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Absence of disturbed axonal transport in spinal and bulbar muscular atrophy

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Spinal and bulbar muscular atrophy (SBMA), or Kennedy's disease, is a late-onset motor neuron disease (MND) caused by an abnormal expansion of the CAG repeat in the androgen receptor (AR) gene on the X-chromosome, encoding a polyglutamine (poly-Q) sequence in the protein product. Mutant poly-Q-expanded AR protein is widely expressed but leads to selective lower motoneuron death. Although the mechanisms that underlie SBMA remain unclear, defective axonal transport has been implicated in MND and other forms of poly-Q disease. Transcriptional dysregulation may also be involved in poly-Q repeat pathology. We therefore examined axonal transport in a mouse model of SBMA recapitulating many aspects of the human disease. We found no difference in the expression levels of motor and the microtubule-associated protein tau, in the spinal cord and sciatic nerve of wild-type (WT) and SBMA mice at various stages of disease progression. Furthermore, we found no alteration in binding properties of motor proteins and tau to microtubules. Moreover, analysis of axonal transport rates both in cultured primary motoneurons *in vitro* and *in vivo* in the sciatic nerve of adult WT and mutant SBMA mice demonstrated no overt axonal transport deficits in these systems. Our results therefore indicate that unlike other motoneuron and poly-Q diseases, axonal transport deficits do not play a significant role in the pathogenesis of SBMA.

INTRODUCTION

Spinal and bulbar muscular atrophy (SBMA), otherwise known as Kennedy's disease, is a hereditary neurodegenerative X-linked disorder which arises from an expansion of the CAG repeat in the first exon of the androgen receptor (AR) gene encoding a polyglutamine (poly-Q) stretch in the translated protein (1). It belongs to a family of poly-Q repeat disorders which includes Huntington's disease (HD) (2). SBMA primarily affects males who experience progressive bulbar dysfunction and proximal limb muscle atrophy and weakness. In the normal population, the polymorphic CAG repeat ranges from 9 to 36 and an expansion of greater than 38 residues results in disease (3). Mutant-expanded AR protein is ubiquitously expressed but leads to selective lower motoneuron loss in the spinal cord and bulbar nuclei of the brainstem. Although the cause of the motoneuron death and dysfunction in SBMA, which is restricted to particular lower and bulbar motoneurons, is largely unknown, current evidence supports the involvement of axonal

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transport dysfunction and transcriptional dysregulation (4,5). Motoneurons, by virtue of their very long axons, are particularly vulnerable to axonal transport defects and disturbances have been observed even in embryonic motoneurons in mouse models of amyotrophic lateral sclerosis (ALS), the most common form of adult motor neuron disease (MND) (6,7). Furthermore, a mutation in the $p150^{Glued}$ subunit of dynactin (dynactin 1), which forms a complex with cytoplasmic dynein, the main retrograde motor complex, causes a slowly progressive form of MND, possibly by disrupting the binding of dynactin to microtubules and thereby impairing axonal retrograde transport (8). In addition, in sporadic ALS patients, mRNA levels of dynactin 1 are reduced (9), signifying the importance of diminished levels of axonal transport proteins in MND pathology.

In the context of SBMA, transcriptional dysregulation by mutant AR has also been shown to decrease $p150^{Glued}$ in a mouse model of SBMA (10). Several *in vitro* studies using squid axon models, in which mutant-expanded AR and poly-Q proteins are over-expressed, have also reported deficits in axonal transport (11–13), which are caused by a loss of kinesin binding (14). *In vivo*, axonal transport deficits have been detected using fluoro-gold tracer analysis in a mouse model of SBMA, likely to be the result of transcriptional-dependent loss of $p150^{Glued}$ (10). However, over-expression of mutant AR in these mice does not result in motoneuron degeneration but instead manifests in an abrupt development of muscle pathology leading to premature death, which is unlike the course and pattern of the disease observed in humans (15). The mouse model used in this study, in which the mutant AR is expressed at endogenous levels, follows more accurately the course of disease seen in humans, resulting in significant motoneuron loss leading to a progressive late-onset neuromuscular phenotype (4). The male mice carry the expanded 100 poly-Q AR repeat mutation (pathogenic AR100 mice) or 20 poly-Q AR repeats (non-pathogenic AR20 mice), which do not possess any phenotype, as less than 38 poly-Q repeats in humans does not cause disease.

Given that transcriptional dysregulation by mutant AR may result in alteration of protein levels, which could account for any transport deficit, in this study we set out to examine in detail the expression of axonal transport-related proteins in an SBMA transgenic mouse model which recapitulates the natural history of the human disease more closely (4). Furthermore, we determined the binding efficiency of motor complexes and the microtubule-associated protein tau, to test the hypothesis that their uncoupling may lead to destabilization and/or transport disturbances. Additionally, we investigated the role of axonal transport kinetics in motoneurons both *in vitro* and *in vivo,* which allowed us to fully characterize the involvement of axonal transport deficits in SBMA mice. We demonstrate both through biochemical and transport assays in living neurons, that axonal transport is not compromised in SBMA transgenic mice (4). Collectively, our observations suggest that unlike several other motor and poly-Q disorders, the underlying pathology in SBMA may not be due to an impairment in axonal transport. Therefore the development of therapeutic strategies for SBMA would be better focused on alternative targets.

