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Wild-type HTT modulates the enzymatic activity of the neuronal palmitoyl transferase HIP14

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Huntington disease (HD) is caused by polyglutamine expansion in the huntingtin (HTT) protein. Huntingtin-interacting protein 14 (HIP14), one of 23 DHHC domain-containing palmitoyl acyl transferases (PATs), binds to HTT and robustly palmitoylates HTT at cysteine 214. Mutant HTT exhibits reduced palmitoylation and interaction with HIP14, contributing to the neuronal dysfunction associated with HD. In this study, we confirmed that, among 23 DHHC PATs, HIP14 and its homolog DHHC-13 (HIP14L) are the two major PATs that palmitoylate HTT. Wild-type HTT, in addition to serving as a palmitoylation substrate, also modulates the palmitoylation of HIP14 itself. *In vivo*, HIP14 palmitoylation is decreased in the brains of mice lacking one HTT allele (*hdh*+/−) and is further reduced in mouse cortical neurons treated with HTT antisense oligos (HTT-ASO) that knockdown HTT expression by ∼95%. Previously, it has been shown that palmitoylation of DHHC proteins may affect their enzymatic activity. Indeed, palmitoylation of SNAP25 by HIP14 is potentiated *in vitro* in the presence of wild-type HTT. This influence of HTT on HIP14 activity is lost in the presence of CAG expansion. Furthermore, in both brains of *hdh*+/− mice and neurons treated with HTT-ASO, we observe a significant reduction in palmitoylation of endogenous SNAP25 and GluR1, synaptic proteins that are substrates of HIP14, suggesting wild-type HTT also influences HIP14 enzymatic activity *in vivo*. This study describes an important biochemical function for wild-type HTT modulation of HIP14 palmitoylation and its enzymatic activity.

INTRODUCTION

Palmitoylation is the posttranslational addition of the saturated 16 carbon lipid palmitate via a thioester linkage to specific cysteine residues (1). Many neuronal proteins are palmitoylated, including ion channels, cell adhesion molecules, scaffolding molecules, neurotransmitter release machinery and signaling proteins. Huntingtin (HTT), which, when in its polyglutamine expanded form, is the genetic basis of Huntington disease (HD), is an important palmitoylated protein (2–4). Palmitoylation is mediated by a group of enzymes called palmitoyl acyl transferases (PATs) characterized by a cysteine-rich domain with a defining Asp–His–His–Cys (DHHC) core motif, which is essential for PAT activity both *in vitro* and *in vivo* (3,5–7).

One of the first identified mammalian DHHC containing PATs was huntingtin-interacting protein 14 (HIP14 or DHHC-17), which palmitoylates HTT (3,8). Accumulating evidence suggests that aberrant palmitoylation of different proteins may contribute to the pathogenesis of HD (3,9,10). The polyglutamine expansion in mutant HTT results in reduced interaction with HIP14 (9) and, in turn, reduced palmitoylation of HTT, which causes accelerated inclusion formation and enhanced neuronal toxicity and cell death (9). In

