PACSIN 1 interacts with huntingtin and is absent from synaptic varicosities in presymptomatic Huntington’s disease brains

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Huntington’s disease (HD) is caused by a pathological expansion of a CAG repeat in the first exon of the gene coding for huntingtin, resulting in an abnormally long polyglutamine stretch. Despite its widespread expression, mutant huntingtin leads to selective neuronal loss in the striatum and cortex. Here we report that the neurospecific phosphoprotein PACSIN 1, which has been implicated as playing a central role in synaptic vesicle recycling, interacts with huntingtin via its C-terminal SH3 domain. Moreover, two other isoforms, PACSIN 2 and 3, which show a wider tissue distribution including the brain, do not interact with huntingtin despite a highly conserved SH3 domain. Furthermore, this interaction is repeat-length-dependent and is enhanced with mutant huntingtin, possibly causing the sequestration of PACSIN 1. Normally, PACSIN 1 is located along neurites and within synaptic boutons, but in HD patient neurons, there is a progressive loss of PACSIN 1 immunostaining in synaptic varicosities, beginning in presymptomatic and early-stage HD. Further, PACSIN 1 immunostaining of HD patient tissue reveals a more cytoplasmic distribution of the protein, with particular concentration in the perinuclear region coincident with mutant huntingtin. Thus, the specific interaction of huntingtin with the neuronal PACSIN isoform, PACSIN 1, and its altered intracellular distribution in pathological tissue, together with the observed differences in the binding behavior, suggest a role for PACSIN 1 during early stages of the selective neuropathology of HD.

INTRODUCTION

Huntington’s disease (HD) is an inherited neurodegenerative disease that is caused by an expanded CAG repeat within the HD gene that translates into a stretch of polyglutamine [poly(Q)] residues in the corresponding protein, huntingtin. An expansion of >39 CAG triplets causes the neurological disorder, with an inverse correlation between repeat length and onset of the disease. It has been demonstrated by several studies that both normal and mutant huntingtin are primarily associated with vesicular structures in the cytoplasm (1–3). Moreover, mutant huntingtin forms insoluble nuclear and extranuclear aggregates in neuronal cells of HD patient brains and those of mouse models (4,5).

Although intensively studied in vitro and in vivo, questions remain whether aggregates directly cause neurodegeneration by somehow interfering with cellular function. More recent findings indicate that aggregate formation and cellular toxicity in culture are uncoupled (6) and that inclusions might even be protective in some cases (7). Furthermore, the tetracycline-regulated overexpression of exon 1 huntingtin with an expanded poly(Q) region induces both formation of inclusions and neuropathological changes in transgenic mice, but inclusions largely disappear and neuropathological signs are mostly reversed when the expression is turned off (8). Other transgenic mouse models also support the notion of dissociation between inclusion formation and cell death. A mouse model expressing only the first exon of the huntingtin gene with either 115 or 150 CAG repeats developed widespread huntingtin inclusions but failed to show neuronal loss (9), whereas a model expressing full-length huntingtin (10) with
89 CAG repeats showed minimal nuclear inclusion formation but extensive neurodegeneration. Therefore, the selective neuronal loss observed in HD might not be caused by the formation of nuclear inclusions, but by aberrant interactions or a gain or loss of function involving protein interaction motifs of huntingtin flanking the poly(Q) expansion and specific binding partners.

Within the last few years, a number of huntingtin-interacting proteins have been identified independently by several groups. Some of these, such as huntingtin-associated protein (HAP1), huntingtin-interacting protein (HIP1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and endophilin A3, clearly bind to N-terminal fragments of huntingtin, and the interactions are modulated by the length of the poly(Q) stretch in huntingtin (11–14). Except for endophilin A3, also known as SH3GL3 protein, which promotes poly(Q) protein aggregation, previous studies identifying interaction partners of huntingtin have been unable to demonstrate a direct link to pathological mechanisms.

