Widespread Expression of Huntington's Disease Gene (IT15) Protein Product

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Summary

Huntington's Disease (HD) is caused by expansion of a CAG repeat within a putative open reading frame of a recently identified gene, IT15. We have examined the expression of the gene's protein product using antibodies developed against the N-terminus and an internal epitope. Both antisera recognize a 350 kDa protein, the predicted size, indicating that the CAG repeat is translated into polyglutamine. The HD protein product is widely expressed, most highly in neurons in the brain. There is no enrichment in the striatum, the site of greatest pathology in HD. Within neurons, the protein is diminished in nuclei and mitochondria and is present in the soluble cytoplasmic compartment, as well as loosely associated with membranes or cytoskeleton, in cell bodies, dendrites, and axons. It is concentrated in nerve terminals, including terminals within the caudate and putamen. Thus, the normal HD gene product may be involved in common intracellular functions, and possibly in regulation of nerve terminal function. The product of the expanded allele is expressed, consistent with a gain of function mechanism for HD at the protein level.

Introduction

Huntington's disease (HD) is a fatal inherited neurodegenerative disorder. Symptoms are notable for a clinical triad of movement disorder, cognitive decline, and emotional disorder (Albin and Tagle, 1995; Folstein, 1989; Hedreen and Ross, 1995; Ross et al., 1993). Movement disorder includes both choreiform involuntary movements and incoordination of voluntary movements. The cognitive dis-
Figure 1. Characterization of Anti-HD Protein Antibodies and Analysis of HD Protein Expression Using Western Blots

(A) Homogenates of tissue from human brain cortex (H) and whole rat brain (R) (150 µg per lane) were subjected to SDS-PAGE, blotted to nitrocellulose membranes, and probed with affinity-purified anti-HD protein antibodies. All lanes shown were from a single gel stained in a single experiment. Antibodies against the N-terminus of the protein (AP78) reacted with both human (H) and rat (R) brain in the absence of the peptide immunogen (–PEP), whereas antibodies against amino acids 650–663 (AP81) reacted only with the human tissue. Immunoreactivity was completely blocked by preincubation of the antibodies with the appropriate peptide immunogen (2 µg/ml; +PEP). A blot probed with a mixture of the two antibodies showed that both antibodies labeled bands of exactly the same size (AP78 + AP81).

(b) The cDNA encoding the N-terminal third of the HD protein (with an expanded glutamine repeat of 44 amino acids) was transfected into HEK 293 cells. Homogenates of control cells (C), transfected cells (T), and monkey brain cortex (M) were subjected to SDS-PAGE and blotted to nitrocellulose membranes. Antibody AP81 recognized a distinct band near the expected size, only in the transfected cell homogenates, as well as the endogenous protein at ~350 kDa. Immunoreactivity was completely blocked by preincubation of the antibody with the peptide immunogen (2 µg/ml).

(C) Homogenates of lymphoblastoid cells prepared from a control subject (C) and a HD patient with 82 repeats (P) were subjected to electrophoresis on a 4% SDS–polyacrylamide gel and the dye front run off to separate the two alleles in the patient material. Proteins were transferred electrophoretically to nitrocellulose paper, and the blot was stained with antibody AP81. A single band was recognized in the control cells, whereas a second, larger band was also recognized in the patient cells.

Results

Antibodies to the HD Protein

Antibodies were raised in rabbits against two peptides within the predicted HD protein sequence (Huntington's Disease Collaborative Group, 1993). One peptide corresponded to the predicted N-terminus, from the initial methionine through the phenylalanine immediately to the N-terminal side of the glutamine repeat (amino acids 1–17), while the other corresponded to amino acids 650–663.

Western blots of brain tissue using affinity-purified antibodies from rabbit 78 (AP78; N-terminal peptide) or from rabbit 81 (AP81; internal peptide) yielded a prominent band at 350 kDa (Figure 1A). Preincubation of antibody AP78 with the N-terminal peptide completely eliminated the 350 kDa band (Figure 1A, AP78 + PEP), whereas preincubation with other peptides, including the internal peptide, had no effect (data not shown). Antibody AP81 also recognized a clear band at 350 kDa (Figure 1A, AP81). However, unlike AP78, AP81 only recognized the 350 kDa band in human and monkey tissue and not in rat. Labeling with AP81 was eliminated by preincubation with the internal peptide. Mixtures of AP78 and AP81 recognized a single band on Western blots of human brain homogenates (Figure 1A, AP78 + AP81).

To confirm the specificity of the antibody for the HD gene product, Western blots were also made from HEK 293 cells transfected with a construct including the first third of the IT15 coding region with an expanded glutamine repeat of 44 repeats (Figure 1B). The blot reveals a band at approximately the expected migration in the transfected cells, but not in the untransfected cells. This band, like the band at ~350 kDa representing the endogenous HD gene product, is completely eliminated by pre-treatment of the antibody with peptide immunogen (Figure 1B, AP81 + PEP).

