease due to its chronic nature in TG-only males. On the other hand, Tfm/TG males

like TG-only females, lack endogenous male levels of androgens postnatally, and only express an acute disease phenotype when provided with exogenous androgens. In sum, the defects in transport and motor dysfunction induced by T treatment of Tfm/TG males, who like TG-only males and females, express the disease-causing AR allele *only* in muscle fibers, is comparable to what is seen in TG-only males and females in which *both* motor function and axonal transport are disrupted only in the presence of male levels of androgens.

Evidence strongly argues that the deficit found in the number of retrogradely labeled motoneurons is not caused by a net loss of motoneurons. Cell counts of motoneuronal cell bodies in Nissl stained sections of the spinal cord show no evidence of motoneuronal loss in the myogenic TG model (Johansen et al., 2009; Monks et al., 2007). Moreover, extending CT transport time to 24 hours rather than the 12 hours used here eliminates the deficit in retrograde labeling of motoneurons (Kemp et al., 2011). Notably, defects in axonal transport observed here are not likely due to secondary effects of disease since deficits in CT-HRP transport occur just 24h after the commencement of T-treatment in female TG mice (Kemp et al., 2011) who display the same time-course of deterioration as the inducible Tfm/TG model used here. Moreover, no muscle atrophy is apparent in 5 day-treated TG females, despite their displaying severe motor impairment and a loss of muscle force that does not involve a loss of muscle mass (Oki et al., 2013). Additionally, no dying-back of the motoneuron occurs in even chronically diseased TG males, as all endplates are contacted by a motor nerve terminal (Kemp et al., 2011). Thus, deficits in axonal transport and not neuronal loss nor muscle atrophy correlate with motor dysfunction, suggesting that axonal transport dysfunction may contribute to early losses in motor function and ultimately motoneuron death in SBMA.

This deficit in retrograde labeling of motoneurons may be due to a number of factors, including dysfunction originating in the distal most aspect of the axon, the synaptic nerve terminal, where material is first taken up, sorted, and packaged into vesicles for either local recycling or retrograde transport to the motoneuronal cell bodies. Deficits could also originate in

more proximal aspects of the axon. Transport of cargo in these two axonal compartments depends on different transport machinery, with local transport of cargo in the synaptic nerve terminal moved along actin by specialized myosin, whereas long distance transport of cargo toward motoneuronal cell bodies is moved along microtubules by dynein and the motor-associated protein complex dynactin (Chevalier-Larsen & Holzbaur, 2006). Our first-level analysis of endosomal transport in living axons of diseased Tfm/TG indicated that endosomal flux, but not net velocity, was perturbed in Tfm/TG males, as found previously in chronically diseased myogenic males and acutely diseased myogenic females (Kemp et al., 2011). Thus, fewer labeled endosomes than normal move along the diseased axon per unit of time, offering an explanation for why fewer motoneurons in diseased Tfm/TG mice are retrogradely labeled after 12 hours of CT transport. Such a deficit in flux suggests that the early endocytotic pathway in the nerve terminal is susceptible to non-cell autonomous regulation, with disease signals originating in muscle perturbing some aspect of this presynaptic pathway, including possibly the uptake, local sorting, packaging/fusion, and/or movement of CT-containing cargo within the synaptic nerve terminal. Moreover, the nerve terminal exhibits accumulation of neurofilament in SBMA patients, further linking the distal axon to defects in transport (Katsuno et al., 2006). Nevertheless, which of these processes is affected is not clear and would require direct monitoring of endocytosis in synaptic nerve endings to determine.

While net velocity was *not* affected by disease in Tfm/TG males, paradoxically instantaneous velocity was, counter to previous findings reported for myogenic TG males (Kemp et al., 2011). A deficit in instantaneous velocity means that when endosomes are in motion, they move slower in diseased axons than in healthy axons, raising questions about whether AR in muscle can also cause defects in the retrograde motor dynein (Alper et al., 2013). A possible explanation for dynein dysfunction *per se* is suggested by a report from Ramzan et al. (2015). Using Cre-lox technology to drive expanded polyglutamine AR expression specifically in muscle fibers (MyoAR), they found reduced nitrotyrosine staining in motoneurons (Ramzan et al.,

455 2015). These data suggest a loss of oxidative homeostasis in motoneurons triggered by
456 diseased muscle can in turn impair dynein. In fact, dynein heavy chain is reduced in ventral
457 spinal root of symptomatic AR97Q SBMA mice, but only in later stages of disease (Katsuno et
458 al., 2006).

459 The effect of disease on instantaneous velocity in Tfm/TG males is reminiscent of
460 previous findings in a knock-in model of SBMA, also involving a deficit in instantaneous velocity
461 (Kemp et al., 2011). While these data suggested that mutant AR expressed in motoneurons can
462 cause defects in dynein/dynactin function in a cell autonomous fashion, the current data suggest
463 an additional hypothesis that endogenous (WT) AR in motoneurons of TG males may have
464 protected against the toxic effects of muscle TG AR on instantaneous velocity. Knock-in males
465 would not have this protection since every cell that expresses AR in this model, expresses only
466 the mutant AR allele. This suggestion is consistent with previous findings that endogenous WT
467 AR can blunt the toxicity of a disease-causing TG AR in a different SBMA model (Thomas et al.,
468 2006). It is also possible that endogenous AR in muscle could have a protective role, but this
469 scenario seems less parsimonious. Finally, while AR aggregates perturbed axonal transport in
470 one SBMA cell model (Piccioni et al., 2002), this cannot explain the defects seen in the current
471 TG model as AR aggregates do not occur in myogenic mice (Monks et al., 2007), nor do they
472 occur in axons of other SBMA models in which axonal transport defects have been found
473 (Katsuno et al., 2006; Szebenyi et al., 2003).

