Partial characterisation of murine huntingtin and apparent variations in the subcellular localisation of huntingtin in human, mouse and rat brain

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Huntington’s disease (HD) is an inherited neurodegenerative disorder caused by the expansion of a CAG repeat in a gene coding for a protein of unknown function. We have raised a polyclonal antibody against a 12 amino acid peptide (residues 2110–2121 of human huntingtin) which specifically recognises huntingtin on Western blots of human, rat and mouse brain. We have characterised huntingtin expression in the mouse. The protein was detected on Western blots of all mouse tissues examined, with the highest expression seen in brain. Human, mouse and rat brain were fractionated by differential centrifugation and discontinuous Percoll gradients. The fractions were analysed by Western blotting for huntingtin and synaptophysin (a synaptic vesicle localised protein). In mouse brain, huntingtin was localised in the soluble S3 fraction; in rat brain it was localised in the soluble S3 fraction and also in the membrane P2 and P3 fractions; in both normal and HD-affected human brain, huntingtin was membrane bound with a distribution essentially the same as that of synaptophysin. These observed differences in the subcellular localisation of huntingtin between mouse and human brain are important in the context of mouse models for HD.

INTRODUCTION

Huntington’s disease (HD) is an autosomal dominant inherited neurodegenerative disorder. The onset of the disease is usually in the third to fifth decade of life and is characterised by choreiform movements, dementia and a range of psychiatric symptoms (1). Death typically results – 15 years after onset. The neuropathology of HD is very specific and involves selective neuronal loss and astrogliosis, primarily in the caudate nucleus, putamen and, in later stages of the disease, specific layers of the cortex (2,3).

Characterisation of antibodies

Antibodies were raised against a synthetic peptide corresponding to residues 2110–2121 of huntingtin. The immune serum was found to react on Western blots with the bacterial huntingtin–glutathione S-transferase fusion proteins BA10, ES and HA6, which contain the peptide sequence, but not with the similar fusion protein H1 which does not contain it (Fig. 1). Fresh homogenates of mouse and rat brains were analysed by Western
Figure 1. Western blot of  *Escherichia coli*  expressed GST–huntingtin fusion proteins probed with anti-HUNT3 serum. The fusion proteins BA10, ES and HA6 contain the sequence used to raise the antibody while H1 does not. Substantial degradation of the fusion proteins is apparent.

Figure 2. Western blot illustrating the specificity of anti-HUNT3 antibodies. Fifty µg of total rat brain homogenate was electrophoresed in a 6% SDS–PAGE gel, transferred to a PVDF membrane and immunostained with anti-HUNT3 serum. Blotting and a single band at ∼340 kDa was obtained (Fig. 2). This molecular weight was obtained by probing tracks containing mouse brain and human fibroblast extracts for huntingtin and fibrillin (330 kDa) respectively. The electrophoretic mobility of huntingtin was slightly less than that of fibrillin (not shown). No reaction was obtained when blots were probed with pre-immune serum from the animals used to raise anti-HUNT3 antibodies. The immunoreactivity was totally competed out with the peptide used to raise the serum (data not shown).

**Distribution of huntingtin in mouse tissues**

Figure 3 shows a Western blot of protein samples prepared from various mouse tissues probed with anti-HUNT3 serum (b is a longer exposure of the blot shown in a). Expression was highest in the brain, intermediate in kidney, liver and lung, and low in heart and skeletal muscle where overexposure was necessary to obtain a signal. We have also performed the experiment on rat tissues where our results are essentially the same as those of Sharp et al. (13) although slightly different from the results of Trottier et al. (15). Overexposure (Fig. 3b) shows other minor immuno-reactive bands in kidney and liver samples, which are probably degradation products, and a cross-reacting band of higher molecular weight in mouse brain.

