Journal of Neurocytology **33**, 517–533 (2004)

Early changes in Huntington's disease patient brains involve alterations in cytoskeletal and synaptic elements

NICHOLAS A. DIPROSPERO[1,2,*,†] ER-YUN CHEN[3,†], VINOD CHARLES[2], MARKUS PLOMANN[4], JEFFREY H. KORDOWER[3] and DANILO A. TAGLE[2]

[1]*Neurogenetics Branch, National Institute of Neurological Disorders and Stroke, NIH, 9000 Rockville Pike, Bethesda, MD 20892, USA;* [2]*National Human Genome Research Institute, NIH, 49 Convent Drive, Bethesda, MD 20892, USA;* [3]*Rush-Presbyterian Medical Center, Chicago, IL 60612, USA;* [4]*Institute for Biochemistry II and Center for Molecular Medicine Cologne (CMMC), University of Cologne, Joseph-Stelzmann-Str. 52, D-50931 Cologne, Germany*
diprospern@ninds.nih.gov

Received 10 May 2004; revised 8 November 2004; accepted 15 November 2004

Abstract

Huntington's disease (HD) is caused by a polyglutamine repeat expansion in the N-terminus of the huntingtin protein. Huntingtin is normally present in the cytoplasm where it may interact with structural and synaptic elements. The mechanism of HD pathogenesis remains unknown but studies indicate a toxic gain-of-function possibly through aberrant protein interactions. To investigate whether early degenerative changes in HD involve alterations of cytoskeletal and vesicular components, we examined early cellular changes in the frontal cortex of HD presymptomatic (PS), early pathological grade (grade 1) and late-stage (grade 3 and 4) patients as compared to age-matched controls. Morphologic analysis using silver impregnation revealed a progressive decrease in neuronal fiber density and organization in pyramidal cell layers beginning in presymptomatic HD cases. Immunocytochemical analyses for the cytoskeletal markers α-tubulin, microtubule-associated protein 2, and phosphorylated neurofilament demonstrated a concomitant loss of staining in early grade cases. Immunoblotting for synaptic proteins revealed a reduction in complexin 2, which was marked in some grade 1 HD cases and significantly reduced in all late stage cases. Interestingly, we demonstrate that two synaptic proteins, dynamin and PACSIN 1, which were unchanged by immunoblotting, showed a striking loss by immunocytochemistry beginning in early stage HD tissue suggesting abnormal distribution of these proteins. We propose that mutant huntingtin affects proteins involved in synaptic function and cytoskeletal integrity before symptoms develop which may influence early disease onset and/or progression.

Introduction

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder that affects 4-10/100,000 individuals usually in mid-adulthood (Harper, 1992). This progressive disease is clinically characterized by disordered movement, psychiatric disturbances, and dementia and is usually fatal within approximately 10 to 15 yrs of onset. HD is a member of a growing family of trinucleotide repeat expansion disorders (Paulson & Fischbeck, 1996; Zoghbi & Orr, 2000). The mutation in HD is a CAG repeat expansion in the coding region of exon 1 of the *IT15* gene that results in an expanded stretch of polyglutamines in its protein product, huntingtin (Huntington's Disease Research Collaborative Group, 1993). While mutant huntingtin is widely expressed in both non-neuronal and neuronal tissues (Strong *et al.*, 1993; Sharp *et al.*, 1995), the pathological hallmark is preferential loss of neurons in the striatum and to a lesser degree in the cortex (Mann *et al.*, 1993). Early postmortem studies of HD brain tissue revealed variability in the degree of neuropathological involvement in the striatum. This observation led to the development of a five-tiered pathological grading system (grades 0–4) that was based upon the degree of neuronal loss and atrophy in the striatum; grade 0 contains no demonstrable cell loss, grade 1 has approximately 50% cell loss, and progresses to 95% neuronal loss in

* To whom correspondence should be addressed.
† These authors contributed equally to this work.

0300–4864

grade 4 cases (Vonsattel *et al.*, 1985). The pathological grade closely correlates with the clinical severity in that HD patients with higher pathological grades noted at autopsy had a longer duration of disease and greater disability.

Based on the clinical manifestations of HD and the marked loss of medium spiny neurons, research has primarily focused on the striatal degeneration in HD. However, several lines of evidence suggest cortical involvement early in the disease. Human postmortem studies have revealed neuronal loss within the cortex (de la Monte *et al.*, 1988) that is specific for the large pyramidal neurons in layers 3, 5, and 6 (Sotrel *et al.*, 1991). The extent of cortical neuronal loss does not correlate with the grade of striatal atrophy (Cudkowicz & Kowall, 1990), suggesting that the loss of cortical neurons is not a consequence of loss of trophic support from target cells. In fact, the converse seems to be the case where loss of functional huntingtin results in decreased levels of cortically supplied brain-derived neurotrophic factor which in turn causes striatal neuron degeneration (Zuccato *et al.*, 2001). This implies that the corticostriatal pathway may play a primary role in HD pathogenesis. Indeed, in a transgenic mouse model of HD, cortical changes which include accumulation of huntingtin in the nucleus and cytoplasm along with the appearance of dysmorphic dendrites predicts disease onset and severity of symptoms (Laforet *et al.*, 2001). This is particularly interesting since in human postmortem tissue nuclear inclusions containing mutant huntingtin are more abundant in the cortex than in the striatum regardless of the extent of striatal pathology, and in low grade cases inclusions are noted in the cortex but not in the striatum (Sapp *et al.*, 1999). Moreover, numerous dystrophic axons projecting to the striatum contain heavy accumulations of mutant huntingtin (DiFiglia *et al.*, 1997) and Golgi impregnation studies of cortical neurons also show abundant dendritic abnormalities (Sotrel *et al.*, 1993). These biochemical and neuroanatomical findings of early cortical involvement are also congruent with clinical findings. Cognitive and behavioral disturbances that are related more to cortical function manifest early in the course of the disease often prior to motor deficits (Foroud *et al.*, 1995; Lawrence *et al.*, 1998). Thus, cortical neuronal dysfunction may underlie the initiation of the HD phenotype.

Mechanisms of neuronal dysfunction and degeneration remain unclear. Several lines of evidence support that the expanded polyglutamine stretch in the huntingtin proteins imparts a toxic gain-of-function (*i.e.* novel function) as well as a loss-of-function directly through the mutation and indirectly by sequestration (*i.e.* dominant negative) of wildtype huntingtin (Suhr *et al.*, 2001). Wild-type huntingtin is widely distributed throughout the cytoplasm and in neuronal processes (Trottier *et al.*, 1995) and appears to have numerous interacting and target proteins (Harjes & Wanker, 2003). Immunohistochemistry has localized huntingtin to vesicle membranes and microtubules (Gutekunst *et al.*, 1995). Further studies have demonstrated an interaction between huntingtin and a number of proteins involved in endocytosis and organelle transport in cell culture (Velier *et al.*, 1998). This is particularly interesting in light of the fact that huntingtin has been shown to be anterogradely and retrogradely transported in the rat sciatic nerve (Block-Galarza *et al.*, 1997). Mutant huntingtin has also been found in neuronal processes and axonal terminals in postmortem tissue (DiFiglia *et al.*, 1997) and was noted to bind to synaptic vesicles and to prevent uptake of the neurotransmitter glutamate *in vitro* (Li *et al.*, 2000). Thus, disruption of cellular systems involved with cell structure and vesicular trafficking may underlie cortical dysfunction. In an effort to identify early changes involved in cortical pathogenesis of HD, we compared early stage, consisting of presymptomatic (asymptomatic individuals carrying the gene mutation) and grade 1, and late stage (grade 3 and 4) human postmortem cortical tissue for alterations in cytoskeletal and synaptic elements.

Materials and methods

MATERIALS

Antibodies utilized in these experiments were obtained from the following sources: mouse anti-neuronal nuclear antigen (NeuN), mouse anti-synaptobrevin, and mouse anti-synaptophysin from Chemicon (Temecula, CA); mouse anti-clathrin heavy chain, mouse anti-complexin 2, mouse anti-dynamin, mouse anti-Rab3A, mouse anti-Rab5, mouse anti-α/β SNAP, and mouse anti-synaptotagmin from BD Biosciences (Lexington, KY); mouse anti-microtubule associated protein 2, MAP2, (SMI-52) and mouse anti-phosphorylated neurofilament (SMI-35) from Sternberger monoclonals (Lutherville, MD); mouse anti-NSF from Exalpha (Boston, MA); mouse anti-SNAP 25 from Oncogene Research Products (Boston, MA); mouse anti-syntaxin from Calbiochem (San Diego, CA); and mouse anti-α tubulin and mouse anti-β tubulin from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-PACSIN 1 antibodies were previously described (Plomann *et al.*, 1998). All other materials and reagents were obtained from Sigma Chemical Co. (St. Louis, MO) and Invitrogen (Carlsbad, CA) except where noted.

HUMAN TISSUE

Formalin-fixed human cortical tissues from Brodmann area 9 used for histology (Table 1) were obtained from the Harvard Brain Tissue Resource Center (McLean Hospital, Boston, MA) in accordance with institutional guidelines (NIH, Office of Human Subjects Research). Specimens were matched for age, postmortem interval (PMI), and divided into groups based upon information from the Harvard Brain Tissue Resource Center: (a) controls ($n = 4$, age $= 60 \pm 11$ yrs, PMI $= 16 \pm 5$ h); (b) presymptomatic HD gene carriers ($n = 2$) and grade 1 ($n = 3$) HD cases (age $= 74 \pm 10$ yrs, PMI $= 18 \pm 9$ h); (c) grade

Table 1. Characteristics of formaldehyde-fixed brain tissue from Brodmann area 9*.

Case number	*Age (years)*	*Gender*	*Post-mortem interval (hours)*	*Grade (based on striatal pathology)*	*Cortical cytoarchitecture*	*Cortical neuronal density*
B4354	68	F	15	Normal	NR	NR
B4379	50	M	15	Normal	Normal	Normal
B4389	70	M	23	Normal	Normal	Normal
B4077	50	M	10	Normal	Normal	Normal
B1632	60	F	30	Presymptomatic	Normal	Normal
B3214	83	M	7	Presymptomatic	Normal	Normal
B3914	75	F	11	1	Normal	Normal
B4296	68	M	22	1	Normal	Normal
B4316	95	F	21	1	Normal	Normal
B4404	72	M	8	3	Normal	Decreased
B4351	78	M	41	3	Normal	Decreased
B4381	55	F	24	4	Normal	Decreased
B4386	55	M	16	4	Normal	Decreased

*The information provided is based upon autopsy and neuropathological reports from donating institutions; NR = not reported.

Table 2. Characteristics of frozen brain tissue from Brodmann area 9*.