To determine whether the protein with the expanded allele is translated, homogenates of lymphoblastoid cells from HD patients and controls were run on a 4% polyacrylamide gel, blotted to nitrocellulose, and probed with antibody AP81. As illustrated in Figure 1C, the expanded allele can be clearly visualized as a band with slower migration than the normal allele. In blots from other cases, the separation between the two alleles was roughly proportional to the size of the expanded repeat (data not shown).

Subcellular Fractionation

Fractionation of rat brain tissue using differential and density gradient centrifugation (Figure 2A) revealed that the 350 kDa band labeled by AP78 was decreased in the crude nuclear fraction (P1) and present in both medium speed (P2) and high speed (P3) pellets, as well as in high speed supernatant (S3). Subfractionation of the P2 fraction using sucrose gradient centrifugation by the method of Gray and Whitaker (1962) indicated that immunoreactivity was low-
Localization of HD Gene Protein Product

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Figure 2. Subcellular Fractionation

(A) Subcellular fractions from rat cerebellar tissue were prepared according to the method of Gray and Whitaker (1962), resolved by SDS–PAGE, blotted to nitrocellulose membranes, and reacted with antibody AP78. Equal amounts of protein (75 μg) were used in each lane. Immunoreactivity was lowest in the crude nuclear pellet (P1) and the mitochondria-enriched sucrose gradient (1.2 M) fraction, and somewhat enriched in the synaptosome-enriched (0.8–1.2 M) sucrose gradient fraction. Immunoreactivity was also present in the high speed pellet (P3) and the high speed supernatant (S3) fractions.

(B) A P2 fraction generated from rat brain striatum was subfractionated after hypotonic lysis essentially according to the method of Huttner et al. (1983). Fractions (65 μg of protein per lane) were subjected to SDS–PAGE, blotted to nitrocellulose membranes, and reacted with antibody AP78. The HD gene product was present in the soluble (high speed supernatant) fraction (LS2), but was most concentrated in the high speed pellet (LP2). The blot was also probed with antibodies to the indicated proteins (p145, dynamin, and synaptophysin), yielding bands at the appropriate indicated molecular masses.

est in the mitochondria-enriched fraction (1.2 M sucrose gradient fraction) and enriched in a synaptosomal fraction (0.8–1.2 M sucrose gradient fraction). The proportion of immunoreactivity associated with membranes could be greatly reduced by washes with 200 mM or 1 M NaCl (data not shown), consistent with a loose association with membranes. When a P2 fraction was subfractionated after hypotonic lysis according to the method of Huttner et al. (1983) (Figure 2B), synaptophysin, an integral synaptic vesicle protein, was highly enriched in the LP2 fraction, as expected. Some of the HD gene product was found in the soluble fraction (LS2), but was more concentrated in the synaptic vesicle–enriched LP2 fraction. The subcellular distribution of the HD protein was similar to those of p145 and dynamin, two hydrophobic neuronal proteins recently shown to be concentrated in nerve terminals and proposed to have roles in synaptic vesicle recycling (McPherson et al., 1994). When the LP2 fraction was further fractionated by sucrose gradient centrifugation (data not shown), most of the HD gene product remained at the top of the gradient (SGS fraction), whereas synaptic vesicles are most enriched in the 200–400 mM sucrose fraction (SGV; Huttner et al., 1983).

Regional Distribution

On Western blots prepared from rat tissues, the band at 350 kDa, detected with AP78, was enriched in brain and testis, but low in thymus, spleen, vas deferens, and intestine and moderate in liver and kidney (Figure 3). A small amount was also present in heart (data not shown). In all these tissues, the immunoreactivity at 350 kDa was eliminated by preincubation with the peptide immunogen at 2 μg/ml. By contrast, a cross-reactive band at about 65 kDa that was restricted to rat testis was unaltered by this pretreatment. Within the brain, the band at 350 kDa (using

Figure 3. Western Blot Analysis of HD Protein Expression in Rat Peripheral Tissues and Brain

The HD protein was present in essentially all tissues. Soluble fractions (high speed supernatants) prepared from various rat tissues were resolved by SDS–PAGE, blotted to nitrocellulose membranes, and probed with antibody AP78 (left) or antibody AP78 preincubated with peptide immunogen (right). Specific labeling at 350 kDa was completely eliminated by preincubation with the peptide.
AP81) was present in all monkey brain regions, with enrichment in the cerebral cortex and cerebellum and slightly less in the striatum and brainstem (Figure 4). Results in rat brain regions with AP78 were similar (data not shown).