RESULTS

Mutant AR expression results in motoneuron degeneration in SBMA mice

The SBMA mouse model has been previously characterized (4) and mice heterozygous for the expanded AR100 mutation (100 poly-Q AR repeats) have been shown to develop a late-onset, gender-specific, progressive muscular phenotype similar to the disease manifested in human SBMA patients. The mice exhibit a motor phenotype and show clear histological evidence of muscle pathology in hindlimb muscles (4). Nonetheless, quantitative analysis of motoneuron number and loss in the spinal cord has not been previously described for these mice. As the disease is gender specific, in this study we examined heterozygous male mice with the expanded AR100 (pathogenic) poly-Q tract which develop histopathological changes in the muscle and spinal cord, consistent with a late-onset neuromuscular phenotype (4). Additionally, heterozygote male AR20 mice, which carry non-pathogenic 20 poly-Q AR repeats and do not develop a phenotype, were compared with the AR100 mice. To further characterize the pathology in the mutant AR mice, we show that as well as the histopathological alterations shown by Sopher *et al.* (4), staining of lumbar spinal cord sections reveals that motoneuron survival in the sciatic motor pool (Fig. 1A, encircled area) was significantly compromised in the mutant expanded AR100 but not in wild-type (WT) or AR20 mice (Fig. 1A and B). In mutant AR100 mice, the total number of surviving motoneurons was only 226.22 ($\pm$13.37, $n = 8$) in 18-month-old mice compared to 365.80 ($\pm$10.91, $n = 7$) in AR20 control mice and 386.37 ($\pm$7.51, $n = 7$; $P < 0.001$ ANOVA, Fig. 1B) in WT mice. The AR100 mice therefore develop a neuromuscular pathology characterized by loss of ~40% of motoneurons in the sciatic motor pool of the lumbar spinal cord by 18 months of age. The natural history of neurodegeneration in these mice, therefore, more closely resembles the observations in humans, compared with other reported SBMA models (15).

Expression levels of axonal transport proteins are not altered in SBMA mice

In MND, it is now well documented that impairment in axonal transport is an early feature of disease and has been observed in motoneurons of SOD1 mice that model ALS, both *in vitro* in isolated embryonic motoneurons (7) and *in vivo* in intact adult sciatic nerves at different disease stages (6,16). We therefore set out to examine the role of axonal transport in an SBMA mouse model. Since deficits in axonal transport may arise from a decrease in levels of transport-related proteins, we examined the expression levels of these proteins in SBMA mice in which mutant-expanded poly-Q AR100 is associated with transcriptional dysregulation (4). Levels of the motor proteins (kinesin 1, cytoplasmic dynein), tau, a microtubule-stabilizing protein and a regulator of axonal transport (17), and the dynein-associated protein $p150^{Glued}$ were established in pre-symptomatic (3 months), symptomatic (12 months) and diseased mice near end stage (18 months) and the results are summarized in Figures 2 and 3. Protein levels were assessed in WT, AR20 and AR100 poly-Q male

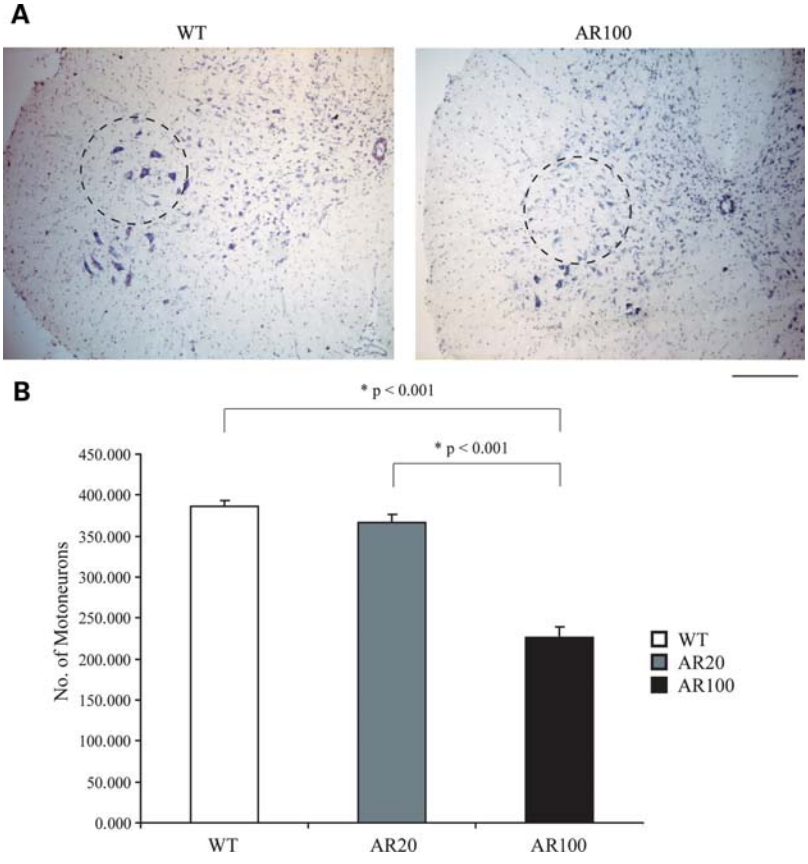

Figure 1. Motoneuron degeneration in SBMA mice. Motoneuron survival was significantly reduced in 18-month-old heterozygous SBMA mice with the mutant AR100 androgen receptor. (**A**) Typical examples of Nissl-stained lumbar spinal cord sections from WT and heterozygous AR100 mice are shown, encircled area represents the sciatic motor pool in the ventral horn (scale bar = 200 μm). (**B**) The total number of surviving motoneurons in the sciatic motor pool of each mouse was determined and the results are summarized in the bar chart, with values expressed as mean ± SEM. For each genotype, $n \geq 7$, with one-way ANOVA and *post hoc* analysis performed to test for significance. $^{*}P < 0.001$, error bars represent SEM.

mice in the spinal cord (Fig. 2) and the sciatic nerve (Fig. 3), two primary regions displaying SBMA pathology. Our findings show that there was no alteration in levels of axonal transport-related proteins, at any stage of the disease process, in either the spinal cord or sciatic nerve of mutant mice. At 18 months (Fig. 2C), there was a general but small decline in the levels of protein examined in the spinal cord in AR100 mice. However, statistical analysis and pairwise comparisons between all genotypes showed there were no statistically significant differences in the expression level of any of the proteins. This general decline in the expression levels of all the examined proteins, observed in the 18 month spinal cord, is likely to be a reflection of the significant loss of motoneurons that has occurred by this stage of the disease (Fig. 1). These results show that the transcriptional dysregulation induced by the mutant AR100 does not impact on the levels of proteins involved in axonal transport.