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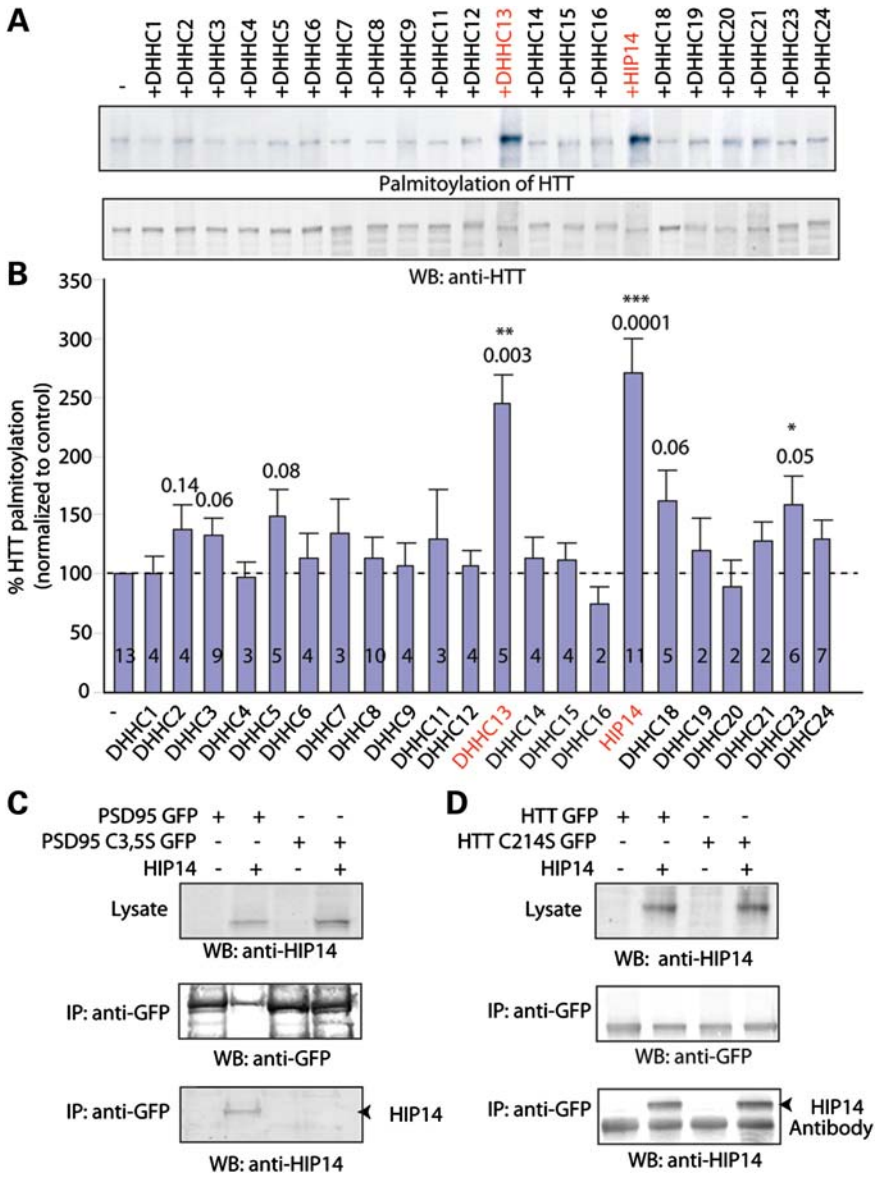

Figure 1. HIP14 and HIP14L are the two major PATs for HTT. (**A**) COS cells expressing either HTT alone or HTT with individual DHHC protein. HTT was immunoprecipitated, followed by the biotin palmitoylation labeling assay. Among the indicated DHHC proteins, HIP14L (DHHC-13) and HIP14 (DHHC-17) significantly increased palmitoylation of HTT. (**B**) Quantification of HTT palmitoylation increase by individual DHHC protein. Palmitoylation of HTT by an individual enzyme was normalized by western blot and compared with control. Number of repeats was indicated in the graph. (**C**) Interaction between PSD95 and HIP14 requires the presence of palmitoylated cysteines C3, 5. Mutation of cysteines 3 and 5 in PSD95 abolished its interaction with HIP14 detected by co-IP. (**D**) In contrast, association of wild-type HTT to HIP14 was independent of palmitoylation of HTT.

addition to that of HTT, palmitoylation and function of glial glutamate transporter-1 (GLT-1) are also reduced in the YAC128 mouse model of HD. Impaired GLT-1 palmitoylation is present early in the pathogenesis of HD and may contribute to decreased glutamate uptake, excitotoxicity and, ultimately, neuronal cell death in HD (10).

Multiple studies in cellular and animal model systems indicate that mutant HTT imparts a novel toxic function, and the mechanism of toxicity involves multiple pathogenic pathways (11). However, the normal function of wild-type HTT, an essential cellular protein in higher vertebrates, is not yet well understood (12). HTT is encoded by a single gene and is ubiquitously expressed in mammals. It is primarily a cytoplasmic protein, known to be both vesicle- and microtubule-associated (13,14). Deletion of the *HTT* gene is lethal early in mouse embryogenesis (15–17). The multidomain structure of HTT indicates that the protein may have multiple distinct cellular roles, including transcriptional regulation, nucleo-cytoplasmic shuttling, vesicular trafficking and intracellular transport in the cell, synaptic function and anti-apoptotic activity (reviewed in 12,18).