474 We also found that retrogradely transporting endosomes in diseased axons had a higher
475 overall incidence of trafficking perturbations, with more endosomes in axons of Tfm/TG males
476 showing reversals, stalls, and velocity changes than endosomes in axons of healthy Tfm males
477 (Figure 3). Such “error” events reflect reduced processivity. An important regulator of
478 processivity is the motor-associated protein dynactin (King & Schroer, 2000). In fact, dynactin is
479 reduced in motoneurons of SBMA patients and in a mouse model of SBMA in which the disease
480 allele is globally expressed and shows early defects in axonal transport (Katsuno et al., 2006),

suggesting it is likely to also be perturbed in the myogenic model. That transport of endosomes in axons of diseased Tfm/TG males is less efficient suggests that dynactin function is also perturbed in a *non-cell autonomous* manner by diseased muscle. The run length of endosomes in axons of diseased Tfm/Tg males is also shorter than in axons of healthy Tfm axons, consistent with reduced processivity, though this difference is not significant, perhaps because of the increased error variance seen in healthy Tfm controls. Nonetheless, together these data suggest that signals from diseased muscle can instigate defects in the core transport machinery involving the retrograde motor and its associated proteins although the effect of diseased muscle on retrograde transport seems to lie predominantly in the motor synapse where cargo is first taken up and packaged. While the current study represents a small step toward recognizing the potential role of non-cell autonomous regulators of axonal transport, this possibility is sufficiently important to merit further investigation.

The fact that instantaneous velocity but not net velocity is perturbed in Tfm/TG diseased males suggests that compensation occurred. One possible explanation is that while reversals are more common in diseased axons, perhaps the distance traversed in the wrong direction is less in diseased than healthy axons. Regardless of the explanation for the apparent discrepancy in instantaneous versus net velocity, the current findings raise the possibility that dynein and dynactin, essential in the retrograde transport of cargo, are each susceptible to non-cell autonomous regulation by the muscle. While there is precedence that both dynein and dynactin are impaired in models of SBMA (Katsuno et al., 2006; Szebenyi et al., 2003), the current data are the first to suggest that such defects in motor proteins could be caused via a non-cell autonomous mechanism.

How might action of a toxic AR in muscle perturb axonal transport in the motoneurons? One attractive scenario is the loss of muscle-derived trophic factors. Skeletal muscle releases many kinds of trophic factors that can act on receptors in motor nerve terminals to promote healthy cell function. Diseased muscle from various SBMA mouse models exhibits deficits in

multiple trophic factors, including brain-derived neurotrophic factor (BDNF), glial cell line-derived factor, insulin-like growth factor 1, neurotrophin-4 (NT-4), and vascular endothelial growth factor (VEGF) (Halievski et al., 2015; Mo et al., 2010; Monks et al., 2007; Sopher et al., 2004; Yu et al., 2006). Moreover, replenishing the supply of such neurotrophic factors can reverse disease-related deficits in axonal transport in models of motoneuron/neuromuscular disease. For example, delivery of CNTF, BDNF, or NT-3 to muscle improves retrograde transport in the *progressive motor neuronopathy* mouse model (Sagot et al., 1998). Additionally, treating diseased muscles of myogenic TG SBMA females with VEGF reverses a disease-related deficit in retrograde labeling of motoneurons and endosomal flux (Kemp et al., 2011). These data are compelling precedence for a cause-effect relationship between muscle-supplied neurotrophic factors and retrograde transport in motoneurons.

Loss of such trophic support from target musculature could lead to defective transport in at least two ways. First, lack of trophic factor signaling might mean a loss of survival signaling, leaving the cell deprived of needed resources for proper maintenance of axonal transport (Chowdary et al., 2012). Alternatively, local neurotrophic signaling may be needed for endocytosis *per se*, since VEGF can reverse a deficit in flux (Kemp et al., 2011). Internalization of the neurotrophin-bound Trk receptor requires PI3K signaling to recruit Rab5 (Christoforidis et al., 1999; also see Harrington & Ginty, 2013 for review). Since internalization and sorting of the CT receptor GM1 also depends on Rab5 (Pelkmans et al., 2004), neurotrophic factors could enhance endocytosis through this pathway. Once internalized, Trk signaling also leads to phosphorylation of dynein intermediate chains through the MAPK/ERK pathway to enhance binding of dynein to Trk-activated signaling endosomes (Mitchell et al., 2012). Defects in this pathway could explain the deficit in flux as well as the reduced efficiency of transport in diseased axons. In short, muscle-derived neurotrophic factors are a strong candidate for how diseased muscle might disrupt the uptake and/or retrograde transport of cargo in motoneuronal

axons of SBMA mice (see Figure 4 for a proposed model). While data are scant, the relevance of these data to SBMA in humans is likely (Katsuno et al., 2006).

While considerable attention has recently been redirected to muscle, recent work also supports the idea that mutant AR acting in motoneurons contributes to SBMA pathogenesis (Ramzan et al., 2015; Sahashi et al., 2015). Given that both motoneurons and muscle normally express AR, and that motoneurons and muscles are interdependent, it would not be surprising if the symptoms of SBMA reflects a complex interplay of mutant AR acting in both motoneurons and skeletal muscles, if not in other cell types too. The important task before us is to better understand where and how mutant AR acts to cause disease and to sort out primary effects of AR from secondary effects of disease. AR acting in muscle to impair axonal transport may be one such early pathogenic event leading to widespread neuromuscular dysfunction.