One postulated mechanism is aberrant neurotransmission (15,16), which could account for the cognitive and emotional symptoms observed in the early stages of HD (17–22) and also possibly contribute to the cell death observed in later stages through excitotoxicity (23). In this regard, disruption of proteins critical to endocytosis could underlie some aspects of altered neurotransmitter levels and directly contribute to the pathology of HD. As the PACSIN proteins such as the endophilins contain a C-terminal SH3 domain and share several common binding partners, we tested whether one of the larger, so-far unidentified, PACSIN 1-interacting proteins could be huntingtin. Here we report the isoform-specific interaction of the synaptic vesicle protein PACSIN 1 with huntingtin and show its mislocalization in neurons of early-stage HD patients, implying a critical role of PACSIN 1 in the development of the early neuronal features of HD, presumably through an impairment of endocytosis.

RESULTS

Interactions of PACSIN 1

We searched for brain-specific binding partners for PACSIN 1 by incubating immobilized fusion protein comprising glutathione-S-transferase (GST) and the complete coding region of PACSIN 1 (GST–PACSIN 1) or GST alone as a control with mouse brain cytosolic extracts. In this assay, the fusion protein containing full-length PACSIN 1, but not GST alone, was able to precipitate protein complexes that resolve to relative molecular masses of 40, 50, 62 and 100 kDa as detected in a Coomassie-stained gel (Fig. 1: left). Furthermore, additional minor bands with molecular masses of 80, 145, 220, 300 and 350 kDa could be identified after silver staining of the gel (Fig. 1: right). Most of these protein bands were identified as corresponding to previously identified synaptic proteins, such as dynamin 1, synaptotagmin 1, synapsin 1, N-WASP and mSOS (24–26). Since another endocytic protein, endophilin A3, was shown to interact with the 350 kDa protein, huntingtin (13), we tested the identity of the 350 kDa protein precipitated with PACSIN 1, and confirmed its identity as huntingtin by western blot analysis (Fig. 2).

The PACSIN 1 SH3 domain interacts with the proline-rich region in exon 1 of huntingtin

To verify direct binding and to identify the protein regions responsible for the interaction between huntingtin (htt) and PACSIN 1, truncated and mutated versions of the proteins were expressed as GST fusion proteins for precipitation experiments and GAL4 fusion proteins in yeast for analysis in a two-hybrid assay (Fig. 2A).

In vitro precipitation experiments using the GST-PACSIN 1 fusion protein and truncated versions of the protein demonstrated that only full-length PACSIN 1 and the isolated SH3 P ACSIN 1 domain were able to precipitate huntingtin out of a mouse brain extract (Fig. 2B). Furthermore we confirmed the results obtained with the precipitation assay by using the yeast two-hybrid system. Only cells expressing an intact PACSIN 1 SH3 domain activated the HIS and lacZ reporter genes, while an SH3 deletion mutant (∆SH3) and a mutant form in which a conserved proline residue was changed to leucine (P434L) were unable to interact (Fig. 2C).
Figure 2. The PACSIN 1 SH3 domain interacts directly with huntingtin. (A) Full-length PACSIN 1, PACSIN 1 with a point mutation in its SH3 domain negative for SH3 interactions (PACSIN 1P434L), PACSIN 1 lacking its SH3 domain (PACSIN 1ΔSH3) and the isolated SH3 domain of PACSIN 1 (SH3PACSIN1) were generated as GST fusion proteins for co-precipitation assays and as GAL4 fusion proteins for two-hybrid interaction studies. (B) In a co-precipitation assay, the 350 kDa protein was identified as huntingtin and interacted with the C-terminal SH3 domain of PACSIN 1. (C) The SH3-specific interaction of PACSIN 1 with huntingtin was confirmed in a yeast two-hybrid assay using PACSIN 1 variants fused to GAL4-BD as bait and huntingtin exon 1 protein fused to GAL4-AD as target. Activation of both the HIS and lacZ reporter genes was only detected when the intact PACSIN 1 SH3 domain was present.
Huntingtin exon 1 protein containing 18 glutamines within the poly(Q) stretch (htt-ex1pQ18) was sufficient for binding, indicating that the proline-rich region in htt-ex1pQ18 (residues 36–75) might be essential for the observed interaction, as has been shown previously for other SH3 domain-containing proteins [e.g. endophilin A3 (13)]. To verify its direct involvement, we used three GST fusion proteins that contained peptides of the N-terminal, central and C-terminal parts of the proline-rich region, respectively (Fig. 3A), in an overlay assay. A lysate of CHO cells transiently transfected with Myc–PACSIN 1 was used to incubate a western blot of the purified electrophoretically separated GST fusion proteins. After immunostaining with an anti-Myc antibody, the central proline-rich peptide was sufficient for the interaction of PACSIN 1 with huntingtin (Fig. 3B).

