Nuclear-targeting of mutant huntingtin fragments produces Huntington’s disease-like phenotypes in transgenic mice

Gabriele Schilling1,*, Alena V. Savonenko1, Alexandra Klevytska1, Johanna L. Morton1, Stina M. Tucker1, Michelle Poirier2, Alexa Gale1, Ning Chan1, Vicky Gonzales1, Hilda H. Slunt1, Michael L. Coonfield1, Nancy A. Jenkins4, Neal G. Copeland4, Christopher A. Ross2,3 and David R. Borchelt1,3

1Department of Pathology, 2Department of Psychiatry and Behavioral Sciences, 3Department of Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA and 4Mouse Cancer Genetics Program, NCI-Frederick Cancer Research and Development Center, Frederick, MD 21702, USA

Received March 24, 2004; Revised and Accepted May 26, 2004

Huntington’s disease (HD) results from the expansion of a glutamine repeat near the N-terminus of huntingtin (htt). At post-mortem, neurons in the central nervous system of patients have been found to accumulate N-terminal fragments of mutant htt in nuclear and cytoplasmic inclusions. This pathology has been reproduced in transgenic mice expressing the first 171 amino acids of htt with 82 glutamines along with losses of motoric function, hypoactivity and abbreviated life-span. The relative contributions of nuclear versus cytoplasmic mutant htt to the pathogenesis of disease have not been clarified. To examine whether pathogenic processes in the nucleus disproportionately contribute to disease features in vivo, we fused a nuclear localization signal (NLS) derived from atrophin-1 to the N-terminus of an N171–82Q construct. Two lines of mice (lines 8A and 61) that were identified expressed NLS–N171–82Q at comparable levels and developed phenotypes identical to our previously described HD–N171–82Q mice. Western blot and immunohistochemical analyses revealed that NLS–N171–82Q fragments accumulate in nuclear, but not cytoplasmic, compartments. These data suggest that disruption of nuclear processes may account for many of the disease phenotypes displayed in the mouse models generated by expressing mutant N-terminal fragments of htt.

INTRODUCTION

Huntington’s disease (HD) is an autosomal-dominant inherited, progressive and fatal neurodegenerative disease that usually starts in midlife (1–3). HD occurs when a CAG (glutamine codon) repeat near the N-terminus of huntingtin (htt) expands to a length greater than 36 consecutive glutamines (1). The symptoms of HD include a movement disorder, with cognitive impairment and psychiatric disturbances (4), that progresses to death over 15–25 years. Neuropathological characteristics of the disease include general atrophy of the brain with dramatic losses of projection neurons in the deeper layers of the cortex and medium spiny neurons in the caudate–putamen (5,6). Other less affected areas include the globus pallidus, subthalamic nucleus and amygdala. Surviving neurons throughout the central nervous system (CNS) harbor inclusion bodies, in both the nucleus and the cytoplasm, that are immunoreactive with antibodies directed against the N-terminal regions of htt (7–11).

To model HD, several laboratories have used different strategies to produce transgenic mice. Three groups have independently used gene targeting strategies to expand the glutamine repeat domain of murine htt (12–14), finding that mice with extremely long expansions (>150 glutamines) develop disturbances in motor function and diminished activity levels (14). Mice that express full-length mutant human htt, through integration of a large segment of human genomic DNA carried in a modified yeast artificial chromosome, develop similar behavioral disturbances (15). In all of these models, CNS neurons develop htt inclusion pathology.
which is most prominent in striatum (caudate–putamen) (12). Our group and the laboratory of Dr Gillian Bates have developed mouse models of HD that express an N-terminal fragment of human htt (16,17). In both of these models (N171–82Q and R6/2 mice), htt-immunoreactive inclusion pathology is the prevalent neuropathological marker (17–19). As observed in humans (7–11), CNS neurons of both the N171–82Q and the R6/2 mice develop htt inclusions in both nuclear and cytoplasmic compartments (17–19). Both the N171–82Q and the R6/2 mice show a more widespread distribution of inclusion pathology (all regions of the CNS develop inclusions) and more severe behavioral abnormalities culminating in premature death.

