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Huntingtin Is a Cytoplasmic Protein Associated with Vesicles in Human and Rat Brain Neurons

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Summary

The gene defective in Huntington's disease encodes a protein, huntingtin, with unknown function. Antisera generated against three separate regions of huntingtin identified a single high molecular weight protein of ~320 kDa on immunoblots of human neuroblastoma extracts. The same protein species was detected in human and rat cortex synaptosomes and in sucrose density gradients of vesicle-enriched fractions, where huntingtin immunoreactivity overlapped with the distribution of vesicle membrane proteins (SV2, transferrin receptor, and synaptophysin). Immunohistochemistry in human and rat brain revealed widespread cytoplasmic labeling of huntingtin within neurons, particularly cell bodies and dendrites, rather than the more selective pattern of axon terminal labeling characteristic of many vesicle-associated proteins. At the ultrastructural level, immunoreactivity in cortical neurons was detected in the matrix of the cytoplasm and around the membranes of the vesicles. The ubiquitous cytoplasmic distribution of huntingtin in neurons and its association with vesicles suggest that huntingtin may have a role in vesicle trafficking.

Introduction

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder that causes progressive motor and cognitive impairments in its victims, ultimately leading to death (Wexler et al., 1991; Folstein, 1989). Although widespread abnormalities in the HD brain have been reported, the striatum is the most severely affected structure, exhibiting marked atrophy and loss of neurons, particularly of medium-sized spiny cells (Bruyn et al., 1973; Graveland et al., 1985; Vonsattel et al., 1985). The link between the genetic change in HD and processes leading to cell death remain elusive, although there is considerable evidence that excitotoxic or disrupted metabolic processes may be involved in HD (DiFiglia, 1990; Beal et al., 1993; Coyle and Puttfarcken, 1993). Recently, the gene for HD, IT15 (for interesting transcript 15), was isolated (The Huntington's Disease Collaborative Research Group, 1993) and was predicted to encode a protein, huntingtin, of 348 kDa molecular weight. The mutation in HD involves an expansion of a trinucleotide (CAG) repeat encoding glutamines near the 5′ end of the coding sequence. IT15 bears no homology to other known genes, and thus the function of huntingtin is not known. Northern analysis and in situ hybridization studies (Li et al., 1993; Strong et al., 1993; Landwehrmeyer et al., 1995) show that IT15 mRNA is widely expressed in all tissues and is highest in brain, testes, and a number of tumor cell lines. In brain, IT15 mRNA is present principally in neurons. IT15 expression does not differ measurably in normal and HD brains, except in the HD striatum in which IT15 mRNA is reduced or absent, owing to the loss of neurons. Thus, how the genetic mutation causes a selective neuronal degeneration is not readily explained by changes in transcription, implicating alterations of the mutant huntingtin protein structure and/or function as a critical factor in the pathogenesis in HD.

Polyglutamine tracts have been identified in a number of proteins known to function as transcription factors, and it was initially speculated that huntingtin may be involved in transcription (Gerber et al., 1994). Consistent with this idea, a recent immunohistochemical study using antipeptide antisera directed to the C-terminus of huntingtin showed both a cytoplasmic and nuclear localization of the protein in neuronal somata (Hoogeveen et al., 1993). However, in peripheral organ tissues, huntingtin appeared to be localized only to the cytoplasm. To elucidate the intraneuronal subcellular localization of huntingtin in brain, we generated polyclonal antisera to different regions of the predicted coding sequence of IT15. Our results show that huntingtin has a cytoplasmic localization in neuronal cell bodies, dendrites, and axons in both human and rat brain; also, the protein appears to associate in part with the membranes of vesicles.

Results

Characterization of Anti-Huntingtin Antisera

To evaluate their specificity, purified antisera directed to residues 1–17 (Ab 1), 585–745 (Ab 585), and 2911–3140 (Ab 2911) of human huntingtin were examined on Western blots of Escherichia coli extracts containing recombinant proteins expressed from IT15 cDNA clones. All of the antisera selectively recognized the relevant protein antigen on Western blot (Figure 1A) and did not recognize unrelated fusion proteins or other proteins present in E. coli extracts.