**Huntingtin is a PACSIN 1-specific binding partner**

To explore whether all three PACSIN isoforms are able to interact with huntingtin, we tested each isoform as bait against htt-ex1pQ18 in a yeast two-hybrid assay. Surprisingly, only PACSIN 1 led to activation of both reporter genes (Fig. 4A), although all three PACSIN members are expressed in the brain and share a conserved SH3 domain (16). To further confirm the specificity of PACSIN 1 interaction with full-length huntingtin, a cytosolic protein extract was prepared from murine brain and used for precipitation experiments using GST–PACSIN fusion proteins. As shown in Figure 4B, only GST–PACSIN 1 specifically precipitated huntingtin. A sequence comparison of the PACSIN SH3 domains revealed that there are only two non-conservative amino acid changes (PACSIN 1 positions 398 and

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**Figure 3.** The proline-rich region of the exon 1 protein of huntingtin interacts directly with PACSIN 1. (A) Three peptides corresponding to the N-terminal (peptide 1), central (peptide 2) or C-terminal (peptide 3) portions of huntingtin’s proline-rich region were generated as GST fusion proteins and, together with GST alone as a control, were purified as recombinant proteins. (B) For an overlay assay equal amounts of all four proteins (see Ponceau staining) were separated by SDS–PAGE and blotted on to a membrane. Myc-tagged PACSIN 1 overexpressed in CHO cells only interacted with GST-Peptide 2 corresponding to the central portion of huntingtin’s proline-rich region.
between PACSIN 1 and 2, whereas all other residues in the SH3 domain are either identical or reflect conservative amino acid changes (Fig. 4C). The SH3 domain of PACSIN 3 shows 10 non-conservative amino acid substitutions compared with PACSIN 1 (Fig. 4C). Taken together, huntingtin represents the first unique binding partner of PACSIN 1, since all other proteins known to interact with the PACSIN 1 SH3 domain also bind to PACSINs 2 and 3 (24,25).

A pathologically elongated poly(Q) stretch in htt-ex1p enhances the binding to PACSIN 1

To determine if PACSIN 1 and huntingtin interaction is poly(Q) repeat-length dependent, we used GAL4AD–htt-ex1pQ18 and GAL4AD–htt-ex1pQ44 with GAL4BD–PACSIN 1 in the yeast two-hybrid system and analyzed the binding in a semiquantitative liquid β-Gal assay (Fig. 5). In the presence of 44 glutamine repeats in GAL4AD–htt-ex1pQ44, the level of β-Gal activity was found to be 2-fold higher than that observed in the presence of GAL4AD–htt-ex1pQ18, indicating that an elongated glutamine repeat in htt-ex1p enhances the binding to the PACSIN 1 protein. No interaction was observed with the control proteins GAL4 and GAL4BD–PACSIN 2.

Co-localization of PACSIN 1 and huntingtin in human brain

To study the expression and localization of PACSIN 1 relative to huntingtin, we performed immunohistochemistry on sections of human cortical tissue (Brodmann area 9) from HD patients and normal controls (Fig. 6). After staining, we treated the tissues with 0.1% Sudan black to quench endogenous autofluorescence caused by lipofuscin, and examined the sections using a DAPI filter to verify that the fluorescent background was successfully abolished (Fig. 6I). Additionally, we stained other sections from the same tissue sets using only the respective secondary antibodies for PACSIN 1 (Cy5) and huntingtin (Cy2) to demonstrate staining specificity (Fig. 6G and H, respectively).