Case number	*Age (years)*	*Gender*	*Post-mortem interval (hours)*	*Grade (based on striatal pathology)*	*Cortical cytoarchitecture*	*Cortical neuronal density*
B4213	69	M	10	Normal	Normal	Normal
B4180	47	M	9	Normal	Normal	Normal
B4030	81	F	12	Normal	Normal	Normal
B0166	27	M	12	Presymptomatic	NR	NR
B3214	83	M	7	Presymptomatic	Normal	Normal
VU01	28	M	20	Presymptomatic	NR	NR
B4296	68	M	22	1	Normal	Normal
B1736	49	F	21	1	NR	NR
B3756	63	F	8	1	Normal	Normal
B4424	67	F	22	3	Normal	Decreased
B4404	72	M	8	3	Normal	Decreased
B4356	61	F	19	4	Normal	Decreased

*The information provided is based upon autopsy and neuropathological reports from donating institutions; NR = not reported.

3 ($n = 2$) and grade 4 ($n = 2$) HD cases (age $= 65 \pm 12$ yrs, PMI $= 22 \pm 14$ h).

Additionally, frozen blocks of human cortical tissue used for Western blotting (Table 2) were obtained from the Harvard Brain Tissue Resource Center and Vanderbilt University Medical Center. The specimens were divided into groups: (a) controls ($n = 3$, age $= 65 \pm 16$ yrs, PMI $= 10 \pm 2$ h); (b) presymptomatic HD gene carriers ($n = 3$, age $= 46 \pm 31$ yrs, PMI $= 13 \pm 6$ h); (c) grade 1 ($n = 3$, age $= 60 \pm 10$ yrs, PMI $= 13 \pm 7$ h) HD cases; (d), grade 3 ($n = 2$), and grade 4 ($n = 1$) HD cases (age $= 67 \pm 6$ yrs, PMI $= 16 \pm 7$ h).

Each sample was examined by a neuropathologist who provided information regarding cytoarchitecture and neuronal density (listed in Tables 1 and 2). Additionally, the neuropathologist commented on the presence of neurofibrillary tangles and neuritic plaques which were reported to be very rare, age related, and not to the level seen in Alzheimer's disease. The mutant alleles in the HD cases ranged from 45 to 50 CAG repeats. These specimens were genotyped and utilized in previous experiments in this lab (Guidetti *et al.*, 2001). Images shown are representative of each experimental group based upon blinded observation.

IMMUNOFLUORESCENCE

Blocks of frontal cortex from Brodmann area 9 were cut from the tissue specimens and cryoprotected by immersion in 0.1 M phosphate buffered saline (PBS), pH 7.4, containing 30% (w/v) sucrose for 48 h. The blocks of tissue were frozen on dry ice and sections were cut on a sliding knife microtome (25 μm thick) and stored in PBS at 4°C until time of use. Sections were washed with PBS containing 0.1% Triton-X100 (PBS-T) and background staining was blocked with a one-hour incubation in PBS-T containing 10% normal serum. Subsequently, the sections were incubated with primary antibodies (mouse anti-SMI52, 1:10,000; mouse anti-SMI35, 1:10,000; mouse anti-αttubulin, 1:100) diluted in PBS with 5% normal serum and 0.4% Triton-X 100 overnight at room temperature. Sections were then extensively washed with PBS-T and incubated with secondary-fluorochrome conjugated antibodies (Cy2

conjugated goat anti-mouse IgG, 1:400; Jackson Immunoresearch Labs, West Grove, PA) diluted in PBS-T for 1 h at room temperature. The tubulin signal was amplified by incubation with a biotinylated antibody followed by incubation with a Cy2-conjugated strepavidin antibody. Sections were washed with PBS-T and incubated with 5 mM CuSO4 in 50 mM ammonium acetate buffer (pH 5.0) for 1.5 h to eliminate background from intrinsic autofluorescent pigments (Schnell *et al.*, 1999). Following several washes in PBS, sections were mounted on gelatin-coated slides, dehydrated through graded alcohols, cleared in xylene, and coverslipped with DPX. The sections were examined using a Zeiss laser scanning confocal microscope and observers were blinded to disease status. Captured digital images were exported and printed using Adobe Photoshop 5.5 software (San Jose, CA).

IMMUNOHISTOCHEMISTRY

Antigen retrieval was performed by incubating the sections in a 10 mM sodium citrate solution (pH 9.0) preheated to and maintained in an 80°C water bath for 30 min (Jiao *et al.*, 1999). Following several washes in the Tris-buffered saline (TBS), pH 7.4, solution containing 0.05% Triton X-100 (TBS-T), sections were incubated for 20 min in a TBS solution containing 0.1 M sodium periodate to eliminate endogenous peroxidase activity. After 3 washes in TBS-T, background staining was then inhibited by a one hour incubation in 5% normal serum and 2% bovine serum albumin (BSA), after which the primary antibodies at the appropriate dilution (mouse anti-NeuN, 1:200; mouse anti-dynamin, 1:1000; rabbit anti-PACSIN 1, 1:5000) were applied overnight at room temperature in a 0.1 M sodium phosphate buffer saline solution containing 0.4% Triton X-100 and 3% normal horse serum. After 6 washes in TBS-T sections were then sequentially incubated in the biotinylated horse anti-mouse IgG (Vector labs, Burlingame, CA; 1:200) for 1 h and the avidin-biotin complex (ABC Elite Kits, Vector labs; 1:500) for 75 min separated by 3 washes in TBS-T. The chromogen solution that completed the reaction consisted of 0.05% 3′3-diaminobenzidine (DAB) and 0.005% H_2O_2. Sections were mounted on gelatin-coated slides, dehydrated through graded alcohol, cleared in xylene, and coverslipped with Permount.

BIELSCHOWSKY SILVER STAIN

Blocks of formalin-fixed human cortical tissue were also processed for paraffin embedding. Sections cut at 8 μm were mounted on poly-L-lysine coated slides and processed as follows by Molecular Histology Labs, Inc. (Montgomery Village, MD). The sections were deparaffinized and rehydrated through a graded series of alcohols. The sections were then washed and incubated in 18% silver nitrate for 15 min at 37°C in the dark. After washing the sections in ddH_2O for 10 min, the sections were transferred to an ammoniacal silver solution for 10 min at 37°C in the dark. Afterwards, the sections were transferred to a 1% ammonium hydroxide for 3 min and then ammoniacal silver solution mixed with developing solution. Sections are checked microscopically for fiber development, and then washed in 1% ammonium hydroxide followed by ddH_2O. The slides were then placed in 5% sodium thiosulfate for 5 min and rinsed off with ddH_2O. The sections were then dehydrated through a graded series of alcohols, cleared in xylene and mounted with glass coverslips using Permount.

NISSL STAINING

Paraffin embedded sections adjacent to those processed for silver staining were deparaffinized and rehydrated through a graded series of alcohols. The sections were incubated in cresyl violet acetate (1%, pH 3.0) for 5 min at room temperature to stain for Nissl substance. Afterwards the sections were rinsed with ddH_2O, dehydrated through a graded series of alcohols, cleared in xylene and mounted with glass coverslips using Permount.

WESTERN BLOTTING

Pieces of frozen cortical tissue were immersed in liquid nitrogen and freeze-fractured using a BioPulverizer™ (Biospec Products, Bartlesville, OK). Homogenization buffer consisting of 25 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1% SDS, and protease inhibitors (mini complete, Boehringer Mannheim, Mannheim, Germany) was added to the powdered tissue at 1:10 w/v. The samples were sonicated on ice for 10 s three times. The samples were then centrifuged at 14,000 g for 10 min at 4°C to remove any particulate matter. The supernatant was transferred to another tube and protein concentrations were determined using the Pierce BCA protein assay (Rockford, IL). Laemmli buffer (BioRad, Hercules, CA) was added to the remaining extract and the sample was heated to 95°C for 5 min. Equal protein amounts of cell extract (20 μg total protein) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose. The blots were then incubated in blocking solution containing 5% non-fat dry milk in PBS containing 0.1% Tween 20 (PBS-T) for 1 h at room temperature. The blocking solution was then removed and the blot was incubated overnight at 4°C in a solution of primary antibody diluted as indicated in PBS-T (anti-clathrin, 1:1000; anti-complexin 2, 1:500; anti-dynamin, 1:2500; anti-PACSIN 1, 1:5000; anti-rab3A, 1:2500; anti-rab5, 1:500; anti-α/β SNAP, 1:5000; anti-SNAP25, 1:5000; anti-synaptobrevin 1, 1:1000; anti-synaptophysin, 1:1000; anti-synaptotagmin, 1:1000; anti-syntaxin, 1:2500). The blots were then washed five times in PBS-T and incubated with either anti-mouse IgG or anti-rabbit IgG horseradish peroxidase-conjugated secondary antibodies (Amersham, Buckinghamshire, England) diluted 1:5000 in PBS-T for 1 h at room temperature. The blots were then washed as above and the incubated with chemiluminescent detection reagents (ECL-Plus, Amersham, Buckinghamshire, England) for 5 min at room temperature. The blot was exposed to autoradiography film (X-OMAT, Kodak, Rochester, NY) for varying amounts of time to visualize the labeled protein. The immunoblots were scanned at 600 dots per inch and 8 bits/channel using an Arcus II AGFA scanner. Relative levels of protein expression were determined by analyzing the pixel intensity of the bands using NIH Image (Version 1.62) and normalized as a percentage of control. The results from three independent sets of tissues (each set consisting of a control, presymptomatic, grade 1, and a grade 3 or 4 specimen) were pooled and examined by one-way ANOVA and Fisher's PLSD for statistical significance.

Results

Examination under darkfield optics of tissue sections from normal human cortex (Brodmann area 9) after silver impregnation revealed a dense meshwork of interdigitating neuronal fibers throughout all layers. Neuronal processes emanating from pyramidal cells in layers 5 and 6 displayed an organized network of fiber bundles running in parallel to one another (Fig. 1A). In contrast, a reduction in fiber density and organization of pyramidal cells in layers 2/3 and 5 was observed in presymptomatic (Fig. 1B) and grade 1 disease cases. A further loss of silver stained fibers was seen in grade 4 cases (Fig. 1C). Previous imaging studies failed to observe degeneration in the frontal cortex of presymptomatic gene carriers (Harms *et al.*, 1997) and even in mildly affected HD patients (Aylward *et al.*, 1998). To examine whether this change in fiber density may be due to cell loss, adjacent sections were stained for Nissl substance and neurons using a specific antibody (NeuN). There was no qualitative loss of cells as revealed by Nissl staining (Fig. 1D and E) or a specific loss of neurons as determined by NeuN staining (Fig. 1G and H) in the same areas that displayed fiber changes in presymptomatic and grade 1 HD tissue, which is in agreement with the neuropathology reports from the donating institutions (Table 1). Moreover, it should be noted that there does appear to be some cell loss by Nissl staining (Fig. 1F) and NeuN staining (Fig. 1I) in late stage HD tissue which is consistent with the pathology information and data previously reported (Sotrel *et al.*, 1991).