Accumulating data on the roles of wild-type HTT in endocytosis, endosomal motility and axonal transport have led to an emerging model for HTT as an integrator of protein

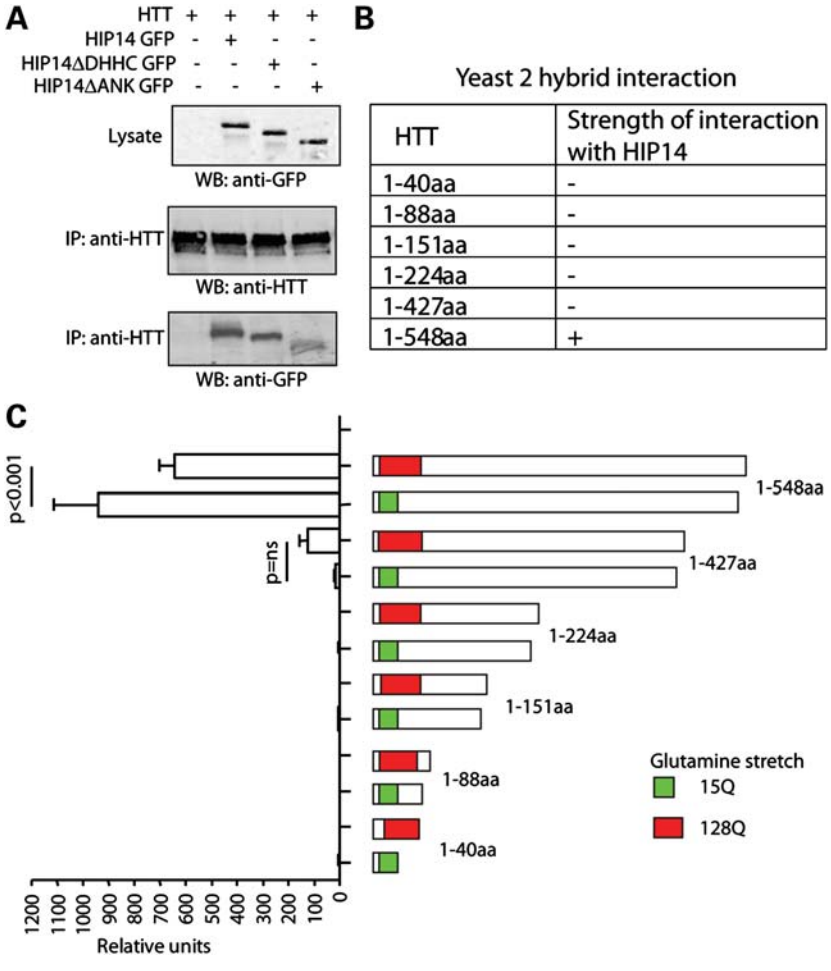

Figure 2. Ankyrin repeats of HIP14 and N-terminus (1–548 amino acids) of HTT are crucial for their interaction. (**A**) HTT was IPed from COS cells expressing HTT alone or HTT with HIP14 GFP, HIP14 ΔANK GFP or HIP14 ΔDHHC GFP. The amount of HIP14 co-IPed was detected by western blotting with anti-GFP antibody. Deletion of ankyrin repeats significantly reduces the association of HIP14 and HTT. In contrast, deletion of DHHC catalytic domain does not affect the interaction. (**B** and **C**) Yeast two-hybrid semiquantitative β-galactosidase assays to map the region of HTT to which HIP14 binds. The strength of interaction is indicated by a semiquantitative scale of colony growth (with +, and − indicating strong and no growth, respectively), indicating that HIP14 interacts specifically with a region of HTT encompassing amino acids 427–548. Interaction between HIP14 and mutant HTT (1–548 amino acids) is significantly decreased compared with that of wild-type HTT.

transport along the cellular cytoskeleton (reviewed in 12). HIP14, which is predominantly expressed in the Golgi apparatus, is also found to be associated with endosomal vesicles in the soma, presynaptic terminals and dendritic spines. It is possible that trafficking of these HIP14-containing vesicles to sites where it executes its enzymatic function may require HTT. Given the multi-domain nature of HTT and its strong interaction with HIP14, HTT may also function as an allosteric activator of HIP14. In this study, we tested the hypothesis that wild-type HTT, in addition to serving as a substrate of HIP14, also directly influences HIP14 enzymatic activity.