**Subcellular fractionation**

The results of marker enzymes for the various subfractions of mouse brain are shown in Table 1. Around 70% of the mitochondrial marker activity (citrate synthase) is located in the P2 fraction as expected. Some mitochondria pelleted with the slowest speed centrifugation and were found in the P1 pellet, while some mitochondria lysed accounting for the activity seen in the S3 fraction. The bulk of the cytosolic marker activity (lactate dehydrogenase) occurred in the S3 fraction with a small amount in the P2S fraction, presumably localised within synaptosomes. The yield of the microsomal marker assay (choline phosphotransferase) activity is low but the bulk of the activity occurs in the P3 fraction. These results indicate that the fractionation procedure is efficient. The results for the human brain fractionation shown in Table 2 are essentially similar, but there appears to have been more lysis of the mitochondria (28% of marker activity in the soluble S3 fraction) and again the yield of the microsomal assay is low, but the bulk of activity occurs in the P3 fraction.
Table 1. Characterisation of subcellular fractions from mouse brain

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Table 2. Characterisation of subcellular fractions from human brain

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Details of fractions as for Table 1. Data were generated in the experiment for which the Western blots are shown in Figure 6.

Figure 4 shows the localisation of synaptophysin and huntingtin after subcellular fractionation of mouse brain. As would be expected, synaptophysin appeared in the P2 and P3 fractions. Huntingtin was slightly enriched in the synaptosomal P2S fraction and highly enriched in the cytosolic S3 fraction. This experiment has been performed on four occasions with consistent results. We have also fractionated whole rat brain (Fig. 5) and normal human temporal cortex (Fig. 6). With rat brain, huntingtin was abundant in the synaptosomal (P2S), microsomal (P3) and cytosolic (S3) fractions. With human temporal cortex, huntingtin was present in the P2 and P3 fractions but diminished in the P1 and S3 fractions. On other occasions with more concentrated fractions using frozen cortical human tissue, huntingtin was readily detected in a human cortex S3 fraction although it was still far more abundant in the membranous fractions (not shown). We have also fractionated human cortex from an HD-affected brain (CAG repeat 44/19) (Fig. 7). Here huntingtin distribution was very similar to that of normal human cortex, with the immunoreactivity in the P2 and P3 fractions and the strongest signal from the synaptosomal (I) fraction. There is also a strong signal in the P1 fraction, and the synaptophysin distribution also shows immunoreactivity in the P1 fraction, unlike the normal brain fractionation shown in Figure 6.

**DISCUSSION**

The huntingtin distribution observed in mouse tissues shows some similarities with those published by other workers for rat tissues, but there are some discrepancies with the previously published data for rats. Sharp et al. (13) examined the distribution of huntingtin in the 100,000 g supernatants from soluble fractions of tissue homogenates and found highest levels in brain (particularly cerebellum), testis, liver and kidney, with lower levels in lung. Trotter et al. (15) used complete homogenates and found strong signals in rat cortex, testis, liver and heart with lower levels in lung and no immunoreactivity in kidney. Gutekunst et al. (16) also found a substantial signal in various brain regions, testis and liver using complete homogenates. In view of our findings with regard to the differential membrane association seen in brain tissue from different species, it is entirely feasible that some of the discrepancies observed in the rat tissue distribution data are a result of sample preparation. Our results for mouse are most similar to those for rat obtained by Sharp et al. (13). Our samples were centrifuged, but only at low speed to remove bulk cell debris and thus if mouse and rat tissues were very similar our results would be expected to look more similar to those of Trotter et al. (15) who used total homogenates. The minor cross-reacting
bands of lower molecular weight seen in kidney and liver are probably degradation products. The faint larger band in brain tissue was only observed in this sample preparation and could be due to incomplete denaturation of the sample.

Figure 4. ImmunobLOTS showing the distribution of huntingtin and synaptophysin after subcellular fractionation of whole mouse brain. Twenty and 10 µg of each fraction was electrophoresed in 6 and 10% SDS–PAGE gels respectively. The resolved proteins were electroblotted onto PVDF membranes and immunostained for (a) huntingtin (blot of 6% gel) and (b) synaptophysin (blot of 10% gel). H = total homogenate; P1 = crude nuclei and debris; P2M = mitochondria-enriched fraction; P2S = synaptosome-enriched fraction; P3 = membrane-enriched fraction; S3 = cytosol-enriched fraction. The synaptosome-enriched fraction (P2S) was further purified on a 10–16% Percoll step gradient: S = supernatant from top of gradient; I = synaptosome-enriched fraction removed from gradient interphase; P = pelleted material.