Figure Captions

Figure 1. Representative kymograph showing various trafficking perturbations measured. Endosomal traces are seen as dark irregular lines that typically slope downward from right to left, indicating the time it takes for a given endosome to traverse a given length (25.26 μm) of axon. Note that the slope of the trace can change over time, going from a downward slope to one that is vertical (parallel to the time axis) indicating a stall (1), to less severe changes in slope (2 versus 3), with the steeper downward slope (3) indicating a transient increase in endosomal velocity. A negative slope in an endosomal trace (4) indicates that the endosome transiently reversed its direction of movement. The shown kymograph is taken from a healthy Tfm male.

Figure 2. Adult male mice with functional AR only in skeletal muscle fibers (Tfm/TG) show defects in motor function, retrograde transport, and endosomal trafficking. (**A**) Myogenic TG male mice with a mutant allele (*testicular feminization mutation*, Tfm) of the endogenous *AR* gene (Tfm/TG) show an androgen-dependent loss of grip strength, with grip strength dropping to basement within 4 days of testosterone (T) exposure. Both groups of males were treated with exogenous T given their negligible levels of endogenous androgen. Note that the grip strength of Tfm controls who lack functional AR is unaffected by T. (**B**) The number of anterior tibialis motoneurons filled with CT-HRP after 12 hrs of retrograde transport is significantly reduced in diseased Tfm/TG males compared to healthy Tfm controls, indicating that transgenic AR acting in muscle fibers induces defects in retrograde transport, independent of endogenous wild-type AR in motoneurons and elsewhere. (**C, D**) Such Tfm/TG mice show deficits in flux, but not net velocity, after 5 days of T treatment, comparable to reported results from TG males on a wild-type background (Kemp et al., 2011). The current data reinforce the idea that muscle AR instigates a disease process that retrogradely impairs endosomal trafficking in motoneurons, possibly by perturbing aspects of the early endocytotic pathway.

568 Graphs represent mean ± SEM (A, B: N = 4-5/group; C, D: N = 6/group). A repeated measures
569 ANOVA indicated a grip strength by group interaction, and further revealed an effect of time on
570 grip strength only for the Tfm/TG genotype. * indicates significant differences (p<0.05) between
571 groups based on a one-way ANOVA (A) and independent t-test (B - D).

572 **Figure 3. Androgen activated AR in skeletal muscle fibers perturbs trafficking kinetics of**
573 **retrogradely transporting endosomes.** Based on live imaging of fluorescently-labeled
574 trafficking endosomes in sciatic nerve axons of Tfm and Tfm/TG males, we find that the
575 instantaneous velocity (**A**) of transporting endosomes is significantly decreased in diseased
576 Tfm/TG males compared to Tfm control males. Endosomes in motor-impaired Tfm/TG males
577 also show elevated levels of trafficking perturbations overall (**B**), including an increased
578 tendency to show velocity changes (**C**), reversals (**D**) and stalls (**E**). The average run length (**F**)
579 of retrogradely transporting endosomes was also shorter in diseased Tfm/TG males, but this
580 difference was not significant, perhaps due to the larger error variance in run length for Tfm
581 males. Because Tfm and Tfm/TG normally have very low levels of circulating androgens, males
582 used in this study were treated with testosterone, mimicking normal male levels of androgens.
583 Such trafficking perturbations demonstrate that non-cell autonomous mechanisms from skeletal
584 muscle can impair trafficking machinery in the axon proper, possibly perturbing the retrograde
585 motor dynein and its associated dynactin complex. Graphs represents mean ± SEM (A-F: N =
586 6/group) and * indicates significant differences (p<0.05) between groups based on independent
587 t-test.

588 **Figure 4. Proposed model of non-cell autonomous influence of muscle on motoneuronal**
589 **axonal transport.** In the healthy neuromuscular system, neurotrophic factors produced and
590 released from skeletal muscle support motoneuronal health and survival. SBMA skeletal muscle
591 is deficient in neurotrophic factors, which may lead to reduced endocytosis in motoneurons,
592 which may manifest as reduced retrograde transport of cholera toxin and reduced endosomal

593 flux, as observed in the present study. Therapies aimed at increasing muscle-derived
594 neurotrophic factors may remedy defective axonal transport and ameliorate disease symptoms.
595

References

Alper, J. D., Tovar, M., & Howard, J. (2013) Displacement-weighted velocity analysis of gliding assays reveals that Chlamydomonas axonemal dynein preferentially moves conspecific microtubules. Biophys. J. 104:1989-1998. doi: 10.1016/j.bpj.2013.03.041

Baniahmad, A. (2015) Inhibition of the Androgen Receptor by Antiandrogens in Spinobulbar Muscle Atrophy. J. Mol. Neurosci. doi: 10.1007/s12031-015-0681-8

Chevalier-Larsen, E., & Holzbaur, E. L. (2006) Axonal transport and neurodegenerative disease. Biochim. Biophys. Acta 1762:1094-1108. doi: 10.1016/j.bbadis.2006.04.002

Chevalier-Larsen, E. S., O'Brien, C. J., Wang, H., Jenkins, S. C., Holder, L., Lieberman, A. P., & Merry, D. E. (2004) Castration restores function and neurofilament alterations of aged symptomatic males in a transgenic mouse model of spinal and bulbar muscular atrophy. J. Neurosci. 24:4778-4786. doi: 10.1523/JNEUROSCI.0808-04.2004

Chowdary, P. D., Che, D. L., & Cui, B. (2012) Neurotrophin signaling via long-distance axonal transport. Annual review of physical chemistry 63:571-594. doi: 10.1146/annurev-physchem-032511-143704

Christoforidis, S., Miaczynska, M., Ashman, K., Wilm, M., Zhao, L., Yip, S. C., Waterfield, M. D., Backer, J. M., & Zerial, M. (1999) Phosphatidylinositol-3-OH kinases are Rab5 effectors. Nat. Cell Biol. 1:249-252. doi: 10.1038/12075