Microtubule-binding efficiency of motor complexes and tau in SBMA mice

Deficits in axonal transport may arise from the uncoupling of motor proteins from microtubules or by abnormal binding of axonal transport regulators (e.g. tau). Therefore, we examined the binding properties of motor proteins, such as kinesin heavy chain (KHC), dynein intermediate chain (DIC) and $p150^{Glued}$, as well as tau in spinal cord (Fig. 4) and sciatic nerve (Fig. 5) samples from mice of each genotype at different stages of disease. The microtubule-binding assay revealed that the mutant-expanded AR did not cause a reduction in the binding properties of the majority of the proteins examined. Although there seemed to be no decrease in the levels of proteins found in the supernatant fraction (indicative of loss of microtubule binding) when normalized to tubulin levels in the fractions (data not shown), densitometric analysis of normalized supernatant/pellet fractions (normalized to protein levels in total spinal cord and sciatic nerve to account for decreased protein levels at 18 months, Fig. 4C) revealed a moderate alteration of the binding of DIC in the spinal cord from 18-month-old AR100 mice compared with AR20 and WT mice (Fig. 4C). As this was present only in 18 month AR100 mice, which exhibit a significant neuromuscular phenotype, this was likely to be a consequence of the disease process and loss of motoneurons rather than a causative pathogenic event. Even at the late stage of disease, no reduction in the binding properties of the other proteins in spinal cord or sciatic nerve tissue was detected. Thus, in this mouse model of SBMA, we did not detect a reduction in the microtubule-

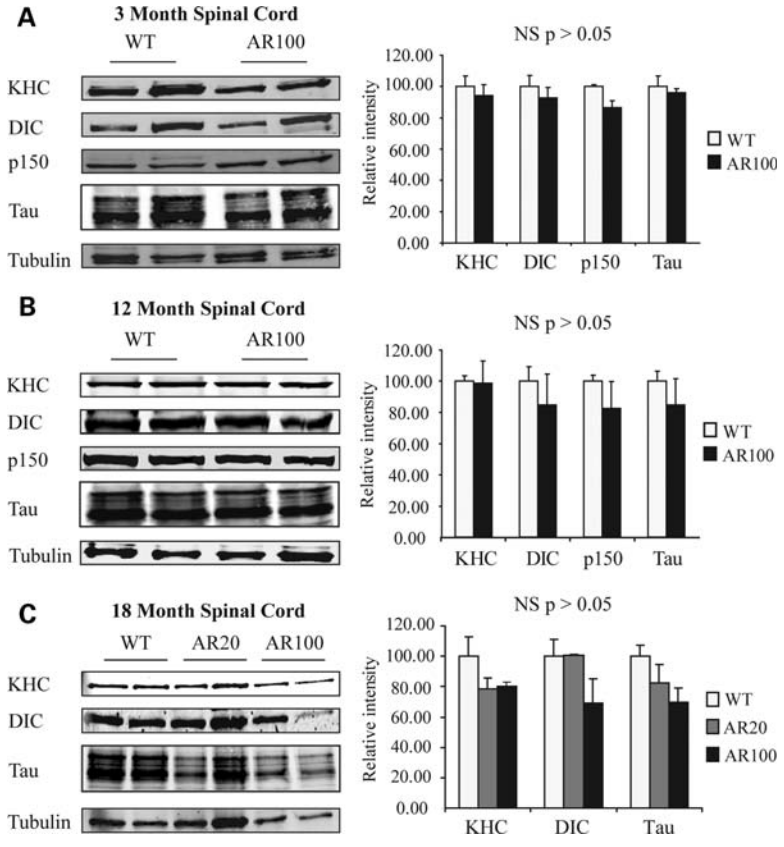

Figure 2. Expression levels of axonal transport proteins in the spinal cord of SBMA mice. Several components of the axonal transport machinery show no change in levels in the spinal cord from WT and AR100 transgenic mice of 3 months (**A**), 12 months (**B**) and 18 months of age (**C**). KHC, kinesin heavy chain; DIC, dynein intermediate chain; p150, dynactin $p150^{Glued}$ subunit. Densitometry was performed as described in Materials and Methods. Values are presented as mean $\pm$ SEM of relative intensities expressed as a percentage of WT. Statistical analysis of data was performed using ANOVA and Student–Newman–Keuls and Tukey's HSD *post hoc* tests with pairwise comparisons between all groups within the experiment, $P < 0.05$, NS, not significant.

binding properties of motor complexes or MAPs, at either the pre-symptomatic or symptomatic phase of the disease.

In view of these findings, we next examined axonal transport in motoneurons of developing and adult SBMA mice.

Kinetics of retrograde axonal transport of TeNT H_C in cultured motor neurons from SBMA mice

Axonal transport deficits are one of the earliest deficits to have been identified in ALS in embryonic motoneurons (7). In addition, deficits in axonal transport have also been implicated in the pathogenesis of SBMA (10,13,14). Therefore, in conjunction with the biochemical analysis shown above, we set out to examine axonal transport kinetics in E13-cultured embryonic motoneurons from WT and AR100 mutant mice. Live motoneurons were used to assess the kinetics of axonal retrograde transport using assays based on an atoxic fluorescent fragment of tetanus toxin (TeNT H_C) (18). TeNT H_C can be used to monitor fast retrograde axonal transport in cultured embryonic motoneurons on the basis of its high-affinity binding to plasma membrane and subsequent entry into endocytic carriers containing neurotrophins and their receptors in motoneurons (19). As ligand binding and subsequent nuclear translocation of AR is thought to underlie pathology in SBMA (20–22) and abnormalities in cells are only present or are exacerbated in the presence of testosterone (23–26), motoneurons from WT and AR100 littermates were treated with AR agonist [dihydrotestosterone (DHT)] before transport assays were performed (Fig. 6). The results showed that there was no significant difference between WT and mutant AR100 mice when the speed of carriers (Fig. 6A), proportion of carriers pausing (Fig. 6B) and the proportion of carriers that pause per axon (Fig. 6C) were measured. Taken together, these findings show that the mutant AR100 does not disrupt axonal transport kinetics in embryonic motoneurons.