The relative contribution of nuclear and cytoplasmic htt aggregates to the pathogenesis of disease is unknown (20,21). In humans, cytoplasmic, but not nuclear, htt aggregates are a prominent pathological feature in the medium spiny neurons of the caudate–putamen and in the deep layers of the cortex, the cells most severely lost in human disease (7,9,11). However, data from several other sources suggest that mutant protein localized to the nucleus may be more toxic. For example, in cell culture models, nuclear localization of the protein is associated with greater toxicity (22,23). Substantial evidence suggests that mutant htt localized to the nucleus can disrupt the activities of transcription factors (24) and alter the normal transcriptional profile of neurons (25,26). However, several recent studies have demonstrated that cytoplasmic mutant htt can inhibit synaptic function and glutamate release and disrupt the axonal transport of protein and vesicles (27–31). Notably, in the vast majority of the diverse disorders caused by expansions of polyglutamine domains, mutant protein and inclusion pathology are confined to the nucleus (32). HD is one of the rare examples where inclusion pathology occurs in both the nucleus and the cytoplasm.

In the present study, we have investigated how the location of mutant htt contributes to the manifestation of disease phenotypes in mice by generating transgenic mice expressing the first 171 amino acids of htt that also encodes a nuclear localization signal (NLS) fused to the N-terminus (NLS–N171–82Q). We used an atrophin-1 NLS, which has been shown to be functional in cell culture models (33) and to induce nuclear inclusion pathology in a mouse model of dentatorubral–pallidoluysian atrophy (34). Otherwise, the construct was identical to the N171–82Q construct in the MoPrP vector we used previously (17,35), allowing us directly to compare the consequences of the htt fragment localized into nucleus (NLS–N171–82Q model) to that of N171–82Q fragments in both nuclear and cytoplasmic compartments (our N171–82Q model). We obtained two lines of mice (NLS–N171–82Q: lines 8A and 61) that express the highest level of NLS–N171–82Q protein. We obtained two lines of mice (NLS–N171–82Q model). We used an atrophin-1 NLS, which has been shown to be functional in cell culture models (33) and to induce nuclear inclusion pathology in a mouse model of dentatorubral–pallidoluysian atrophy (34). Otherwise, the construct was identical to the N171–82Q construct in the MoPrP vector we used previously (17,35), allowing us directly to compare the consequences of the htt fragment localized into nucleus (NLS–N171–82Q model) to that of N171–82Q fragments in both nuclear and cytoplasmic compartments (our N171–82Q model). We obtained two lines of mice (NLS–N171–82Q: lines 8A and 61) that expressed the mutant protein at levels comparable with that of our previously characterized HD–N171–82Q mice. Immunoblot analysis of nuclear and cytoplasmic fractions prepared from symptomatic mice showed a strict localization of NLS–N171–82Q protein to the nucleus. Moreover, pathologic analyses confirmed that the brains of affected NLS–N171–82Q transgenic mice developed prominent nuclear pathology (diffuse accumulation of htt immunoreactivity with inclusions) with no evidence of cytoplasmic inclusions. These pathologic abnormalities correlated with the appearance of a progressive neurological phenotype, which included hunched posture, loss of weight, decreased motoric performance, lower spontaneous activity and premature death. In all aspects that we analyzed, the phenotypes of the NLS–N171–82Q mice could not be distinguished from that of HD–N171–82Q mice. We conclude that disturbances in nuclear processes are sufficient to alter neuronal function profoundly and induce disease-related phenotypes in mice.

RESULTS

Construction of NLS–N171–82Q vectors and generation of transgenic mice

A consensus NLS sequence lies within the first 30 amino acids of atrophin-1 and is responsible for targeting atrophin-1 to the nucleus (33). Using PCR-based strategies, we amplified cDNA encoding the first 30 amino acids of atrophin-1 in a manner that permitted us to fuse the amplified segment, in-frame, to the N-terminus of a cDNA that encoded the first 171 amino acids of htt (based on 23Q number) with an expanded glutamine tract (82 consecutive glutamines) (Fig. 1A). This cDNA construct was inserted into the mouse prion protein vector, the same vector used for our previous HD transgenic (17,35). Initially, expression of the transgene vector was confirmed in cell culture by transient transfection and immunoblot analysis (data not shown). The transgene construct was injected into fertilized mouse embryos from crosses of C57BL/6J × C3H/HeJ F1 mice (the same strain used to create our original HD–N171–82Q mice) and 35 distinct founders, with varying numbers of transgene copies, were identified. Ten founders that appeared to have transgene copy numbers, which varied by PCR analysis (data not shown), similar to HD–N171–82Q 81 were bred to generate lines of mice. Of these 10 founders we were able to establish two lines 8A and 61 that express transgene mRNA at levels similar to our previously described HD–N171 mice (Fig. 1B). One line (131) expressed somewhat lower levels of the transgene. All other lines of mice expressed at levels equal to or lower than mice from line 131 and were discontinued. Northern blot analysis of transgene-derived mRNA in the brain of these new NLS mice indicated that mice from lines 8A and 61 accumulate as much transgene-derived mRNA as mice that harbor HD–N171–82Q transgenes (Fig. 1B). Because we do not know how the addition of atrophin-1 sequence to the transgene mRNA will affect translation of the mRNA, we cannot conclude that the NLS mice from line 8A express greater levels of transgene protein than our HD mice from lines 81 or 6 (Fig. 1B). However, the three lines of NLS mice, line 8A would be expected to express the highest level of NLS–N171–82Q protein.