De Vos, K. J., Grierson, A. J., Ackerley, S., & Miller, C. C. (2008) Role of axonal transport in neurodegenerative diseases. Annu. Rev. Neurosci. 31:151-173. doi: 10.1146/annurev.neuro.31.061307.090711

Goldstein, J. L., & Wilson, J. D. (1972) Studies on the pathogenesis of the pseudohermaphroditism in the mouse with testicular feminization. J. Clin. Invest. 51:1647-1658. doi: 10.1172/JCI106966

Halievski, K., Henley, C. L., Domino, L., Poort, J. E., Fu, M., Katsuno, M., Adachi, H., Sobue, G., Breedlove, S. M., & Jordan, C. L. (2015) Androgen-dependent loss of muscle BDNF mRNA in two mouse models of SBMA. Exp. Neurol. 269:224-232. doi: 10.1016/j.expneurol.2015.04.013

Hamburger, V., & Levi-Montalcini, R. (1949) Proliferation, differentiation and degeneration in the spinal ganglia of the chick embryo under normal and experimental conditions. The Journal of experimental zoology 111:457-501.

Harrington, A. W., & Ginty, D. D. (2013) Long-distance retrograde neurotrophic factor signalling in neurons. Nat. Rev. Neurosci. 14:177-187. doi: 10.1038/nrn3253

He, W. W., Kumar, M. V., & Tindall, D. J. (1991) A frame-shift mutation in the androgen receptor gene causes complete androgen insensitivity in the testicular-feminized mouse. Nucleic Acids Res. 19:2373-2378.

Ilieva, H., Polymenidou, M., & Cleveland, D. W. (2009) Non-cell autonomous toxicity in neurodegenerative disorders: ALS and beyond. J. Cell Biol. 187:761-772. doi: 10.1083/jcb.200908164

Johansen, J. A., Troxell-Smith, S. M., Yu, Z., Mo, K., Monks, D. A., Lieberman, A. P., Breedlove, S. M., & Jordan, C. L. (2011) Prenatal flutamide enhances survival in a myogenic mouse model of spinal bulbar muscular atrophy. Neurodegener Dis 8:25-34. doi: 10.1159/000313682

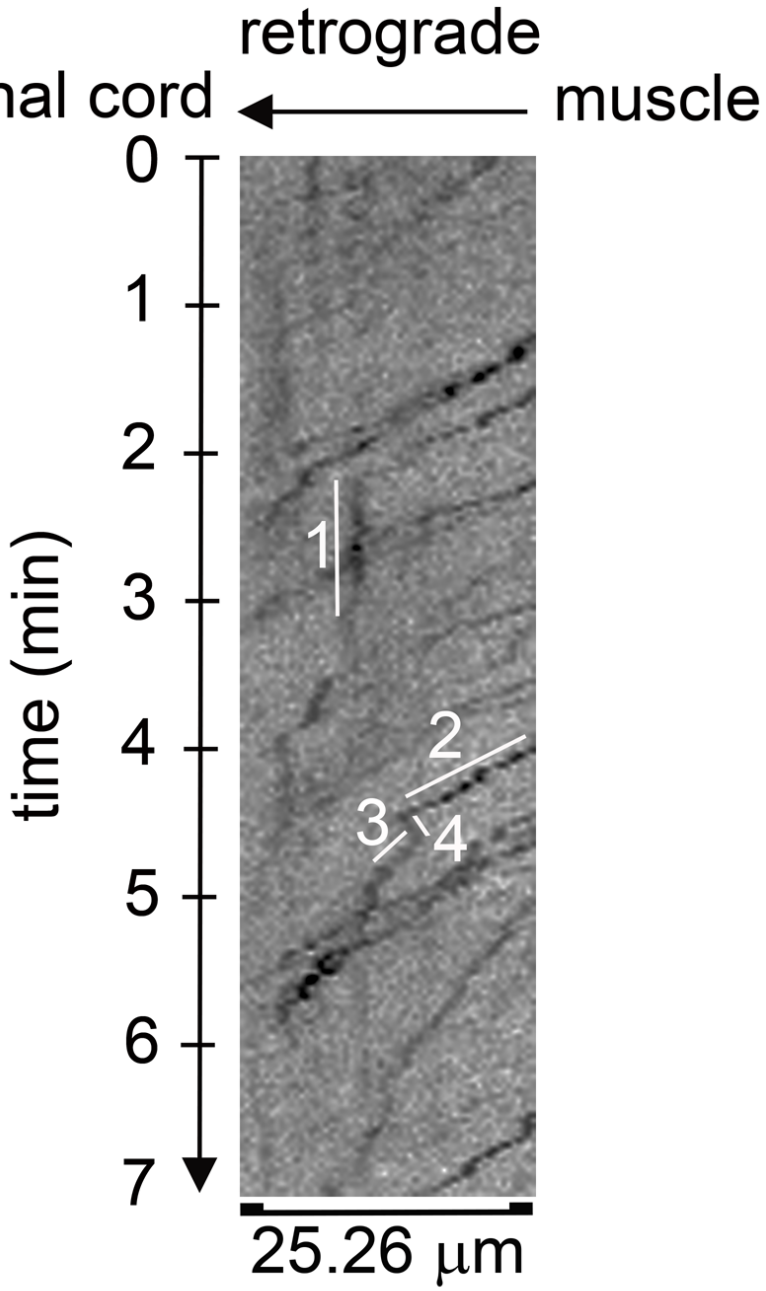
Johansen, J. A., Yu, Z., Mo, K., Monks, D. A., Lieberman, A. P., Breedlove, S. M., & Jordan, C. L. (2009) Recovery of function in a myogenic mouse model of spinal bulbar muscular atrophy. Neurobiol Dis 34:113-120. doi: 10.1016/j.nbd.2008.12.009