Retrograde transport is unaffected in adult SBMA mice *in vivo*

Since SBMA is a progressive late-onset neurodegenerative disorder, it is possible that deficits in axonal transport may only manifest in adult motoneurons. We therefore examined axonal transport *in vivo* in anaesthetized adult SBMA mice. Following intramuscular injection of fluorescently labelled TeNT H_C in both 13-month-old WT and heterozygous AR100 mice, we were able to monitor its axonal transport in the intact sciatic nerve (16). *In vivo* transport of TeNT H_C occurred predominantly in the retrograde direction in the

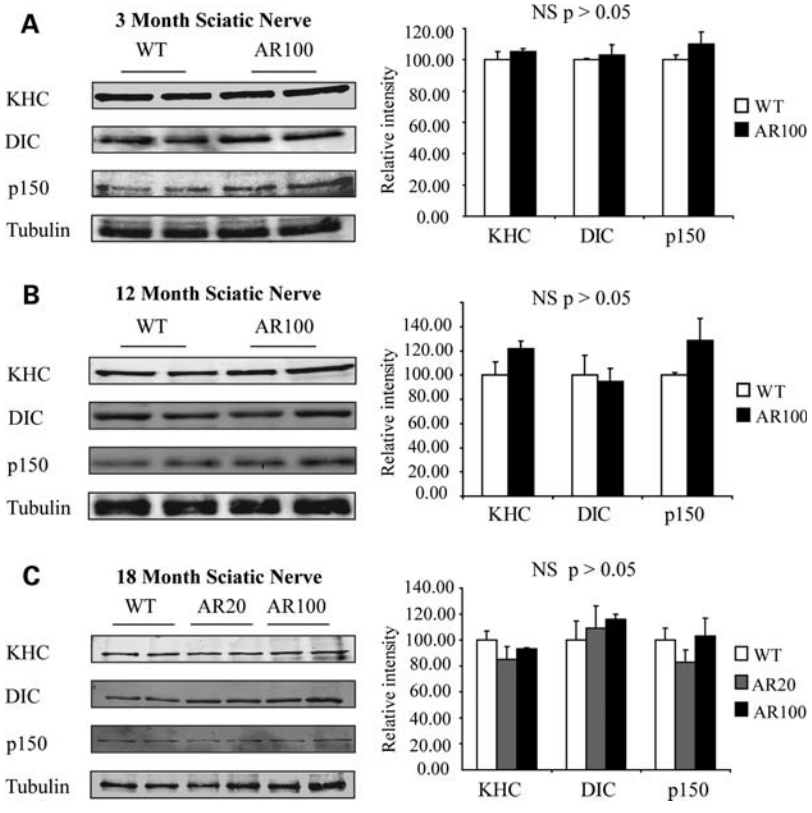

Figure 3. Expression levels of axonal transport proteins in the sciatic nerve in SBMA mice. Levels and densitometric analysis of axonal transport proteins were not changed in the sciatic nerve from WT and mutant AR-expressing transgenic mice of 3 months (**A**), 12 months (**B**) and 18 months of age (**C**). KHC, kinesin heavy chain; DIC, dynein intermediate chain; p150, dynactin p150^{Glued} subunit. Densitometry was performed as described in Materials and Methods. Values are presented as mean $\pm$ SEM of relative intensities expressed as a percentage of WT. Statistical analysis of data was performed using ANOVA and Student–Newman–Keuls and Tukey's HSD *post hoc* tests with pairwise comparisons between all groups within the experiment, $P < 0.05$, NS, not significant.

sciatic nerve (Fig. 7A), as previously described (16), with very rare anterograde movements. Quantitative and statistical analysis of the retrograde transport of these carriers *in vivo* in single axons revealed no significant difference between the heterozygous AR100 mice and their age-matched WT littermates (Fig. 7B).

Taken together, our results therefore show that retrograde transport is not altered in motoneurons at any stage of disease progression in AR100 mice that model SBMA.

DISCUSSION

Failure in the transport of vital cargoes and nutrients along axons has been shown to arise early in ALS (7,27). In addition, expanded poly-Q mutant proteins have also been reported to disrupt axonal transport in several models of poly-Q diseases (10,13,14). In this study, we therefore set out to investigate the role that axonal transport may play in the pathology of SBMA. Our findings show that in SBMA mice, there was no change in the expression levels of components of the axonal transport machinery in either the spinal cord or sciatic nerve at any stage of the disease. Furthermore, changes in the microtubule-binding properties of motor proteins and tau, which might have caused deficits in axonal transport, were also not observed in SBMA mice. Accordingly, functional analysis of axonal transport kinetics, both *in vitro* in developing embryonic motoneurons and *in vivo* in adult symptomatic mice, confirmed that axonal transport was not impaired in mutant SBMA mice. Therefore, our results show that, contrary to other neurodegenerative diseases such as ALS, Alzheimer's disease and HD, there is no evidence that impaired axonal transport plays a significant role in the initiation or progression of SBMA.

The role of axonal transport deficits in motoneuron disorders have been extensively studied (6,7,16,28–31). Motoneurons are particularly dependent for their survival and function on an efficient bidirectional transport system for important cargoes including mitochondria, vesicles and nutrients. Deficits in axonal transport have been shown to be one of the very earliest pathogenic manifestations of disease in ALS mutant SOD1 embryonic motoneurons (7) and have also been observed *in vivo* in motoneurons of adult SOD1 mice prior to symptom onset (6,16). Studies in ALS patients (32) and transgenic mouse models (7,16,27,32–35) confirm that impairments of axonal transport occur in both retrograde and anterograde directions. Moreover, the presence of transport deficits in mutant SOD1 motoneurons during embryonic development (7,27) and at pre-symptomatic stages of disease (16) suggests that these deficits may play a causative role in disease initiation in ALS.