NLS–N171–82Q mice exhibit a progressive neurological phenotype

The life expectancies of the mice from the three lines of NLS mice correlated well with the levels of mutant mRNA. Mice with higher levels of mutant mRNA (line 8A) had the most abbreviated life-spans, living to 7–9 months of age (Fig. 2).
Mice with lower mutant mRNA levels (line 61) live considerably longer, dying between 11 and 16 months of age (Fig. 2). Mice with the lowest levels of mutant mRNA (line 131) had apparently normal life-span (data not shown). Towards the end of their life expectancy, NLS mice from line 8A exhibited reduced weight, hunched posture, poor grooming and hindlimb clasping when suspended by the tail (Fig. 3A–C). Mice from line 61 display a similar appearance as they approach the end of their life expectancy (data not shown).

Among the more HD-like behavioral changes seen in our original HD–N171–82Q mice are reductions in coordination and motor skills, which are assessed by measuring the ability of mice to remain on a rotating and accelerating rod (17). To assess whether the NLS mice show reduced performance in the rotarod task, six NLS–N171–82Q line 8A mice and non-transgenic littermates were tested at two ages: 3.5 and 4.5 months of age (Fig. 4). We used a paradigm in which performance was measured in three 4 min trials, daily, on three consecutive days. Latencies to fall, averaged for testing at 3.5 and 4.5 months, are shown in Figure 4. At 3.5 months of age, NLS line 8A mice performed at a level similar to non-transgenic littermates, but by 4.5 months of age the NLS–N171–82Q mice exhibited a significant reduction in performance when compared with non-transgenic littermates. Three-way (genotype × age × day of testing) ANOVA for latencies confirmed this observation and showed a significant effect of genotype [F(1,17) = 7.52, P < 0.01]. LSD post hoc revealed that significant between-group differences were due to an impairment in 4.5-month-old NLS–N171–82Q mice when compared with age-matched non-transgenic littermates (P < 0.001). These data suggest that the NLS mice exhibit a loss of coordination and/or motor skills as we have described previously in HD–N171–82Q mice (17,36,37).

Another prominent phenotype of HD–N171–82Q mice is that these animals become profoundly hypactive (37). To determine whether the NLS–N171–82Q mice develop similar phenotypes, we used 6- to 7-month-old transgenic mice (line 8A) and non-transgenic littermates to measure circadian changes in motor activity. Noteworthy, at this age the NLS line 8A mice begin to show some of the overt signs of disturbed neurological reflexes illustrated in Figure 3 and are comparable in disease-stage to 4-month-old NLS–N171–82Q mice that we have previously demonstrated to exhibit reduced spontaneous activity (37). Horizontal motor activity was measured in 2 h increments in an activity chamber over a total of 22 h of testing (37). As shown in Figure 5, non-transgenic mice were more active during dark cycles, showing a normal circadian rhythm (mice are predominantly nocturnal animals exhibiting exploratory and maintenance activities during dark periods and sleep during light periods). NLS–8A mice also demonstrated a diurnal pattern of spontaneous activity, becoming quiescent in the hours just prior to and just after the transition from dark to light (Fig. 5). However,
in the dark period where activity levels would be expected to be highest, the NLS transgenic mice demonstrated far less horizontal beam breaks than non-transgenic littermates (Fig. 5). Two-way (group \( \times \) period) analysis of variance (ANOVA) confirmed these observations with a significant effect of period \( F_{(10,100)} = 5.05, P < 0.0001 \) and group \( \times \) period interaction \( F_{(10,100)} = 2.31, P < 0.02 \). LSD post hoc test revealed that NLS transgenics were significantly less active than NTG mice during the dark periods of circadian cycle \( (P < 0.01) \). Thus like the HD mice, the NLS line 8A mice become hypoactive towards the end of their life-span (Supplementary Material, Fig. S1).

Nuclear inclusions predominant in NLS–N171–82Q mice

Histological analysis of brain sections from our two NLS–N171–82Q lines were performed at two different ages. In total, six NLS–82Q line 8A and six NLS–82Q line 61 mice were analyzed. Similar to what we have reported previously in HD–N171–82Q mice, the brains of severely affected mice appeared grossly normal (data not shown). To assess the prevalence and location of htt inclusion pathology, we analyzed sections collected from transgenic mice that were about half way through their disease course and from mice at the end of their life-span. We used a monoclonal antibody (mEM48) that is similar to the originally described rabbit antiserum rEM48, reported to demonstrate preference to detect cytoplasmic aggregates (11,18).