Since cytoskeletal proteins underlie neuronal process growth and morphology, we performed immunohistochemistry to visualize phosphorylated neurofilament, microtubule-associated protein 2 (MAP2), and α-tubulin in sections of control and HD cortical tissues. The SMI 35 antibody reacts with neurofilaments (primarily heavy and medium chains) of both high and moderate degrees of phosphorylation. Staining of cortical tissue from control cases using SMI 35 and a fluorescent secondary antibody revealed intense labeling of fibers throughout all layers of the cortex (Fig. 2A). Higher magnification of the upper layers revealed a rich network of neuronal processes (Fig. 2B). In contrast, presymptomatic tissues did not label as intensely, especially in the upper layers of the cortex (Fig. 2C). Closer examination revealed the robust fiber density was replaced by scattered immunopositive fibers (Fig. 2D). This loss of staining appeared to be progressive, since the grade 4 tissues displayed a further decrease in labeling that involved all cortical layers (Fig. 2E) with only a few distinct fibers noted at higher magnification (Fig. 2F).

Microtubules, which serve a multiplicity of functions, are composed of heterodimers of α and β tubulins. Staining for α-tubulin in control brain sections revealed a diffuse staining pattern (Fig. 3A), which was most intense in the upper layers (Fig. 3B). Examination of presymptomatic tissue showed a modest reduction in intensity of labeling in the upper layers (Fig. 3C and D), whereas, staining in grade 4 tissue was markedly reduced (Fig. 3E) with a more punctate appearance at higher magnification (Fig. 3F). These results were also confirmed and appeared more dramatic using the avidin-biotin system of staining most likely because of its amplification step (data not shown) but we chose to show similar staining methods for all cytoskeletal proteins as well as the more conservative image.

Microtubule-associated proteins (MAPs) regulate the polymerization, bundling, and stability of microtubules. MAPs are differentially distributed with MAP2 found almost exclusively in perikarya and dendrites. Staining for MAP2 using SMI 52 in control tissue revealed uniform pyramidal cell body staining with well-labeled apical and basilar dendritic processes (Fig. 4A and B). However, staining of presymptomatic tissue demonstrated a complete loss of dendritic labeling (Fig. 4C). Moreover, the soma staining did not appear uniform, exhibiting a polarized appearance with the staining accumulating disproportionately within the cell (Fig. 4D). This pattern was observed in grade 4 tissues as well. There was no obvious dendritic staining in the grade 4 tissues, and there was a further reduction in overall cell body staining (Fig. 4E and F).

Three sets of protein lysates from human cortical tissue were analyzed for expression of synaptic elements involved in neurotransmitter release and reuptake. Each set contained a control, one presymptomatic case, a grade 1 case, and a grade 3 or 4 case, which were matched as best as possible for age and post mortem interval. Proteins examined included the synaptic membrane protein synaptobrevin (v-SNARE) and the plasma membrane proteins syntaxin and SNAP-25 (t-SNARE), which collectively make up the fusion machinery known as the SNARE complex. We also examined proteins involved in SNARE complex dynamics (NSF, α/β-SNAP, rab3A, rab 5, synaptotagmin, synaptophysin, and complexin 2) as well as some involved in endocytosis (clathrin, dynamin, and PACSIN 1). The expression of almost all the proteins appeared similar across all samples with the exception of complexin 2 (Fig. 5); semi-quantitative analysis confirmed these results (Table 3). While the expression of complexin 2 appeared decreased in some early stage HD tissues, semi-quantitative analysis only demonstrated a significant reduction in protein levels in late-stage tissues. However, it is possible that a larger sample size may be able to discriminate whether a reduction in complexin 2 occurs in early stage HD and thus may be involved with the etiology of the disease.

Although the levels of almost all synaptic proteins were unchanged, it is possible that the cytoskeletal perturbations observed could alter the localization of these proteins. To examine this possibility, we performed

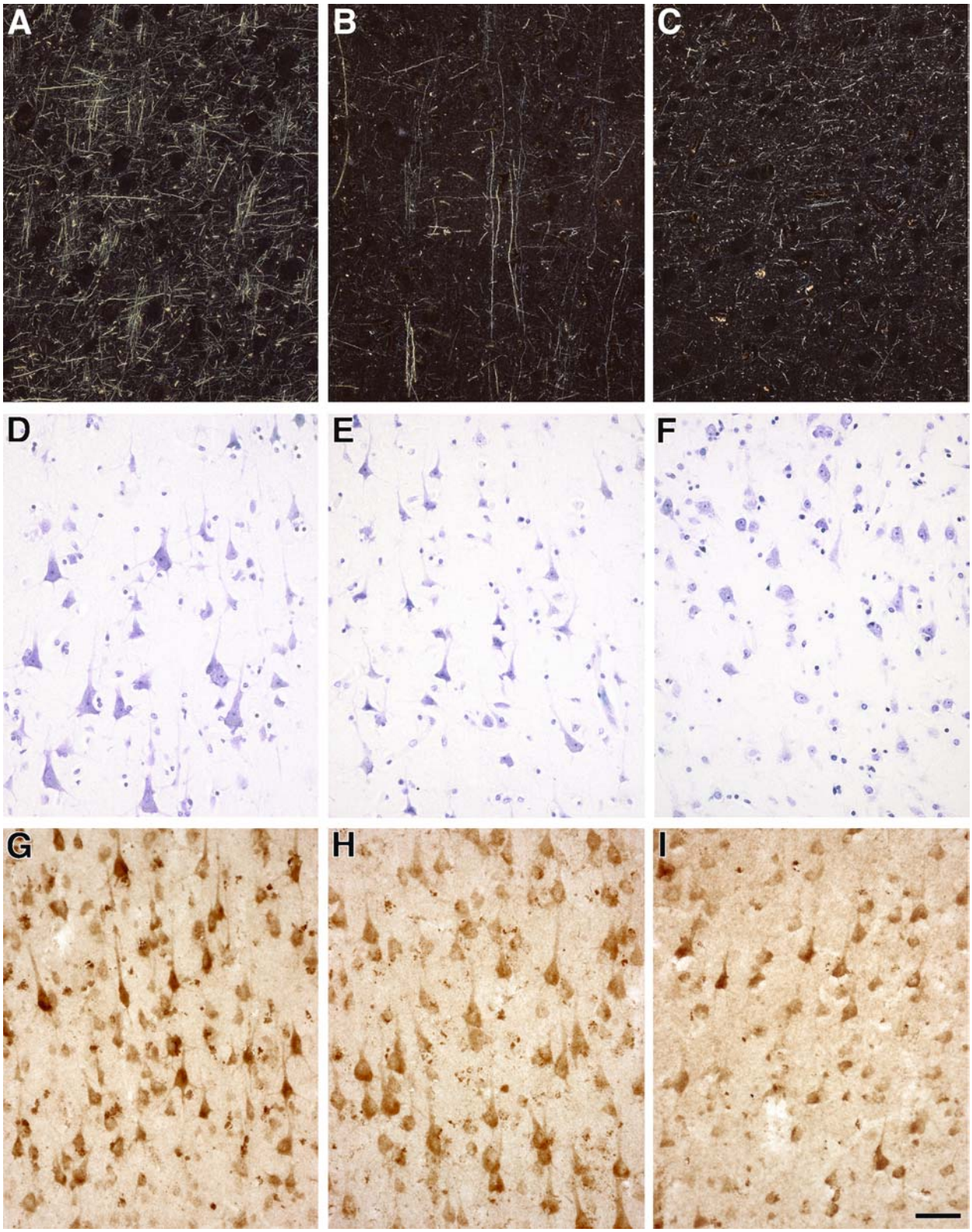

Fig. 1. Loss of nerve fiber density in early stage HD cases. Silver staining of nerve fibers in control human cortical tissue (formaldehyde fixed, paraffin embedded, 8 μm thick sections) examined by darkfield optics reveals a dense meshwork of fibers in layer 5 (A) that is diminished in presymptomatic cases (B) and more noticeable in grade 4 tissue (C). The loss of fiber density is not likely due to a loss of cells in this area since serial sections of tissue stained for Nissl substance and an antibody specific for neuronal cells (NeuN) reveals an approximately equal cell density between presymptomatic (E and H) and control cases (D and G). However, Nissl staining of grade 4 tissue reveals shrunken cells with less intense staining (F) and a noticeable loss of neurons (I) in agreement with previous observations. Bar = 50 μm for all panels.

