Ultrastructural localization and progressive formation of neuropil aggregates in Huntington’s disease transgenic mice

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Received February 5, 1999; Revised and Accepted April 9, 1999

How aggregates of polyglutamine proteins are involved in the neurological symptoms of glutamine repeat diseases is unknown. We show that huntingtin aggregates are present in the neuronal processes of transgenic mice that express exon 1 of the Huntington’s disease (HD) gene. Unlike aggregates in the nucleus, these neuropil aggregates are usually smaller and are not ubiquitinated. Electron microscopy reveals many neuropil aggregates in axons and axon terminals. Huntingtin aggregates in the axon terminal are co-localized with some synaptic vesicles, implying that they may affect synaptic transmission and neuronal communication. The formation of neuropil aggregates is highly correlated with the development of neurological symptoms. The present study raises the possibility that neuropil aggregates may cause a dysfunction in neuronal communication and contribute to the neurological symptoms of HD.

INTRODUCTION

Huntington’s disease (HD) is characterized by psychological, motor and cognitive symptoms. The motor symptoms are defined as chorea and excessive spontaneous movement, which is irregularly timed, randomly distributed and abrupt. The neuropathology of HD has been defined as selective neuronal loss occurring most prominently in the striatum and deep layers of the cortex (1). Striatal degeneration is consistent with the observed motor symptoms, as this region of the brain is important for movement coordination (2,3).

The genetic basis of HD has been identified as a CAG expansion in the first exon of the HD gene, which encodes a large 350 kDa protein, huntingtin (4). This CAG repeat encodes a polyglutamine tract that varies in length from six to 39 units in normal individuals and expands from 35 to 180 units in HD patients (5). The first HD animal model, the R6/2 mouse, was established by introducing a transgene consisting of exon 1 of the HD gene with 141–157 CAG/glutamine repeats (6). These mice develop a progressive neurological phenotype with onset at ~8 weeks on the basis of home cage behavior. The phenotype includes pronounced motor symptoms, such as a resting tremor, rapid, abrupt shuddering movements, stereotypic grooming movements and epileptic seizures in some cases. In addition, brain weight loss starts after 4 weeks and body weight decrease begins at 8 weeks of age. Most importantly, the transgene protein forms neuronal intranuclear inclusions (NIIs), which consist of huntingtin aggregates (7). Despite the appearance of NIIs prior to 4 weeks, selective neurodegeneration is not found until 14 weeks in R6/2 mice (M. Turmain, G.P. Bates and S.W. Davies, unpublished data) and therefore the symptoms are thought to be primarily caused by neuronal dysfunction rather than by cell death (8). In support of this, changes in the function and expression of specific neurotransmitter receptors can be detected between 4 and 12 weeks (9).

Intranuclear aggregates formed by expanded polyglutamine proteins have also been reported in the brains of patients with HD or other glutamine repeat disorders (10–13). These studies clearly indicate that expanded polyglutamine tract can cause huntingtin and other proteins to form aggregates in the nucleus. However, the role of nuclear aggregates in the diseases is still unclear. First, the regional distribution of the nuclear aggregates in the brains of HD patients does not correspond to the selective neuropathology (14). Second, recent studies show that intranuclear localization of polyglutamine protein, not aggregated protein, is sufficient to induce neurodegeneration in a mouse model of spinocerebellar ataxia type 1 (SCA1) (15) and in a transient transfection assay (16). Therefore, it is possible that the neurodegeneration observed in HD is caused by additional or alternative factors to NIIs.

Our recent studies have revealed abundant neuropil aggregates in the cortex of a pre-symptomatic HD patient (14), suggesting that these aggregates in the cortex could affect cortical input to the striatal neurons and be involved in early striatal neuropathology. However, we could not localize these aggregates to the axons of post-mortem HD brains. Therefore, HD transgenic mice may provide a better model system; they should have well-preserved brain morphology and they will allow us to examine the association between brain morphology and neurological symptoms. This study describes the formation and localization of aggregates in the neuronal processes of R6/2 mice. Many neuropil aggregates were found in axons and axon terminals. More importantly, their progressive appearance is highly correlated with the development of neurological symptoms. We propose that neuropil aggregates may affect...
nerve terminal function or neuronal communication and contribute to the neurological symptoms of HD.