RESULTS

HIP14 and HIP14L are the two major HTT PATs

We have previously shown that HTT is palmitoylated by HIP14 and HIP14L (DHHC-13), a HIP14 homolog (3,8,9). In fact, palmitoylation of HTT by HIP14 is essential for its trafficking and function (9). The identification of 23 DHHC proteins in this PAT family raised the question whether other DHHC proteins palmitoylate HTT (5). We therefore applied the acyl biotin exchange (ABE) labeling method to examine the palmitoylation level of HTT upon overexpression of individual DHHC proteins along with HTT in COS cells. We found that HIP14 and HIP14L are the two major PATs that significantly increase palmitoylation of HTT (Fig. 1A and B).

Interaction of HIP14 and HTT is unique and distinct from other palmitoylation enzyme–substrate interactions

Co-transfection of a palmitoylation substrate protein with its cognate PAT has been found in a number of cases to result in a long-lived enzyme–substrate interaction seen both by co-localization and co-immunoprecipitation (co-IP) (5,8,19). These interactions typically require that both the substrate protein and PAT be competent for palmitoylation, with

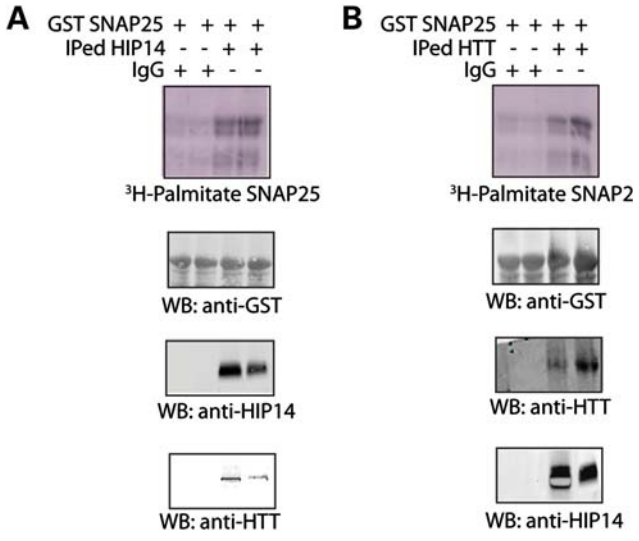

Figure 3. HIP14 and HTT form a tight complex, exhibiting palmitoyl-transferase activity. (**A**) IPed HIP14 from rat whole brain exhibited palmitoyl-transferase (PAT) activity toward GST-SNAP25. Unimmunized rabbit serum (control) or HIP14 antibody bound to sepharose beads was used to IP HIP14 from rat whole brain lysate. Then the beads containing IgG control or IPed HIP14 were added to reaction mix containing ^{3}H-palmitoyl-CoA to palmitoylate GST-SNAP25 *in vitro*. As previously shown, IPed HIP14 is capable of increasing palmitoylation of SNAP25 compared with serum control (top panel). HIP14 IP was confirmed by western blot with HIP14 antibody and co-IPed HTT was detected by HTT antibody (bottom two panels). (**B**) IPed wild-type HTT from rat whole brain also exhibited PAT activity toward GST-SNAP25. Similar to (A), unimmunized mouse serum (control) or HTT antibody bound to sepharose beads was used to IP HTT from rat whole brain lysate. IPed HTT was confirmed by western blot with HTT antibody and co-IPed HIP14 was detected by HIP14 antibody (bottom two panels).