Figure 5. ImmunobLOTS showing the distribution of huntingtin and synaptophysin after subcellular fractionation of whole rat brain. Fifty and 10 µg of each fraction was electrophoresed in 6 and 10% SDS–PAGE gels respectively. The resolved proteins were electroblotted onto PVDF membranes and immunostained for (a) huntingtin (blot of 6% gel) and (b) synaptophysin (blot of 10% gel). H = total homogenate; P1 = crude nuclei and debris; P2M = mitochondria-enriched fraction; P2S = synaptosome-enriched fraction; P3 = membrane-enriched fraction; S3 = cytosol-enriched fraction. The synaptosome-enriched fraction (P2S) was further purified on a 10–16% Percoll step gradient: S = supernatant from top of gradient; I = synaptosome-enriched fraction removed from gradient interphase; P = pelleted material.

Figure 6. Western blots showing the distribution of huntingtin and synaptophysin after subcellular fractionation of human temporal cortex. Twenty and 10 µg of each fraction was electrophoresed in 6 and 10% SDS–PAGE gels respectively. The resolved proteins were electroblotted onto PVDF membranes and immunostained for (a) huntingtin (blot of 6% gel) and (b) synaptophysin (blot of 10% gel). H = total homogenate; P1 = crude nuclei and debris; P2M = mitochondria-enriched fraction; P2S = synaptosome-enriched fraction; P3 = membrane-enriched fraction; S3 = cytosol-enriched fraction.

On subcellular fractionation murine huntingtin was most abundant in the soluble fraction (Fig. 4). Sharp et al. (13) fractionated rat cerebellar tissue and rat brain striatum and found that huntingtin was abundant in the synaptosomal subfraction of P2, P3 and S3, a very similar result to our result in whole rat brain (Fig. 5). Our results on fractionation of human cortex shown in Figure 6 are slightly different from those reported by DiFiglia et al. (14). They observed substantial huntingtin and synaptophysin immunoreactivity in the P1 fraction which includes cell debris and nuclei from a low speed centrifugation. We did not see such immunoreactivity and this was probably the result of slight procedural differences. We filtered the brain homogenate through nylon gauze before centrifugation, but DiFiglia et al. (14) apparently did not do so. This filtration allows nuclei to pass into the filtrate but prevents this fraction containing larger cell debris and is particularly effective with fresh tissue. We observed P1 huntingtin immunoreactivity in fractionations from previously frozen human brain tissue. The HD sample in Figure 7 shows this immunoreactivity in P1 clearly, and this is a frozen sample. DiFiglia et al. (14) did not indicate whether their human brain cortical sample had been obtained fresh or if it had been frozen prior to fractionation and analysis, but this could account for the observed differences. DiFiglia et al. (14) also observed some huntingtin immunoreactivity in the S3 soluble fraction; we observed very low levels of huntingtin in the soluble fraction in HD or normal brain tissue fractionations. This was not usually visible on SDS–PAGE followed by Western blotting unless the S3 sample was very concentrated. However, we found, as did DiFiglia et al. (14), that in both HD and normal human brain,
fractions containing synaptophysin also contain huntingtin, and this is an approximately proportional relationship.

The fractionation of HD brain material also showed that there was no detectable huntingtin immunoreactivity in the soluble fraction. The frozen brain material from HD patients gave a weaker signal than fresh normal brain (compare Fig. 7 with Fig. 6), but this is in line with our previous observations (unpublished) that frozen brain samples and post-mortem samples did generally give a poorer signal and also showed a series of degradation products which were not observed when fresh brain tissue was used. The huntingtin immunoreactivity observed in the P1 fraction is probably also due to tissue degradation, and it should be noted that synaptophysin immunoreactivity also occurred in the P1 fraction. These results, however, clearly indicate that in normal and HD human cortex huntingtin is a membrane associated protein with only a very small proportion of the immunoreactivity found in the soluble fraction. It should be added that the gel conditions used in the experiment shown in Figure 7 did not separate the proteins containing 19 and 44 glutamines. We have used different gel conditions to separate them and used cortex from HD brains with greater differences between the normal and expanded proteins, but have not observed any differences between the partitioning of the proteins to date.