RESULTS

EM48 labels aggregates in the nucleus and neuropil

We developed a polyclonal antibody, EM48, against the N-terminal region of huntingtin. The antigen used for generation of EM48 was a glutathione S-transferase (GST) fusion protein containing the first 256 amino acids of huntingtin but lacking the polyglutamine and polyproline tracts. This antibody reacts with neuropil aggregates in the human brain (14). To examine whether HD transgenic mice also have neuropil aggregates, we performed an EM48 immunocytochemistry study on R6/2 transgenic mice. EM48 intensely labels numerous puncta or aggregates in brain sections of R6/2 mice (Fig. 1). The labeling is specific to the huntingtin transgene protein, since there is no such punctate or aggregate-like labeling in the brain of a littermate control (Fig. 1). Expression of the R6/2 transgene is 75% of the endogenous level (6); therefore, the intense staining of the transgene protein and the very light staining in the control brain suggest that EM48 reacts more strongly with transgenic huntingtin than with the endogenous rodent huntingtin. Similarly, EM48 also preferentially reacts with mutant huntingtin in HD patient brain (14). Thus, EM48 offers a unique probe to examine the distribution of mutant huntingtin.

Both the cerebral cortex and striatum in R6/2 mice have the most EM48-labeled puncta or aggregates. In other brain regions, such as the hippocampus, cerebellum and spinal cord, less punctate or aggregate-like staining is seen (data not shown). The distribution of EM48 immunolabeled aggregates is very similar to that of NIs which were previously identified using other antibodies against the N-terminus of huntingtin (7). However, EM48 appears to label more aggregates than did previous antibodies.

High magnification light micrographs confirm that EM48 labels many small aggregates which are outside the cell body (Fig. 2). Again, the control brain from wild-type mice did not show this small punctate labeling (data not shown). NIs were intensely labeled by EM48 in the same manner as previously described using other anti-huntingtin antibodies (7). Interestingly, the EM48 immunoreactive small aggregates were arranged in linear arrays. The linear distribution of aggregates resembles the immunolabeling pattern of neuropil aggregates in the brain of HD patients (14).

Most neuropil aggregates are not ubiquitinated

As NIs in 12-week-old R6/2 mice are always labeled by antibodies to ubiquitin (7), we examined whether neuropil aggregates were also ubiquitin immunoreactive. To perform

![Figure 1. Huntingtin aggregates immunolabeled by EM48. Low magnification micrographs of brain sections from wild-type mice (A and B) and R6/2 transgenic mice (C and D) at 12 weeks of age. Sections of the cerebral cortex (Cx) (A and C) and striatum (Str) (B and D) were labeled with EM48. Note the numerous puncta or aggregates in brain sections from R6/2 mice, but not in those from wild-type mice. Scale bar, 100 µm.](http://hmg.oxfordjournals.org/)

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immunofluorescent double labeling with a rabbit antibody to ubiquitin, we used a guinea pig anti-huntingtin antibody, EM73, that was generated with the same antigen used for EM48. This guinea pig antibody labeled NIIs and neuropil aggregates as did EM48 (Fig. 3). In 6-week-old R6/2 mice, some NIIs were ubiquitinated but almost no neuropil aggregates were labeled by the antibody to ubiquitin. In 8- and 12-week-old mice, most NIIs were ubiquitin immunoreactive. However, only a subpopulation of neuropil aggregates was labeled by the antibody to ubiquitin (Fig. 3). Some ubiquitinated aggregates displayed very weak huntingtin staining. Thus, similar to our observations in HD patient brain, less neuropil aggregates than NIIs became ubiquitinated (14). The ubiquitin-immunoreactive neuropil aggregates were often larger than the ubiquitin-negative aggregates, suggesting that ubiquitin was only bound to the larger aggregates. These results also suggest that most neuropil aggregates do not have the same composition or modification as NIIs, although both types of aggregate contain the same transgene protein.