PACSIN 1 is normally localized throughout the cytoplasm and along neuronal processes (arrows in Fig. 6A). Huntingtin immunoreactivity using a monoclonal antibody (Chemicon 2166) reveals a similar cytoplasmic staining pattern, but little fiber staining (arrow in Fig. 6B). Merging of these images demonstrates that PACSIN 1 and huntingtin co-localize within the cytoplasm in cells expressing both proteins (Fig. 6C). However, this pattern is different in HD cortical tissue. Staining for PACSIN 1 in presymptomatic HD brains reveals a more concentrated, polarized appearance of the protein within the cytoplasm, which in some cells appears perinuclear (arrows), as well as an absence of staining in the neuronal processes (Fig. 6D). In presymptomatic HD tissue, huntingtin is largely cytoplasmic, but in some cases appears concentrated in a perinuclear position (arrows in Fig. 6E) that co-localizes with PACSIN 1 (Fig. 6F). Indeed, whereas in control tissues PACSIN 1 is found throughout the cell, in HD tissues, it predominantly appears co-localized with huntingtin (compare Fig. 6C with 6F).

Relocalization of PACSIN 1 in HD patients

Our data obtained thus far demonstrate that PACSIN 1 and huntingtin interact in vitro and in vivo, suggesting that mutant huntingtin may alter the normal distribution of PACSIN 1.
We therefore compared human frontal cortex sections of patients at different disease stages with normal controls. Examination of PACSIN 1 at low magnification in control tissue reveals immunoreactivity throughout the cortex (Fig. 7: left panel), which is most intense along fibers and in varicosities (inset). However, staining for PACSIN 1 is markedly lost, beginning in presymptomatic HD tissue (Fig. 7: middle panel), and while some fiber staining is still noted, there is conspicuous absence of varicosity staining (Fig. 7: middle panel, inset). In grade 1 and later-stage HD tissue, there is almost complete loss of PACSIN 1 staining (Fig. 7: right panel). These results suggest that PACSIN 1 is absent from its normal site of action within synaptic boutons—possibly resulting in disruption of endocytic function in early-stage HD.

To further analyze the intracellular re-localization of PACSIN 1, we performed subcellular fractionation. Upon differential centrifugation of HD brain homogenate, most of the PACSIN 1 was recovered in the microsomal pellet (P3), whereas it was primarily recovered in the synaptosomal pellet (P2) when a neurologically normal control brain homogenate was used as starting protein preparation (Fig. 8A). This redistribution was not observed after reprobing the same membrane for other three synaptic proteins: synapsin, synaptophyisin and 14-3-3. Furthermore, no obvious enrichment of PACSIN 1 in the nuclear fractions (P0 and P1) could still be detected in the HD brain sample, which agrees with the fact that we rarely observed intranuclear localization of PACSIN 1 with aggregated huntingtin (data not shown).

To complement these results, equal amounts of both human brain homogenates were subjected to SDS–PAGE and analyzed by western blotting. As judged from the signal strength of PACSIN 1 in comparison with actin and β-tubulin, the expression level of PACSIN 1 in the patient sample appeared not to be significantly reduced (Fig. 8B). These results are consistent with PACSIN 1 being re-localized in HD neurons, most likely by direct interaction with mutant huntingtin, and exclude the possibility that PACSIN 1 redistribution is due to a lack or reduction of protein expression.

**DISCUSSION**

Previous studies have proposed a role for PACSIN 1 in linking the recruitment of vesicles and endocytosis with the induction of actin rearrangements mediated by the Arp 2/3 complex (24,25,27). The identification of huntingtin as an interaction partner of PACSIN 1 allows us to further examine the role of huntingtin in the endocytic machinery and how PACSIN 1 may contribute to HD pathophysiology. Our in vitro and in vivo binding experiments demonstrate that the SH3 domain in PACSIN 1 and the proline-rich region in htt-ex1p are essential for the interaction of these proteins. Furthermore, we found that the interaction is dependent on the length of the poly(Q) tract in htt-ex1p. With increasing length of the poly(Q) region, the affinity of htt-ex1p for PACSIN 1 increases as well. The binding of PACSIN 1 to htt-ex1p containing a glutamine repeat in the pathological range (44 glutamines) was ~2-fold stronger than the binding to the same protein containing a poly(Q) repeat in the normal range (18 glutamines). The elongation of the poly(Q) repeat beyond a critical length could lead to the formation of hairpins in the poly(Q) stretch or in the oligomerization of the protein by a ‘zipper’ mechanism (28).