Each antibody was evaluated by immunoblotting total protein extracts of human neuroblastoma cell line SY5Y. This cell line had been previously reported to express high levels of IT15 mRNA (Li et al., 1993). To compare the size

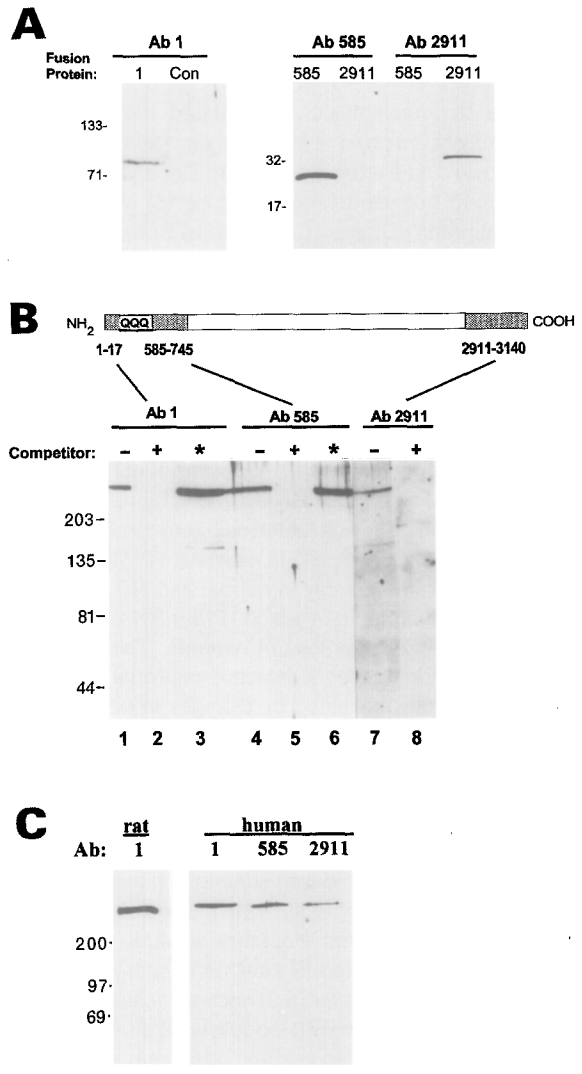

Figure 1. Characterization of Anti-Huntingtin Antisera

(A) Western blots of E. coli expressed huntingtin fusion proteins with Ab 1, Ab 585, and Ab 2911. Fusion protein (1), which contains the N-terminal 1–590 residues of normal human huntingtin, is detected by the antipeptide antibody Ab 1 (1 μg/ml). Ab1 detects no protein in E. coli extract without IT15 insert (Con). Fusion protein (585) containing residues 585–745 of huntingtin is detected with Ab 585 (2 μg/ml) but not with Ab 2911 (2 μg/ml). Fusion protein (2911) containing residues 2911–3140 is seen by Ab 2911 but not by Ab 585. Numbers to the left of the Western blots identify positions of molecular weight standards in kDa.

(B) Immunoblots of human neuroblastoma extracts probed with antisera directed against three regions of huntingtin. Diagram (above) shows the location and number of amino acid residues in huntingtin used to generate Ab 1, Ab 585, and Ab 2911. QQQ identifies the polyglutamine region. Nitrocellulose strips (below) containing 200 μg total protein transferred from a wide-lane gel were immunoblotted with Ab 1 (1 μg/ml; lanes 1–3), Ab 585 (1 μg/ml; lanes 4–6), and Ab 2911 (3 μg/ml; lanes 7–8). The immune serum was used alone without competitor (–) in lanes 1, 4, and 7. Detection was blocked in the presence of a 5-fold excess concentration of the appropriate antigen (+) in lanes 2, 5, and 8. Huntingtin immunoreactivity was unaffected in the presence of a 5-fold excess concentration of an unrelated peptide or protein (asterisk) in lanes 3 and 6 (and also 9, which is not shown). Positions of molecular weight standards in kDa are shown on the left.

(C) Immunoreactive huntingtin in synaptosomes of rat and human cortex. Western blots of synaptosomes (fraction C) are shown from rat (left) and human cortex (right) probed with Ab 1 (rat and human) and Ab585 and Ab 2911 (human). Human synaptosomes were prepared from the same control brain. Molecular weight standards in kDa are shown on the left.

of the protein species recognized by each of the immune sera, nitrocellulose strips containing protein transferred from a wide-lane gel were treated with the preimmune or immune sera. Results showed that Ab 1, Ab 585, and Ab 2911 each recognized a similar size, high molecular weight band in neuroblastoma SY5Y extracts (Figure 1B). Detection of the huntingtin-like protein was blocked when the immune sera were preincubated with the appropriate antigen and present when the immune sera were preincubated with an unrelated peptide or protein antigen (Figure 1B). Respective preimmune sera were examined similarly (data not shown) and did not detect the high molecular weight species. The huntingtin-immunoreactive protein migrated slightly faster than immunoreactive MAP1b (migrating as an apparent molecular weight 320 kDa) and thyroglobulin (apparent molecular weight 330 kDa).