**Immunohistochemistry**

Antibody AP81 gave specific immunohistochemical labeling in human and monkey brain tissue, but failed to label rat tissue, reflecting its ability to detect a 350 kDa band on Western blots of human and monkey tissue but not of rat tissue. Antibody AP78 gave a similar pattern of labeling, but with considerably less intensity in both rat and human tissues; therefore, AP81 was used for further studies in monkey and human tissues (Figure 5). In sections of monkey striatum, specific labeling was evident throughout the caudate and putamen as well as in the adjacent claustrum, but not within the internal capsule. In the cerebral cortex, specific labeling was evident throughout all cortical layers but was most striking in layers II–VI. Within the cerebellar cortex, label was present in the granule cell layer, the Purkinje cell layer, and the molecular layer, with a band of less intense label immediately superficial to the Purkinje cell somata. The Purkinje cells were moderately labeled, whereas their nuclei were clearly unlabeled.

At higher magnification (Figure 6), labeling was detected in all regions of the brain in neuronal cell bodies, dendrites, axons, and terminals, but not in nuclei. In many areas of brain, label was most striking in small (commonly 1 µm or smaller in diameter), punctate structures, often having the appearance of beads on a string suggestive of nerve terminals. This label could be seen most clearly in regions of the brain in which they stood out against an unlabeled background, such as in the nucleus of the tractus solitarius (Figure 6A, NTS). Pyramidal cells in the deep layers of the cerebral cortex were clearly labeled with a cytoplasmic pattern. Labeling extended into the dendrites, but was absent from cell nuclei (Figure 6B, arrows). In the superficial layers, labeled processes appeared to represent distal dendrites. Furthermore, there was fine granular labeling in all layers of cortex, suggestive of neuronal terminal labeling.

In the striatum, there was fine neuropil labeling, suggestive of terminal boutons; label in cell bodies was present as well, though weaker. As could most clearly be seen when the label was visualized using the biotinyl-tyramide immunohistochemistry protocol, there was intense label in small punctate structures (Figure 6C). In the globus pallidus, lightly labeled cell bodies and dendrites often had punctate, beaded structures adjacent to them, suggestive of presynaptic terminals (Figure 6E). In the cerebellar cortex, there was homogeneous dense labeling of granule cells with unlabeled nuclei, and labeling of punctate structures that appeared to be terminals, consistent with the appearance at low magnification (Figure 5). Purkinje cells were moderately labeled, while their proximal dendrites were lightly labeled. In addition, there were scattered densely labeled punctate structures outside of the Purkinje cell somata. By contrast, the Bergmann glia were unlabeled. In the molecular layer, there was dense labeling of neuropil throughout, with numerous densely stained, punctate structures.

**Electron Microscopic Immunohistochemistry**

Sections from the striatum were processed with antibodies...
or with antibodies preabsorbed with the peptide immunogen, and then examined using electron microscopy. Immunostained sections exhibited reaction product having a distribution consistent with that observed with light microscopy. Some cell bodies and dendritic profiles were lightly labeled. Occasionally, more densely labeled dendrites were observed, whereas most were unlabeled. Myelinated axons also occasionally contained reaction product. Much of the label was found in presynaptic terminals, though not all were labeled. This result was consistent with light level results showing a larger number of structures per unit area labeled with antibodies against synaptophysin, a marker of presynaptic terminals, compared with the HD gene product (data not shown). The morphology was best observed in lightly labeled structures, where synaptic vesicles and postsynaptic densities were unob-

strued by the presence of reaction product and clearly identifiable (Figures 7B and 7C). This pattern of labeling was not present in tissue processed with antibodies preabsorbed with peptide (Figure 7A). Some nonspecific staining (not blanked by preabsorption with antigen) was present in astrocytic processes (data not shown). Stained glia were observed on sections processed for light microscopy only when the peroxide in methanol pretreatment was omitted. This pretreatment could not be used on sections processed for electron microscopy because it resulted in inadequate morphologic preservation.