Ultrastructural localization of neuropil aggregates

To define the subcellular localization of neuropil aggregates, we performed electron immunogold labeling of brain sections of R6/2 mice. The weak immunoreaction of EM48 with rodent huntingtin allows a specific immunogold labeling of the transgene huntingtin. This method provides high resolution micrographs showing two types of immunogold labeling. One is scattered immunogold particles that are mainly seen in the nuclear region. The other is clustered immunogold particles that represent immunogold huntingtin aggregates. These aggregates were either localized in the nucleus or in the neuronal processes. The neuronal processes containing huntingtin aggregates often show synaptic vesicles, indicating that huntingtin aggregates are in the axon (Fig. 4A). Very few immunogold particles were found in other organelles and the perikaryal region, suggesting that huntingtin aggregates are mainly distributed in the nucleus and neuronal processes (Fig. 4A). As intranuclear aggregates or NIIs have been well described in previous studies (7), we will mainly describe EM48 immunogold-labeled aggregates in the neuronal processes.

Occasionally, we observed large aggregates in dendrites. Figure 4B shows a longitudinal dendrite containing a large aggregate that was intensely labeled by immunogold particles. The length of this aggregate is ~3.1 µm. The aggregate is composed of filamentous materials that are arrayed along the dendrite. Its shape and contents are similar to those of NIIs with a lower density and a fibrous composition (7). Most neuropil aggregates identified, however, were much smaller and did not have an obviously filamentous structure. These small aggregates could only be observed with EM48 immunolabeling.

Further EM immunogold examination also shows that NIIs are single, large aggregates (1.5 µm on average) in the nucleus (Fig. 5A). In addition, many diffuse immunogold particles are evident within the nucleus. We did not observe any small aggregates (<0.5 µm) in the nucleus in the brain of 12-week-old R6/2 mice. Instead, most small aggregates (0.1–0.25 µm) were localized to axons in the cortex (Fig. 5B and C) and striatum (Fig. 5D). Immunogold particles were clustered in axonal terminals in which synaptic vesicles and pre-synaptic junctions can be seen (Fig. 5B–D). Interestingly, these immunogold particles were co-localized with some synaptic vesicles. In the post-synaptic junction, which shows prominent post-synaptic densities, no immunogold particles were seen, suggesting that huntingtin aggregates were mainly distributed in the pre-synaptic terminals and associated with synaptic vesicles. Approximately 10–15% of identified axons were found to contain huntingtin aggregates in our electron microscopic examination, suggesting a selective accumulation of transgene protein in the nerve terminals of different types of neuron. However, the number of axons having aggregates must be higher than that seen by electron microscopy, because electron microscopic immunogold labeling often reduces the labeling sensitivity. In addition, light immunocytochemistry
has indicated a high density of neuropil aggregates in the brain (Fig. 2). The density and size of clustered immunogold particles varied in the neuronal processes, also suggesting that the extent of huntingtin aggregation varied in the neuropil. Among all neuropil aggregates examined under electron microscopy, ~60% of these aggregates were localized to axons or axon terminals, which could be identified by the presence of synaptic vesicles and synapses. The rest were localized to unidentified processes that could be either axons or dendrites. Thus, >60% of neuropil aggregates are likely present in axons.