This could then result in a better accessibility of the proline-rich region in htt-ex1p for the interaction with the SH3 domains of binding partners. On the other hand, there have also been reports describing huntingtin interaction partners, such as HIP1, whose affinity to bind to mutant huntingtin is profoundly reduced (29). Recently, HIP1 has been independently identified as an interaction partner of clathrin and adaptin proteins, further implicating huntingtin as playing a role in clathrin-mediated endocytosis (30–32). Our results presented here further add to the possibility that the early neurological features in HD could be caused by changes in the composition of endocytosis-related interaction partners in complexes with huntingtin.

We and others have identified dynamin 1, synaptopojatin 1, N-WASP, synapsin 1 and mSos as interaction partners for all the PACSIN isoforms, and have shown that these interactions are mediated by the C-terminal SH3 domain (24–26). In contrast to these binding partners, huntingtin interacts specifically with PACSIN 1 and does not bind to PACSINs 2 and 3. This is a surprising finding, since PACSINs 1 and 2 differ by only two non-conservative substitutions in their SH3 domains. Furthermore, we and others have shown that huntingtin also binds to the SH3 domains of several members of another protein family, the endophilins (13; unpublished results, manuscript in preparation), which most likely play a role in clathrin-mediated endocytosis and participate in membrane dynamics (33–35). Endophilins A1 and A3 and also the less conserved endophilin B1b are able to interact with huntingtin (13; unpublished results, manuscript in preparation), which suggests a more general function for the endophilin–huntingtin interaction in membrane dynamics. Furthermore, endophilin A3 promotes the formation of insoluble poly(Q)-containing
aggregates *in vivo*, implicating an involvement in the progressive pathology of this disorder (13).

Huntingtin, whose primary function remains unknown, has been shown to associate with vesicles, particularly in cell bodies and dendrites (1,36). It localizes to both clathrin-coated and non-coated vesicles and pits along membranes of the trans-Golgi network and endosomal vesicles (37). The PACSIN proteins are believed to act as an important linker between endocytic proteins and actin polymerization-related proteins (27) at different intracellular locations. In the case of PACSIN 1, its presynaptic localization suggests a role in recruiting the actin polymerization machinery to freshly formed synaptic vesicles that have just been pinched off their presynaptic donor membrane outside of active zones. These vesicles are very likely being removed from these sites via actin comet tail propulsion, and PACSIN 1 may be necessary for the correct attachment of the motor to the vesicle at the former neck. Our immunohistochemistry in human postmortem tissues demonstrates PACSIN 1 in varicosities, along fibers and in the cell bodies of cortical neurons, and huntingtin co-localizing

Figure 6. Co-localization of PACSIN 1 and huntingtin *in vivo*. Using a PACSIN 1-specific antiserum, the distribution of the protein was determined in 30 μm-thick free-floating human frontal cortex sections derived from a healthy control (A) and presymptomatic HD tissue (D), respectively. The endogenous distribution of huntingtin in both sections (B and E) was visualized using a specific antibody. In the overlay, the partial co-distribution of both proteins in healthy (C) and presymptomatic tissue (F) is shown. As controls, (G) and (H) show comparable sections stained only with secondary antibodies as indicated. Additionally, the last panel (I) was examined with a DAPI filter, showing that autofluorescence by lipofuscin was successfully quenched using Sudan black.
with the non-synaptic pool of PACSIN 1 in the cell bodies of those neurons expressing both proteins. In presymptomatic HD tissue, PACSIN 1 appeared polarized and in some cases perinuclear, and was only found in areas where huntingtin was located, indicating that the association with pathological huntingtin leads to a mislocalization of PACSIN 1. Indeed, during the progression of the disease, PACSIN 1 immunoreactivity throughout healthy cortex is gradually removed along fibers and varicosities, which histologically resemble areas of synaptic activity, suggesting an early impairment of PACSIN 1 functions in affected cells. It may be that in these neurons, new synaptic vesicles formed by endocytosis could no longer be actively removed from their donor membranes, but, if at all, would rather move by undirected diffusion. Furthermore, the GTPase dynamin, which plays an essential role at the fission step of nascent clathrin-coated vesicles from the plasma membrane (for a review, see 38) also was found to be gradually removed in presymptomatic tissues (unpublished data, manuscript in preparation). This suggests that pathological huntingtin, by its strong interaction with PACSIN 1, also re-localizes other interaction partners of PACSIN 1 essential for endocytosis in synapses.