Discussion

Evidence that the immunoreactivity represents identification of the HD gene product includes the following. First, Western blots of material from animals or normal humans yield a single band of $\sim 350$ kDa, as predicted by the open reading frame in the IT15 cDNA (Huntington's Disease Collaborative Group, 1993). Antibodies generated against two distinct peptides recognize bands of identical migration. Furthermore, each of these bands is blocked by preincubation of the antibody with the appropriate peptide, but not by preincubation with other peptides. The antibody to the N-terminal peptide recognizes a fusion protein containing the HD N-terminal portion of the protein product when transfected into HEK 293 cells. Second, the localization by immunohistochemistry is only seen in the presence of primary antibody and is blocked by preincubation of the antibody with the peptide antigen. Immunohistochemical labeling with antibody AP78 (though considerably weaker than that with antibody AP81) shows a similar distribution to that of AP81. Third, the cellular pattern of labeling by immunohistochemistry matches that shown by the subcellular fractionation using Western blots. There is very low label in the crude nuclear fraction corresponding to the lack of label in the nuclei by immunohistochemistry. By contrast, Hoogeveen et al. (1993) observed nuclear labeling in many cells and no labeling of nerve terminals, but no preabsorption controls were included. Fourth, the regional pattern of labeling in the brain by immunohistochemistry corresponds to that by Western blot, with higher labeling in cerebral and cerebellar cortex than in caudate and putamen, and low labeling in the brainstem. Fifth, the data in the present studies correspond well to previous studies of IT15 expression using mRNA techniques. Both Northern blots and in situ hybridization data indicated that IT15 mRNA is widely expressed in neurons within the brain, but expressed to a much lower level in glia (Li et al., 1993; Strong et al., 1993). The regional distributions of protein and mRNA are quite similar.

Thus, this study indicates that the HD gene product is expressed as an approximately 350 kDa protein with widespread distribution in both brain and peripheral tissue. Within the brain, this protein is present but not enriched in caudate and putamen, where the pathology is greatest in HD. It is expressed in neurons at moderate to high levels and in glia at a much lower level, if at all. In neurons, it is expressed in cell bodies, dendrites, axons, and terminals, but not in nuclei. It is present in a subpopulation...
of neuronal terminals and preterminal axons, including a population in the caudate and putamen. In HD patients, the HD protein with the expanded glutamine repeat is expressed and has a distinctly different rate of migration on SDS-PAGE than the protein with the normal repeat.

The presence of the HD gene product in soluble as well as particulate fractions is compatible with the hydrophobicity plot of the protein sequence, which shows no hydrophobic domains suggestive of membrane-spanning regions (Huntington's Disease Collaborative Group, 1993). Therefore, the portion in the synaptosomal fraction seen by Western blot might represent a low estimate because soluble proteins might be partially lost from this compartment during homogenization, before the synaptosomal membranes reseal. Furthermore, since immunohistochemistry revealed that not all terminals appear to be labeled to the same degree, the synaptosomal fraction presumably represents a mixture of terminals with relatively high levels of HD protein and terminals with relatively low levels.

The subcellular distribution of the HD protein is similar to that of the recently described p145 (McPherson et al., 1994). This protein is also present in both high speed supernatant and high speed pellet, and moderately enriched in the LP2. Minor differences in the subcellular distribution of p145 shown here and that published by McPherson et al. (1994) are probably due to subtle differences in technique, e.g., in homogenization. Both the HD protein and p145 are enriched in the LP2 fraction but decreased in further purified synaptic vesicles. Both the HD protein and p145 appear to be loosely associated with membranes or cytoskeleton, since they can be partially washed from the pellets with 200 mM or greater salt. By immunohistochemistry, both are present in a generally cytoplasmic distribution in neurons as well as in punctate structures appearing to be nerve terminals, although the HD protein appears to give somewhat more prominent cytoplasmic labeling than p145, as especially seen in the cerebral cortex. Both proteins contain a proline-rich region, though unlike p145 there is no evidence that the HD protein can bind SH3
domains. The function of p145 is currently unknown, but has been hypothesized to be involved with synaptic vesicle endocytosis and recycling.

Because Western blots with AP78 (which recognizes a sequence immediately to the N-terminal side of the triple repeat) showed a band of 350 kDa, the CAG repeat indeed appears to be translated into polyglutamine. In this respect, the HD gene resembles the genes for spinocerebellar ataxia type I (SCA-1; Orr et al., 1993), spinobulbar and muscular atrophy (the androgen receptor; La Spada et al., 1991), Smith's disease or dentato-rubro and pallido-locysian atrophy (DRPLA; atrophin-1; Li et al., 1993; Nagauchi et al., 1994, Margolis et al., 1994, Soc. Neurosci., abstract), and SCA-3 (Kawaguchi et al., 1995), which all have CAG repeats believed to code for glutamine; and differs from those for myotonic dystrophy (Brook et al., 1992) and fragile X syndrome (Verheij et al., 1993), in which the repeats are outside the reading frame. All five of the CAG glutamine repeat diseases are notable for being progressive neurodegenerative disorders. All have selective neuronal vulnerability, but messages that are widely expressed.