We noticed that most axons containing aggregates have an asymmetric synapse (Fig. 5B–D). By examining 137 cortical and 53 striatal synapses that contain huntingtin aggregates in their axon terminals, we observed that 90 and 100% of them were asymmetric synapses, respectively. In these asymmetric synapses, the synaptic vesicles are often small and round; cytoplasmic densities are more prominent in the post-synaptic membrane than in the pre-synaptic one. This morphology is distinct from that of a symmetric synapse, which often has elongate or flattened vesicles and almost equal densities in both pre-synaptic and post-synaptic membranes. In brain, axonal terminals forming asymmetric synapses are likely to be excitatory and those forming symmetric synapses are inhibitory (17–19). Thus, the transgene protein appears to preferentially form aggregates in excitatory axons.

Progressive formation of huntingtin aggregates

To examine the formation of neuropil aggregates over time, we used light immunohistochemistry to examine brain sections from R6/2 mice at 4, 6, 8 and 12 weeks. In 4-week-old mice (Fig. 6A and D), the majority of the immunoreaction product was diffuse in the nucleus. A few neurons had a single inclusion in the nucleus and some small aggregates were in the region outside the cell body, suggesting that both NIIs and neuropil aggregates were formed at 4 weeks. Although NIIs are larger than neuropil aggregates, the numbers of NIIs and neuropil aggregates per microscopic field are very similar. In 8-week-old mice (Fig. 6B and E), the percentage of neurons having NIIs increased slightly and diffuse intranuclear labeling became less intense. In contrast, neuropil aggregates were significantly increased and became more frequent than NIIs. In 12-week-old mice (Fig. 6C and F), the majority of EM48 immunolabeling was associated with neuropil aggregates and the density of neuropil aggregates was much higher than that of NIIs. Intranuclear huntingtin labeling, on the other hand, consisted mainly of NIIs, with no diffuse nuclear labeling. The number of NIIs was also slightly increased. Neuropil aggregates, however, were noticeably more abundant by 12 weeks of age. These results suggest that the formation of neuropil aggregates is more progressive than that of NIIs.
**Aggregation of the HD exon 1 protein**

In humans with HD, neuropil aggregates form even before intranuclear aggregates (14). We reasoned that R6/2 mice form NIIs more rapidly because the small size of the HD exon 1 protein and the expanded polyglutamine tract allow it to enter and accumulate in the nucleus more easily. As aggregated proteins can be seen in the stacking gel on western blots (20,21), we examined the aggregation of the HD exon 1 protein in R6/2 mice by western blotting. The exon 1 HD protein from 6-week-old HD mice formed aggregates in addition to a soluble form (75 kDa band; Fig. 7). In 12-week-old R6/2 mice, aggregated protein was abundant, whereas the soluble form was not detected. Thus, western blot analysis further confirms that aggregation of the HD exon 1 protein is a time-dependent process which is likely to associate with disease progression.

**Neuropil aggregate formation is correlated with disease progression**

To quantitatively compare the formation of neuropil aggregates and NIIs, we used light EM48 immunostaining microscopy to measure the density and percentage of these aggregates in the brains of 4-, 6-, 8- and 12-week-old transgenic mice. We chose the striatum and cerebral cortex for examination because these two regions are the most enriched in aggregates and are the most affected areas in HD patients. The density of aggregates was counted as the number of aggregates per microscopic field and the percentage of each type of aggregate was also calculated. The small size of neuropil aggregates and their localization outside the cell body made it easy to distinguish them from NIIs. A quantitative assay confirmed that the densities of NIIs and neuropil aggregates were similar in 4-week-old mice (Fig. 8A). As mice became older, both NIIs and neuropil aggregates increased in density and number. However, neuropil aggregates had a much greater increase than NIIs as age increased. The greater increase in neuropil aggregates was further confirmed by examination of the percentage of each type of aggregate. The percentage of neuropil aggregates increased from 45–50% of the total number of aggregates at 4 weeks to 80–85% at 12 weeks in the cortex and striatum (Fig. 8B). The increase in neuropil aggregate formation is also significantly higher in the cerebral cortex than in the striatum. Thus, the cortex contained more neuropil aggregates than the striatum.