The most important conclusion of this study is that, unlike human and rat huntingtin, the murine homologue is a predominantly cytosolic protein. Knowledge concerning the subcellular and tissue distribution of huntingtin in the mouse is important when considering potential mouse models for HD. In this context, differences between mouse and human huntingtin will need to be understood.

It is possible that the observed apparent differences in the membrane affinity of huntingtin may be due to differences in the primary structure between species. Alternatively, the human protein may interact with another, membrane-bound protein not present in mouse brain, thus giving the apparent membrane localisation in human brain. This observation is very relevant to any transgenic mouse models. The most striking difference between human and rodent huntingtin is the length of the polyglutamine stretch, but it is difficult to see how a single extra glutamine could account for the observed differences between mouse and rat huntingtin (see Figs 4 and 5) (12,17) and other differences do exist (17). It seems more likely that differences between mouse, rat and human tissue, other than differences in huntingtin itself, account for the observed results. It is also possible that different areas of the brain may show different huntingtin fractionation properties. However, our result for whole rat brain is essentially the same as that of Sharp et al. (13) in rat striatum and cerebellum. It is interesting that while subcellular fractionation of brain from human, rat and mouse gave quite different results, immunohistochemical staining of cortex from all three species gave very similar results (13,16 and MacMillan et al., unpublished results).

Interpretation of these differences is complicated by the fact that these fractionations were not performed under physiological saline conditions. Sharp et al. (13) reported that a high degree of huntingtin immunoreactivity could be removed from membranes by washing with 200 mM or 1 M NaCl. We have observed that unwashed mouse brain P3 membrane fractions contained a substantial amount of huntingtin immunoreactivity (not shown). A single wash with buffer A greatly reduced this; enzyme assays indicated that 3.2% of the initial lactate dehydrogenase activity was found in an unwashed P3 membrane fraction, which fell to 0.9% on washing (Table 1). We have also observed that a further wash of rat brain P3 membranes with 150 mM or 500 mM NaCl does not remove all of the huntingtin immunoreactivity. This implies that some of the protein may be firmly attached to membranes. This difference may reflect the existence of the protein in different states and it may move between membrane-associated and cytosolic localisation in response to specific signalling mechanisms.

The widespread expression of huntingtin in neural and non-neural tissues (13,15,16), its cytoplasmic localisation (13–15), its association with membranes (13) and the lethality of targeted disruption of the mouse Hdh gene in the homozygous state (18–20) are all consistent with huntingtin playing an essential role in cellular membrane and/or protein trafficking (14). The mouse knockout data (18–20) illustrates that huntingtin is expressed at an early stage in rodent embryonic development, while we have detected huntingtin in 16 week human foetal brain tissue (unpublished results).

How does the HD mutation cause the disease phenotype? Polyglutamine tracts may be involved in protein–protein interactions (21,22). The polyglutamine expansion seen in HD may change the binding properties of huntingtin for some as yet unidentified microtubular or vesicular protein component. It is possible that this could perturb the balance of neurotransmitter release and uptake in neurones leading to the pathology associated with HD. To look for evidence of novel interactions, we have fractionated brain tissue from a number of HD patients.
and unaffected controls but, as yet, we have not found any major differences in the subcellular distribution of the normal and expanded proteins. Although in both normal and HD brain fractionations huntingtin appears to be membrane bound (Figs 6 and 7), there is some soluble protein. Is it the membrane-bound or the soluble form which causes the pathology when an expanded polyglutamine tract is present, or do both forms play a role in the pathology? More information is required before the mechanism underlying the pathogenesis of the polyglutamine expansion is understood.

MATERIALS AND METHODS

Preparation of antibodies

Oligopeptide HUNT3 (CWTRSDSALLEG, residues 2110–2121 of human huntingtin) was synthesised by Severn Biotech. Ltd., Kidderminster, UK. The peptide was conjugated to keyhole limpet haemocyanin with glutaraldehyde and antibodies raised in adult male NZW rabbits. Primary inoculations were with 300 µg in incomplete Freund’s. The first boost was given 2 weeks after the primary inoculation then two further boosts given at 6 and 10 weeks. Serum was collected 12–14 days after boosts. Serum was used routinely for Western blotting. As a control, antibodies were raised against limpet haemocyanin with glutaraldehyde and antibodies raised in adult male mice by the same procedure. Serum was collected 2 weeks after the primary inoculation then two further boosts given at 6 and 10 weeks. Serum was collected 12–14 days after boosts. Serum was used routinely for Western blotting.