Immunohistochemical staining of symptomatic NLS–N171–82Q mouse brains revealed that nuclear pathology was fairly widespread and present in most neurons of the CNS, similar to what we have described previously in our HD mice (17,37). However, in either line of NLS mice at any age, we failed to detect cytoplasmic inclusion pathology. To illustrate the pathologic differences in NLS versus HD mice, we focused on the cortex of these animals (Fig. 6 and Supplementary Material, Fig. S2), which in HD–N171–82Q mice has a high frequency of cytoplasmic inclusion pathology (17,37). In both lines of NLS mice, diffuse nuclear accumulation of mutant htt was the predominant pathologic abnormality. In older mice, nuclear inclusions were prominent and often larger and more defined than what were found in the HD–N171–82Q mice (Fig. 6). When compared with the HD–N171–82Q mice, where cytoplasmic aggregates were frequently found in neuropil throughout the cortex (Fig. 6, bottom row), we could not detect cytoplasmic inclusions in the NLS mice (Fig. 6, rows 2 and 3). Immunostaining of tissue sections with an affinity-purified rabbit polyclonal antibody [AP360 (17,37)] to the N-terminus of htt (data not shown) also failed to detect cytoplasmic aggregates (data not shown). Higher magnification images of cortical neurons from the HD line 81 mice at 4 months of age demonstrate more clearly the presence of NII (white arrows) and cytoplasmic aggregates (black arrows) (Fig. 7, left panel). However, the same high magnification of cortical neurons from the NLS line 8A mice, at 8 months of age, revealed nuclear aggregates (white errors) without evidence of cytoplasmic labeling (Fig. 7, right panel). Collectively, these data demonstrate that the NLS–82Q mice do not develop cytoplasmic inclusion bodies that are visible by standard light microscopy.

Subcellular fraction of tissues from NLS–N171–82Q line 8A mice

To determine biochemically whether NLS–N171–82Q protein preferentially accumulates in the nucleus, we harvested brain tissues from symptomatic mice and prepared nuclear and cytoplasmic fractions. In the NLS mice, NLS–N171–
82Q protein was detected in total homogenate (H) and in the nuclear fraction (N), but not in cytoplasmic fractions (C) (Fig. 8, lanes 4–6). In contrast, cytoplasmic fractions of brain tissue from HD–N171–82Q line 81 mice contained a prominent 46 kDa transgene protein band (Fig. 8, lane 8). As expected, nuclear fractions of brain tissue from HD–N171–82Q mice also accumulated transgene product (Fig. 8, lane 9). The slightly slower migration of the NLS–N171–82Q protein is because of the slightly higher molecular weight added by the NLS sequence. As a control of fractionation, parallel blots were probed with anti-histone antibody and revealed an expected enrichment in nuclear fractions (Fig. 8, middle panel). Protein loading was assessed by Coomassie-staining of gels loaded with identical amounts of each fraction used in immunoblot analysis (Fig. 8, lower panel). Assessing the total levels of NLS–N171–82Q and N171–82Q protein is complicated by the tendency of both proteins to aggregate, but it appears that the accumulated level of NLS–N171–82Q is slightly less than that of the HD–N171–82Q mice, possibly explaining the longer life expectancy of the NLS mice.

DISCUSSION

In previous studies of transgenic mice that express N-terminal fragments of mutant htt (N171–82Q), we have demonstrated that the mutant protein accumulates as inclusion-body structures in both the nucleus and the cytoplasm (17,37). The accumulation of the protein in the nucleus of these mice has been associated with alterations in the function of transcription factors, including CREB-binding protein (24) and altered patterns of gene transcription (25). The accumulation of N171–82Q in the cytoplasm has been associated with changes in synaptic function (28,30). These pathologic features appear in mice showing a number of disturbances in behavior, including hunched postures, loss of coordination, diminished motor function and hypoactivity (17,37). Ultimately, the N171–82Q mice die prematurely. Here, we demonstrate that targeting N171–82Q to the nucleus, through the engineering of an NLS into the N-terminus of the coding sequence, induces the same debilitating phenotypes we observed in our HD–N171–82Q mice. These data indicate that a disruption of nuclear processes may be the dominant mechanism of toxicity in N171–82Q mouse models of HD. Clearly, the accumulation of mutant htt in the nucleus causes sufficient toxicity to elicit debilitating behavioral abnormalities.