We then compared the formation of huntingtin aggregates with phenotype development as described in previous studies (6,7). Because the numbers of neuropil aggregates and NIIs per microscopic field are similar at 4 weeks (Fig. 6), we can express the increase in the number of these aggregates as a multiple of the number of aggregates at 4 weeks. The comparison revealed that neuropil aggregate formation increased by 3.3-, 7.3- and 13.6-fold at 6, 8 and 12 weeks, respectively (Table 1). However, NIIs increased by 1.4-, 1.6- and 1.7-fold at these time points. Since NIIs are present as a single inclusion in the nucleus, the results suggest that the number of neurons with aggregates does not increase greatly, but the number of aggregates in the processes of these neurons are increased dramatically. R6/2 mice display a complex progressive neurological phenotype. Recent studies have shown that significant motor deficits can be detected as early as 5–6 weeks, at which time these mice have difficulty in swimming, traversing a narrow square beam and maintaining their balance on an accelerating rotarod (22). The time at which deficits can be detected depends on the nature and difficulty of the task that has been imposed. Our study shows that the transgenic huntingtin formed aggregates prior to the onset of symptoms and progressively formed more aggregates as the disease developed. Although the number of NIIs remained fairly steady from 4 to

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**Table 1. Increase in neuropil or nuclear aggregate formation during disease progression**

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuropil aggregates</td>
<td>1</td>
<td>3.3</td>
<td>7.3</td>
<td>13.6</td>
</tr>
<tr>
<td>Nuclear aggregates</td>
<td>1</td>
<td>1.4</td>
<td>1.6</td>
<td>1.7</td>
</tr>
<tr>
<td>Brain weight loss</td>
<td>–</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Body weight loss</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Symptoms*</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

The increase in aggregate formation is expressed as fold density of aggregates in 4-week-old R6/2 mice.

* Symptoms are motor deficits and disorders that were described by Davies et al. (7) and Carter et al. (22).
12 weeks, R6/2 mice developed neurological symptoms from 5 weeks and all aspects of the motor disorder and non-motor symptoms (decrease in brain and body weight) progressed throughout the lifetime of the mouse. Similarly, neuropil aggregates continuously and significantly increased until death. Thus, the progressive formation of neuropil aggregates is more closely associated with disease progression than is that of NIIs.

**DISCUSSION**

In this study, we present two major findings to show that neuropil aggregates may have a role in HD. First, many neuropil aggregates are localized to axons and axon terminals, suggesting that they may affect neurotransmitter release. Second, the formation of neuropil aggregates is more progressive than that of NIIs and is better correlated with disease development in R6/2 mice, suggesting a causative role for them in HD.

Polyglutamine aggregates have been observed as NIIs in the HD R6 transgenic lines, post-mortem brains from HD (10,11,14), DRPLA (11.23), SCA1 (24), SCA3 (12), SCA7 (25) and SBMA (26) patients, and in SCA1 transgenic mice (24) and two other more recently described HD transgenic mouse models (27,28). It would appear logical that polyglutamine aggregation would be the cause of neuronal dysfunction and neurodegeneration in vivo. However, the frequency of NIIs found in HD post-mortem striatum is considerably lower than might be expected given that this is a

![Figure 6. Progressive formation of huntingtin aggregates. Light micrographs of sections of the cerebral cortex (A–C) and striatum (D–F), stained with EM48. Sections were obtained from R6/2 transgenic mice at 4 (A and D), 8 (B and E) and 12 (C and F) weeks. Intranuclear aggregates or NIIs are indicated by arrows. Note that EM48 immunoreactive neuropil aggregates are more prominent in 12-week-old mice, whereas 4-week-old mice display more diffuse intranuclear labeling. Scale bar, 25 µm.](image-url)
major site of cell loss in the disease (14). The distribution of
NIIs in R6/2 mice is ubiquitous from a comparatively early
time point and yet a selective cell death that is restricted to the
cortex, striatum and cerebellum does not occur until these mice
are very close to the end stage of the disease (M. Turmain, G.P.
Bates and S.W. Davies, unpublished data). In addition, NIIs
have been observed in a number of peripheral tissues in the R6
lines (29; H. Li and X.-J. Li, unpublished data) and SBMA
patients (26), in which they are never associated with cell
death. The appearance of NIIs in pancreatic islets, however,
does cause cellular dysfunction (29) resulting in the diabetes
that has been described in the R6/2 line (30) and which may be
one of the contributing factors to the weight loss in these mice.