interactions not seen either with the mutational removal of substrate protein cysteinyl acceptors or when the co-transfected PAT is mutationally inactivated, suggesting that the interaction may reflect a continued association of enzyme and substrate that may persist following the productive palmitoylation (5,8,19). As an example of this phenomenon, here, we find by co-IP that wild-type PSD95, but not the non-palmitoylatable PSD95 C3,5S mutant interacts with its cognate PAT, HIP14 (Fig. 1C). Interestingly, this is not the case for HTT. The strong HTT interaction with HIP14 persists even when HTT palmitoylation is severely impaired by the C214S mutation of the primary HTT cysteinyl acceptor site (9) (Fig. 1D). Thus, in this regard, the HTT–HIP14 interaction appears to be fundamentally different from the interactions of other palmitoylation substrates with their PATs, in that the stable HTT–HIP14 association does not require the palmitoylated HTT cysteine.

HIP14 and HIP14L are unique among the 23 DHHC proteins in that both contain ankryin repeat domains, mapping within the cytoplasmically disposed N-terminal domains of these two PATs (20). We have constructed two HIP14 deletion mutants that remove either the ankryin repeat domain (HIP14 ΔANK) or the catalytic DHHC domain (HIP14 ΔDHHC). Both mutants were tested for their effects on the HTT–HIP14 interaction (Fig. 2A). While HIP14 ΔANK shows a markedly impaired interaction with HTT, HIP14 ΔDHHC shows a surprisingly robust interaction, indistinguishable from that seen with wild-type HIP14. This lack of reliance on DHHC domain integrity and thus on PAT activity again indicates that the HTT–HIP14 interaction is fundamentally different from those documented for other DHHC PAT–substrate interactions (5,8,19).

Next, we used yeast-two-hybrid analysis to delineate the HTT domains that are required for this interaction (Fig. 2B and C). A series of HTT N-terminal fragments were examined. The 548 residue-long N-terminal fragment showed a quite strong interaction. Furthermore, as previously documented (20), reduced interaction is seen for mutant HTT which contains the glutamine expansion. The HTT–HIP14 interaction is quite severely impaired when the HTT fragment is shortened from 548 to 427, indicating that elements within the HTT 427–548 amino acids likely play a key role in this interaction. With further shortening, HIP14–HTT interaction was fully abolished (Fig. 2B and C). Note that the N-terminal HTT fragment 1–88 amino acids that contain exon 1 is unable to interact with HIP14.

HTT and HIP14 form a tight complex that has enzymatic activity

We have previously showed that HIP14 is a PAT for SNAP25 (3). Consistent with this, immunoprecipitated (IP) HIP14 from rat brain lysate exhibited palmitoyl-transferase activity toward GST-SNAP25 *in vitro* (Fig. 3A). When HTT was IPed from rat brain lysate, the resulting protein surprisingly also increased palmitoylation of GST-SNAP25 (Fig. 3B). This suggested to us that HTT's binding partner, HIP14, was co-IPed resulting in PAT activity. Western blots (Fig. 3A and B, bottom panels) confirmed that, in all IPs, the respective binding partner was co-IPed. These results suggest that HTT and HIP14 form a tight complex that is enzymatically active.

Wild-type HTT influences palmitoylation of HIP14 itself

DHHC PATs themselves are palmitoylated, which is crucial for their enzymatic activity (5,19,21). To determine the effect of wild-type HTT on the catalytic activity of HIP14, we first set out to test whether wild-type HTT may affect palmitoylation of HIP14 itself *in vivo* using the following two approaches: (i) using *hdh*+/− mice that lack one allele of the HTT gene and express approximately half of the endogenous HTT protein (Fig. 4A) and (ii) primary mouse cortical neurons treated with HTT antisense oligos (HTT-ASO), in which endogenous HTT expression is reduced by over 95% (Fig. 4D). In both approaches, palmitoylation of HIP14 itself was significantly decreased, with more evident reduction shown in neurons treated with HTT-ASO (Fig. 4B, C, E, F). These results suggest wild-type HTT modulates palmitoylation of HIP14 itself in a dose-dependent manner (Fig. 4G).