Antisera directed against all three epitopes of huntingtin were used to probe extracts of human and rat cortex on Western blots (Figure 1C). All antisera recognized a single high molecular weight protein of approximately 320 kDa in human cortex synaptosomes (fraction C; see Experimental Procedures) and in total protein extracts of human cortex (data not shown). In rat cortex synaptosomes, Ab1, but not Ab 585 and Ab 2911, detected a similar size protein (Figure 1C). These results provided evidence that huntingtin is synthesized in the rat and human brain as a large molecular weight protein, which by epitope mapping, spans the protein sequence as predicted by the IT15 cDNA. Additionally, huntingtin was readily detected in synaptosomes.

Subcellular Localization of Huntingtin in Brain: Biochemical Studies

Subcellular differentiation was used to determine whether huntingtin was distributed with membranes. Huntingtin immunoreactivity was readily detected in fractions containing P1, P2, P3, LP1, and LP2 (see Experimental Procedures), along with immunoreactive synaptophysin, a vesicle membrane protein (Figure 2A). Supernatants (S3 and LS2) exhibited little or no huntingtin immunoreactivity (Figure 2A). These results raised the possibility that huntingtin was associated with a vesicle compartment. The distribution of huntingtin was then compared with vesicle and nonvesicle proteins on continuous sucrose density gradients. Analysis of sucrose density gradients of the vesicle-enriched LP2 fractions showed a nearly identical correspondence between the distribution of immunoreactive huntingtin and immunoreactivity of the vesicle membrane–associated proteins SV2, synaptophysin, and transferrin receptor (Cameron et al., 1991; Volknandt, 1995; White et al., 1992). In contrast, the pattern of huntingtin immunoreactivity differed from that of the plasma membrane protein, Na^+/K^+ ATPase (α subunit). Huntingtin was

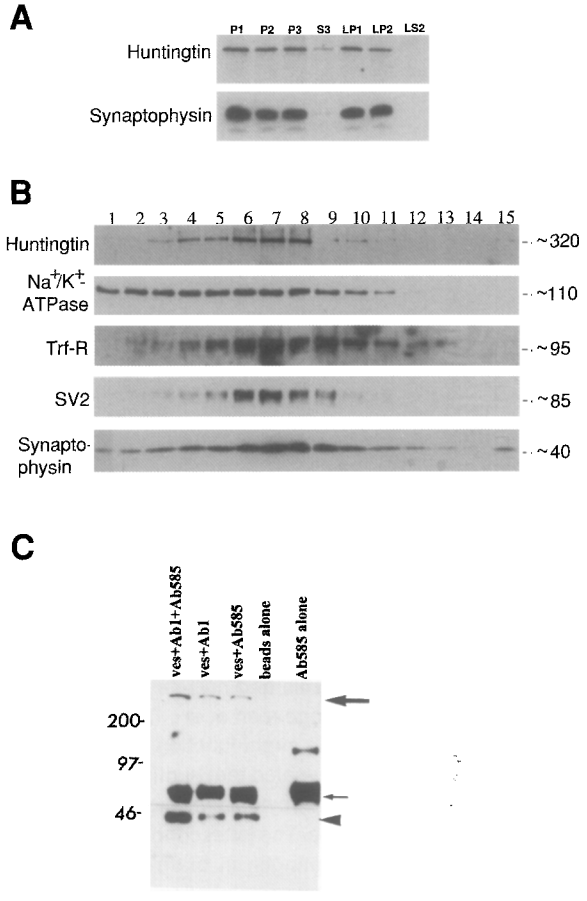

Figure 2. Huntingtin in Human Brain: Biochemical Analysis

(A) Immunoblots of subcellular fractionation of human cortex extracts. Subcellular fractions (20 μg/ml) isolated from human cortex were probed with Ab 1 (1 μg/ml) and then with anti-synaptophysin monoclonal antibody (0.5 μg/ml). Fractions are marked as P1, P2, P3, S3, LP1, LP2, and LS2.

(B) Distribution of huntingtin immunoreactivity with vesicle- and non-vesicle-associated proteins after sucrose density gradient fractionation. LP2 extracts were applied to sucrose density gradients; equal volumes of each fraction were evaluated by Western blot analysis. Antisera used are noted to the left of the figure. Approximate molecular weights are denoted on the right margin. The sucrose density gradient fractions are marked at the top, from 0.35 M sucrose (lane 1) to 2.0 M sucrose (lane 15).