Our findings are in agreement with several previous examinations of mutant htt toxicity in cultured cell models and with examinations of polyglutamine toxicity in the context of other proteins in both mouse and cell models. With some discrepancy, cell culture studies have generally demonstrated that N-terminal fragments of mutant htt are more toxic when targeted to the nucleus, or delivered directly to the nucleus by microinjection, whereas cytoplasmic targeting diminishes toxicity (20,22,23,38). Similarly, in studies of transgenic mice engineered to express recombinant forms of ataxin-1 (the protein mutated in SCA-1), inactivating the NLS sequence by site-directed-mutagenesis diminished its toxicity (39). These data were interpreted to suggest that the primary mechanism of mutant ataxin-1 toxicity involves a disruption of nuclear events. More recently, Jackson and colleagues (40) studied strains of mice in which the hypoxanthine phosphoribosyl transferase (HPRT) locus has been modified to encode a polyglutamine tract. In a previous study of mice harboring HPRT–polyQ, animals developed abnormalities in spontaneous motor activity and died prematurely. Pathologically, neurons throughout the brains of these animals were found to contain HPRT- and ubiquitin-immunoreactive inclusions in nuclei (40). Cytoplasmic inclusions were not described. Building on this model, Jackson and colleagues further modified the HPRT–polyQ gene to harbor functional NLS and Nuclear export signal (NES) sequences. The addition of an NLS accelerated the appearance of NII pathology, shortened the age of onset of various behavioral abnormalities and reduced life expectancy. Adding an NES sequence delayed the appearance of NII pathology and produced modest to significant delays in the appearance of behavioral abnormalities and a modest lengthening of life expectancy. Collectively, these data are consistent with the notion that the experimentally mutated HPRT protein exerts its toxic activities in the nucleus. However, because neither the HPRT–polyQ mice nor the ataxin-1 mice normally show cytoplasmic inclusion pathology, these studies do not shed significant insight into the potential contribution that the cytoplasmic pathology in mouse models of HD may have in the development of behavioral abnormalities. The present study directly addresses this issue in which we demonstrate that nuclear pathology and, more importantly, nuclear localization of N171–82Q is associated with profound behavioral phenotypes and premature death. Moreover, as the overall phenotypes of the NLS–82Q mice are similar to that of mice with both cytoplasmic and nuclear pathology, we conclude that it is unlikely...
that any specific behavioral abnormality in our original HD–N171–82Q mice can be attributed to cytoplasmic mutant htt. Rather, we believe that a disruption of nuclear processes is the likely origin of behavioral manifestations and premature death in the HD–N171–82Q mice. What is not known is whether disturbances in cytoplasmic processes exacerbate nuclear toxicity in the HD–N171–82Q; a scenario that would provide an explanation for the delayed appearance of behavioral disturbances and longer life expectancy of the NLS–82Q mice.

Our study also provides insight into the mechanisms of mutant htt localization. Normally, the vast majority of full length, non-mutant, htt is localized to cytoplasmic compartments (41,42). Htt does not appear to encode a functional NLS (21,43), but does encode a functioning NES (43) (not contained in our N171 amino acids constructs). Hence, it may be critical that htt be kept out of the nucleus. Studies of mice where the HD mutation has been introduced into the mouse htt gene have similarly demonstrated that the vast majority of full-length mutant htt is localized to the cytoplasm (12). However, early in the evolution of pathologic abnormalities in these knock-in mice, some full-length htt protein is translocated to the nucleus (12). Later in disease, nuclear compartments accumulate large amounts of N-terminal fragments of mutant htt (12,19).

There are three ways in which N-terminal fragments of mutant htt may enter the nucleus. First, it is possible that truncation of mutant htt creates a protein fragment that can diffuse passively into or out of the nucleus, depending on whether
truncation occurs predominantly in the nucleus or cytoplasm. Second, it is possible that mutant htt may associate with another protein, which is localized to the nucleus normally, and thus be carried in. Third, the polyglutamine domain itself might function as a weak nuclear localization signal. These later two scenarios would explain how some full-length mutant htt is translocated to the nucleus because full-length htt is too large to diffuse passively across the nuclear membrane (12). Our data demonstrate that adding a functional NLS can shift the distribution of mutant htt to a predominately nuclear localization. Active translocation of NLS–N171–82Q protein to the nucleus may lower cytoplasmic concentrations below the critical concentration necessary to induce aggregation. In contrast, the appearance of both nuclear and cytoplasmic aggregates in the original HD–N171–82Q and in the R6/2 mice could be construed as evidence that the htt fragments produced in these mice equilibrate between nucleus and cytoplasm prior to aggregation.