In HD, a poly-Q disease similar to SBMA, expression of full-length mutant huntingtin (Htt) has been shown to impair axonal

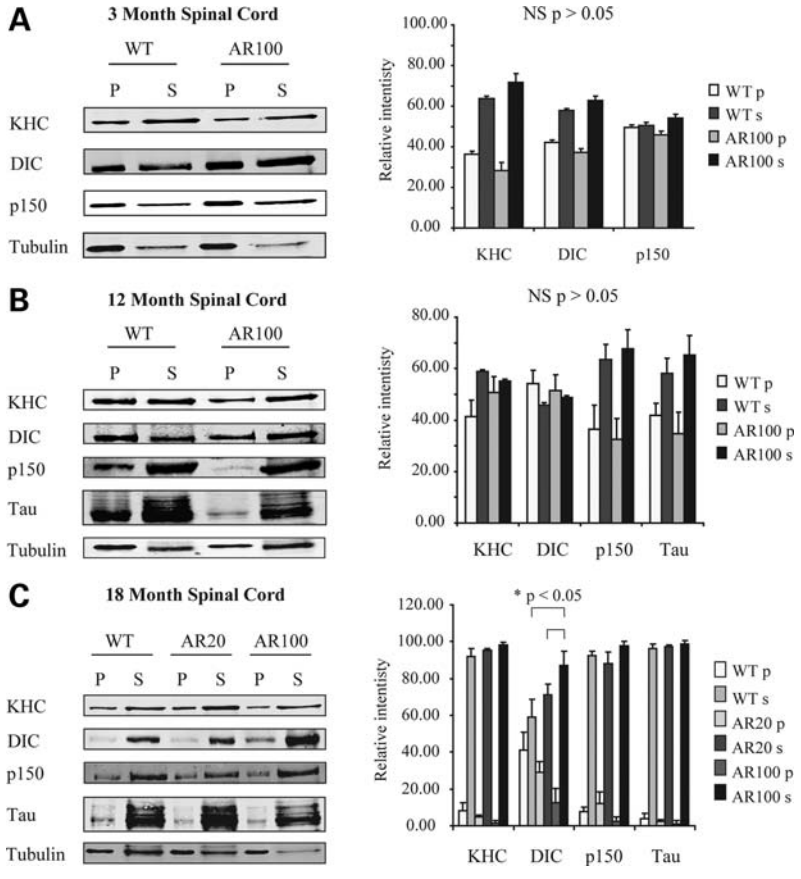

Figure 4. Microtubule-binding properties of axonal transport proteins in the spinal cord in SBMA mice. Microtubule-binding assay and densitometric analyses of representative western blots of microtubule pellets (P) and supernatants (S) were performed using the spinal cord from WT and mutant AR mice of 3 months (**A**), 12 months (**B**) and 18 months of age (**C**). KHC, kinesin heavy chain; DIC, dynein intermediate chain; p150, dynactin $p150^{Glued}$ subunit. Densitometry was performed as described in Materials and Methods. Values are presented as mean $\pm$ SEM of relative intensities expressed as a percentage of WT. Statistical analysis of data was performed using ANOVA and Student–Newman–Keuls and Tukey's HSD *post hoc* tests with pairwise comparisons between all groups within the experiment, $P < 0.05$, NS, not significant.

transport in embryonic neurons both *in vitro*, by measurement of vesicle motility, and *in vivo*, by use of the retrograde tracer fluoro-gold (36). In view of the studies concluding that in neurodegenerative disorders, pathology is secondary to disruption of transport (7,16,36,37), we initially hypothesized that axonal transport disturbances would be observed in SBMA mice. Indeed, several studies have previously suggested the involvement of axonal transport deficits in SBMA *in vitro*. Expanded AR added to squid axoplasm preparations inhibits fast axonal transport (13) via a mechanism thought to involve the inhibition of kinesin binding to microtubules (14,38). Neuropil aggregate formation in mutant AR-transfected NSC34 cells leads to alteration of axonal trafficking and mitochondrial distribution in cell processes (11). *In vivo*, Katsuno *et al* (10) reported disruption of transport in a mouse model of SBMA using the fluoro-gold tracer and attributed this dysfunction to the loss of $p150^{Glued}$ through transcription-dependent mechanisms. Loss of $p150^{Glued}$ mRNA may also occur in sporadic forms of ALS (9).

Earlier evidence therefore argues that axonal transport deficits may be involved in the pathology of SBMA. In this study, however, using several biochemical and transport assays, we found no impairment in transport in SBMA mice. There was no decrease in the expression levels of axonal transport proteins, nor a significant impairment of their microtubule binding. We did however observe a minor alteration in the binding of DIC in the spinal cord of 18-month-old AR100 mice at advanced stages of the disease. However, this is unlikely to be a primary factor in SBMA pathogenesis, and is more likely to arise as a consequence of the disease.

Measurement of transport kinetics verified that axonal transport rates were not compromised in SBMA mice, despite the mice displaying the characteristic pathology of lower motoneuron degeneration associated with a neuromuscular phenotype. These results were surprising, since SBMA is both an MND and a poly-Q disease. However, unlike in HD, the affected protein is not normally involved in intracellular transport. Htt assists in the transport of brain-derived neurotrophic factor (BDNF)-containing vesicles (39), and interacts with dynein and htt-associated protein-1 (HAP1), which complexes with $p150^{Glued}$ and kinesin 1 (39–41). In the mutant SOD1 mouse model of ALS, cytoplasmic dynein has been reported to interact with mutant superoxide dismutase (SOD1) (30). Thus, neurodegenerative diseases in which disruption of axonal transport has been shown to play a role in the development of the pathology are similar in that the affected proteins are transported in the axon and can impair transport, for