Jordan, C. L., & Lieberman, A. P. (2008) Spinal and bulbar muscular atrophy: a motoneuron or muscle disease? Curr Opin Pharmacol 8:752-758. doi: 10.1016/j.coph.2008.08.006

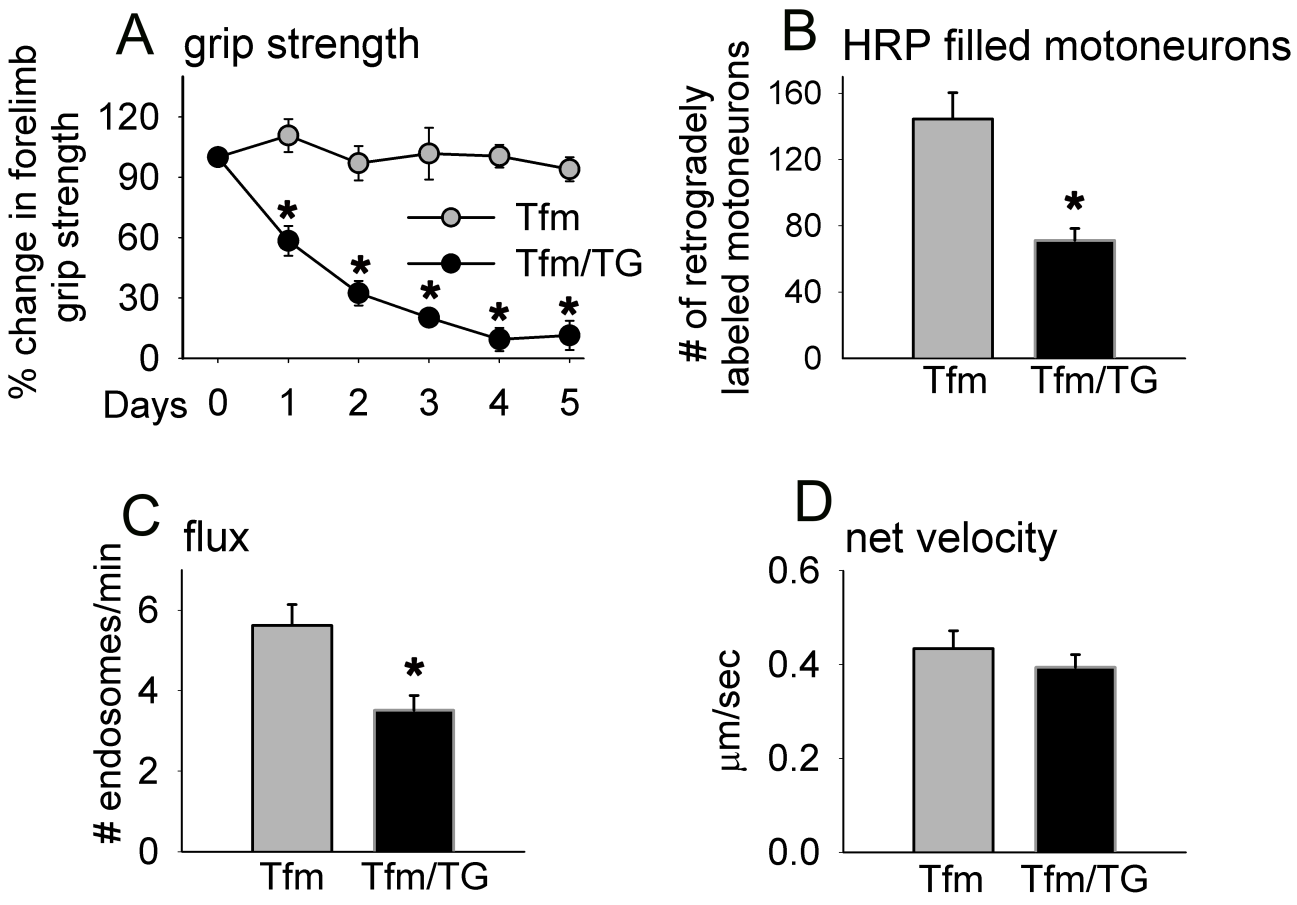
642 Katsuno, M., Adachi, H., Kume, A., Li, M., Nakagomi, Y., Niwa, H., Sang, C., Kobayashi, Y., Doyu, M., &
643 Sobue, G. (2002) Testosterone reduction prevents phenotypic expression in a transgenic mouse
644 model of spinal and bulbar muscular atrophy. Neuron 35:843-854.
645 Katsuno, M., Adachi, H., Minamiyama, M., Waza, M., Tokui, K., Banno, H., Suzuki, K., Onoda, Y., Tanaka,
646 F., Doyu, M., & Sobue, G. (2006) Reversible disruption of dynactin 1-mediated retrograde axonal
647 transport in polyglutamine-induced motor neuron degeneration. J. Neurosci. 26:12106-12117.
648 doi: 10.1523/JNEUROSCI.3032-06.2006
649 Katsuno, M., Tanaka, F., Adachi, H., Banno, H., Suzuki, K., Watanabe, H., & Sobue, G. (2012)
650 Pathogenesis and therapy of spinal and bulbar muscular atrophy (SBMA). Prog. Neurobiol.
651 99:246-256. doi: 10.1016/j.pneurobio.2012.05.007
652 Kemp, M. Q., Poort, J. L., Baqri, R. M., Lieberman, A. P., Breedlove, S. M., Miller, K. E., & Jordan, C. L.
653 (2011) Impaired motoneuronal retrograde transport in two models of SBMA implicates two sites
654 of androgen action. Hum. Mol. Genet. 20:4475-4490. doi: 10.1093/hmg/ddr380
655 King, S. J., & Schroer, T. A. (2000) Dynactin increases the processivity of the cytoplasmic dynein motor.
656 Nat. Cell Biol. 2:20-24. doi: 10.1038/71338
657 La Spada, A. R., Wilson, E. M., Lubahn, D. B., Harding, A. E., & Fischbeck, K. H. (1991) Androgen receptor
658 gene mutations in X-linked spinal and bulbar muscular atrophy. Nature 352:77-79. doi:
659 10.1038/352077a0
660 Malik, B., Nirmalananthan, N., Bilsland, L. G., La Spada, A. R., Hanna, M. G., Schiavo, G., Gallo, J. M., &
661 Greensmith, L. (2011) Absence of disturbed axonal transport in spinal and bulbar muscular
662 atrophy. Hum. Mol. Genet. 20:1776-1786. doi: 10.1093/hmg/ddr061
663 Mitchell, D. J., Blasier, K. R., Jeffery, E. D., Ross, M. W., Pullikuth, A. K., Suo, D., Park, J., Smiley, W. R., Lo,
664 K. W., Shabanowitz, J., Deppmann, C. D., Trinidad, J. C., Hunt, D. F., Catling, A. D., & Pfister, K. K.