Production of fusion proteins

Restriction fragments of clone IT15B were cloned into the prokaryotic expression vector pGEX-2T (Pharmacia Biotech) to generate fusion proteins. The following fusion proteins were generated: H1, a 1.2 kb HindIII fragment (bases 3401–4613) corresponding to amino acids 1030–1432; HA6, a 2.9 kb HindIII fragment (bases 4613–7570) corresponding to amino acids 1434–2418; ES1a, a 1.65 kb EcoRI fragment (bases 5345–6988) corresponding to amino acids 1678–2224; BA10, a 1.8 kb BamHI fragment (bases 6257–8052) corresponding to amino acids 1980–2579. Fusion proteins were used in bacterial cultures with 0.1 mM IPTG and inclusion bodies purified according to Castro et al. (23).

Western blotting

Proteins were separated by SDS–PAGE using the discontinuous buffer system described by Laemmli (24) in either 6% T, 2.6% C or 10% T, 2.6% C polyacrylamide gels, then electroblotted onto a PVDF membrane (Immobilon-P, Millipore) according to Towbin et al. (25). Ten percent gels were transferred using a semi-dry blotter while 6% gels were wet-blotted. The transfer of high molecular weight proteins (>100 kDa) was found to be very inefficient with the semi-dry apparatus. Blots of 6% gels were probed with anti-HUNT3 serum at a 1:750 dilution, while blots of 10% gels were probed with a monoclonal anti-synaptophysin antibody at 20 ng/ml (Calbiochem). Blots were developed using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Amersham).

Tissue distribution of huntingtin

Fresh tissue samples were homogenised in 5 volumes of 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM benzamidine, 10 µg/ml pepstatin A, 2 µg/ml aprotinin, 50 µg/ml leupeptin. Debris was pelleted by centrifugation at 3000 g for 1 min. The protein concentrations of the supernatants were determined according to Peterson (26) and equivalent loadings of the samples subjected to Western blot analysis.

Subcellular fractionation of brain tissue

Fresh, unfrozen brain tissue was used, except for HD-affected tissues, which were from samples with 9–72 h post-mortem intervals, subsequently stored frozen at –70°C or in liquid nitrogen. Whole mouse and rat brains were fractionated. The fresh human material was normal temporal cortex obtained from a young adult male with an astrocytoma. Tissue was homogenised in 10–20 volumes of buffer A (5 mM HEPES (pH 7.4), 0.32 M sucrose, 1 mM PMSF, 1 mM benzanidine, 10 µg/ml pepstatin A, 2 µg/ml aprotinin, 50 µg/ml leupeptin) using a motorised glass–teflon Potter homogeniser and filtered through nylon gauze. Frozen tissue was defrosted in buffer A before homogenisation. The homogenate was centrifuged at 100 000 g for 5 min and the nuclear pellet (P1) washed by resuspending in buffer A and recentrifuging. The S1 supernatants were pooled and centrifuged at 10 000 g for 10 min to give a mitochondrial/synaptosomal P2 pellet which was washed by resuspending in buffer A and recentrifuging. On some occasions, the cream upper portion of P2 (P2S) which is more enriched in synaptosomes was scooped off the brownish lower portion (P2M) which is more enriched in mitochondria. The S2 supernatants were pooled and centrifuged at 105 000 g for 1 h. This gave a microsomal P3 pellet which was washed to reduce the level of cytosolic contamination. The supernatants from the high speed spins were combined to give the cytosolic S3 fraction which was concentrated by ultrafiltration (10 kDa cut off membrane) when necessary. Synaptosomes were further purified on a 10–16% Percoll step gradient according to Gordon-Weeks (27). The concentration of protein in each fraction was determined according to Peterson (26). Fractions were characterised using marker enzymes. Citrate synthase was used as a mitochondrial marker (28), lactate dehydrogenase as a cytosolic marker (29) and choline phosphotransferase as a microsomal marker (30). Fractions were then subjected to Western blot analysis.

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REFERENCES