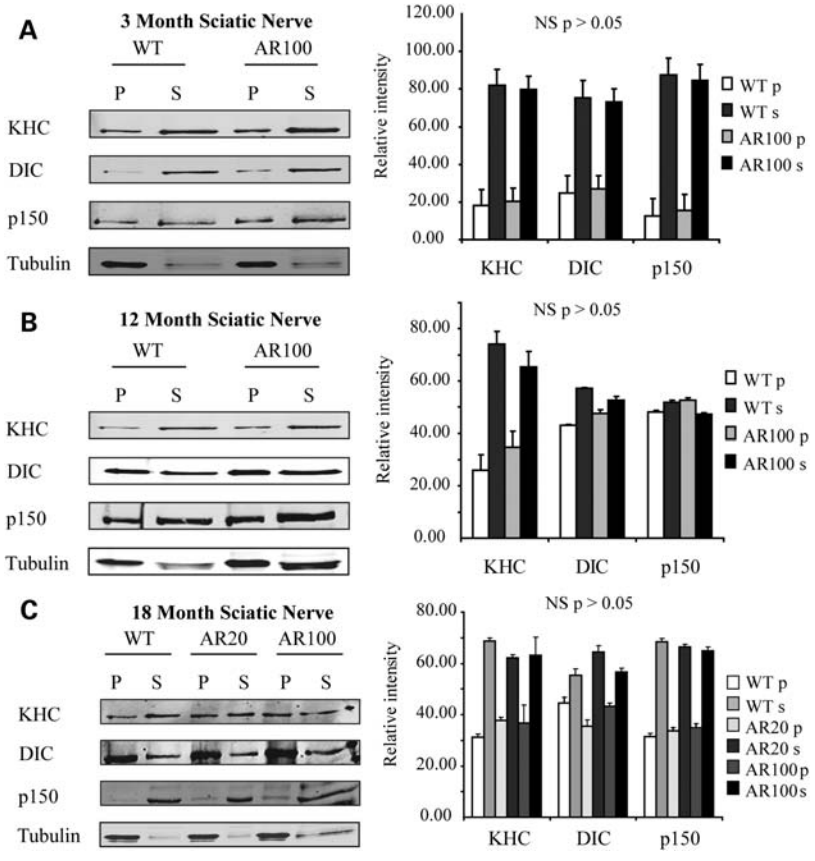

Figure 5. Microtubule-binding properties of axonal transport proteins in the sciatic nerve of SBMA mice. Microtubule-binding assay and densitometric analyses of representative western blots of microtubule pellets (P) and supernatants (S) were performed using the sciatic nerve from WT and mutant AR mice of 3 months (**A**), 12 months (**B**) and 18 months of age (**C**). KHC, kinesin heavy chain; DIC, dynein intermediate chain; p150, dynactin p150^{Glued} subunit. Densitometry was performed as described in Materials and Methods. Values are presented as mean $\pm$ SEM of relative intensities expressed as a percentage of WT. Statistical analysis of data was performed using ANOVA and Student–Newman–Keuls and Tukey's HSD *post hoc* tests with pairwise comparisons between all groups within the experiment, $P < 0.05$, NS, not significant.

example SOD1^{G93A} in ALS, poly-Q Htt in HD and presenilin 1 and amyloid precursor protein in Alzheimer's disease (42). Although AR has been reported to interact with HAP1 (23), there is no evidence as yet of any complex being formed with the molecular motors directly or AR transport within the axon. This suggests that mutant AR would not necessarily have any deleterious impact on axonal transport in SBMA.

Furthermore, most of the studies examining axonal transport in SBMA have been conducted *in vitro* squid axoplasm (13,14) or cell lines (11), which may not be completely relevant to the pathology observed *in vivo*. Additionally, over-expression of proteins in such models may lead to protein mislocalization and result in a pathology that does not reflect poly-Q disease (5). Examination of axonal transport deficits *in vivo* has been previously undertaken in another mouse model of SBMA (10). However, this study did not involve kinetic analysis of transport, and rather focused on end-point tracer analysis. It did however identify transcriptional-dependent loss of p150^{Glued} levels. The mouse model used in the Katsuno study involved massive over-expression of full-length AR bearing 97 poly-Q repeats under the control of the chicken β-actin promoter (22). This strain develops an unusually rapid neuromuscular phenotype in the absence of motoneuron degeneration, resulting in a reduced life span. This is in contrast to the human disease, where SBMA is a disease of the middle age involving loss of lower motoneurons and a slowly progressive natural history with motor disability, but rarely associated with early mortality (15). Importantly, a characteristic and defining pathological feature of SBMA is the loss of motoneurons, which is fundamental to the development of disease (43). The yeast artificial chromosome (YAC) transgenic mice used in the present study (4) develop a late-onset progressive muscular phenotype similar to that observed in human SBMA patients, associated with a significant loss of motoneurons from the sciatic motor pool (Fig. 1). In addition, the expression patterns and levels of the AR expressed in this model recapitulate the endogenous gene expression of the AR (4). As a result, this mouse model represents an excellent genetic reproduction of SBMA (44).

In conclusion, in this study, we provide evidence that there is no disruption of axonal transport in a well-characterized mouse model of SBMA, which reproduces key features and natural history of the human disease more accurately. Although SBMA shares many features with other MND and poly-Q diseases, our findings indicate that compromised

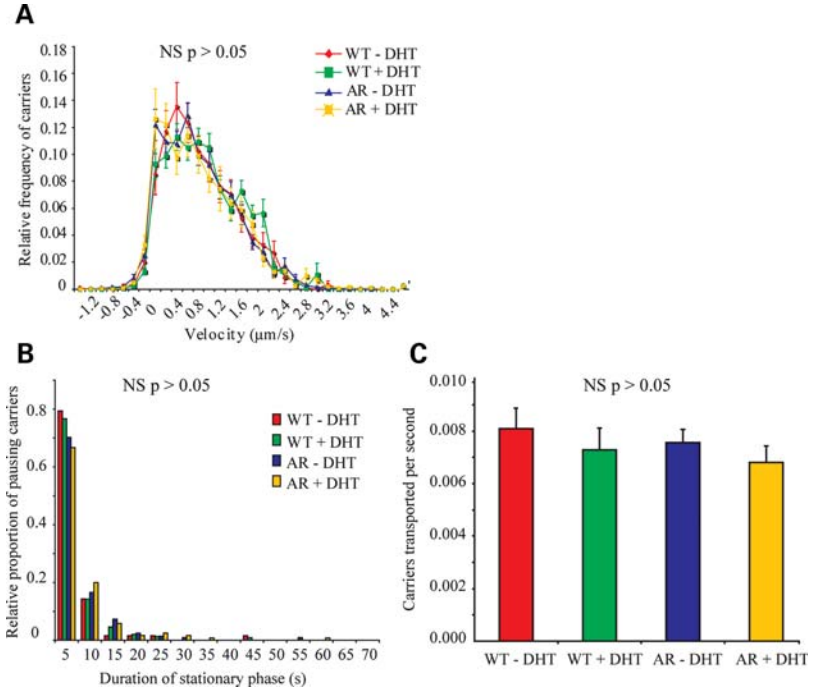

Figure 6. Axonal transport kinetics in cultured embryonic motoneurons from SBMA mice. (**A**) Speed distribution profiles of axonal retrograde TeNT H_C-labelled carriers in WT and AR100 motoneurons. No significant difference was found in the kinetic profiles of carriers in AR100 and WT motoneurons in the presence or absence of DHT. In total, 116 and 115 carriers were imaged for WT motoneurons exposed to and not exposed to DHT, respectively. For AR100 motoneurons, 107 and 231 carriers were counted with and without exposure to DHT, respectively. Experiments were carried out using at least three separate embryos for each genotype ($n \geq 3$). (**B**) Frequency distribution of pauses observed in TeNT H_C carriers as a function of the duration of the observed stationary period. (**C**) Proportion of TeNT H_C-labelled carriers per axon in which a pause was observed. Error bars represent SEM. Statistical analysis of data was performed using ANOVA and Student–Newman–Keuls and Tukey's HSD *post hoc* tests with pairwise comparisons between all groups within the experiment, $P < 0.05$, NS, not significant.