665 (2012) Trk activation of the ERK1/2 kinase pathway stimulates intermediate chain
666 phosphorylation and recruits cytoplasmic dynein to signaling endosomes for retrograde axonal
667 transport. J. Neurosci. 32:15495-15510. doi: 10.1523/JNEUROSCI.5599-11.2012
668 Mo, K., Razak, Z., Rao, P., Yu, Z., Adachi, H., Katsuno, M., Sobue, G., Lieberman, A. P., Westwood, J. T., &
669 Monks, D. A. (2010) Microarray analysis of gene expression by skeletal muscle of three mouse
670 models of Kennedy disease/spinal bulbar muscular atrophy. PLoS One 5:e12922. doi:
671 10.1371/journal.pone.0012922
672 Monks, D. A., Johansen, J. A., Mo, K., Rao, P., Eagleson, B., Yu, Z., Lieberman, A. P., Breedlove, S. M., &
673 Jordan, C. L. (2007) Overexpression of wild-type androgen receptor in muscle recapitulates
674 polyglutamine disease. Proceedings of the National Academy of Sciences of the United States of
675 America 104:18259-18264. doi: 10.1073/pnas.0705501104
676 Morfini, G., Pigino, G., Szebenyi, G., You, Y., Pollema, S., & Brady, S. T. (2006) JNK mediates pathogenic
677 effects of polyglutamine-expanded androgen receptor on fast axonal transport. Nat. Neurosci.
678 9:907-916. doi: 10.1038/nn1717
679 Morfini, G. A., Burns, M., Binder, L. I., Kanaan, N. M., LaPointe, N., Bosco, D. A., Brown, R. H., Jr., Brown,
680 H., Tiwari, A., Hayward, L., Edgar, J., Nave, K. A., Garberrn, J., Atagi, Y., Song, Y., Pigino, G., &
681 Brady, S. T. (2009) Axonal transport defects in neurodegenerative diseases. J. Neurosci.
682 29:12776-12786. doi: 10.1523/JNEUROSCI.3463-09.2009
683 Murphy, L., & O'Shaughnessy, P. J. (1991) Testicular steroidogenesis in the testicular feminized (Tfm)
684 mouse: loss of 17 alpha-hydroxylase activity. J. Endocrinol. 131:443-449.
685 Navarro, X., Vivo, M., & Valero-Cabre, A. (2007) Neural plasticity after peripheral nerve injury and
686 regeneration. Prog. Neurobiol. 82:163-201. doi: 10.1016/j.pneurobio.2007.06.005
687 Oki, K., Wiseman, R. W., Breedlove, S. M., & Jordan, C. L. (2013) Androgen receptors in muscle fibers
688 induce rapid loss of force but not mass: implications for spinal bulbar muscular atrophy. Muscle
689 Nerve 47:823-834.