The cause of the selective degeneration of specific neurons in distinct brain regions is unknown. Protein aggregates are a shared feature of a wide range of neurodegenerative disorders. In the case of Huntington’s disease, some reports have raised the possibility that nuclear inclusions of mutant huntingtin might not be directly related to striatal cell death, and two recent studies provide new evidence in support of this dissociation. Martín-Aparicio and co-workers (39) have shown that switching off the expression of mutant huntingtin in an inducible transgenic mouse model leads to the disappearance of both huntingtin inclusions and motor impairments in these mice. Despite the reversal of many disease features, a significant reduction in striatal volume was reported. Expression of mutant huntingtin in mechanosensory neurons of Caenorhabditis elegans, an organism that lacks an endogenous version of this protein, led to touch insensitivity and the formation of inclusions (particularly in axons), but not to cell death (40). As PACSIN 1 is selectively expressed in different brain regions affected by HD, including striatum and cortex, the interaction of PACSIN 1 with huntingtin containing an elongated poly(Q) stretch and its re-localization early in the disease could provide another parameter that, over time, leads to the impairment of synaptic functions of distinct neuron populations. With the progression of the disease, the removal of PACSIN 1 could then contribute to the selective vulnerability of neuronal cells and early pathological features in HD disease brains.

Taken together, we have identified huntingtin \textit{in vitro} and \textit{in vivo} as a novel, PACSIN 1-specific interaction partner. The binding appears to be enhanced with pathologically enlarged polyglutamine regions in huntingtin, which leads to re-localization of PACSIN 1 away from varicosities towards the cell body of the neurons, indicating a critical role for PACSIN 1 in the early and progressive neuropathological changes of Huntington’s disease.
MATERIALS AND METHODS

Yeast two-hybrid interaction assays

The experiments were performed using the Matchmaker 2 two-hybrid system (Clontech, Heidelberg, Germany). The full-length open reading frames (ORFs), deletion and point mutants of Pacsin were cloned in frame with the GAL4 DNA-binding domain into the pAS2-1 vector and sequenced. Furthermore cDNA fragments corresponding to exon 1 of human huntingtin (htt-ex1p) with 18 or 44 CAG repeats, respectively, were amplified from a patient’s genomic DNA by PCR using the oligonucleotides 5'-TGG CTA GCC CCC TGG AAA AGC TGA TAGG-3' and 5'-TGG GAT CCC TAG AAT TCC GGT AGC AGC GCC TCC TCA GCC ACA GC-3', adding a BamHI restriction site (indicated here in italics). Both huntingtin fragments were cloned in frame with the GAL4 transcription activation domain into the pACT2 vector. After co-transformation of the yeast strain Y190 with both plasmids, transformed yeast cells expressing interacting GAL4 fusion proteins were selected by their ability to grow on SD medium lacking L-tryptophan, L-leucine and L-histidine. Yeast clones expressing the lacZ gene were detected by a β-galactosidase filter assay. Activation of the lacZ gene was quantified by a liquid culture β-galactosidase assay using o-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate.