The sequence of the HD protein contains one possible leucine zipper, raising the possibility that the protein could function as a dimeric transcription factor. However, the present study indicates that the HD gene product is not present in nuclei. Another hypothesis has suggested that it could be involved in mitochondrial function. However, the 350 kDa band is considerably less dense in the sucrose gradient mitochondrial fraction (Figure 2A) than in other fractions, indicating that the HD gene product is not selectively localized to mitochondria. This observation does not rule out the possible involvement of mitochondrial dysfunction in the pathophysiology (Beal et al., 1993b), but appears to rule out a mitochondrial function for the HD protein. The widespread distribution of the protein in neurons and cells in the periphery suggests that the normal role of the HD protein may be in intracellular functions of common importance. In addition, the concentration in nerve terminals is consistent with a role in nerve terminal function. It has recently been proposed that the HD protein product associates with microtubules, raising the possibility of a role in cytoskeletal function (Steiner et al., 1994, Soc. Neurosci., abstract). Neuronal terminals of the cortico–striatal pathway participate in the generation of excitotoxicity in the striatum (McGeer et al., 1978, Biziare and Coyle, 1979). Further work will be required to determine whether the HD protein is present in these terminals. The expansion of the expanded allele in HD patients is consistent with earlier predictions that HD results not from a loss of function, but rather from a toxic gain of function or conceivably a dominant-negative alteration (Wexler et al., 1987; Ross et al., 1993; Ambrose et al., 1994; Albin and Tagle, 1995). This may involve interactions with other proteins within the cell, as suggested by the transglutaminase hypothesis or possible polar zipper interactions (Green, 1993; Perutz et al., 1994), or may involve interactions with as yet uncharacterized proteins.

**Experimental Procedures**

**Preparation of Antibodies against Synthetic HD Protein Peptides**

Peptide sequences for immunogens were chosen from the HD protein predicted amino acid sequence (Huntington's Disease Collaborative Group, 1993) using hydrophilicity and antigenicity profiles (MacVector sequence analysis program, International Biotechnologies, Inc.). The first peptide corresponded to the first 17 amino acids of the N-terminus, from the initial methionine to the phenylalanine immediately before the glutamine repeat (MATLEKLMAFGLKSF). The second peptide corresponded to amino acids 650–663 (VLDDEATEPDQDEN). The N-terminal peptide was coupled to keyhole limpet hemocyanin (KLH), BSA, and ovalbumin via a C-terminal cysteine that was added to the peptide during synthesis. Coupling was accomplished using sulfo-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC; Pierce) by an adaptation of the methods provided by the manufacturer and methods described for a similar reagent, N-maleimidobenzy-N-hydroxysuccinimide ester (MBS, Harlow and Lane, 1988). Carrier proteins (10 mg) were dissolved in 1 ml of 100 mM sodium phosphate buffer (pH 7.4). Sulfo-SMCC was added (2.5–3 mg) and the mixture was stirred for 1 hr at room temperature. Unreacted sulfo-SMCC was removed by gel filtration using prepacked PD10 Sephadex G25 columns (Pharmacia) pre-equilibrated with 100 mM sodium phosphate buffer (pH 7.2). Column fractions were monitored by absorbance at 280 nm, and the peak corresponding to the void volume was collected in 2 ml. Peptide (6 mg) was suspended in 0.5 ml of degassed 100 mM sodium phosphate buffer (pH 7.2) and brought into solution by the addition of 100 μl of 10% SDS. The clear peptide solution was added to 2 ml (10 mg of protein) of the activated carrier protein solution, and the pH was readjusted to 7.2 by the addition of a small volume of 10% NaOH solution. The mixture was allowed to react overnight at 4°C with stirring. Unconjugated peptide was removed by extensive dialysis, using 50 kDa exclusion limit dialysis tubing (Spectrum), against 0.1% SDS in HBS (20 mM HEPES, 0.9% NaCl) followed by HBS alone. The internal peptide, corresponding to amino acids 650–663, was coupled to bovine thyroglobulin, BSA, and ovalbumin with 0.1% glutaraldehyde essentially as described (Harlow and Lane, 1988). The reaction was stopped by reaction of remaining glutaraldehyde with 100 mM ethanolamine, and free peptide was removed by extensive dialysis against HBS. Peptide–carrier conjugates were assayed for protein and stored at −20°C until used for immunization of rabbits (Cocalico Biologicals, Inc.).