axonal transport is not a pathological feature of SBMA. Our results therefore challenge the view that the pathogenesis of SBMA involves deficits in axonal transport. These data suggest that alternative disease mechanisms and molecular targets for potential therapies in SBMA need to be identified.

MATERIALS AND METHODS

Materials

All materials were purchased from Sigma (Sigma-Aldrich, Poole, UK) unless indicated otherwise. The following antibodies were used: KHC (1:1000; Chemicon, UK) and DIC (1:1000; Chemicon), $p150^{Glued}$ (1:2000; BD Biosciences, UK), tau (1:20 000; Dako, UK) and α-tubulin DM1A (1:5000; Sigma-Aldrich).

AR mutant mice-breeding protocol and characterization

The experimental procedures described in this study were carried out under licence from the UK Home Office (Scientific procedures Act 1986) and were approved by the Ethical Review Panel of the Institute of Neurology, University College London (UCL). The generation, genotyping and characterization of the YAC mice containing the human AR transgene have been described previously (4,45). The mice were originally provided by Albert R. La Spada and were subsequently bred and maintained at the UCL Institute of Neurology Biological Services. To maintain the colony, heterozygote males carrying the 100 poly-Q AR repeats (pathogenic AR100 mice) or 20 poly-Q AR repeats (non-pathogenic AR20 mice) were mated with WT C57BL6J females. Only male offspring were used for all experiments to ensure gender specificity of the disease and all AR mutant mice (AR20 and AR100) were heterozygote for the mutation. Genotyping of mice was carried out by PCR amplification on ear punches on weaned animals and repeated on tail snips on completion of all experiments, using a forward (5′-catctgagtccaggggaacagc-3′) and reverse primer (5′-gcccaggcgctgccgtagtcc-3′) with digestion of the PCR product with *Bgl*1 (New England Biolabs, UK). To collect tissue, mice were terminally anaesthetized with phenobarbitone, transcardially perfused with 0.9% saline and the whole spinal cord and sciatic nerves were rapidly dissected, snap frozen in liquid nitrogen and stored at −80°C.

Microtubule-binding assay

Microtubules from the spinal cord and sciatic nerve were isolated from WT and mutant AR mice and a microtubule-binding assay performed as described (46). Briefly, tissue was homogenized in microtubule assembly buffer (pH 6.8: 100 mM PIPES, 1 mM $MgSO_4$, 1 mM EGTA) and centrifuged. The supernatant was recentrifuged at 180 000 g for 90 min at 4°C and the new supernatant (total protein fraction) used to

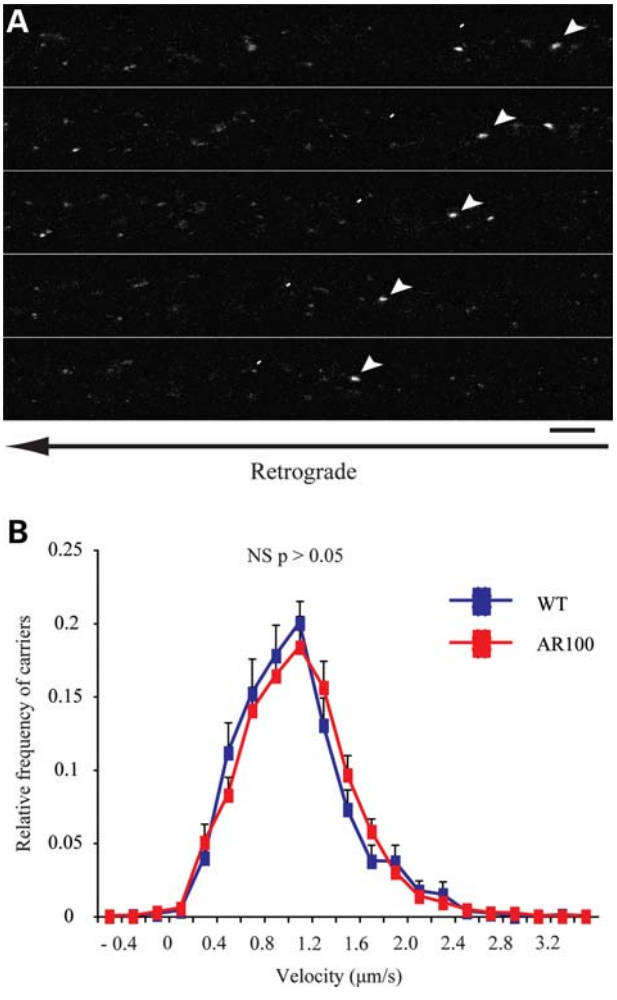

Figure 7. Retrograde transport kinetics in heterozygous AR100 mice *in vivo*. Retrograde transport was assessed *in vivo* at 13 months of age in both symptomatic heterozygous AR100 mice and age-matched WT littermates. (**A**) Images of axonal transport of TeNT H_C in the sciatic nerve acquired by time-lapse confocal microscopy (representative movie stills from an SBMA mouse are shown, scale bar = 5 μm). (**B**) Analysis revealed no significant difference between the retrograde transport of TeNT H_C *in vivo* in single axons in the sciatic nerve of heterozygous AR100 mice (167 carriers, 1204 single movements) and WT littermates (201 carriers, 1415 single movements). Error bars represent SEM. Statistical analysis of data was performed as described in the Materials and Methods, $P < 0.05$, NS, not significant.

assemble microtubules by adding 20 μM taxol, 1 mM GTP in the presence of 1 mM AMP-PNP and incubating for 30 min at 37°C. Polymerized microtubules were pelleted by centrifuging over a 5% sucrose cushion. MAPs and motor proteins were released by adding 0.35 M NaCl and 2 mM Mg-ATP, incubating at 37°C and centrifuging at 30 000 g for 30 min at 37°C. The final supernatant contained released MAPs and motor proteins, while the pellet contained microtubules. The fractions were analysed using western blot. Experiments were performed using three different replicate samples, each sample consisting of tissue pooled from five different animals. Thus, the results represent protein levels assessed in 15 mice per genotype per age, for both the spinal cord and sciatic nerve. Representative western blot images are shown in corresponding figures.