(C) Immunoprecipitation and immunoblot analysis of human brain vesicle fraction with anti-huntingtin antisera. LP2 fractions from human cortex were treated with a mixture of Ab 1 and Ab 585 (ves + Ab1 + Ab585), Ab 1 alone (ves + Ab1), and Ab 585 (ves + Ab585). Controls included omission of anti-huntingtin antisera (beads alone) and omission of beads (Ab585 alone). In this assay, magnetic beads conjugated to anti-rabbit antibody (Dynal) were used to isolate components of subcellular fractions, with anti-huntingtin antisera recognizing either an N-terminal (Ab 1) or internal (Ab 585) epitope. The blots were probed with either Ab 1 (shown) or Ab 585 (data not shown; identical results). A single high molecular weight band appears under all three immunoprecipitation conditions (arrow). Protein is absent in the controls in which beads alone or Ab 585 alone were used. The blots were reprobed with anti-synaptophysin antibody. Detection of synaptophysin (arrowhead) in the immunoprecipitates is shown in the panel below. The small arrow indicates the detection of immunoglobulin.

therefore enriched in compartments containing vesicle-associated proteins. Finally, immunoprecipitation of vesicles in LP2 fractions was also achieved with the huntingtin antisera (Figure 2C). Ab 1 recognized the same size protein in Western blots of vesicles immunoprecipitated by either Ab 1 or Ab 585, or a mixture of Ab 1 and Ab 585. These results were also obtained in Western blots probed with Ab 585 following vesicle immunoprecipitation with Ab 1, Ab 585, or a mixture of both (data not shown). The anti-huntingtin antisera also immunoprecipitated structures containing synaptophysin (Figure 2C). Huntingtin, therefore, copurified with synaptophysin, providing further support for the association of huntingtin with vesicles.

Huntingtin Localization in Brain: Immunohistochemical Analysis

Consistent with the recently reported distribution of IT15 mRNA in rat and human brain (Li et al., 1993; Strong et al., 1993; Landwehrmeyer et al., 1995), huntingtin immunoreactivity detected with Ab 1 was found in neurons throughout the rat brain. In the cortex (Figures 3a–3e), neurons in all cortical layers were stained. Reaction product appeared within the cytoplasm (Figures 3a and 3d) of neuronal cell bodies and could also be detected in dendrites (Figure 3d) and axons (Figure 3e) traversing the neuropil. Preadsorption with peptides 1–17 eliminated the staining from cell bodies and neuropil (Figure 3b), whereas preadsorption with an unrelated peptide did not reduce the staining (Figure 3c). At the ultrastructural level, reaction product was present in the matrix of the cytoplasm and along the membranes of vesicles. In cell bodies, discrete labeling appeared on the membranes and vesicles of the Golgi complex (Figure 4a). Small patches of labeling intermixed with clusters of vesicles were seen in dendrites (Figure 4b), unmyelinated axon fibers (Figure 4c), and axon terminals (Figure 4d). Within the matrix, immunoreactivity appeared on microtubules, often in association with labeled vesicles (Figure 4b).

The localization of huntingtin in the human brain with Ab 585 produced a widespread labeling of neurons similar to that of Ab 1 in rat brain. In the cortex, Ab 585 reacted with neurons in all layers (Figure 5a). Reaction product appeared in the cytoplasm (Figure 5a) of cell bodies, dendrites, and axons; in the neuropil, the apical and basal dendrites of pyramidal cells were prominently labeled (Figure 5a, arrows). Preadsorption with the purified fusion protein 585–745 eliminated most staining from cell bodies and within the neuropil (Figure 5b). Neuronal staining in other regions included the Purkinje cells and granule cells of the cerebellum (Figure 5c) and the medium-sized cells of the caudate nucleus (Figure 5d). At the ultrastructural level, peroxidase reaction product in human cortical neurons was dispersed in patches within the matrix of the cytoplasm and was concentrated on and around the membranes of vesicles in cell bodies, proximal and distal dendrites (Figure 5g), and myelinated axons and axon terminals (Figure 5e). Some of the labeled terminals formed synaptic contacts (Figure 5f). Reaction product appeared on microtubules, particularly in the dendrites. Immunogold

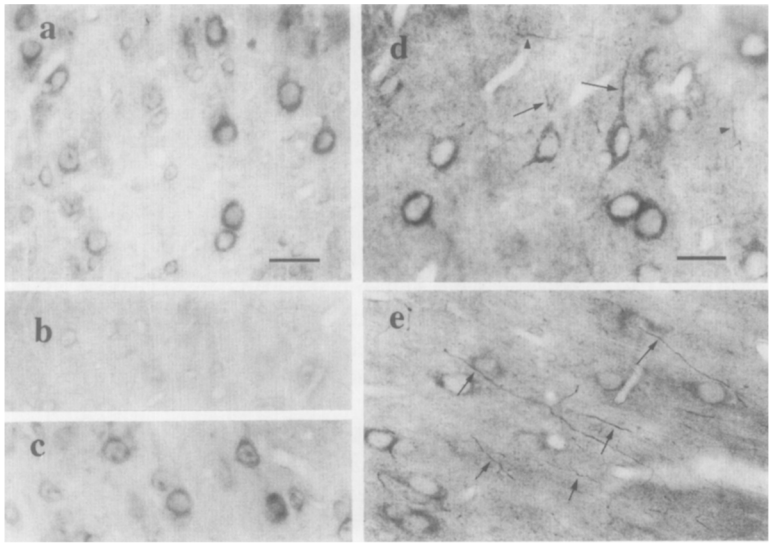

Figure 3. Huntingtin Immunohistochemistry with Ab 1 in Rat Cortex: Light Microscopy