Although the behavioral features of our new NLS–N171–82Q mice closely resemble those of the HD–N171–82Q mice, the onset of disease does not appear to be accelerated in the NLS mice. Comparisons of the levels of transgene mRNA in the NLS mice versus the HD mice indicate that the NLS mice from line 8A have higher levels of mRNA than either line of HD–N171–82Q that we studied (lines 81 and 6) (Fig. 1B). However, because we do not know how the addition of sequences encoding the NLS of atrophin-1 to domains adjacent to the start codon of the message influences protein production, we cannot conclude that the level of NLS–N171–82Q protein in either line of NLS–N171–82Q mice is higher than that of the HD–N171–82Q mice. Unfortunately, the high aggregation propensity of both the NLS and the HD N171–82Q fragments makes absolute quantification of the transgene protein difficult. However, we note that the levels of SDS-soluble protein detected in older, symptomatic, NLS (line 8A) mice were somewhat lower than the levels in both cytoplasmic and nuclear compartments of the HD–N171–82Q mice (Fig. 8). We therefore believe that the relative age to onset and death in our NLS mice is fairly consistent with that of the HD–N171–82Q mice, with regard to levels of protein that accumulate in the nucleus. As mentioned above, however, it is possible that the delayed onset of behavioral disturbances and longer life expectancy of the NLS mice is due to the absence of a cytoplasmic alteration, which in some manner...
exacerbates nuclear dysfunction, rather than a simple difference in protein levels.

In cell culture models, the toxicity of over-expressed mutant htt is often manifested as the induction of programmed cell death (22,23). However, we have not noted substantial apoptotic cell death in either our HD–N171–82Q mice or our new NLS–N171–82Q mice. In the mice, death of the organism seems to be due to neuronal dysfunction rather than the loss of critical populations of neurons. One process that has been shown to be dysfunctional in cultured cells expressing mutant htt is transcriptional regulation of gene expression (44,45). Similarly, evidence of transcriptional dysregulation has been obtained by analysis of tissues from transgenic mouse models of HD or other polyglutamine models (25,26,46). Indeed, proteins containing expanded polyglutamine tracts in other disease settings have been shown to acquire activities that disrupt transcription (25,47–53). Several studies have demonstrated that mutant htt, or proteins with expanded glutamate tracts, can interact with transcription factors, including CREB-binding protein (24,49,54), p53 (49), Sp1 (45,55), TAFI130 (45,56,57), TATA-binding protein (56), REST/NRSF (53,58), NCoR (59,60), NF-kB/Rel/dorsal family member (61), co-repressor C-terminal-binding protein (53) and CA150 (62). Whether one of these pathways is most affected in HD or whether the disease results from a general disruption in many pathways is uncertain. However, in work focusing on the CBP pathway, several studies have demonstrated that restoring this pathway by augmenting CBP function through transgene expression (24) or by inhibiting histone deacetylases pharmacologically (CBP is a histone acetylase) (50,63) diminishes the toxicity of mutant htt. Recent transcriptional profile studies of an inducible system suggesting that the CBP pathway may be one of the major pathways altered by mutant htt (44,54,64).

Although we have focused on potential nuclear pathways of mutant htt toxicity, there is ample data to suggest that mutant htt in the cytoplasm may also disrupt critical processes. Recent studies of our HD–N171–82Q mouse model and the R6/2 HD mouse model have provided evidence that cytoplasmic mutant htt can inhibit synaptic function and glutamate release (27,29,65). Forms of htt and androgen receptor that encode long polyglutamine tracts form cytoplasmic inclusions that can inhibit fast axonal transport (30,31). Htt has also been reported to interact with HAP-1 and HIP-1 (66–68), which play roles in microtubule-based vesicle transport (69).

The present study is the first in vivo study of the consequences of actively transporting mutant htt to the nucleus. Our data strongly argue that the toxicity of nuclear-targeted mutant htt is sufficient to cause behavioral changes closely resembling our original HD–N171–82Q transgenic model. Therefore, we conclude that in mice expressing N-terminal fragments of mutant htt, the disruption of nuclear processes may be the predominant mechanism of toxicity. It remains possible that alterations in some cytoplasmic process, mediated by cytoplasmic mutant htt, exacerbate nuclear toxicity. It also remains possible that in humans, a disruption of cytoplasmic processes plays a more significant role in the pathogenesis of disease than that occurs in the mouse models. Our results provide evidence that a disruption of nuclear processes could account for the majority of toxicity. Deciphering the relative contribution of nuclear and cytoplasmic abnormalities to the pathogenesis of the human disease may be resolved through future pharmacologic interventions, for example, if drugs targeting nuclear processes prove to be very effective in humans. Our NLS–N171–82Q mice may provide a useful model for comparison to our original HD–N171–82Q, the R6/2 model and full-length htt mouse models in therapeutic studies because we would presume that the NLS–82Q mice should show the greatest benefit from drugs that target nuclear processes. Through such comparisons, it should also be possible to determine the extent to which cytoplasmic dysfunction participates in disease pathogenesis.