The KLH or thyroglobulin conjugates were used for the first two injections (days 0 and 14), and the BSA conjugates were used for subsequent boosts (days 21 and 51, and each 1–2 months thereafter). Antisera were initially screened by Western blot (Lassen, 1970; Towner et al., 1979) against the ovalbumin–peptide conjugates and shown to react with the peptide used for immunization, but not with other peptides. Antisera were subsequently screened by Western blot against homogenates of human and rat brain. The best antisera were selected for affinity purification of specific antibodies. Antibodies against the N-terminal peptide were affinity purified using an affinity column consisting of the peptide coupled via the C-terminal cysteine to SulfoLink sulfhydryl-reactive agarose resin (Pierce) according to the manufacturer. Antibodies against the internal peptide were affinity purified using the ovalbumin–peptide conjugate immobilized on a solid phase matrix. The ovalbumin conjugate (2.5–10 mg of total protein) was mixed with 2 ml of Affigel-15-activated agarose resin (Bio-Rad) in PBS at 4°C overnight with end over end mixing. After coupling, affinity resins were poured into columns and washed with PBS. Reactive groups remaining on the columns were blocked by reaction with 100 mM glycine overnight at 4°C with end over end mixing. The resins were then poured into columns and washed extensively with PBS. Ovalbumin conjugates of peptides unrelated to the HD gene product were produced using 0.1% glutaraldehyde as for the IT15 peptides and were used to produce affinity matrices using identical methods. Crude antisera (15–20 ml) were first mixed batchwise for 4 hr at 4°C in the presence of 1 mM EDTA and a cocktail of protease inhibitors (1 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, 0.5 μg/ml antipain, 1 μg/ml chymostatin) with the unrelated peptide–ovalbumin
affinity matrices to reduce the concentration of contaminating nonspecific antibodies. The treated antisera was then batched with the appropriate peptide and affinity matrix overnight at 4°C. The affinity matrix was poured into a column and then washed sequentially with 25 ml of 0.1% Triton X-100 in 50 mM Tris-HCl (pH 7.4), 0.9% NaCl, with 25 ml of 50 mM Tris-HCl (pH 7.4), 1 M NaCl, with 25 ml of 50 mM Tris-HCl (pH 7.4), 0.9% NaCl, and finally with 10 ml of 5 mM Tris-HCl (pH 7.4), 0.09% NaCl. Specific antibodies were then eluted with either 0.5 M glycine (pH 2.5), 10% ethylene glycol (AP78), or 4 M MgCl2 (AP81). Fractions (1 ml each) of the eluate containing significant absorbance at 280 nm were pooled and dialyzed extensively against HBSS. Antibodies were then dialyzed into 40% glycerol in the same buffer, collected, and stored at -20°C.

Monoclonal antibodies against synaptophysin were purchased from Boehringer Mannheim, and antibodies against P145 and dynamin were the generous gift of Peter McPherson and Pietro De Camilli.

**Tissue Preparation, SDS-PAGE, and Immunoblot Analyses**

For initial immunochemical characterization of antibodies and analysis of protein distribution, cells or tissues were homogenized in ice-cold 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM 2-mercaptoethanol, with a cocktail of protease inhibitors (0.4 mM PMSF, 1 mM benzamidine, 0.5 μg/ml antipain, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml chymostatin, 1 μg/ml pepstatin A) using a Brinkmann Polytron set at 5 for 20 strokes. Preparations were then washed sequentially with the homogenates at 100,000 × g for 1 hr. All steps were performed at 4°C. Protein was assayed using the Coomassie Plus reagent (Pierce) and subjected to electrophoresis on SDS-polyacrylamide (5%-12% gradient) gels (Laemmli, 1970). Prestained high molecular weight protein standards (Bio-Rad-LRL) were run in adjacent lanes. Because the dye used to label the standards affects their mobility on SDS-PAGE, the manufacturer standardizes each lot. We have reported the apparent molecular masses as indicated by the manufacturer. Proteins were transferred electrophoretically to nitrocellulose membranes (Towbin et al., 1979). Blots were preincubated in 5% nonfat dry milk in PBS for 1 hr at room temperature or overnight at 4°C to block nonspecific sites. Antibodies were diluted in 5% nonfat dry milk or 3% BSA in PBS. Blots were incubated overnight at 4°C with antibody AP78 at a dilution of 1:30,000 (approximately 100 μg/ml) or with antibody AP81 at ~1 μg/ml. For preblockade controls, antibodies were preincubated overnight at 4°C with the appropriate peptide (2 μg/ml). The N-terminal peptide originally synthesized proved to be poorly soluble in aqueous medium. Therefore, a soluble version of the peptide (MATLEKLMKAFESLTFKDDDDDK) was synthesized for use in preblockade controls. Preblockade resulted in complete loss of immunoreactivity in immunobLOTS. Unrelated, nonimmunoreactive. After incubation in antibody, blots were washed for 15 min in 5% nonfat dry milk in PBS, followed by three more washes of 5 min each in the same solution. Blots were then incubated with secondary antibodies (peroxidase-linked, affinity-purified goat anti-rabbit from Boehringer Mannheim) at a dilution of 1:10,000 for 1 hr at room temperature. After three washes (one for 15 min and two for 5 min) in 5% nonfat dry milk in PBS, the blots were washed two more times for 5 min each in 0.1% Tween-20 in PBS. Blots were then developed using the Lumi Glo enhanced chemiluminescence reagent of Kirkegaard and Perry.