Using the antibody EM48, we have recently identified
neuropil aggregates in the brains of HD patients (14), extend-
ing the previous report of dystrophic neurites in HD (10).
EM48 appears to be much more sensitive to aggregated hunt-
ingtin than previous antibodies. EM48 was raised against a
GST–huntingtin fusion protein that contains the first 256
amino acids of huntingtin, but lacks the polyglutamine and
polyproline stretches. It is most likely that huntingtin protein
aggregation confers a conformation preferentially recognized
by EM48. The neuropil aggregates revealed by EM48 were
also frequent in the brain of a pre-symptomatic HD patient,
suggesting that they may play an important role in the early
neuronal dysfunction that occurs before neurodegeneration.
In addition, neuropil aggregates were more frequent in the cortex
than in the striatum. As a large number of cortical neurons
project to the striatum (31), it is possible that cortical aggre-
gates may impair axonal transport in these cortical neurons and
affect corticostriatal transmission. However, because of the
poor preservation of post-mortem tissue, we were unable to
localize the aggregates to the axons in human post-mortem
brains to support this idea (14).

In this report we have used EM48 immunostaining to
identify numerous neuropil aggregates in R6/2 mice that were
not detected with other N-terminal huntingtin antibodies (7).
This has allowed us to localize neuropil aggregates to specific
neuronal compartments as the HD transgenic mouse brains can
be perfusion fixed and ultrastructural morphology can be better
preserved than that of post-mortem tissue. Although EM48 did
not label well endogenous normal huntingtin, which was found
to associate with synaptic vesicles in other studies (32–34), its
intense labeling of many small aggregates in axons indicates
that transgene huntingtin accumulates and aggregates in axons.
These aggregates may also associate with synaptic vesicles,
raising the possibility that aggregates could mechanically
interfere with synaptic vesicle recycling or regeneration and
lead to altered synaptic transmission and neuronal communi-
cation. Corticostriatal glutamatergic fibers represent the major
excitatory input to the striatum (31,35). It is conceivable that a
disruption caused by huntingtin aggregates in the pre-synaptic
terminals could result in increased glutamate release, in turn
resulting in excitotoxicity within the striatum, which would be
consistent with earlier proposals of mechanisms for HD patho-
genesis (36–38). The finding that most neuropil aggregates are
seen in excitatory axons (Fig. 5) provides a morphological basis for this hypothesis. In support of this proposal, a downregulation of pre-synaptic mGlur2 receptors in the cortex of R6/2 mice may result from an impairment of axonal trafficking and lead to an increase in glutamate release (9).

If neuropil aggregates contribute to early neurological dysfunction, we should see an association between the development of neuropil aggregates and neurological symptoms. The comparison of neuropil aggregates and NIIs confirms that neuropil aggregate formation is much more progressive than NIIs formation. This conclusion is based on the following observations: (i) neuropil aggregates appear in 4-week-old mice that are pre-symptomatic; (ii) neuropil aggregates become more frequent than NIIs beginning at 6 weeks, a time when mice show significant motor deficits (22); and (iii) neuropil aggregates continue to progressively increase until 12 weeks, a time close to the end stage of the disease; in contrast, the number or density of NIIs is fairly steady from 4 to 12 weeks. These results suggest that neuropil aggregates are more closely associated with the progression of disease symptoms in R6/2 mice.