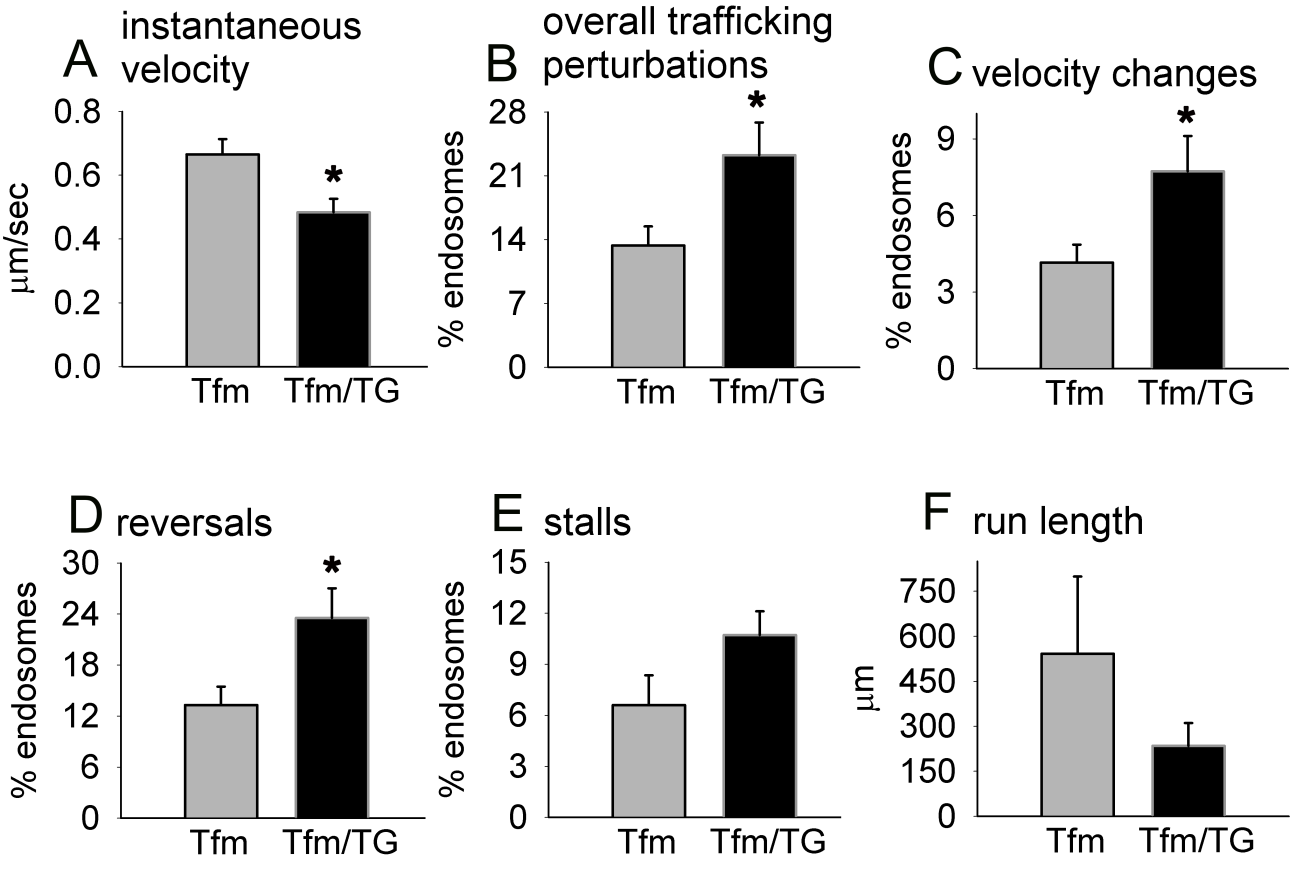
690 Park, J. J., Zup, S. L., Verhovshek, T., Sengelaub, D. R., & Forger, N. G. (2002) Castration reduces
691 motoneuron soma size but not dendritic length in the spinal nucleus of the bulbocavernosus of
692 wild-type and BCL-2 overexpressing mice. J. Neurobiol. 53:403-412. doi: 10.1002/neu.10103
693 Pelkmans, L., Burli, T., Zerial, M., & Helenius, A. (2004) Caveolin-stabilized membrane domains as
694 multifunctional transport and sorting devices in endocytic membrane traffic. Cell 118:767-780.
695 doi: 10.1016/j.cell.2004.09.003
696 Piccioni, F., Pinton, P., Simeoni, S., Pozzi, P., Fascio, U., Vismara, G., Martini, L., Rizzuto, R., & Poletti, A.
697 (2002) Androgen receptor with elongated polyglutamine tract forms aggregates that alter
698 axonal trafficking and mitochondrial distribution in motor neuronal processes. FASEB J. 16:1418-
699 1420. doi: 10.1096/fj.01-1035fje
700 Ramzan, F., McPhail, M., Rao, P., Mo, K., Halievski, K., Swift-Gallant, A., Mendoza-Viveros, L., Cheng, H.
701 Y., & Monks, D. A. (2015) Distinct Etiological Roles for Myocytes and Motor Neurons in a Mouse
702 Model of Kennedy's Disease/Spinobulbar Muscular Atrophy. J. Neurosci. 35:6444-6451. doi:
703 10.1523/JNEUROSCI.3599-14.2015
704 Renier, K. J., Troxell-Smith, S. M., Johansen, J. A., Katsuno, M., Adachi, H., Sobue, G., Chua, J. P., Sun Kim,
705 H., Lieberman, A. P., Breedlove, S. M., & Jordan, C. L. (2014) Antiandrogen flutamide protects
706 male mice from androgen-dependent toxicity in three models of spinal bulbar muscular atrophy.
707 Endocrinology 155:2624-2634. doi: 10.1210/en.2013-1756
708 Sagot, Y., Rosse, T., Vejsada, R., Perrelet, D., & Kato, A. C. (1998) Differential effects of neurotrophic
709 factors on motoneuron retrograde labeling in a murine model of motoneuron disease. J.
710 Neurosci. 18:1132-1141.
711 Sahashi, K., Katsuno, M., Hung, G., Adachi, H., Kondo, N., Nakatsuji, H., Tohnai, G., Iida, M., Bennett, C.
712 F., & Sobue, G. (2015) Silencing neuronal mutant androgen receptor in a mouse model of spinal
713 and bulbar muscular atrophy. Hum. Mol. Genet. 24:5985-5994. doi: 10.1093/hmg/ddv300
714 Sambataro, F., & Pennuto, M. (2012) Cell-autonomous and non-cell-autonomous toxicity in
715 polyglutamine diseases. Prog. Neurobiol. 97:152-172. doi: 10.1016/j.pneurobio.2011.10.003
716 Sopher, B. L., Thomas, P. S., Jr., LaFevre-Bernt, M. A., Holm, I. E., Wilke, S. A., Ware, C. B., Jin, L. W.,
717 Libby, R. T., Ellerby, L. M., & La Spada, A. R. (2004) Androgen receptor YAC transgenic mice
718 recapitulate SBMA motor neuronopathy and implicate VEGF164 in the motor neuron
719 degeneration. Neuron 41:687-699.
720 Szebenyi, G., Morfini, G. A., Babcock, A., Gould, M., Selkoe, K., Stenoien, D. L., Young, M., Faber, P. W.,
721 MacDonald, M. E., McPhaul, M. J., & Brady, S. T. (2003) Neuropathogenic forms of huntingtin
722 and androgen receptor inhibit fast axonal transport. Neuron 40:41-52.
723 Thomas, P. S., Jr., Fraley, G. S., Damian, V., Woodke, L. B., Zapata, F., Sopher, B. L., Plymate, S. R., & La
724 Spada, A. R. (2006) Loss of endogenous androgen receptor protein accelerates motor neuron
725 degeneration and accentuates androgen insensitivity in a mouse model of X-linked spinal and
726 bulbar muscular atrophy. Hum. Mol. Genet. 15:2225-2238. doi: 10.1093/hmg/ddl148
727 Yu, Z., Dadgar, N., Albertelli, M., Gruis, K., Jordan, C., Robins, D. M., & Lieberman, A. P. (2006) Androgen-
728 dependent pathology demonstrates myopathic contribution to the Kennedy disease phenotype
729 in a mouse knock-in model. J. Clin. Invest. 116:2663-2672. doi: 10.1172/JCI28773
730 Zuloaga, D. G., Morris, J. A., Jordan, C. L., & Breedlove, S. M. (2008) Mice with the testicular feminization
731 mutation demonstrate a role for androgen receptors in the regulation of anxiety-related
732 behaviors and the hypothalamic-pituitary-adrenal axis. Horm. Behav. 54:758-766. doi:
733 10.1016/j.yhbeh.2008.08.004