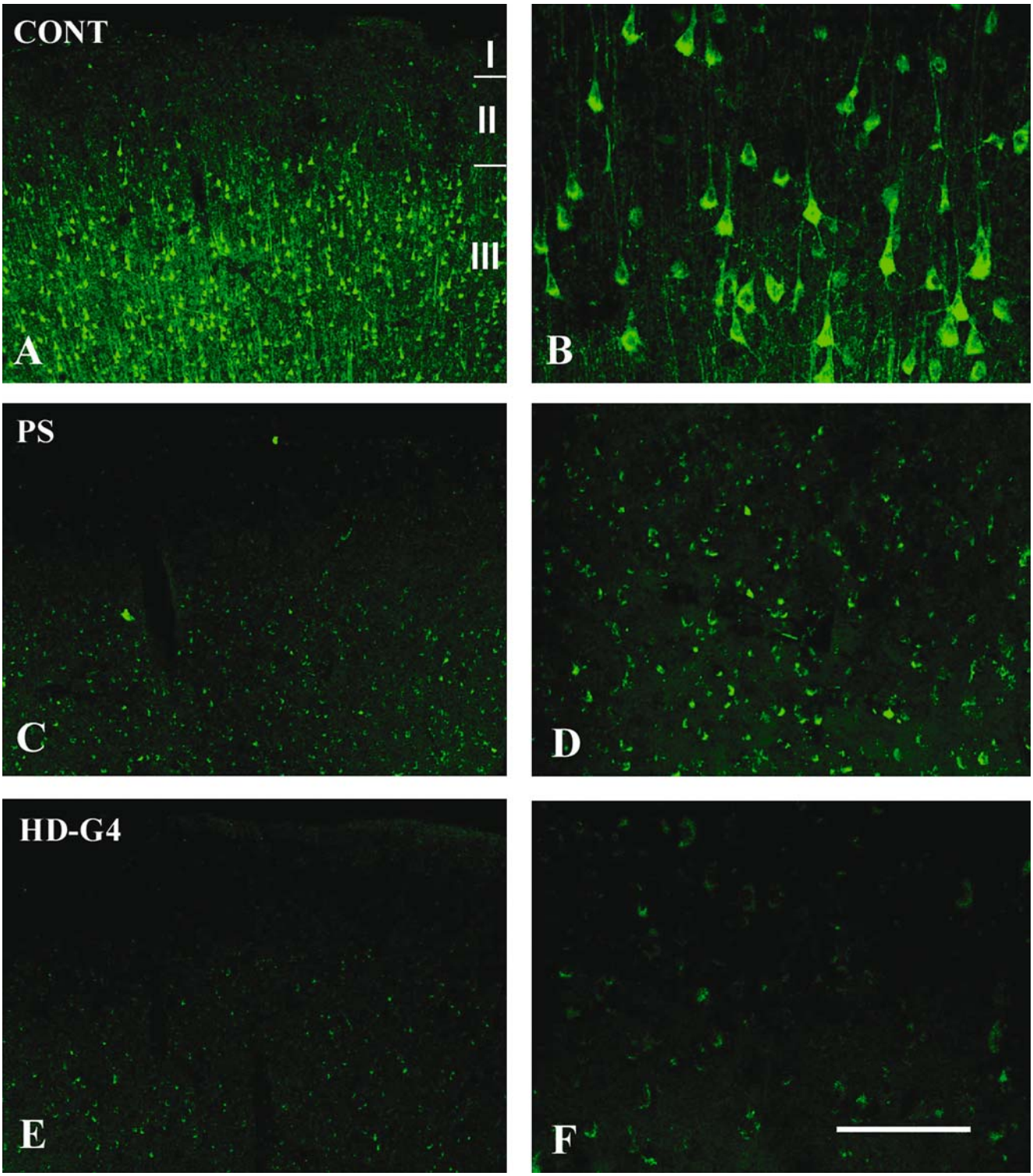

Fig. 2. Progressive loss of neurofilament staining. Sections of human cortical tissue (formaldehyde fixed, 25 μm thick, free-floating sections) were immunostained with SMI 35 and examined using immunofluorescence confocal microscopy. Control tissue shows intense staining of neurofilaments throughout all layers of the cortex (A) with dense networks of fibers noted at higher magnification (B). Staining of presymptomatic tissue shows a reduction in the intensity of labeling mostly in the upper layers of the cortex (C) with fewer immunopositive fibers (D). Late-stage HD tissue is almost devoid of staining (E) with even fewer labeled fibers (F). Bar = 800 μm (A, C, and E); Bar = 200 μm (B, D, and F).

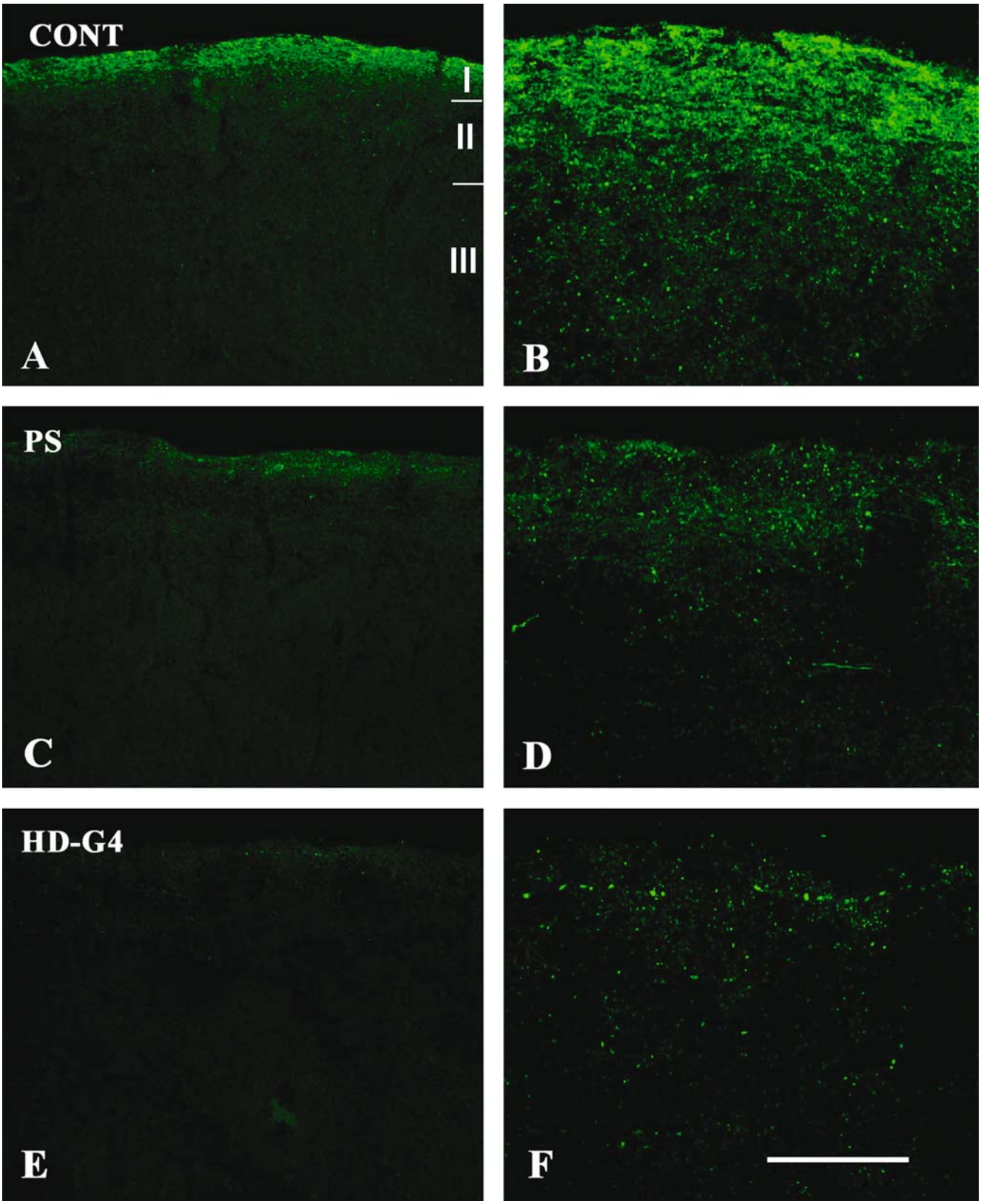

Fig. 3. Decreased tubulin staining in HD cases. Sections of human cortical tissue (formaldehyde fixed, 25 μm thick, free-floating sections) were immunostained with anti-tubulin antibodies and viewed using immunofluorescence confocal microscopy. Examination of control tissue reveals intense staining mostly in layers 1 and 2 (A and B; higher magnification) that is diminished in presymptomatic tissue (C and D) and further reduced in grade 4 tissue (E and F). Bar = 800 μm (A, C, and E); Bar = 200 μm (B, D, and F).

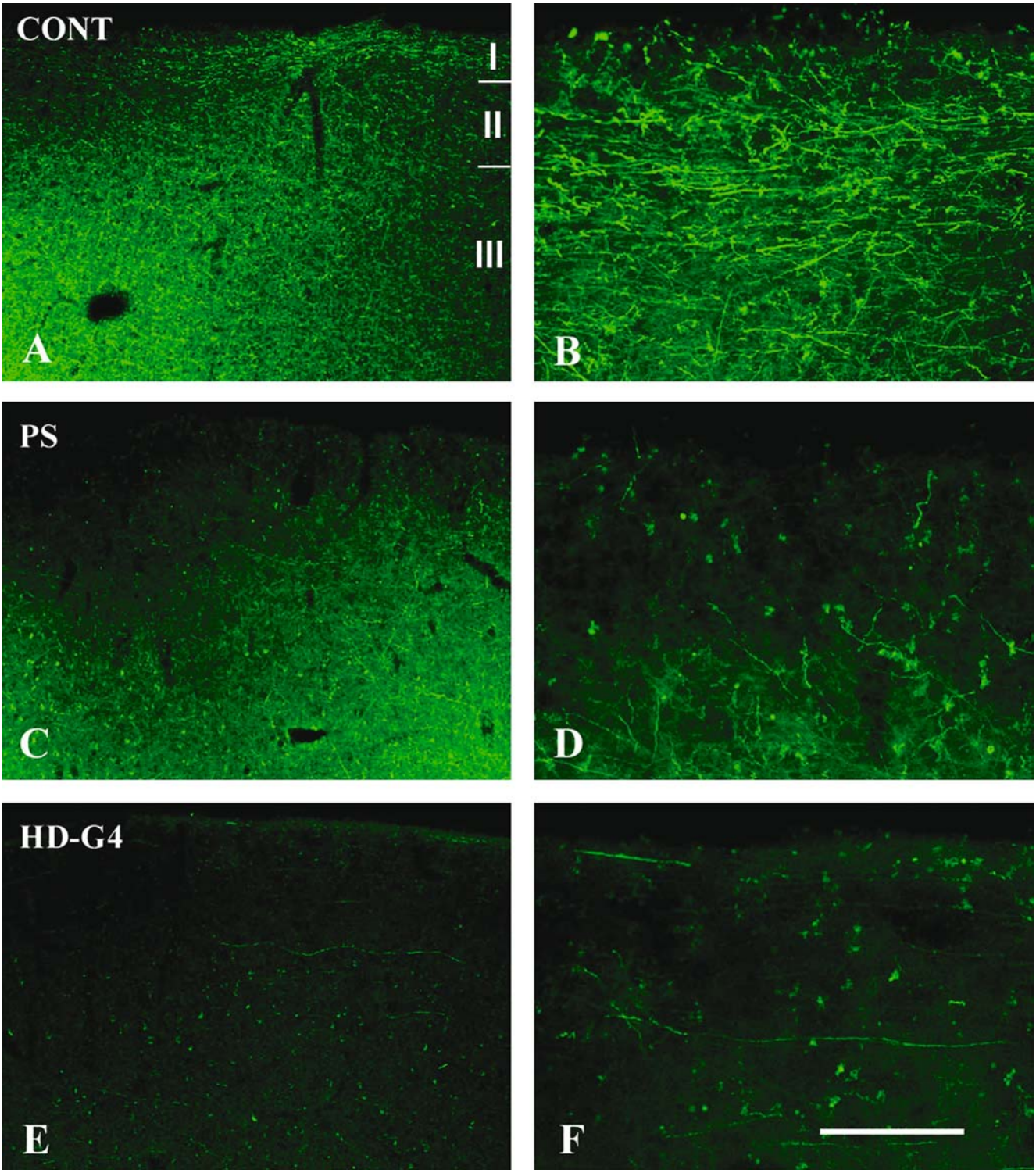

Fig. 4. MAP2 staining is lost in early stage HD cortex. Sections of human cortical tissue (formaldehyde fixed, 25 μm thick, free-floating sections) were immunostained with SMI 52 and examined using immunofluorescence confocal microscopy. In control tissue, MAP2 staining is robust and noted in the pyramidal cell layers of the cortex (A) with intense labeling of cell bodies and dendrites (B). Examination of presymptomatic tissue demonstrates a striking loss of overall staining (C) with complete loss of dendrite labeling and patchy staining within the soma (D). The intensity of MAP2 staining was further reduced in grade 4 tissue (E) with only slight, eccentric staining in the cell body (F). Bar = 800 μm (A, C, and E); Bar = 200 μm (B, D, and F).