**MATERIALS AND METHODS**

**Generation of MoPrP·NLS–N171–82Q**

The decision to use the atrophin-1 NLS was based on two observations. First, in transgenic mice expressing full-length atrophin-1 with a 65 glutamine repeat, a truncated fragment of mutant atrophin-1 (containing the putative NLS) was found to accumulate in the nucleus (37). Second, in cell transfection studies, mutagenesis of the putative NLS sequence was found to diminish the nuclear localization of recombinant atrophin-1 proteins (33). To generate MoPrP·NLS–N171–82Q, the sequence encoding the atrophin-1–NLS (RRKEAPG-PREEILRSRGR—the consensus residues R = arginine and K = lysine for the NLS are bolded) was amplified by PCR using an atrophin-1 cDNA construct with the following primers: AT-NLS-5′: 5′-ACCGAATTCATGCATGAGACAC-3′ and AT-NLS-3′: 5′-ACCGAATT CAGGGGAGGCCGCCCCTCGATCT-3′. The amplified atrophin-1 sequence was engineered to encode EcoR1 sites at each end and encompassed the N-terminal start codon of atrophin-1. This PCR product was ligated into an EcoR1 site just 5′ of the start codon in an HD–N171–82Q cDNA construct carried in pBluescript, which was used to generate our original HD mice and has been described elsewhere (17). The NLS–N171–82Q construct was cut with SalI and XhoI and ligated into a unique XhoI site in the MoPrP·Xho vector (35). The sequence MoPrP·NLS–N171–82Q was confirmed by sequencing and expression of the protein was confirmed by transient transfection into HEK293 cells and immunoblot analysis with rabbit polyclonal antibody AP360 (70). The construct was then linearized with NotI, separated from pBluescript carrier plasmid by gel electrophoresis, and purified for injection as previously described (17).

**Production, identification, and housing of transgenic mice**

Purified DNA for the NLS construct was microinjected into the male pronucleus of C3H/HeJ × C57BL/6J F2 embryos. Lines of NLS transgenic mice were identified by extracting the DNA from tail for analysis by PCR (17). Primers for the genotyping were: PrP-sense: 5′-CCTCTTGTGACTAGT
TGGACTGATGTCGG-3', PrP-antisense: 5'-GTGGATA CCCCTCCCCCA GCCTAGACC-3' and HD–5'-AGAATTTCAGCTACCAAGAAAGACCGTG-3'. The PrP-sense and antisense primers amplify a portion of the endogenous PrP gene for a positive control (750 bp). The HD-sense and PrP-antisense primers amplify a portion of the transgene construct (250 bp). We maintained transgenic mouse lines in the hybrid strain background by breeding males harboring the desired transgene to non-transgenic female C57BL/6J x C3H/HeJ (F1) mice purchased from the Jackson Laboratories (Bar Harbor, ME, USA).

Mice for behavioral experiments were transferred from a microisolator facility to a conventional facility at about 8 weeks of age. The dark–light cycle in this room was set on 12 h light/dark cycles (8 am to 8 pm, light) and maintained by automatic switched. All the mice for this study had ad libitum access to food and water.

Rotarod trials
Six naive mice, each, from NLS line 8A and non-transgenic littersmates were tested on an accelerating rotarod device (Rotamex 4/8, Columbus Instruments International Corporation, Columbus, OH, USA). We use a paradigm in which the device was set to accelerate from 5 to 30 rpm over a period of 4 min. We ran this trial three times a day on three consecutive days. The first 2 days of testing were considered training days and data for the all three trials of the third day were averaged and graphed. Asterisk indicates a significant difference from NTG group as a result of LSD post hoc test (P < 0.001) applied to significant effect of genotype (ANOVA).

Activity chambers
Spontaneous activity measures were conducted in a separate conventional room where the light–dark cycle had been rotated to a 2pm to 2am (light) period. Mice to be tested in the activity chambers were moved to the experimental rooms 6 days before the test to allow them to adjust to the changes in the dark–light periods. Six mice from the NLS–N171–82Q line 8A mice between 6 and 7 months of age and age-matched non-transgenic littersmates were placed in the optical animal activity monitoring cages (Digiscan, XRYZCM, Columbus Instruments) 24 h prior to the experiments to allow them to adjust to the new cages. The paradigm we used to assess spontaneous activity was identical to that used in a previous study of the HD–N171–82Q mice (37). Beam-breaks for horizontal activity were measured for 22 h, starting 2 h before the dark period. Measures were taken every 120 min.