Co-precipitation assays

GST–PACSIN fusion proteins were produced by cloning cDNAs corresponding to the complete ORF of wild-type PACSIN and point and deletion mutants into the pGEX-2T, pGEX-3X or pGEX-4T1 vectors (Amersham Pharmacia Biotech, Freiburg, Germany), followed by expression in Escherichia coli (BL21). For precipitation experiments, the GST fusion proteins were immobilized on Glutathione–Sepharose 4B (Amersham Pharmacia Biotech, Freiburg) in PBS. Mouse brains were homogenized on ice in 2.5 ml/g wet weight preparation buffer (150 mM NaCl, 1 mM EGTA, 0.1 mM MgCl2 and 10 mM HEPES, pH 7.4) containing 1% detergent (CHAPS or deoxycholate) and supplemented with a protease inhibitor cocktail (Sigma, Taufkirchen, Germany), 50 μl/g tissue) using a Dounce homogenizer and centrifuged for 30 min at 21 000 g. The supernatant was decanted and recentrifuged. Triton X-100 was added to a final concentration of 0.05%, and the supernatant was dialyzed against preparation buffer lacking detergents for 48 h. Glutathione–Sepharose 4B beads saturated with GST–PACSINs were incubated overnight with the supernatant containing 1 mg of brain protein extracts in 400 μl at 4°C with end-over-end rotation. The beads were washed extensively with preparation buffer and eluted with double-concentrated SDS–PAGE sample buffer. Proteins were separated by SDS–PAGE on a 3–15% gel and transferred to PVDF membrane for subsequent immunodetection with specific antibodies and the ECL kit (Amersham Pharmacia Biotech, Freiburg).

Overlay assays

GST fusion proteins containing short peptides corresponding to the N-terminal, central and C-terminal portions of the human exon 1 proline-rich region of huntingtin were produced by synthesis of the corresponding oligonucleotides and cloning them into the pGEX-6P1 vector (Amersham Pharmacia Biotech, Freiburg, Germany) followed by expression in E. coli (BL21). The GST fusion proteins were purified and eluted using Glutathione–Sepharose 4B (Amersham Pharmacia Biotech, Freiburg, Germany).
Prepared tissues or cells in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris, pH 7.5) containing protease inhibitors (Sigma, Taufkirchen), 50 µl/g tissue). Aliquots of lysates corresponding to 80 µg of protein were resolved on 10% SDS–polyacrylamide gels, followed by transfer to PVDF membranes (Millipore, Bedford, USA). Blots were blocked at room temperature for 1 h with 5% skimmed milk in TBS containing 0.1% Tween-20 (TBST), rinsed with TBST, and incubated at room temperature for 1 h with diluted antibodies. After three 5 min washes with TBST, bound antibodies were detected using peroxidase-conjugated anti-rabbit IgG antibodies (Dako, Hamburg) and the ECL kit (Amersham Pharmacia Biotech, Freiburg).

Antibodies

Polyclonal antibodies against PACSIN 1 were described earlier (41). Monoclonal antibodies against huntingtin (MAB2166) used for the immunoblots were purchased from Chemicon (Hofheim, Germany), polyclonal antibodies against synapsin 1 from Sigma (St Louis, USA), polyclonal antibodies against the Myc epitope from BD Transduction Laboratories (San Diego, USA), polyclonal antibodies against synaptophysin (clone H-8) from Santa Cruz (Santa Cruz, USA), monoclonal antibodies against 14-3-3 proteins (clone N93) and monoclonal antibodies against actin (clone C-4) from ICN (Aurora, USA) and a monoclonal antibody against β-tubulin (N356) from Amersham Pharmacia Biotech (Freiburg).

For visualization of primary antibodies, peroxidase-conjugated goat antibodies against rabbit and mouse immunoglobulins (IgG) were purchased from Dako (Hamburg, Germany).