Western blot analysis

Protein levels were determined using western blotting as described (47). Proteins were separated by SDS–PAGE, transferred onto nitrocellulose membrane, incubated with indicated primary antibodies followed by either anti-mouse AlexaFluor 680 (Invitrogen, Paisley, UK) or anti-rabbit IR dye 800 (Rocklard, UK) secondary antibodies. Blots were examined using the Odyssey detection system (Licor, UK) and densitometry was performed using the Odyssey software. For the microtubule-binding assay, densitometry values of bands in the fractions were normalized to tubulin levels in each fraction, which were subsequently normalized to tubulin levels in the total protein lysates (the input material used for generating microtubule fractions).

Motoneuron cultures

Mixed primary motoneuron cultures were prepared from WT and AR100 mice as described previously (48). Spinal cords from E13 embryonic mice were removed, ventral horns isolated and tissue dissociated with 0.025% trypsin. Motoneurons were plated onto poly-ornithine- and laminin-coated plates and cells were grown in neurobasal medium supplemented with 2% B27 supplement, 0.5 mM glutamine, 0.05% mercaptoethanol and 2% horse serum (all Invitrogen), 0.1 ng/ml glial-derived neurotrophic factor (GDNF), 0.5 ng/ml ciliary neurotrophic factor and 0.1 ng/ml BDNF (all Caltag, Silverstone, UK), 50 U/ml penicillin and 50 μg/ml streptomycin (all Sigma-Aldrich). Motoneurons were allowed to differentiate for 7 days before use. All plates were maintained at 37°C in 5% CO_2 and 95% air. For all axonal transport experiments, motoneurons were treated with 5 μM DHT or vehicle for 3 days before transport assays were performed.

Measurement of axonal transport

Transport kinetics were analysed as described previously (18). An atoxic fluorescent fragment of tetanus toxin (TeNT H_C) was used to visualize fast retrograde transport in cultured embryonic motoneurons from E13 WT and mutant AR100 mice. TeNT H_C has been shown to bind with high affinity to the plasma membrane of neurons and can enter endocytic carriers containing neurotrophins and their receptors in motoneurons (19). The TeNT H_C-labelled endosome carriers can therefore be used in the monitoring of retrograde axonal transport (16). Fluorescent density of carriers was quantified using Metamorph (Universal Imaging Corporation, Downingtown, PA, USA).

In vivo retrograde transport assay in intact sciatic nerve

An *in vivo* transport assay was employed to test for axonal transport deficits in the sciatic nerve of symptomatic SBMA mice (16). Thirteen-month-old heterozygous AR100 mice and age-matched male WT littermates were anaesthetized with isoflurane (National Veterinary Services, Stoke-on-Trent, UK) and their tibialis anterior and gastrocnemius muscles exposed in one hindlimb. AlexaFluor555-TeNT H_C (13 μg) and BDNF (50 ng), which has been reported to increase

TeNT H_C internalization (49), were slowly injected intramuscularly using a Hamilton microsyringe as described previously (16). The mice were re-anaesthetized 6 h later and the sciatic nerve exposed and kept moist with physiological saline. Mice were placed on a heated stage, covered with a heated blanket and axonal transport was imaged in single axons in the intact sciatic nerve by time-lapse, high-resolution *in vivo* confocal neuroimaging (16,50). Images were acquired every 5 s with an inverted Zeiss LSM 510 confocal laser scanning microscope (Oberkocken, Germany) equipped with a plan apochromat 63× water immersion objective lens (N.A. 1.2), following the excitation of selected regions of interest with the 543 nm line of a helium–neon laser.

Tracking and data quantification

Tracking was undertaken using the Motion Analysis software (Kinetic Imaging, Liverpool, UK) as described previously (7,16,18). Moving carriers that could be tracked for at least four consecutive time points were considered. The distance covered by a carrier between two consecutive frames (single movement) was used to determine speed. The speed distribution of transport organelles from different mouse genotypes and age groups was obtained using a 0.2 μm/s binning interval.

Motoneuron survival

Motoneuron counts were performed as described previously (51). Mice were anaesthetized, transcardially perfused with 4% PFA and the lumbar region of the spinal cord removed. Serial 20 μm transverse sections were cut, stained with gallocyanin (a Nissl stain) and the number of positively stained motoneurons in the sciatic motor pool between the L2 and L5 levels of the spinal cord was counted in every third section ($n = 40$). The total number of surviving motoneurons in the sciatic motor pool of each mouse was determined for all genotypes (for each genotype, $n \geq 7$). Only large, polygonal neurons with a clearly identifiable nucleus and nucleolus were counted.

Statistical analysis

Statistical analysis and curve fitting of axonal transport data were performed using Kaleidagraph (Synergy Software), Microsoft Excel and SPSS v15 (SPSS Inc.) and as described previously (16). Mean velocity of TeNT H_C-labelled endosome carriers was calculated using non-linear regression and tested for significance using a Student's *t*-test or ANOVA as described (16). Other statistical analysis was performed using SPSS v15. To test for significance, non-parametric data analysis was performed using the Kruskal–Wallis test and for parametric data, one-way ANOVA with Tukey's HSD and Student–Newman–Keuls *post hoc* tests carried out for pairwise comparison between all groups within the experiment. A *P*-value of <0.05 was considered statistically significant.

Conflict of Interest statement. None declared.

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