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Huntingtin is associated with cytomatrix proteins at the presynaptic terminal

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ABSTRACT

Huntington's disease (HD) is a single gene disorder produced by expansion of the gene encoding *huntingtin* (htt), a large protein with features of a multi-functional scaffold. Expansion of htt's polyglutamine domain induces novel, toxic interactions and likely also disrupts normal htt function. Because of its predicted role as a scaffold, pursuit of huntingtin function and HD pathogenesis has focused on identifying htt-interacting proteins. Here we present a focused screen designed to identify htt-interacting proteins in the presynaptic terminal. To identify interactions that occur in situ, synaptosomes (isolated nerve terminals) from cerebral cortices, striata and hippocampi were subjected to chemical crosslinking followed by denaturation, immunoprecipitation using an anti-htt antibody, and nano-flow liquid chromatography tandem mass spectrometry (nanoLC–MS/MS) analyses. The presynaptic cytomatrix proteins Bassoon, Piccolo/Aczonin and Ahnak were among the most consistently identified binding partners. Co-immunoprecipitation and co-fractionation studies support the conclusion that huntingtin is a component of the presynaptic cytomatrix, a complicated network of proteins that regulates the positioning and priming of synaptic vesicles. These findings implicate htt in presynaptic functioning, and suggest that aberrant organization of presynaptic components may contribute to the neurological pathology associated with HD.

1. Introduction

Affecting one in every 10,000 people in the U.S., Huntington's disease (HD) is a devastating neurodegenerative disorder that is characterized by progressive loss of neurons in the striatum, hippocampus and cerebral cortex (Reiner et al., 1988; Rosas et al., 2002, 2005). HD is an autosomal dominant disease. It is caused by expansion of the gene that encodes the protein *huntingtin* (htt) (Group, 1993), a multi-functional scaffolding protein that regulates multiple cellular processes. The normal human huntingtin protein contains a domain of 6–35 consecutive glutamine residues near its amino-terminus. Expansion of this tract beyond 40 glutamines results in the clinical symptoms of HD including middle-age onset of motor, cognitive and psychiatric deficits (Zuccato et al., 2010).

HD is considered to be a gain-of-function disorder caused by a novel function(s) of mutated htt. This idea follows from the observation that a single copy of the expanded gene is sufficient to induce disease. Furthermore, introduction of an expanded copy of the gene into wild-type mice, with two normal htt genes, also produces an HD-like phenotype.

Loss of wild type htt function cannot be excluded in HD pathogenesis, however. Global disruption of the htt gene in mice results in early embryonic lethality, and mice heterozygous for a disrupted htt gene display a phenotype consistent with a mild form of HD (Duyao et al., 1995; Nasir et al., 1995). Moreover, inactivation of htt expression in the forebrain of mice produces progressive neuronal degeneration along with motor abnormalities (Dragatsis et al., 2000). Thus, it appears likely that loss of normal htt action also contributes to the clinical manifestations of HD, and determining htt's normal function may identify new ways to treat the disorder.

htt contains several HEAT repeat sequences (Andrade and Bork, 1995), domains that function as molecular scaffolds. As is true for other scaffolds like the A-Kinase Anchoring Proteins (AKAPS) (Carnegie et al., 2009), htt appears to serve multiple clients throughout the cell. To date it has been implicated in transcription, vesicle transport, and endocytosis (Cattaneo et al., 2005). The possibility that htt functions as a scaffold has led to interest in its binding partners, and several large proteomic screens have been performed (Culver et al., 2012; Kalchman et al., 1997; Shirasaki et al., 2012). The functional range of the proteins identified in these screens supports the concept of htt influencing multiple cellular functions. Among the classes of proteins identified are proteins of the synapse.

The idea that aberrant htt action at presynaptic terminals is an important contributor to HD is supported by a preponderance of genetic interactions between an HD phenotype and genes encoding presynaptic proteins (Kaltenbach et al., 2007; Nollen et al., 2004; Parker et al., 2007; Ravikumar et al., 2008; Romero et al., 2008; Scappini et al., 2007). It is further supported by evidence indicating that synaptic dysfunction is one of the early pathophysiological changes in HD (Milnerwood and Raymond, 2010; Smith et al., 2005).

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Here we report a focused screen for htt-interacting proteins in presynaptic terminals of the central nervous system. Unlike previous screens, all of which employed *in vitro* binding or immunoprecipitation from whole brain lysates, we used synaptosomes (presynaptic nerve terminals) as a starting material. In addition, we biased our screen for interactions that occur *in situ* by treating synaptosomes with a membrane permeant chemical crosslinker followed by denaturation. Our results indicate that htt is a component of presynaptic cytomatrix complex, suggesting that htt may be involved in regulating neurotransmitter release.

2. Results

2.1. htt is present at presynaptic terminals

Genetic interactions between the htt gene and genes encoding presynaptic proteins suggest that htt contributes to presynaptic functioning (Kaltenbach et al., 2007). Indeed, htt is reported to be associated with synaptic vesicles in rat brain, suggesting a role in vesicle exo- and/or endocytosis (DiFiglia et al., 1995; Velier et al., 1998). To identify possible effectors of htt in an animal model for which htt-mutant strains are available, we first asked if htt protein is present in presynaptic terminals isolated from mouse brain. Somewhat surprisingly, we found that the standard synaptosome preparation protocol developed for rat brain (Schivell et al., 1996) did not work consistently with mouse brain. Therefore, we adapted two methods (Dodd et al., 1981; Palaty et al., 1994) and established a two-step sucrose density ultracentrifugation procedure (Fig. 1A) that consistently purified presynaptic markers.