(a) Section treated with Ab 1 exhibits immunoreactivity in the cytoplasm of neurons.
(b) Preadsorption of Ab 1 with 10-fold excess concentration of peptide residues 1–17 blocks the staining.
(c) Presence of 10-fold excess concentration of unrelated peptide does not block the staining.
(d) Immunoreactive dendrites (arrows) and axons (arrowheads) appear in regions of heavily labeled cortical neuropil.
(e) Immunoreactive fibers (arrows) traverse the neuropil. Bar, 40 μm (a, b, c); 25 μm (d and e).

labeling of P2 fractions showed gold particles in presynaptic profiles adjacent to vesicle membranes but not superimposed on the vesicles (Figure 5h). Gold labeling was also seen in the cytoplasmic matrix of postsynaptic profiles (Figure 5h). In sections in which the anti-huntingtin antisera was omitted, gold particles were rarely present.

The localization of huntingtin using immunohistochemical approaches revealed a broad intraneuronal cytoplasmic distribution of huntingtin in dendrites, somata, and axons; additionally, immunohistochemistry supported the association of huntingtin with vesicles.

Discussion

The recent discovery that the gene defective in HD, IT15, is widely expressed in brain and in many internal organ tissues raised speculation that the encoded protein, huntingtin, might serve a common housekeeping function in a diversity of cells. The lack of homology to other known genes, however, left open the search for the normal role of huntingtin in cells. Recent immunohistochemical evidence for both a cytoplasmic and nuclear localization of huntingtin in neurons (Hoogeveen et al., 1993) led to the suggestion that this protein might function as a transcription factor. Our findings revealed that huntingtin is distributed throughout the neuronal cytoplasm and is associated, at least in part, with vesicles. The latter conclusion is based on the enrichment of huntingtin in brain fractions containing vesicles, the immunoprecipitation of vesicles by anti-huntingtin antisera, and the ultrastructural evidence for huntingtin immunoreactivity around vesicles. It is important to emphasize that huntingtin immunoreactvity in brain did not exhibit a restricted pattern of axon terminal labeling characteristic of synaptic vesicle proteins. Thus, huntingtin may contribute to protein trafficking or membrane cycling throughout the neuron. The presence of

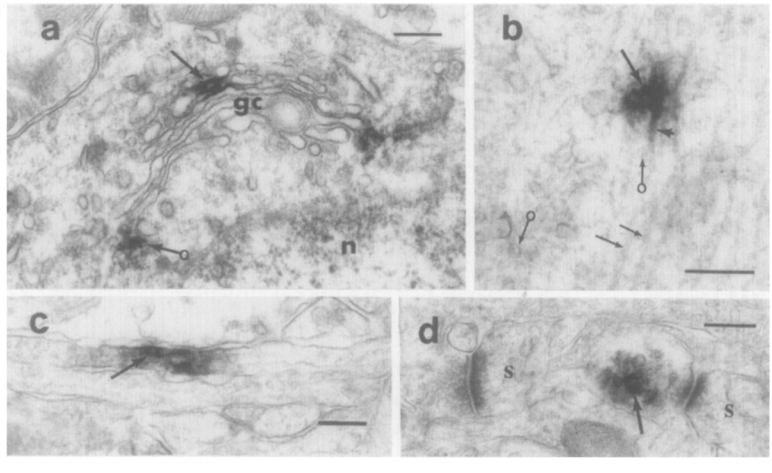

Figure 4. Immunohistochemical Localization of Huntingtin with Ab 1 in Rat Cortex: Electron Microscopy

(a) A portion of a pyramidal cell is shown. Immunoreactivity is present in the cisternae (left arrow) and vesicles (ringed arrow) of the Golgi complex (gc); n, nucleus.
Patches of reaction product are shown in a dendrite (b), axon fiber (c), and axon terminal (d).
(b) Longitudinal section through the dendrite of a pyramidal cell is shown. Reaction product is present on the membranes of vesicles (arrow) and the adjacent microtubule (short arrow). Unlabeled microtubules and vesicles are identified by small arrows and ringed arrows, respectively.
(c) Immunoreactive vesicles (arrow) in transit through a section of a small caliber axon.
(d) The immunoreactivity in the axon terminal on the right is associated with vesicles (arrow). The labeled terminal forms an asymmetric synapse with a dendritic spine (s) on the right. The terminal on the left has no immunoreactivity and also contacts a spine (s).
Bars, 0.25 μm.