Neuropil aggregates are likely to contribute to the early neuronal dysfunction that occurs before neurodegeneration in both HD patients and in the R6 transgenic lines. However, the extent of dysfunctional neurons in both the transgenic mice and in patients is unknown. Dysfunction may arise because neuropil aggregates interfere with organelle or vesicle transport in the neuronal processes. Aggregates in axon terminals could also affect neurotransmitter release and function of the innervated neurons. Given that the cortex contains the highest density of aggregates and that the striatum receives numerous projections from the cortex (31,35), cortical aggregates could affect corticostriatal communication or transmission. However, how could widespread neuropil aggregates form the basis of the selective neuropathology? It is possible that degeneration occurs only in neurons that have some specific vulnerability to the effects of aggregates, which may be an intrinsic property of either the neuron that degenerates or of one with which it interacts. The generation of an excitotoxic effect in the striatum as a result of neuropil aggregates in the cortex is an attractive hypothesis.

Our interpretation of the relationship between aggregation, neuronal dysfunction and cell death is necessarily limited by the reagents at our disposal. The EM48 antibody identifies a far greater extent of huntingtin aggregation than previous antibodies and reveals a considerable fraction of aggregates in axon terminals. These findings have enabled us to propose that neuropil aggregates are central in causing neurodysfunction in HD patients and a transgenic model of HD. It is now important to establish whether similar neuropil aggregates are also seen in excitatory axons (Fig. 5) provides a morphological basis for this hypothesis. In support of this proposal, a downregulation of pre-synaptic mGlur2 receptors in the cortex of R6/2 mice may result from an impairment of axonal trafficking and lead to an increase in glutamate release (9).

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**MATERIALS AND METHODS**

**Antibodies and western blot analysis**

Rabbit polyclonal antibody EM48, specific to the N-terminal region of huntingtin, was generated using a GST fusion protein containing the first 256 amino acids of human huntingtin with an in-frame deletion of the polyglutamine and polyproline stretches (14,21). The same antigen was also used to generate guinea pig antibody EM73, for immunofluorescent double labeling. The antibodies were affinity purified by incubation with a nitrocellulose strip containing transfected GST–huntingtin. Antibodies bound to the strip were then eluted with 0.2 mM glycine (pH 2.8) and neutralized with 1 M Tris–HCl (pH 8). For western blotting, protein samples were solubilized in SDS sample buffer and resolved by 8 or 10% SDS–PAGE. Blots were incubated with EM48 (1:500) and immunoreactive bands were visualized using a chemiluminescence kit (Amer sham, Arlington Heights, IL). The immunoreactivity on the blots could be eliminated by overnight pre-absorption of the antibody with 20 µg/ml GST–huntingtin but not by GST alone. A rabbit polyclonal antibody to ubiquitin (Dako, Carpinteria, CA) was also used in the study.

**Light microscopic examination**

R6/2 mice [B6CBA-TgN (HDexon1)62], which express exon 1 of the human mutant HD gene, were obtained from the Jackson Laboratories (Bar Harbor, ME) or Dr Gillian Bates (King’s College, London, UK). Mice were anesthetized with an overdose of 4% chloral hydrate, injected i.p., and then perfused intracardially with phosphate-buffered saline (PBS, pH 7.2) for 30 s followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.2) for 5 min. Brains were removed and cryoprotected in 30% sucrose at 4°C. Brains were sectioned at 40 µm using a freezing microtome. Sections were collected in PBS and rinsed in 0.05 M Tris-buffered saline (TBS, pH 7.2). Some brain sections of R6/2 mice were prepared in Dr Gillian Bates’ laboratory using the same procedure. Free-floating sections were pre-blocked in 4% normal goat serum (NGS) in TBS, 0.1% Triton-X and avidin (10 g/ml) for 1 h at 4°C. Sections were incubated with primary antibodies in TBS containing 2% NGS, 0.1% Triton-X and biotin (50 µg/ml) at 4°C for 48 h. Sections were then rinsed in TBS for a total of 1 h and incubated overnight in biotinylated goat anti-rabbit antibody containing 2% NGS. Following several rinses in TBS, the sections were incubated in avidin–biotin complex (Vector ABC Elite, Burlingame, CA) for 1 h and rinsed several times in TBS. Immunoreactivity was visualized by incubation in 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St Louis, MO) and 0.01% hydrogen peroxide in 0.05 M Tris buffer, pH 7.6, until a dark brown reaction product was evident (5–10 min). Controls included brain sections from wild-type mice of the same strain (B6CBAF1/J).