Life-span
Survival curves for the NLS transgenic were determined in mice housed in a satellite conventional facility so that we could closely monitor changes in health. These animals were transferred from the microisolator facility at 8 weeks of age. Mice that were monitored for survival were not utilized in any other behavioral study. Animals were housed in standard microisolator cages throughout their life-span. In most cases, animals were simply found dead on morning inspection. Occasionally, animals that were moribund were perfused under anesthesia for pathological analyses. The data were calculated for each of the NLS lines and plotted as a percent surviving a given age. Non-transgenic littersmates were terminated at 18 months of age.

Northern blot analysis
The level of transgene mRNA expression was analyzed by northern blot as described previously (17). Total RNA was extracted from mouse hemibrain, using TRIZOL reagent (Gibco BRL, Gaithersburg, MD, USA): 5 μg of total RNA was fractionated on a formaldehyde–agarose gel, transferred to nylon membrane (Genescreen, NEN Lifescience Products, Boston, MA, USA) and probed with a random primed radio-labeled fragment of the htt cDNA.

Nuclear fractions and western blots
Nuclear and cytoplasmic fractions of neural tissues from the brains of transgenic mice were prepared following a previously published protocol, except that the final centrifugation to isolate nuclei was performed in buffer supplemented with 1.9 M sucrose (34). Protein concentrations were determined by a dye-binding method (Pierce, Rockford, IL, USA) using bovine serum albumin to generate a standard curve. Proteins were solubilized in SDS–PAGE loading buffer at 0.5 μg/μl, sonicated for 30 s with a probe sonicator, heated to 70°C for 10 min and then centrifuged for 1 min at 12 000g. Proteins (15 μg) were resolved by SDS–PAGE in a 4–15% polyacrylamide–SDS gel (Biorad, Hercules, CA, USA) and transferred to nitrocellulose membrane (BA85, Schleicher and Schuell, Keene, NH, USA) in the cold at 25 °C for 8 h. The transfer buffer contained 10% methanol, 25 mM Tris, 192 mM glycine and 0.1% SDS, which previously had been found by Dr J.D. Wood (University of Sheffield, UK) to work well when transferring proteins containing polyglutamine (34). Bound htt proteins were detected with a polyclonal rabbit anti-serum (AP360, which is an antibody generated against GST-fusion protein consisting of the first 17 amino acids of htt, followed by six glutamines, the second poly-proline tract, and extending to amino acid 80). The best results were obtained when the antibody was pre-incubated with a blot from a preparative gel of non-transgenic mouse brain (cut to remove the region containing endogenous mouse htt) at a concentration of 1:250 for 3 h prior to an overnight incubation with the NLS immunoblots. Bound antibodies were visualized with horseradish-peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Amersham, Buckinghamshire, UK). Identical amounts of each protein fraction were analyzed by immunoblotting with a monoclonal anti-histone antibody (MAB052, Chemicon, Temecula CA, USA) at a concentration of 1:1000 to determine the purity of cytoplasmic fractions. The samples were also analyzed in a third gel by SDS–PAGE and staining with Coomassie blue to assess whether each fraction contained similar amounts of protein.
Histology
Animals were anesthetized in ether and perfused with PLP [2% paraformaldehyde, 10 mM sodium periodate and 75 mM lysine buffered in phosphate-buffered saline (PBS)]. The brains were removed and post-fixed overnight in PLP before transfer to PBS for storage at 4°C. The brains were cut sagittally and processed for embedding in paraffin and immunocytochemistry as described previously (17). Immunohistochemical staining of 8 μm paraffin-sections was performed with mEM48 (MAB5374, Chemicon) at a dilution of 1:50 and AP560 at 1:100 (22,70). A subset of immunostained sections were lightly counterstained with hematoxylin and cosin (H&E) to better visualize sub-cellular compartments. Representative images from a total of six symptomatic mice are shown in Figures 6 and 7 (Supplementary Material, Fig. S2).

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS
We thank Dr Timothy Moran for providing the activity cages and advice on procedures for measuring spontaneous locomotor activity. This work was supported by grants from the Huntington’s Disease Society of America. Disease Center Without Walls, NS 38144 and NS 34177) and advice on procedures for measuring spontaneous locomotor activity. This work was supported by grants from the Huntington’s Disease Society of America.

REFERENCES