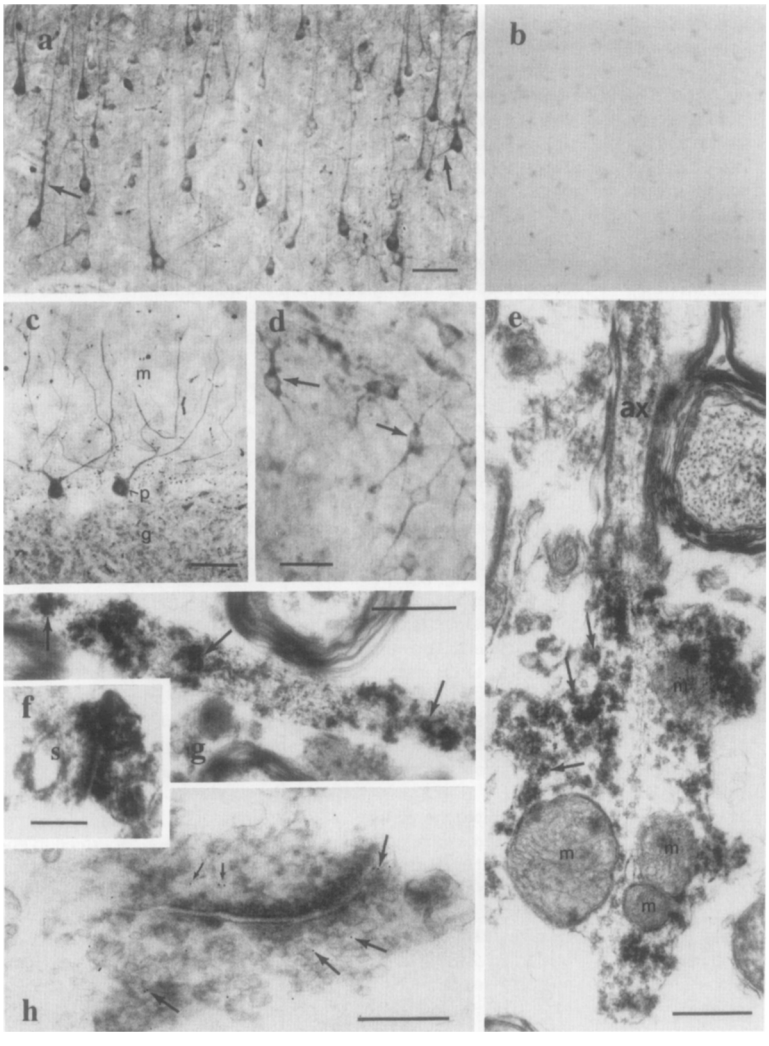

Figure 5. Immunohistochemical Localization of Huntingtin with Ab 585 in Human Brain

(a) Neuronal cell bodies and dendritic processes (arrows) are labeled throughout the cortex.

(b) Preadsorption with a 10-fold excess concentration of fusion protein markedly diminishes the staining of cells and neuropil.

(c) In the cerebellum, huntingtin immunoreactivity is seen in Purkinje cell bodies (p) and their dendrites in the molecular layer (m) and also in the granule cell layer (g).

(d) Labeled neurons (arrows) appear throughout the caudate nucleus.

(e) An axon terminal arising from a myelinated fiber (ax) contains clusters of immunoreactive vesicles (arrows). The mitochondria (m) are unlabeled.

(f) An immunoreactive axon terminal makes an asymmetric contact with a dendritic spine (s).

(g) A labeled dendrite shows patches of reaction product associated with the membranes of vesicles (arrows).

(h) Immunogold labeling of a synaptosome fraction from the human cortex demonstrates the presence of gold particles around vesicles (arrows) in a presynaptic profile and within the matrix of the cytoplasm (small arrows) postsynaptically. Bars, 40 μm (a and b); 60 μm (c); 30 μm (d); 0.5 μm (e and g); 0.25 μm (f and h).

huntingtin within the matrix of the cytoplasm may position it to interact with cytoskeletal elements and thereby influence vesicle movement along microtubule tracks. In any event, further studies of huntingtin that elucidate the vesicle compartments (synaptic, endosomal, transport) with which it associates and its interactions with proteins involved with membrane transport, docking, and fusion (see review by Jahn and Südhof, 1993) would seem especially worthwhile.

Importantly, results in this study showed that the three antisera generated to huntingtin epitopes from the predicted N-terminal region flanking the polyglutamine site (Ab 1 and Ab 585) and the C-terminus (Ab 2911) recognized the same size protein. Our data indicated that the principal protein encoded by the IT15 gene includes the polyglutamine region. These same antisera also detect the mutant form of huntingtin isolated from synaptosomal preparations in HD brains, the mutant protein having a larger molecular weight, consistent with the expanded CAG repeat (Aronin and DiFiglia, unpublished data).