**Cell Transfection Experiments**

A construct including the N-terminal third of the IT15 sequence was obtained by screening brain cDNA libraries and PCR using primers based on the published IT15 sequence (Huntington's Disease Collaborative Group, 1993). This was ligated in-frame using BamHI and BglII into the pEVBHIS vector (Invitrogen) to form a vector expressing a fusion protein containing 930 amino acids of HD protein product with 44 glutamine repeats. This vector was transfected into HEK 293 EBNA cells (1×106). Epoetin-Beta virus using lipofectamine transfection according to the instructions of the manufacturer. Blots were prepared from these cells as described above. Immunohistochemical experiments showed dense cytoplasmic labeling of about 1%-2% of transfected cells, with no labeling in nuclei and little labeling in control cells (data not shown).

**Subcellular Fractionation**

Subcellular fractions of rat cerebellum were prepared essentially as described by Gray and Whitaker (1962). Rats were killed by decapitation, and cerebellar were rapidly collected. Cerebella were homogenized in 10-20 ml per gram wet weight ice-cold 0.32 M sucrose in 4 mM HEPES (pH 7.4) and a cocktail of protease inhibitors (0.4 mM PMSF, 0.5 μg/ml antipain, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml chymostatin, 1 μg/ml pepstatin A [homogenization buffer]) using nine strokes of a glass/teflon homogenizer. The homogenate was centrifuged for 10 min at 1000 × g to produce a pellet (P1), which was washed by resuspension in an equal volume of a homogenization buffer and recentrifuged for 10 min at 10,000 × g. The original supernatant combined with the wash (S1) was then centrifuged at 17,500 × g for 20 min to produce a pellet (P2) and a supernatant (S2). The P2 fraction was resuspended in an amount of homogenization buffer equal to the original homogenization buffer and layered on two-step sucrose gradients consisting of 12 ml each of 0.8 and 1.2 M sucrose. The gradients were centrifuged at 25,000 rpm for 2 hr in an SW28 rotor. Three fractions were collected corresponding to material at the 0.32-0.8 M sucrose interface (myelin-enriched fraction), at the 0.8-1.2 M sucrose interface (synaptosomes-enriched fraction), and below 1.2 M sucrose (mitochondria-enriched fraction). The S2 fraction was centrifuged at 100,000 × g for 60 min to produce a high speed pellet (P3) and a high speed supernatant (S3). All steps were performed at 4°C.

Subcellular fractions of rat brain striatum were prepared essentially as described by Hutten et al. (1985). Briefly, rat striata were homogenized in buffered saline (0.3 M sucrose, 4 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.4 mM PMSF, 1 mM benzamidine, 10 mg/ml leupeptin, 10 mg/ml pepstatin, 1 mg/ml aprotinin, 0.5 mg/ml antipain). The homogenate (Homog) was centrifuged for 10 min at 800 × g, yielding a pellet (P1) and supernatant (S1). The supernatant (S1) was collected and centrifuged at 9000 × g for 15 min, yielding a pellet (P2) and a supernatant (S2). S2 was centrifuged at 100,000 × g for 90 min to give pellet (P3) and solubil (S3) fractions. The P2 fraction was washed by resuspension in buffered sucrose and centrifugation for 15 min at 9200 × g. The washed P2 fraction was resuspended in a small volume of buffered sucrose and hypotonic lysed by addition of 10 vol of ice-cold water containing the above protease inhibitors and homogenization in a glass/telphon homogenizer (three strokes). The P2 lysate was poured into a beaker containing 1 ml of 1 M HEPES (pH 7.4), incubated on ice for 30 min, and centrifuged for 20 min at 25,000 × g to yield the lysate pellet (P1) and lysate supernatant (LS1). The LS1 fraction was then centrifuged at 105,000 × g for 2 hr to give a crude synapic vesicle pellet (LP2) and supernatant (LS2). LP2 was resuspended in a small volume of 40 mM sucrose, 4 mM HEPES (pH 7.4) with the above protease inhibitors. SDS-polyacrylamide gels were loaded with equal amounts of protein from each fraction. The resolved proteins had a pI ranging from 3.0 to 10.0, and were detected electrophoretically to nitrocellulose membranes and probed with antibody AP78 as described above.