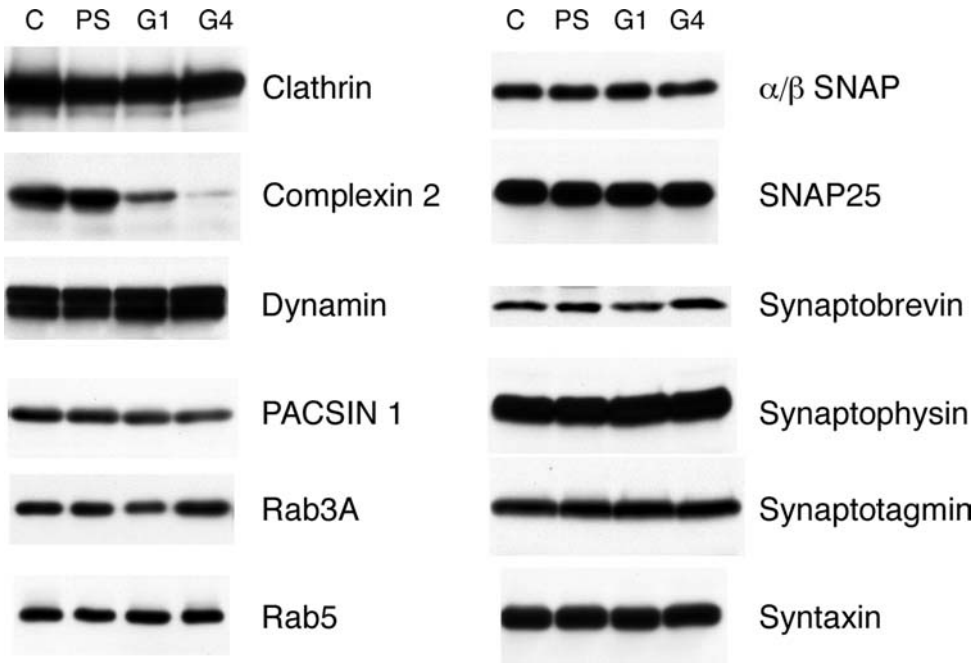

Fig. 5. Levels of synaptic proteins in HD cortical tissue. In one representative experiment of three independent experiments, protein extracts from homogenized cortical tissue were resolved by SDS-PAGE, transferred to nitrocellulose and probed for the proteins listed. Bound antibodies were visualized using HRP-conjugated secondary antibodies and chemiluminescent detection reagents. The band intensity between control (C), presymptomatic (PS), grade 1 (G1), and grade 3/4 (G4) appears relatively uniform for all proteins examined with the exception of complexin 2 which shows a modest reduction in early grade cases and a more striking loss in late stage tissues.

immunohistochemistry with antibodies directed against a number of synaptic proteins on human cortical sections using the avidin-biotin staining method. Staining for SNAP-25 and syntaxin was diffuse and homogenous throughout all layers. Staining for these proteins did not differ between samples and was only slightly more intense than background staining despite antigen retrieval (data not shown). In contrast, staining for both dynamin and PACSIN 1 showed diffuse immunoreactivity throughout all layers of the cortex that appeared to involve both cell bodies and processes (Fig. 6A and C respectively). Interestingly, there was a striking loss of this staining pattern in early stage HD specimens. Dynamin immunoreactivity which stained mostly processes demonstrated a progressive loss beginning in the deeper cortical layers of presymptomatic cases (Fig. 6D). PACSIN 1 showed a more dramatic loss of staining in the presymptomatic tissues where there appeared to be a global loss of staining throughout all layers of the cortex in both cell bodies and processes (Fig. 6B). Thus, while the expression levels of these proteins appeared unchanged, immunohistochemistry revealed a significant change in their staining patterns in HD tissues.

Table 3. Semiquantitative analysis of vesicular proteins in human HD cortex

	Presymptomatic (%)	*Grade 1 (%)*	*Grade 3/4 (%)*
Clathrin	105 ± 5	107 ± 14	108 ± 13
Complexin 2	70 ± 19	65 ± 13	38 ± 18*
Dynamin	93 ± 4	97 ± 7	100 ± 6
PACSIN 1	92 ± 8	84 ± 10	90 ± 9
Rab3A	94 ± 6	97 ± 15	117 ± 13
Rab5	84 ± 5	96 ± 8	82 ± 10
α/βSNAP	98 ± 7	100 ± 9	91 ± 5
SNAP 25	102 ± 1	100 ± 3	100 ± 6
Synaptobrevin	101 ± 9	95 ± 5	108 ± 10
Synaptophysin	104 ± 3	103 ± 6	105 ± 7
Synaptotagmin	106 ± 3	97 ± 9	101 ± 9
Syntaxin	107 ± 3	99 ± 6	100 ± 1

Protein extracts from genotyped HD patient cortical tissue were age and PMI matched and subjected to Western blotting for various synaptic elements as shown in Figure 5. Immunoblots were scanned at a resolution of 600 dpi, and the intensities of the bands were analyzed by NIH Image gel analysis macroroutine. Relative intensities were calculated as a percentage of control for three independent experiments. Only levels of complexin 2 in grade 3/4 tissues were significantly different from controls (* $p < 0.01$ by Fisher's PLSD).

Discussion

HD is classically characterized by extensive striatal atrophy, although there are also numerous reports of pathological changes in the cortex. The observation that some changes in cortical neurons occur prior to alterations in the striatum leads to the speculation that

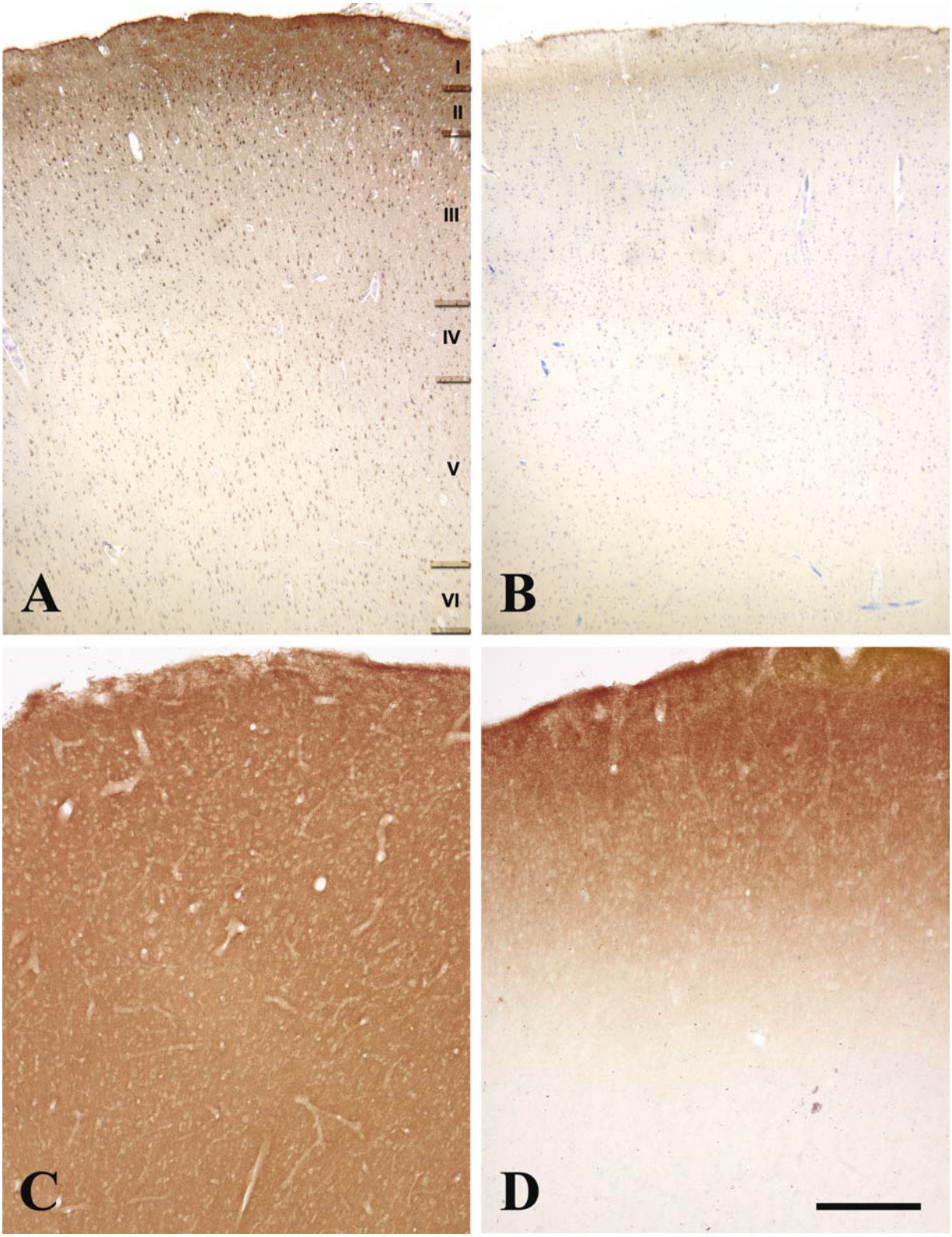

Fig. 6. PACSIN 1 and Dynamin staining are progressively lost in HD cortical tissue. Staining of human cortical tissue (formaldehyde fixed, paraffin embedded, 8 μm thick sections) with antibodies to the vesicular protein PACSIN 1 and visualized with DAB reveals a diffuse, widespread staining pattern noted through all layers of the cortex in controls (A) that is diminished in all cell layers in presymptomatic tissue (B). Staining of human cortical tissue (formaldehyde fixed, 25 μm thick, free-floating sections) for dynamin also revealed a diffuse, widespread staining pattern noted through all layers of the cortex in controls (C). However, dynamin staining in the deep cortical layers shows a progressive decrease beginning in presymptomatic tissue (D) opposed to PACSIN 1 which was diminished in all cell layers. Bar = 500 μm.

cortical pathology may contribute in a primary fashion to the onset of the HD phenotype. Experiments using transgenic HD mouse models have noted alterations in long-term potentiation (Usdin *et al.*, 1999), synaptic currents (Cepeda *et al.*, 2003), and neurotransmitter levels (Nicniocaill *et al.*, 2001) within the striatum of these models suggesting a disturbance in the corticostriatal input. This suggestion of cortical neuron dysfunction is underscored by clinical observations that show that psychiatric and cognitive symptoms often precede the onset of motor dysfunction (Dewhurst, 1969; Folstein, 1983; Lawrence *et al.*, 1996, 1998; Jason *et al.*, 1997; Berrios *et al.*, 2001).

The mechanism of cortical neuronal dysfunction is not clear but may be related to morphological alterations. Dendritic abnormalities are present in pyramidal neurons in early stage (grade 1) HD human cortex (Sapp *et al.*, 1997) and display a characteristic "wavy" morphology that correlates with the presence of cytoplasmic and nuclear accumulations of mutant huntingtin. Recently, this morphological change has been observed in a transgenic mouse model for HD (Laforet *et al.*, 2001) in which cortical changes, including accumulation of mutant huntingtin and the appearance of dysmorphic dendrites, correlate with the onset and severity of behavioral abnormalities. Moreover, brain slice preparations from these mice demonstrate electrophysiological abnormalities following cortical stimulation and N-methyl-D-aspartate receptor activation. This observation is consistent with the findings that alterations in dendritic morphology in cortical neurons can influence neuronal discharge patterns (Henze *et al.*, 1996). Thus, morphological abnormalities may contribute to altered neuronal function in HD. Our results demonstrating a loss of staining of various cytoskeletal elements in early stage HD tissue may be related to changes in neuronal morphology and consequently function.