Western blotting results revealed that a significant proportion of htt was present in the synaptosome fraction, identified by the presence of the presynaptic plasma membrane protein Cav2.1 (a P/Q type calcium channel) (Lautermilch et al., 2005) and the synaptic vesicle protein synaptophysin (Fig. 1B). These data indicate that htt is a component of presynaptic terminal in mouse brain.

2.2. A proteomic screen of htt-associated proteins in synaptosomes identifies components of the presynaptic cytomatrix

Based on the idea that htt functions as a scaffold, we set out to identify its client proteins in the presynaptic terminal using an unbiased, proteomic approach. This screen differed from previous proteomic analyses of htt interactions in three ways. First, we limited our analysis to synaptic terminals from cerebral cortex, hippocampus, and striatum, which are the regions of the brain most affected by HD. Second, we employed chemical crosslinking followed by denaturation. This step allowed us to detect weak interactions. Third, to ensure that we were analyzing interactions that occur *in situ*, we treated crosslinked synaptosomes with denaturing reagents to disrupt non-covalent interactions and discourage interactions that occur upon solubilization. Protein complexes containing htt were then isolated from diluted extracts using anti-htt antibodies.

Because the screen started with a sub-fraction of selected brain regions, we needed to optimize each step to generate sufficient material for mass spectrometric analysis. We screened three membrane-permeant crosslinking reagents, five detergents and four anti-htt antibodies recognizing different regions of the protein. In each case we selected the reagent that provided the best yield of immunoisolated htt-containing protein complexes. The optimized procedure included 1) crosslinking with DSP ([Dithiobis[succinimidyl propionate]), a membrane permeant, thiol-cleavable, homobifunctional and amine-reactive crosslinking agent, 2) solubilization in SDS and removal of insoluble material by centrifugation, and 3) immunoisolation of htt and associated proteins using mAb 2166, an antibody that recognizes an epitope in the middle of the htt protein (a.a. 443–457). Control precipitations were run concurrently in the absence of antibody.

In the studies reported here, synaptosomes isolated from tissue pooled from approximately 6–12 mice were subjected to chemical crosslinking with DSP followed by quenching and solubilization in the denaturing detergent SDS. Insoluble material was removed by centrifugation and the remaining material diluted in a pH buffered potassium acetate solution containing 1% Triton X-100. htt and associated proteins were then immunoprecipitated.

Based on results of four biological replicates, we generated a list of htt-interacting proteins at presynaptic terminals. In all experiments, mass spectrometric results indicated that a substantial amount of htt was recovered in the immunoprecipitated samples whereas control samples with no antibody generated limited to no detectable htt signal. The total extracted ion intensity of peptides identifying a protein in a sample was used to calculate fold-enrichment over the matched control run. If the ratio was ≥ 2, the protein was regarded as a protein enriched in the anti-htt antibody sample. If a protein was determined to be positive in two of the four experiments, it was considered a potential htt-associated protein. Based on these criteria we identified proteins potentially associated with htt at presynaptic terminals (Supplemental Table 1).

2.3. Validation of the interaction between htt and presynaptic cytomatrix proteins

Among the most consistently identified proteins in our proteomic screen were three proteins considered to be part of a large scaffolding

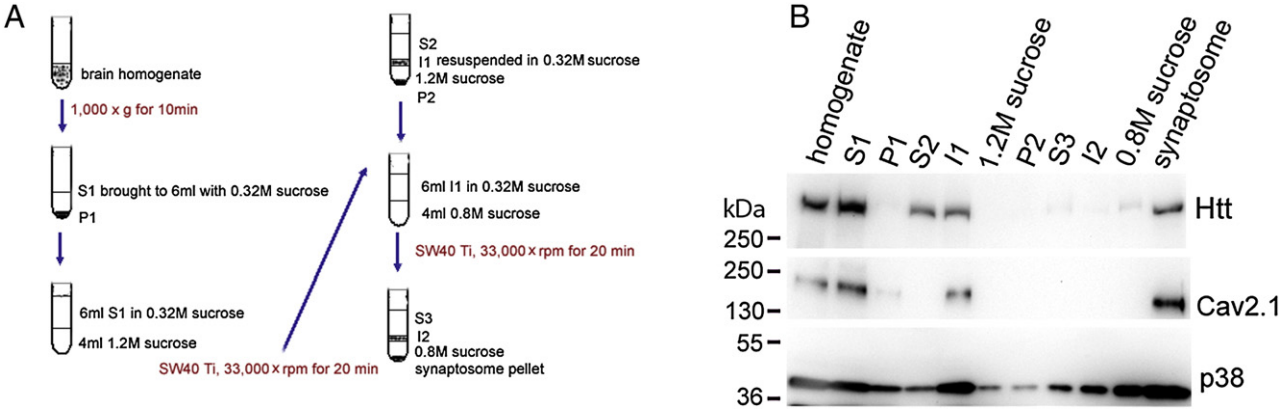

Fig. 1. htt is present in brain synaptosomes. A) Schematic of an optimized preparation of mouse brain nerve terminals (synaptosomes) using sucrose density ultracentrifugation. B) Western blotting analyses of htt, the presynaptic calcium channel Cav2.1 and the synaptic vesicle protein synaptophysin/p38 in fractions across the synaptosome preparation. 0.2% of each sample was used to detect htt and 0.05% to detect Cav2.1 and synaptophysin/p38. htt is present in the synaptosome (presynaptic terminal) fraction. Shown are representative Western blotting results from 6 independent experiments.