**Immunohistochemistry**

Brain tissue was obtained from a monkey that had been deeply anesthetized, perfused with 4% paraformaldehyde in 50 mM sodium phosphate buffer (pH 7.3), and postfixed for 4-6 hr in 4% paraformaldehyde in the same buffer. Some tissue blocks were cut into sections immediately (50-60 μm thick) using a Vibratome. Other blocks were cryoprotected by incubation in 20% glycerol in PBS for 2 days at 4°C, frozen on dry ice, and stored at -80°C. Sections of frozen material (40 μm thick) were cut on a sliding microtome. Vibratome and sliding microtome sections were stored in antifreeze solution (50 mM sodium phosphate [pH 7.4], 1% [w/v] PVP-40, 30% [w/v] sucrose, 30% [w/v] ethylene glycol) at -10°C until use. Human cortical material (surgical specimen) was immersed in 10% formalin in PBS for 3 days and then in 20% sucrose in PBS overnight, frozen on dry ice, and stored at -80°C before sectioning (40 μm thickness) on a sliding microtome. Human striatal autopsy tissue was fixed by immersion in 4% paraformaldehyde for 48 hr at 4°C, then cryoprotected by overnight immersion in 20% glycerol in PBS before freezing on dry ice and sectioning on a sliding microtome. Sections were stored at -10°C as above before use. Sections were washed twice for 10 min each in PBS and then permeabilized by incubation for 30 min at room temperature in 0.3% Triton X-100 in PBS with gentle agitation. Sections were then washed in PBS and treated by Immersion in 0.3% H2O2 in methanol for 15 min at room temperature. After rinsing twice (5 min each) in
PBS, sections were incubated for 20 min in 100 mM ammonium bicarbonate in PBS at room temperature to block unreacted aldehydes. Sections were then washed in 50 mM Tris-HCl (pH 7.4), 1.5% NaCl (TBS) before incubation in 5% normal goat serum (NGS) in TBS for 30 min. Affinity-purified antibodies (AP61; 5–10 μg/ml) were diluted in 1% NGS in TBS. For controls, diluted antibodies were preincubated with 2–10 μg/ml free peptide overnight at 4°C before use. Sections were incubated with antibody solutions at 4°C with gentle agitation for 36–48 hr, washed three times in 1% NGS in TBS for 10 min each, and then processed using the Vectastain Elite avidin/biotinyl-peroxidase reagents (Vector). Sections were incubated 1 hr with biotinylated goat anti-rabbit secondary antibody (Vector) in 1% NGS at a dilution of 1:200 for 1 hr at room temperature, washed 10 min in 1% NGS in TBS, followed by three washes of 7 min each in TBS alone, and then incubated with preformed avidin-biotin peroxidase complex (ABC; 1:50 dilution of each reagent) for 1 hr at room temperature in TBS. After washing three times for 10 min each, sections were developed using 0.5 mg/ml diaminobenzidine (DAB) and 0.1% H2O2 in TBS. Sections were then mounted on subbed glass slides, dehydrated in graded ethanol solutions, cleared with xylene, and coverslipped.

Some sections were stained using the biotinyl-tyramide amplification procedure (Adams, 1992; Lee et al., 1993). In this protocol, a low-concentration of primary antibody was used (1–2 μg/ml), and sections were incubated with secondary antibody at a dilution of 1:2,000, washed as in Figure 3, and incubated with ABC (1:400 dilution of each reagent) for 30 min. Sections were then washed three times for 5 min each in PBS and incubated 20 min in 1 μM biotinyl tyramide (BLAST HRP blot kit, NEN/DuPont), 0.005% H2O2 in PBS. Sections were washed three times for 5 min each in PBS and again incubated in ABC (1:400 dilution of each reagent) for 1 hr at room temperature. The sections were washed three times for 5 min each in PBS and then washed briefly in 175 mM sodium acetate buffer before being developed in 25 mg/ml NISO, 0.5 mg/ml DAB, 0.01% H2O2 in 175 mM sodium acetate. Sections were mounted, dehydrated in graded ethanol solutions, cleared with xylene, and coverslipped.

EM Immunohistochemistry
After immunoprocessing vibratome sections as described above (except without Triton X-100) using the biotinyl-tyramide amplification procedure, selected sections of tissue immunostained with antibodies or with antibodies preabsorbed with peptide (control) were rinsed in TBS, then rinsed in a solution containing 6% dextrose, 0.008% CaCl2, and 0.12M sodium phosphate (pH 7.4), and placed in 1% osmium tetroxide for 15 min. The sections were then washed five times for 10 min each in 0.1 M maleate buffer (pH 5.2), stained en bloc in 1% uranyl acetate overnight at 4°C, dehydrated in increasing concentrations of ethanol, infiltrated with Epon, and flat-embedded between two sheets of Acclar (Allied Chemical Co.). Relevant sections were dissected and re-embedded in BEEM capsules for sectioning. Ultrathin sections were collected on Formvar-coated slitted grids, stained with 7% uranyl acetate, and examined with a JEOL 100CX electron microscope. Methods have been previously described in more detail (Ryugo et al., 1991).

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References