We observed a dramatic, progressive loss of neurofilament staining throughout the cortex. Stabilized neurofilaments provide mechanical support and influence axonal diameter and thereby conduction velocities (Elder *et al.*, 1998a, b). Thus, alterations in neurofilament levels or structure can alter conduction velocities of particular neurons, and these changes could play a role in HD pathology. Indeed, characteristics of neurofilament pathology such as neuronal atrophy and reduction in axonal caliber have been noted in HD tissue (Dom *et al.*, 1976) as well as other polyglutamine diseases (Li *et al.*, 1995a). Moreover, ultrastructural examination of dystrophic neurites from HD cortex revealed an accumulation of granulofilamentous material similar to the neurofilament aggregates observed in a number of neurodegenerative diseases (Jackson *et al.*, 1995) which can result from polyglutamine domains binding to neurofilaments and disrupting the neurofilament assembly (Nagai *et al.*, 1999).

Another cytoskeletal complex which is critical to dendritic shape and function is the microtubule system. Microtubules are polymers composed of α and β tubulins that undergo rapid changes in length and stability in an energy dependent manner (Desai & Mitchison, 1997). These dynamic changes are regulated by microtubule associated proteins or MAPs (reviewed in Johnson & Jope, 1992). Here we noted a loss of α tubulin staining, which may be due to its interaction with mutant huntingtin (Tukamoto *et al.*, 1997) or due to alterations in the level or activity of the regulatory protein, MAP2. MAPs are differentially distributed, with the high molecular weight MAP2 found almost exclusively in the perikarya and dendrites (Matus *et al.*, 1986). While MAP2 is not the only dendritic protein, its expression and function is critical to directional dendritic growth (Kaech *et al.*, 1996) that appears altered in HD cortical neurons (Sotrel *et al.*, 1993). Its function is also directly linked to its phosphorylation state, since phosphorylation of different domains leads to reduced tubulin binding (Brugg & Matus, 1991). MAP2 levels and conformation can be mediated by neurotransmitter systems (Halpain & Greengard, 1990; Diaz-Nido *et al.*, 1993), whereby synaptic-mediated changes in the balance of kinases and phosphatases may alter MAP2 activity and, in turn, the microtubule network, allowing for synaptic plasticity (Avila *et al.*, 1994; Woolf, 1998). Thus, a loss of MAP2 or an aberrant phosphorylation state may impair synaptic plasticity, as is noted in a murine model of HD (Usdin *et al.*, 1999) and results in cognitive dysfunction. Loss of MAP2 expression has been demonstrated in models of excitotoxicity, presumably through the activation of calpain by excess calcium (Hicks *et al.*, 1995; Bordelon & Chesselet, 1999). This possibility is particularly intriguing since excitotoxicity is a postulated mechanism for HD (Beal *et al.*, 1986) and could explain our observation of loss of MAP2 staining. The theory of excitotoxicity also raises the issue of synaptic function and neurotransmitter release in HD.

While the normal function of huntingtin remains elusive, its interaction with synaptic vesicles and other transport proteins raises the possibility that one aspect of HD pathogenesis may lay in altered synaptic function (DiFiglia *et al.*, 1995; Li *et al.*, 1995b; Metzler *et al.*, 2001). The factors involved with neurotransmitter release have not all been completely identified, however; a number of proteins have been characterized. Among these proteins are synaptobrevin, which is found associated with the vesicle membrane, and syntaxin and SNAP-25, which are found associated with the plasma membrane, and collectively make up the SNARE complex (Sollner *et al.*, 1993). These proteins interact to bring together the vesicles with the plasma membrane to allow for fusion and exocytosis of the vesicular contents. The movement of vesicles and mediation of SNARE complex assembly and disassembly are regulated by a number of other accessory proteins, including, Rab3A,

Rab5, α/β SNAP, synaptotagmin, synaptophysin, and complexin 2 (reviewed in Sollner, 2003). Our results using Western blotting for these proteins in HD tissue revealed similar levels in presymptomatic cases as well as samples from grades 1, 3, and 4 cases with the exception of complexin 2. These results are in agreement with a similar study in a transgenic (R6/2) HD mouse model where a progressive loss of complexin 2 was noted in various brain regions including the cortex while most other synaptic proteins examined remained unchanged (Morton & Edwardson, 2001). More recently, the same group of researchers also examined striatal tissue from human postmortem HD cases (Morton *et al.*, 2001). While they noted some minor changes in other proteins, complexin 2 was most markedly reduced. However, they noted a decrease in complexin 2 levels beginning as early as grade 0, whereas we only found a statistical difference in grade 3 and 4 tissues. This may be due to differences in the samples or regions used in the two studies or simply in sample size examined, since we observed a reduction in grade 1 tissues but the results were not significant with the number of specimens available. Overall, both studies support a loss of complexin 2 in HD, which may result in altered neurotransmission. Indeed, complexin 2 is expressed predominantly in excitatory neurons (Harrison & Eastwood, 1998), where it has been shown that expression of the recombinant protein inhibits exocytosis while administration of antibodies to complexin 2 can stimulate release (Ono *et al.*, 1998). Thus, loss of complexin 2 may result in excessive neurotransmitter release, possibly contributing to excitotoxicity. Further, complexin 2 knockout mice show a reduction in LTP (Takahashi *et al.*, 1999) which is similar to what is seen in the R6/2 HD mouse (Murphy *et al.*, 2000). More extensive studies will be needed to address the mechanistic role of complexin 2 in HD pathology.

Another critical event in neurotransmission is the reuptake of neurotransmitters that occurs through endocytosis. Huntingtin has been shown to be associated with both clathrin and non-clathrin coated membranes (Velier *et al.*, 1998), but has not been directly linked to endocytic machinery. Yeast two-hybrid screens revealed an interaction between huntingtin and HIP1 (Wanker *et al.*, 1997), which has recently been shown to directly interact with components of the endocytic machinery (Engqvist-Goldstein *et al.*, 2001; Metzler *et al.*, 2001). These studies raise the possibility that the HD mutation may disrupt the endocytic pathway. Results of our Western blotting experiments for three endocytic proteins, clathrin heavy-chain, dynamin, and PACSIN 1, showed no alteration in their levels in any stage of HD. However, immunohistochemistry for dynamin and PACSIN 1 showed a progressive loss of staining beginning in presymptomatic tissue. Various explanations could account for this observation. First, these proteins may no longer localize to their normal cellular compartments due to sequestration with mutant huntingtin or impaired transport. Indeed, differential centrifugation of HD brain homogenate recovered most of the PACSIN 1 protein in the microsomal pellet rather than the synaptosomal pellet as seen in normal controls (Modregger *et al.*, 2002). The cytoskeleton is critical to cellular transport, and our results demonstrating the disruption of cytoskeletal components in presymptomatic HD tissue may suggest that transport is no longer efficient. *In vitro* studies with striatal cells expressing mutant huntingtin demonstrated neuritic aggregates, which blocked protein transport in neurites and caused degeneration (Li *et al.*, 2001). Moreover, mutant huntingtin was recently shown to impede fast axonal transport in isolated axoplasm (Szebenyi *et al.*, 2003) as well as in Drosophila models of HD (Gunawardena *et al.*, 2003; Lee *et al.*, 2004). Second, the epitopes of these proteins may be masked due to aberrant interaction with mutant huntingtin. Many proteins have been found to be sequestered in polyglutamine aggregates including neurofilament (Suhr *et al.*, 2001), but not all bands found in the aggregates were analyzed, suggesting that there may still be a number of proteins that interact and are sequestered with expanded polyglutamine tracts. Third, alteration in the activity of signal transduction pathways by mutant huntingtin could result in the attachment of protein side groups that mask antigen-binding sites or result in the degradation of particular proteins. Lastly, transcriptional dysregulation appears to be an early event in HD pathogenesis (Cha, 2000; Luthi-Carter *et al.*, 2000). In this regard, altered levels of a variety of proteins could directly or indirectly affect any of the aforementioned mechanisms resulting in loss or mislocalization of proteins in the synaptic compartment. Overall, our data suggest that certain synaptic proteins may be altered which may lead to impaired exocytosis/endocytosis in HD patients.

In this study we have sought to elucidate morphological and molecular alterations in early stage HD patients with emphasis on presymptomatic gene carriers. Our findings demonstrate dendritic and axonal abnormalities and loss of staining of cytoskeletal elements that could account for the disruption in the cellular architecture and morphology. Moreover, we have observed a decrease in the protein levels for complexin 2 and altered staining patterns for two other synaptic proteins, dynamin and PACSIN 1. The loss of synaptic proteins may contribute to aberrant neuronal neurotransmission, as has been observed in models of HD. Moreover, disruption of the cytoskeleton could result in altered trafficking of organelles and vesicles, reduced neurite outgrowth and plasticity, decreased transcription, and apoptosis, all of which are suspected to occur in HD. In fact, other neurodegenerative diseases such as Alzheimer's disease and amyotrophic lateral sclerosis are also suspected to involve cytoskeletal dysfunction (McMurray, 2000). Further examination of these alterations in cytoskeletal and synaptic elements in model

systems may provide insight into the early cellular pathology of HD.

Acknowledgments

This work supported by HDF Cure HD Initiative to Dr. Dan Tagle. Tissues were provided by the Harvard Brain Tissue Resource Center which is supported in part by PHS grant number MH/NS 31862. The authors thank Dr. Kurt Fischbeck, Dr. Charlotte Sumner, Dr. J. Paul Taylor (NINDS, NIH) and Dr. Herbert M. Geller (NHLBI, NIH) for critical reading of this manuscript.

References

AVILA, J., DOMINGUEZ, J. & DIAZ-NIDO, J. (1994) Regulation of microtubule dynamics by microtubule-associated protein expression and phosphorylation during neuronal development. *International Journal of Developmental Biology* **38**, 13–25.

AYLWARD, E. H., ANDERSON, N. B., BYLSMA, F. W., WAGSTER, M. V., BARTA, P. E., SHERR, M., FEENEY, J., DAVIS, A., ROSENBLATT, A., PEARLSON, G. D. & ROSS, C. A. (1998) Frontal lobe volume in patients with Huntington's disease. *Neurology* **50**, 252–258.

BEAL, M. F., KOWALL, N. W., ELLISON, D. W., MAZUREK, M. F., SWARTZ, K. J. & MARTIN, J. B. (1986) Replication of the neurochemical characteristics of Huntington's disease by quinolinic acid. *Nature* **321**, 168–171.