734

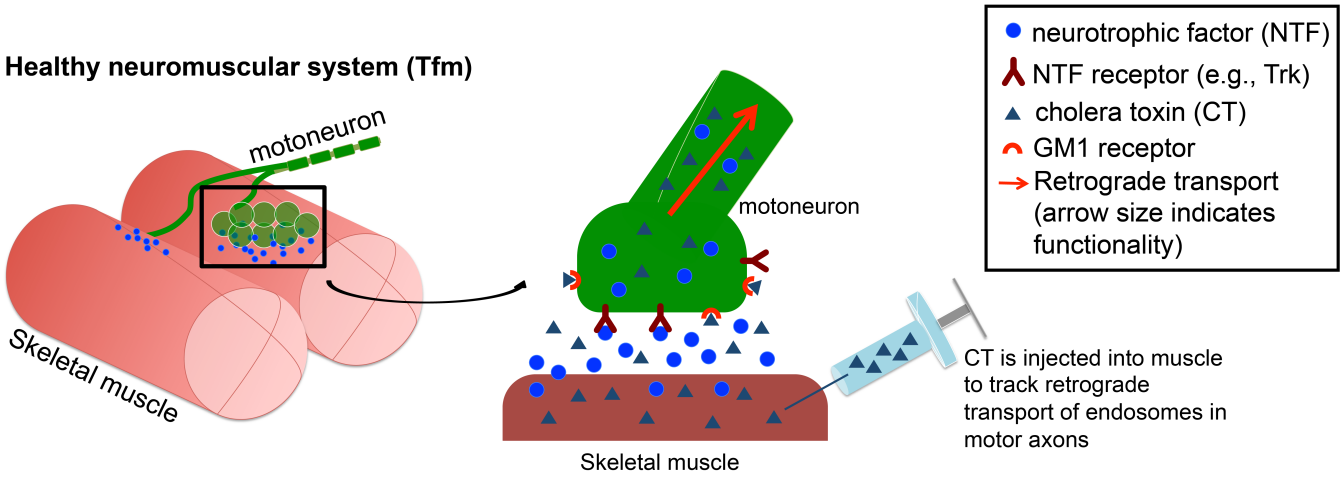


1 = stall
2, 3 = velocity change
4 = reversal





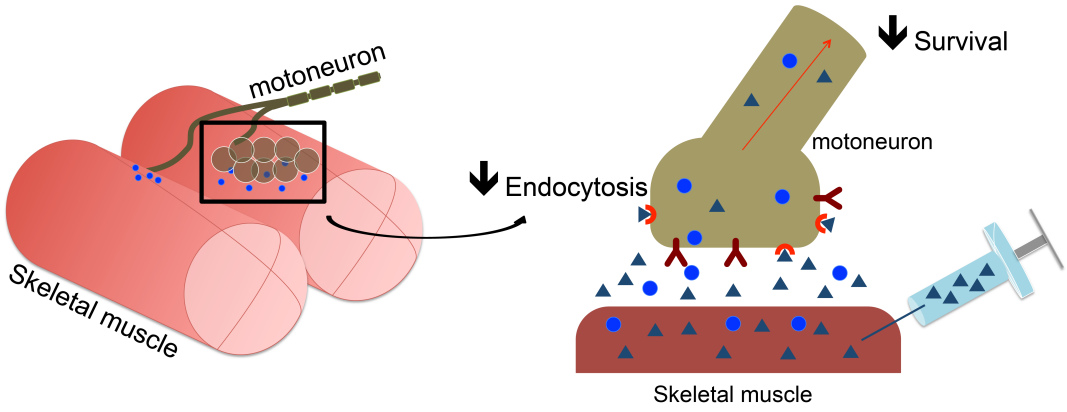
Healthy neuromuscular system (Tfm)



1. Normal production and release of NTFs from skeletal muscle

2. Muscle-derived NTFs enhance the uptake and retrograde transport of needed cargo to motoneuronal cell bodies to maintain their health and survival

Unhealthy (SBMA) neuromuscular system (Tfm/TG)



1. Reduced expression and release of NTFs from skeletal muscle

2. Deficits in muscle-derived NTFs impairs endocytosis (local, early event) and trafficking machinery (later event via possibly effects on gene expression) leading to long term demise and eventual death of motoneurons