For immunofluorescent double labeling, guinea pig antibody EM73 and rabbit anti-ubiquitin antibody were incubated with brain tissue sections. After washing the sections with PBS three times, fluorescent FITC- or rhodamine-conjugated secondary antibodies (1:400: Jackson Immunoresearch Laboratories, West Grove, PA) were added.
Electron microscopic immunocytochemistry

Immunogold labeling was performed as described previously (14,39). Briefly, tissues were fixed by perfusion with PBS containing 4% paraformaldehyde and 0.2% glutaraldehyde. After perfusion, the brain was removed, post-fixed with 4% paraformaldehyde in PB for 6–8 h and then sectioned using a vibratome. Brain sections were incubated with EM48 in PBS containing 4% NGS for 24–60 h at 4°C and then with Fab fragments of goat anti-rabbit secondary antibodies (1:50) conjugated to 1.4 nm gold particles (Nanoprobes, Stony Brook, NY) in PBS with 4% NGS overnight at 4°C. After rinsing in PBS and PB, sections were fixed again in 2% glutaraldehyde in PB for 1 h, silver intensified using the IntenSEM kit (Amersham International, Little Chalfont, UK), osmicated in 1% OsO4 in PB and stained overnight in 2% aqueous uranyl acetate.

All sections used for electron microscopy were dehydrated in ascending concentrations of ethanol and propylene oxide/Eponate 12 (1:1) and embedded in Eponate12 (Ted Pella, Redding, CA). Ultrathin sections (60 nm) were cut using a Leica Ultracut S ultramicrotome. Thin sections were cut counterstained with 5% aqueous uranyl acetate for 5 min followed by Reynolds lead citrate for 5 min and examined using a Hitachi H-7500 electron microscope.

Quantification of aggregates in the brain

We quantified the immunoreactive aggregates in sections of cortex and striatum using light microscopy. Immunoreacted sections (40 µm) were visualized at 63x using a Zeiss microscope (Axioskop 2) and video system (Dage). All huntingtin aggregates within the ocular grid (40940 µm2/frame) were counted. The aggregates were categorized as intranuclear or neuropil aggregates. Neuropil aggregates are localized outside the cell body and their size is usually smaller, whereas nuclear aggregates are localized inside the cell body and their size is usually larger, whereas nuclear aggregates or NIs are a single inclusion within the nucleus. Aggregates in 76–80 frames of the cortex and striatum were counted. The percentage of NIs and neuropil aggregates in the total number of aggregates counted was calculated. Data analysis was performed using SigmaPlot 4.11 f- and t-tests.

To confirm the accuracy of the results, we also captured images at random using a video system (Dage). Images were then stored and processed using Adobe Photoshop. Counting aggregates on the captured images gave the same results as counting them with an ocular grid.

ACKNOWLEDGEMENTS

We thank Dr Steve Hersch for providing electron microscopy facilities and Hong Yi for her technical assistance. We also thank Amarbirpal Mahal for mouse genotype analysis. This work was supported by National Institutes of Health grant NS36232, the Hereditary Disease Foundation Cure HD Initiative, the Wills Foundation (X.-J.L.) and the Wellcome Trust (G.P.B.).

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