BERRIOS, G. E., WAGLE, A. C., MARKOVA, I. S., WAGLE, S. A., HO, L. W., RUBINSZTEIN, D. C., WHITTAKER, J., FFRENCH-CONSTANT, C., KERSHAW, A., ROSSER, A., BAK, T. & HODGES, J. R. (2001) Psychiatric symptoms and CAG repeats in neurologically asmptomatic Huntington's disease carriers. *Psychiatry Research* **102**, 217–225.

BLOCK-GALARZA, J., CHASE, K. O., SAPP, E., VAUGHN, K. T., VALLEE, R. B., DIFIGLIA, M. & ARONIN, N. (1997) Fast transport and retrograde movement of huntingtin and HAP 1 in axons. *NeuroReport* **8**, 2247–2251.

BORDELON, Y. M. & CHESSELET, M. F. (1999) Early effects of intrastriatal injections of quinolinic acid on microtubule-associated protein-2 and neuropeptides in rat basal ganglia. *Neuroscience* **93**, 843–853.

BRUGG, B. & MATUS, A. (1991) Phosphorylation determines the binding of microtubule-associated protein 2 (MAP2) to microtubules in living cells. *Journal of Cell Biology* **114**, 735–743.

CEPEDA, C., HURST, R. S., CALVERT, C. R., HERNANDEZ-ECHEAGARAY, E., NGUYEN, O. K., JOCOY, E., CHRISTIAN, L. J., ARIANO, M. A. & LEVINE, M. S. (2003) Transient and progressive electrophysiological alterations in the corticostriatal pathway in a mouse model of Huntington's disease. *Journal of Neuroscience* **23**, 961–969.

CHA, J. H. (2000) Transcriptional dysregulation in Huntington's disease. *Trends in Neuroscience* **23**, 387–392.

CUDKOWICZ, M. & KOWALL, N. W. (1990) Degeneration of pyramidal projection neurons in Huntington's disease cortex. *Annals of Neurology* **27**, 200–204.

DE LA MONTE, S. M., VONSATTEL, J. P. & RICHARDSON, E. P., JR. (1988) Morphometric demonstration of atrophic changes in the cerebral cortex, white matter, and neostriatum in Huntington's disease. *Journal of Neuropathology & Experimental Neurology* **47**, 516–525.

DESAI, A. & MITCHISON, T. J. (1997) Microtubule polymerization dynamics. *Annual Review of Cell & Developmental Biology* **13**, 83–117.

DEWHURST, K. (1969) Neuro-psychiatric aspects of Huntington's disease. *Confinia Neurologica* **31**, 258–268.

DIAZ-NIDO, J., MONTORO, R. J., LOPEZ-BARNEO, J. & AVILA, J. (1993) High external potassium induces an increase in the phosphorylation of the cytoskeletal protein MAP2 in rat hippocampal slices. *European Journal of Neuroscience* **5**, 818–824.

DIFIGLIA, M., SAPP, E., CHASE, K., SCHWARZ, C., MELONI, A., YOUNG, C., MARTIN, E., VONSATTEL, J. P., CARRAWAY, R., & REEVES, S. A. (1995) Huntingtin is a cytoplasmic protein associated with vesicles in human and rat brain neurons. *Neuron* **14**, 1075–1081.

DIFIGLIA, M., SAPP, E., CHASE, K. O., DAVIES, S. W., BATES, G. P., VONSATTEL, J. P. & ARONIN, N. (1997) Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. *Science* **277**, 1990–1993.

DOM, R., MALFROID, M. & BARO, F. (1976) Neuropathology of Huntington's chorea. Studies of the ventrobasal complex of the thalamus. *Neurology* **26**, 64–68.

ELDER, G. A., FRIEDRICH, V. L., JR., BOSCO, P., KANG, C., GOUROV, A., TU, P. H., LEE, V. M. & LAZZARINI, R. A. (1998a) Absence of the mid-sized neurofilament subunit decreases axonal calibers, levels of light neurofilament (NF-L), and neurofilament content. *Journal of Cell Biology* **141**, 727–739.

ELDER, G. A., FRIEDRICH, V. L., JR., KANG, C., BOSCO, P., GOUROV, A., TU, P. H., ZHANG, B., LEE, V. M. & LAZZARINI, R. A. (1998b) Requirement of heavy neurofilament subunit in the development of axons with large calibers. *Journal of Cell Biology* **143**, 195–205.

ENGQVIST-GOLDSTEIN, A. E., WARREN, R. A., KESSELS, M. M., KEEN, J. H., HEUSER, J. & DRUBIN, D. G. (2001) The actin-binding protein Hip1R associates with clathrin during early stages of endocytosis and promotes clathrin assembly *in vitro*. *Journal of Cell Biology* **154**, 1209–1223.

FOLSTEIN, S. E. (1983) Psychiatric features of Huntington's disease: Recent approaches and findings. *Psychiatric Developments* **1**, 193–205.

FOROUD, T., SIEMERS, E., KLEINDORFER, D., BILL, D. J., HODES, M. E., NORTON, J. A., CONNEALLY, P. M. & CHRISTIAN, J. C. (1995) Cognitive scores in carriers of Huntington's disease gene compared to noncarriers. *Annals of Neurology* **37**, 657–664.

GUIDETTI, P., CHARLES, V., CHEN, E.-Y., REDDY, P. H., KORDOWER, J. H., WHETSELL, W. O., JR., SCHWARCZ, R. & TAGLE, D. A. (2001) Early degenerative changes in transgenic mice expressing mutant

huntingtin involve dendritic abnormalities but no impairment of mitochondrial energy production. *Experimental Neurology* **169**, 340–350.

GUNAWARDENA, S., HER, L. S., BRUSCH, R. G., LAYMON, R. A., NIESMAN, I. R., GORDESKY-GOLD, B., SINTASATH, L., BONINI, N. M. & GOLDSTEIN, L. S. (2003) Disruption of axonal transport by loss of huntingtin or expression of pathogenic polyQ proteins in Drosophila.[see comment]. *Neuron* **40**, 25–40.

GUTEKUNST, C. A., LEVEY, A. I., HEILMAN, C. J., WHALEY, W. L., YI, H., NASH, N. R., REES, H. D., MADDEN, J. J. & HERSCH, S. M. (1995) Identification and localization of huntingtin in brain and human lymphoblastoid cell lines with anti-fusion protein antibodies. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 8710–8714.

HALPAIN, S. & GREENGARD, P. (1990) Activation of NMDA receptors induces rapid dephosphorylation of the cytoskeletal protein MAP2. *Neuron* **5**, 237–246.

HARJES, P. & WANKER, E. E. (2003) The hunt for huntingtin function: Interaction partners tell many different stories. *Trends in Biochemical Science* **28**, 425–433.

HARMS, L., MEIERKORD, H., TIMM, G., PFEIFFER, L. & LUDOLPH, A. C. (1997) Decreased N-acetyl-aspartate/choline ratio and increased lactate in the frontal lobe of patients with Huntington's disease: A proton magnetic resonance spectroscopy study. *Journal of Neurology, Neurosurgery & Psychiatry* **62**, 27–30.

HARPER, P. S. (1992) The epidemiology of Huntington's disease. *Human Genetics* **89**, 365–376.

HARRISON, P. J. & EASTWOOD, S. L. (1998) Preferential involvement of excitatory neurons in medial temporal lobe in schizophrenia. *Lancet* **352**, 1669–1673.

HENZE, D. A., CAMERON, W. E. & BARRIONUEVO, G. (1996) Dendritic morphology and its effects on the amplitude and rise-time of synaptic signals in hippocampal CA3 pyramidal cells. *Journal of Comparative Neurology* **369**, 331–344.

HICKS, R. R., SMITH, D. H. & MCINTOSH, T. K. (1995) Temporal response and effects of excitatory amino acid antagonism on microtubule-associated protein 2 immunoreactivity following experimental brain injury in rats. *Brain Research* **678**, 151–160.

HUNTINGTON'S DISEASE COLLABORATIVE RESEARCH GROUP (1993) A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell* **72**, 971–983.

JACKSON, M., GENTLEMAN, S., LENNOX, G., WARD, L., GRAY, T., RANDALL, K., MORRELL, K. & LOWE, J. (1995) The cortical neuritic pathology of Huntington's disease. *Neuropathology & Applied Neurobiology* **21**, 18–26.

JASON, G. W., SUCHOWERSKY, O., PAJURKOVA, E. M., GRAHAM, L., KLIMEK, M. L., GARBER, A. T. & POIRIER-HEINE, D. (1997) Cognitive manifestations of Huntington disease in relation to genetic structure and clinical onset. *Archives of Neurology* **54**, 1081–1088.

JIAO, Y., SUN, Z., LEE, T., FUSCO, F. R., KIMBLE, T. D., MEADE, C. A., CUTHBERTSON, S. & REINER, A. (1999) A simple and sensitive antigen retrieval method for free-floating and slide-mounted tissue sections. *Journal of Neuroscience Methods* **93**, 149–162.

JOHNSON, G. V. & JOPE, R. S. (1992) The role of microtubule-associated protein 2 (MAP-2) in neuronal growth, plasticity, and degeneration. *Journal of Neuroscience Research* **33**, 505–512.

KAECH, S., LUDIN, B. & MATUS, A. (1996) Cytoskeletal plasticity in cells expressing neuronal microtubule-associated proteins. *Neuron* **17**, 1189–1199.

LAFORET, G. A., SAPP, E., CHASE, K., MCINTYRE, C., BOYCE, F. M., CAMPBELL, M., CADIGAN, B. A., WARZECKI, L., TAGLE, D., REDDY, P. H., CEPEDA, C., CALVERT, C. R., JOKEL, E. S., KLAPSTEIN, G. J., ARIANO, M. A., LEVINE, M. S., DIFIGLIA, M. & ARONIN, N. (2001) Changes in cortical and striatal neurons predict behavioral and electrophysiological abnormalities in a transgenic murine model of Huntington's disease. *Journal of Neuroscience* **21**, 9112–9123.

LAWRENCE, A. D., HODGES, J. R., ROSSER, A. E., KERSHAW, A., FFRENCH-CONSTANT, C., RUBINSZTEIN, D. C., ROBBINS, T. W. & SAHAKIAN, B. J. (1998) Evidence for specific cognitive deficits in preclinical Huntington's disease. *Brain* **121**, 1329–1341.

LAWRENCE, A. D., SAHAKIAN, B. J., HODGES, J. R., ROSSER, A. E., LANGE, K. W. & ROBBINS, T. W. (1996) Executive and mnemonic functions in early Huntington's disease. *Brain* **119**, 1633–1645.

LEE, W. C., YOSHIHARA, M. & LITTLETON, J. T. (2004) Cytoplasmic aggregates trap polyglutamine-containing proteins and block axonal transport in a Drosophila model of Huntington's disease. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 3224–3229.

LI, H., LI, S. H., JOHNSTON, H., SHELBOURNE, P. F. & LI, X. J. (2000) Amino-terminal fragments of mutant huntingtin show selective accumulation in striatal neurons and synaptic toxicity [see comments]. *Nature Genetics* **25**, 385–389.