Implications for HD

Proposed defects in protein trafficking have been identified in a number of recessive inherited disorders, including cystic fibrosis, hereditary emphysema, and familial hypercholesterolemia (Amara et al., 1992). Many of these defects appear to occur at the level of the endoplasmic reticulum, where incorrectly assembled proteins are degraded, resulting in a failure or error in intracellular targeting of the protein. The association of huntingtin with vesicle membranes suggests that this protein may participate in the process of protein targeting. Alteration in huntingtin protein structure caused by the CAG expansion in HD might lead to impaired vesicle function and protein delivery. In neurons, especially those of the striatum, the failure of huntingtin to participate in the proper transport, removal, binding, or recycling of vesicles could lead to a metabolic stress and/or abnormalities in neurotransmission, resulting in cell death. Further analysis should now be possible to test this hypothesis.

Experimental Procedures

Antisera Production

A peptide containing residues 1–17 of huntingtin was synthesized to greater than 95% purity on a multiple peptide synthesizer (Symphony, Rainin). Amino acid analysis was performed by high pressure liquid chromatography to verify the sequence. A tyrosine residue was added to the C-terminus of residue 17; coupling to keyhole limpet hemocyanin

was achieved using bis-diazo-tolidine. White New Zealand Rabbits (n = 3) were injected with a 1:1 ratio of Freund's complete adjuvant for the first injection and incomplete adjuvant for subsequent boosts at 4–6 week intervals. Antisera were affinity purified on AffiGel 15 (BioRad), to which the peptide conjugated to bovine serum albumin was coupled. Preimmune and immune sera were run over separate columns and eluted with 0.1 M glycine (pH 2.5) into Tris–HCL (pH 8.0). Protein concentrations were determined at OD280 for the preimmune and the immune sera. For the production of recombinant proteins, cDNA fragments encoding residues 585–745 and 2911–3140 were generated by PCR from IT15 phage clones. The fragments were purified and cloned in-frame into bacterial expression vector pQE (Qiagen), which encodes six histidines upstream of the polylinker. Cloned fragments were verified by restriction digestion and sequencing. Protein expression and purification on Ni-bound resin columns were performed according to the recommendations of the supplier. Purified fractions were verified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and those with greater than 95% purity were pooled and concentrated (Centricon, Amicon). The purified protein was injected without a carrier protein into rabbits using the immunization procedures described above. The immune serum generated using the six histidine–tagged huntingtin fusion proteins was purified on a protein A column (Ab 585) or by affinity purification against the antigen on nitrocellulose strips (Ab 2911).

Monoclonal antisera to synaptophysin (Boeringer-Mannheim), polyclonal antisera to SV2 (gift from Dr. Kathleen M. Buckley, Harvard Medical School), monoclonal antisera to human transferrin receptor (gift from Dr. Ian Trowbridge, Salk Institute), and monoclonal antisera to Na^+/K^+ ATPase α subunit (Affinity Bioreagents) were also employed.

Preparation of Protein Extracts from Neuroblastoma Cells

Human neuroblastoma SY5Y cells were harvested from the culture dish into phosphate-buffered saline (PBS) and transferred to RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris [pH 8.0]) containing freshly added protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 1 μg/ml pepstatin, 70 μg/ml n-tosyl-l-phenylalanine chloromethyl ketone, 0.5 mg/ml leupeptin) for 30 min at 4°C. Cells were passed through a syringe several times, then centrifuged at 8000 rpm for 5 min at 4°C. The supernatant was collected, and the protein concentration determined by Bradford assay (BioRad). Aliquots were stored at −20°C.

Preparation of Synaptosomal Fractions

Synaptosomal proteins were prepared and separated on a discontinuous sucrose density gradient, as adapted from Koenig et al. (1974). Rats were decapitated and the cortex rapidly removed and placed in 0.32 M sucrose (pH 7.5) containing 0.1 mM phenylmethylsulfonyl fluoride and 2 μg/ml pepstatin A. Human cortex (from the Brain Tissue Resource Center, McLean Hospital, Belmont, MA and Dr. Tom Smith, University of Massachusetts Medical Center, Worcester, MA), which had been stored at −70°C, was thawed in 0.32 M sucrose with the same protease inhibitors. Synaptosomal fractions at the 0.8–1.0, 1.0–1.2 (fraction C), and 1.2–1.4 M sucrose interfaces were collected, 2 v water was added, the fractions were spun at 37,000 × g for 20 min, and the pellets were resuspended in 0.32 M sucrose, 1 mM HEPES with protease inhibitors. The fractions were aliquoted and stored at −70°C until further analysis.