Subcellular fractionation

For the isolation and analysis of cell membranes derived from human brain tissue, differential centrifugation was used (42). The tissue samples (caudatus) were obtained from the Hirngewebebank München, Institut für Neuropathologie, Ludwig-Maximilians-Universität, München, Germany. All steps were performed at 4°C. Equal amounts of both tissue samples (~200 mg) were each homogenized in 1 ml of ice-cold 5 mM HEPES (pH 7.4) supplemented with a protease inhibitor cocktail (Sigma), using nine strokes of a glass homogenizer. The homogenate was centrifuged for the removal of large cell debris for 5 min at 500 g to produce a pellet (P0), which was washed by resuspension in an equal volume of homogenization buffer and re-centrifuged for 10 min at 1000 g to produce a pellet (P1). The original supernatant and wash were combined (S1), and were then centrifuged at 10,000 g for 20 min, yielding a pellet (P2) and a supernatant (S2). The S2 fraction was centrifuged at 105,000 g for 60 min to give a high-speed pellet (P3) and a high-speed supernatant (S3). The subcellular fractions obtained by this method were analyzed by western blotting.

Immunoblotting

Proteins were extracted by mechanical disruption of freshly prepared tissues or cells in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris, pH 7.5) containing protease inhibitors (Sigma, Taufkirchen), 50 µl/g tissue). Aliquots of lysates corresponding to 80 µg of protein were resolved on 10% SDS–polyacrylamide gels, followed by transfer to PVDF membranes (Millipore, Bedford, USA). Blots were blocked at room temperature for 1 h with 5% skimmed milk in TBS containing 0.1% Tween-20 (TBST), rinsed with TBST, and incubated at room temperature for 1 h with diluted antibodies. After three 5 min washes with TBST, bound antibodies were detected using peroxidase-conjugated anti-rabbit IgG antibodies (Dako, Hamburg) and the ECL kit (Amersham Pharmacia Biotech, Freiburg).

Immunohistochemistry

Formalin-fixed human cortical tissue was obtained from the Harvard Brain Tissue Resource Center (McLean Hospital, Boston, USA) in accordance with institutional guidelines (NIH, Office of Human Subjects Research). Specimens were matched for age and postmortem interval (PMI), and were divided into groups: (i) controls (n = 4; age = 60 ± 11 years and PMI = 16 ± 5 h); (ii) presymptomatic HD gene carriers (n = 2) and grade 1 (n = 3) HD cases (age = 74 ± 10 years and PMI = 18 ± 9 h); (iii) grade 3 (n = 2) and grade 4 (n = 2) HD cases (age = 65 ± 12 years and PMI = 22 ± 14 days). Blocks of frontal cortex from Brodmann area 9 were cut from the tissue specimens and cryoprotected by immersion in 0.01 M PBS containing 30% w/v sucrose for 48 h. Afterwards, the blocks of tissue were frozen on dry ice, and sections were cut on a sliding knife microtome (30 μm thick) and stored in PBS at 4°C until time of use. Sections were washed with PBS and then subjected to antigen retrieval by incubation in 10 mM sodium citrate buffer (pH 9.0) for 30 min in an 80°C water bath (43). The sections were then washed again with PBS, and background staining was blocked with a 1 h incubation in 3% normal serum and 2% bovine serum albumin (BSA). Subsequently, the sections were incubated with rabbit anti-PACSIN 1 antibodies (1 : 5000) and mouse anti-huntingtin antibodies (clone 2166 at 1 : 200, Chemicon, Temecula, USA) diluted in PBS containing 1% normal serum, 1% BSA and 0.4% Triton-X100 overnight at room temperature. Sections were washed six times (10 min each wash) with PBS containing 0.5% Triton-X100, and were then incubated with secondary-fluorochrome-conjugated antibodies (Cy3-conjugated goat anti-rabbit at 1 : 200 and Cy2-conjugated goat anti-mouse IgG at 1 : 200; Jackson Immunoresearch Labs, West Grove, USA) for 1 h at room temperature, diluted in the same media as that used for the primary antibodies. Sections were washed with PBS containing 0.5% Triton-X100 and incubated with 0.1% Sudan black for 5 min to eliminate background from intrinsic autofluorescent pigments (44). The cortical sections were then washed, mounted on slides and dried overnight in the dark. Coverslips were mounted with 50% PBS/glycerol and sealed with nail enamel. The sections were examined using a Zeiss Axiovert 100 M laser scanning confocal microscope. Captured digital images were exported and merged for co-localization images using Photoshop 5.5 software (Adobe Systems, San Jose, USA).

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