LI, H., LI, S. H., YU, Z. X., SHELBOURNE, P. & LI, X. J. (2001) Huntingtin aggregate-associated axonal degeneration is an early pathological event in Huntington's disease mice. *Journal of Neuroscience* **21**, 8473–8481.

LI, M., SOBUE, G., DOYU, M., MUKAI, E., HASHIZUME, Y. & MITSUMA, T. (1995a) Primary sensory neurons in X-linked recessive bulbospinal neuropathy: Histopathology and androgen receptor gene expression. *Muscle & Nerve* **18**, 301–308.

LI, X. J., LI, S. H., SHARP, A. H., NUCIFORA, F. C., JR., SCHILLING, G., LANAHAN, A., WORLEY, P., SNYDER, S. H. & ROSS, C. A. (1995b) A huntingtin-associated protein enriched in brain with implications for pathology. *Nature* **378**, 398–402.

LUTHI-CARTER, R., STRAND, A., PETERS, N. L., SOLANO, S. M., HOLLINGSWORTH, Z. R., MENON, A. S., FREY, A. S., SPEKTOR, B. S., PENNEY, E. B., SCHILLING, G., ROSS, C. A., BORCHELT, D. R., TAPSCOTT, S. J., YOUNG, A. B., CHA, J. H. & OLSON, J. M. (2000) Decreased expression of striatal signaling genes in a mouse model of Huntington's disease. *Human Molecular Genetics* **9**, 1259–1271.

MANN, D. M., OLIVER, R. & SNOWDEN, J. S. (1993) The topographic distribution of brain atrophy in Huntington's disease and progressive supranuclear palsy. *Acta Neuropathologica* **85**, 553–559.

MATUS, A., BERNHARDT, R., BODMER, R. & ALAIMO, D. (1986) Microtubule-associated protein 2 and tubulin are differently distributed in the dendrites of developing neurons. *Neuroscience* **17**, 371–389.

MCMURRAY, C. T. (2000) Neurodegeneration: Diseases of the cytoskeleton? *Cell Death & Differentiation* **7**, 861–865.

METZLER, M., LEGENDRE-GUILLEMIN, V., GAN, L., CHOPRA, V., KWOK, A., MCPHERSON, P. S. & HAYDEN, M. R. (2001) HIP1 functions in clathrin-mediated endocytosis through binding to clathrin and adaptor protein 2. *Journal of Biological Chemistry* **276**, 39271–39276.

MODREGGER, J., DIPROSPERO, N. A., CHARLES, V., TAGLE, D. A. & PLOMANN, M. (2002) PACSIN 1 interacts with huntingtin and is absent from synaptic varicosities in presymptomatic Huntington's disease brains. *Human Molecular Genetics* **11**, 2547–2558.

MORTON, A. J. & EDWARDSON, J. M. (2001) Progressive depletion of complexin II in a transgenic mouse model of Huntington's disease. *Journal of Neurochemistry* **76**, 166–172.

MORTON, A. J., FAULL, R. L. & EDWARDSON, J. M. (2001) Abnormalities in the synaptic vesicle fusion machinery in Huntington's disease. *Brain Research Bulletin* **56**, 111–117.

MURPHY, K. P., CARTER, R. J., LIONE, L. A., MANGIARINI, L., MAHAL, A., BATES, G. P., DUNNETT, S. B. & MORTON, A. J. (2000) Abnormal synaptic plasticity and impaired spatial cognition in mice transgenic for exon 1 of the human Huntington's disease mutation. *Journal of Neuroscience* **20**, 5115–5123.

NAGAI, Y., ONODERA, O., CHUN, J., STRITTMATTER, W. J. & BURKE, J. R. (1999) Expanded polyglutamine domain proteins bind neurofilament and alter the neurofilament network. *Experimental Neurology* **155**, 195–203.

NICNIOCAILL, B., HARALDSSON, B., HANSSON, O., O'CONNOR, W. T. & BRUNDIN, P. (2001) Altered striatal amino acid neurotransmitter release monitored using microdialysis in R6/1 Huntington transgenic mice. *European Journal of Neuroscience* **13**, 206–210.

ONO, S., BAUX, G., SEKIGUCHI, M., FOSSIER, P., MOREL, N. F., NIHONMATSU, I., HIRATA, K., AWAJI, T., TAKAHASHI, S. & TAKAHASHI, M. (1998) Regulatory roles of complexins in neurotransmitter release from mature presynaptic nerve terminals. *European Journal of Neuroscience* **10**, 2143–2152.

PAULSON, H. L. & FISCHBECK, K. H. (1996) Trinucleotide repeats in neurogenetic disorders. *Annual Review of Neuroscience* **19**, 79–107.

PLOMANN, M., LANGE, R., VOPPER, G., CREMER, H., HEINLEIN, U. A., SCHEFF, S., BALDWIN, S. A., LEITGES, M., CRAMER, M., PAULSSON, M. & BARTHELS, D. (1998) PACSIN, a brain protein that is upregulated upon differentiation into neuronal cells. *European Journal of Biochemistry* **256**, 201–211.

SAPP, E., PENNEY, J., YOUNG, A., ARONIN, N., VONSATTEL, J. P. & DIFIGLIA, M. (1999) Axonal transport of N-terminal huntingtin suggests early pathology of corticostriatal projections in Huntington disease. *Journal of Neuropathology and Experimental Neurology* **58**, 165–173.

SAPP, E., SCHWARZ, C., CHASE, K., BHIDE, P. G., YOUNG, A. B., PENNEY, J., VONSATTEL, J. P., ARONIN, N. & DIFIGLIA, M. (1997) Huntingtin localization in brains of normal and Huntington's disease patients. *Annals of Neurology* **42**, 604–612.

SCHNELL, S. A., STAINES, W. A. & WESSENDORF, M. W. (1999) Reduction of lipofuscin-like autofluorescence in fluorescently labeled tissue. *Journal of Histochemistry & Cytochemistry* **47**, 719–730.

SHARP, A. H., LOEV, S. J., SCHILLING, G., LI, S. H., LI, X. J., BAO, J., WAGSTER, M. V., KOTZUK, J. A., STEINER, J. P., LO, A., *et al.* (1995) Widespread expression of Huntington's disease gene (IT15) protein product. *Neuron* **14**, 1065–1074.

SOLLNER, T. H. (2003) Regulated exocytosis and SNARE function (Review) *Molecular Membrane Biology* **20**, 209–220.

SOLLNER, T., WHITEHEART, S. W., BRUNNER, M., ERDJUMENT-BROMAGE, H., GEROMANOS, S., TEMPST, P. & ROTHMAN, J. E. (1993) SNAP receptors implicated in vesicle targeting and fusion. *Nature* **362**, 318–324.

SOTREL, A., PASKEVICH, P. A., KIELY, D. K., BIRD, E. D., WILLIAMS, R. S. & MYERS, R. H. (1991) Morphometric analysis of the prefrontal cortex in Huntington's disease. *Neurology* **41**, 1117–1123.

SOTREL, A., WILLIAMS, R. S., KAUFMANN, W. E. & MYERS, R. H. (1993) Evidence for neuronal degeneration and dendritic plasticity in cortical pyramidal neurons of Huntington's disease: A quantitative Golgi study. *Neurology* **43**, 2088–2096.

STRONG, T. V., TAGLE, D. A., VALDES, J. M., ELMER, L. W., BOEHM, K., SWAROOP, M., KAATZ, K. W., COLLINS, F. S. & ALBIN, R. L. (1993) Widespread expression of the human and rat Huntington's disease gene in brain and nonneural tissues. *Nature Genetics* **5**, 259–265.

SUHR, S. T., SENUT, M. C., WHITELEGGE, J. P., FAULL, K. F., CUIZON, D. B. & GAGE, F. H. (2001) Identities of sequestered proteins in aggregates from cells with induced polyglutamine expression. *Journal of Cell Biology* **153**, 283–294.

SZEBENYI, G., MORFINI, G. A., BABCOCK, A., GOULD, M., YOUNG, M., FABER, P. W., MACDONALD, M. E., MCPHAUL, M. J. & BRADY, S. T. (2003) Neuropathic forms of huntingtin and androgen receptor inhibit fast axonal transport. *Neuron* **40**, 41–52.

TAKAHASHI, S., UJIHARA, H., HUANG, G. Z., YAGYU, K. I., SANBO, M., KABA, H. & YAGI, T. (1999) Reduced hippocampal LTP in mice lacking a presynaptic protein: Complexin II. *European Journal of Neuroscience* **11**, 2359–2366.

TROTTIER, Y., DEVYS, D., IMBERT, G., SAUDOU, F., AN, I., LUTZ, Y., WEBER, C., AGID, Y., HIRSCH, E. C. & MANDEL, J. L. (1995) Cellular localization of the Huntington's disease protein and discrimination of the normal and mutated form [see comments]. *Nature Genetics* **10**, 104–110.

TUKAMOTO, T., NUKINA, N., IDE, K. & KANAZAWA, I. (1997) Huntington's disease gene product, huntingtin, associates with microtubules *in vitro*. *Brain Research. Molecular Brain Research* **51**, 8–14.

USDIN, M. T., SHELBOURNE, P. F., MYERS, R. M. & MADISON, D. V. (1999) Impaired synaptic plasticity in mice carrying the Huntington's disease mutation. *Human Molecular Genetics* **8**, 839–846.

VELIER, J., KIM, M., SCHWARZ, C., KIM, T. W., SAPP, E., CHASE, K., ARONIN, N. & DIFIGLIA, M. (1998) Wild-type and mutant huntingtins function in vesicle trafficking in the secretory and endocytic pathways. *Experimental Neurology* **152**, 34–40.

VONSATTEL, J. P., MYERS, R. H., STEVENS, T. J., FERRANTE, R. J., BIRD, E. D. & RICHARDSON, E. P., JR. (1985) Neuropathological classification of Huntington's disease. *Journal of Neuropathology and Experimental Neurology* **44**, 559–577.

WANKER, E. E., ROVIRA, C., SCHERZINGER, E., HASENBANK, R., WALTER, S., TAIT, D., COLICELLI, J. & LEHRACH, H. (1997) HIP-I: A huntingtin interacting protein isolated by the yeast two-hybrid system. *Human Molecular Genetics* **6**, 487–495.

WOOLF, N. J. (1998) A structural basis for memory storage in mammals. *Progress in Neurobiology* **55**, 59–77.

ZOGHBI, H. Y. & ORR, H. T. (2000) Glutamine repeats and neurodegeneration. *Annual Review of Neuroscience* **23**, 217–247.

ZUCCATO, C., CIAMMOLA, A., RIGAMONTI, D., LEAVITT, B. R., GOFFREDO, D., CONTI, L., MACDONALD, M. E., FRIEDLANDER, R. M., SILANI, V., HAYDEN, M. R., TIMMUSK, T., SIPIONE, S. & CATTANEO, E. (2001) Loss of huntingtin-mediated BDNF gene transcription in Huntington's disease. *Science* **293**, 493–498.