Preparation of Vesicles, Separation of Vesicles on Continuous Sucrose Gradients, and Immunoprecipitation

Brain cell fractionation was adapted from Brose et al. (1990) to obtain fractions P1, P2, P3, S3, LP1, LP2, and LS2. (P1, initial pellet of homogenate; P2, crude synaptosomes; P3, high-spin pellet of P2 supernatant; S3, supernatant of P3 spin; LP1, pellet after hypotonic lysis of P2; LP2, pellet of LP1 supernatant; LS2, supernatant of LP2 spin.) Brain tissue (either human or rat) was homogenized in 10 v (ml/g tissue) of 0.32 M sucrose buffer containing 0.05 M Tris (pH 6.8) plus protease inhibitors as above and separated using differential centrifugations. Fractions were stored at −70°C until further analysis.

The protocol for the sucrose density gradient spanning 0.35–2.0 M sucrose in a 10 ml total volume followed the method of Walch-Solimena et al. (1993). Samples (0.75 ml) were collected from the top of the gradient. Aliquots of sample were taken for refractive index calculation for verification of the density gradient and for protein analysis.

For immunoprecipitation, either Ab 1, Ab 585, or both were added to freshly prepared vesicles (LP2). Concentration of purified antisera to vesicle was 5 μg Ab protein to 40 μg vesicle protein; antisera 585 was used at 1:500 dilution to 40 μg vesicle protein. PBS buffer containing 5% bovine serum albumin was used; total volume of the mixture was 500 μl. The mixture was incubated at room temperature for 2 hr; then, 20 μl Dynabeads (Dynal) were added (conjugated to sheep anti-rabbit antisera), and the mixture was incubated overnight at 4°C and separated using a magnetic tube rack. The beads were washed three times with binding buffer. Protein was eluted off the beads with 0.5% SDS buffer and submitted to protein electrophoresis. Controls included omission of beads and omission of anti-huntingtin antisera.

Protein Separation and Western Blot

Protein samples (20–30 μg/lane) were separated by SDS-PAGE, either using a 4% to 20% gradient gel or 10% acrylamide gel containing 0.05% bis-acrylamide. Proteins were transferred onto nitrocellulose; all blots were treated with 5% dry milk in Tris-buffered saline plus 0.1% Tween-20 (TBST) for 1–3 hr at room temperature or overnight at 4°C. Filters were incubated in Ab 1 (0.5–2.0 μg), Ab 2911 (1–3 μg), or Ab 585 (1:4000) overnight at 4°C, washed three times 15 min each in 200 ml TBST, and then treated with anti-rabbit antisera conjugated to horseradish peroxidase at 1:10,000 in 5% dry milk in TBST for 1 hr at room temperature. After washing three times for 15 min each in 200 ml buffer, filters were processed for enhanced chemiluminescence according to the recommendations of the supplier (ECL Kit, Amersham), and the blots were exposed to Hyper-film (Amersham). Western blots with antisera to synaptophysin, SV2, transferrin receptor, and Na^+/K^+ ATPase α subunit were carried out with the above buffers at antibody concentrations recommended by the suppliers.

Immunohistochemistry

Immunoperoxidase and immunogold labeling were performed in human and rat brain tissues as previously described (Aronin and DiFiglia, 1992; Sapp et al., 1995). Human brain tissue from three normal control cases was obtained from the Brain Tissue Resource Center at McLean Hospital, Belmont, MA. Rats (n = 4) were deeply anesthetized with a 1:1 ratio of ketamine to xylazine (5 mg/1 mg per 100 g body weight) and perfused through the heart with 300–400 ml of 4% paraformaldehyde in PBS (n = 2 rats) or the same fixative with 0.1% glutaraldehyde added (n = 2 rats). Ab 585 was used at a 1:500–1:2000 dilution in the human tissue, and Ab 1 was used at 2.5–5 μg/ml in the rat. Controls for the immunohistochemistry consisted of omission of the primary antibody and preadsorption of the primary antibody with a 10-fold concentration of antigen. An unrelated peptide or protein was also used in the blocking experiments as a control. For immunogold labeling, freshly prepared P2 (50 μg) fractions were fixed in 2% paraformaldehyde-lysine-periodate fixative on ice for 15 min. Ab 585 was used at a 1:100 dilution, and 6 nM colloidal gold conjugated to Protein A/G (Jackson ImmunoResearch Laboratories) was used at a 1:20 dilution in PBS/bovine serum albumin for 6–12 hr at 4°C. Following the immunohistochemistry, the pellet was postfixed in 2% glutaraldehyde containing 0.1 M cacodylic acid (pH 7.4) for 1 hr on ice. The tissue was washed in buffer and then treated serially with 1% osmium, 1% uranyl acetate in 70% alcohol, dehydrated in alcohol, and embedded in Epon.

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