Palmitoylation of GST-SNAP25 by GST-HIP14 is potentiated *in vitro* in the presence of wild-type but not mutant HTT

Several lines of evidence show that palmitoylation of PATs is required for their enzymatic activity (3,5,21). To examine

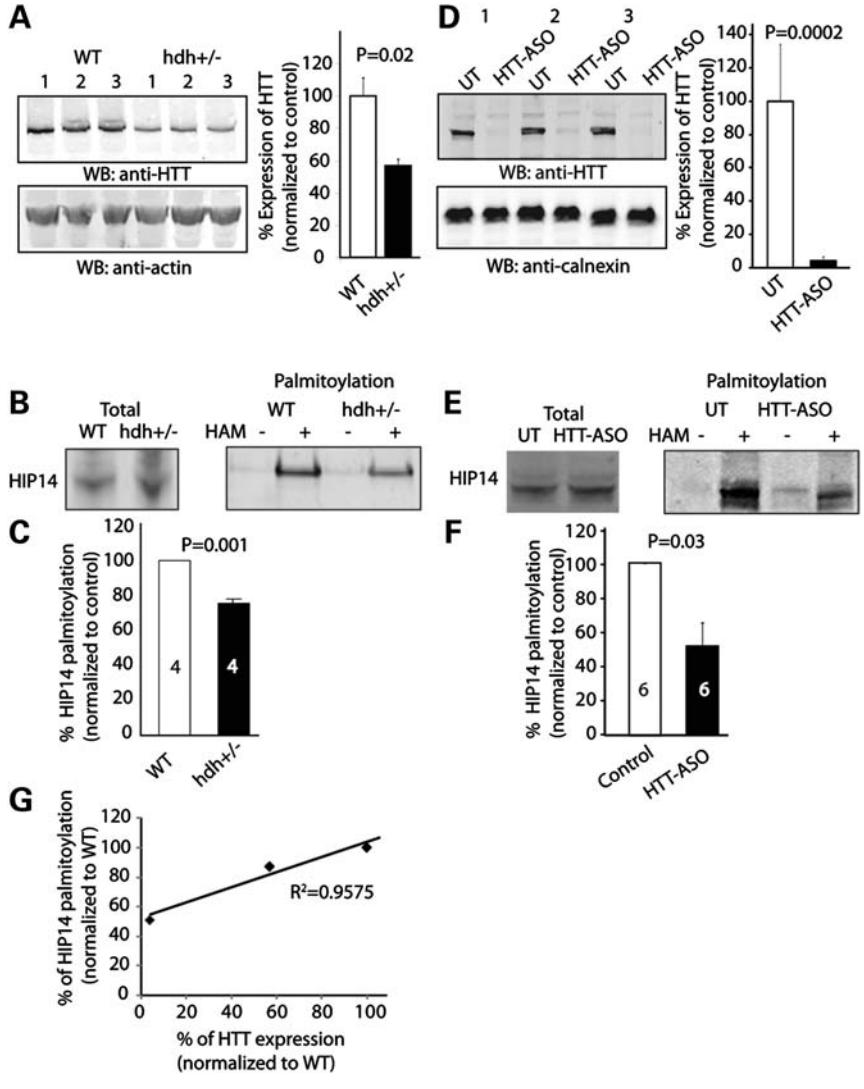

Figure 4. Palmitoylation of HIP14 itself is reduced in *hdh*+/− mice and HTT-ASO-treated neurons. (**A**) Western blot and quantification of endogenous HTT expression in brain extract of *hdh* +/− mice compared with that of WT mice. (**B** and **C**) After ABE palmitoyl-protein purifications of wild-type and *hdh*+/− mice (Materials and Methods), extracted palmitoylated proteins were subject to protein electrophoresis and western blotting with HIP14 antibody. Representative image and quantification showing palmitoylation of endogenous HIP14 was reduced in brain extract of *hdh*+/− mice compared with that of wild-type mice (87.1 ± 0.7%). Number of repeats was indicated in the graph. (**D**) Western blot and quantification of endogenous HTT expression in HTT-ASO-treated cortical neurons. (**E** and **F**) Same as described in (B) and (C), representative image and quantification showing palmitoylation of endogenous HIP14 is reduced in HTT-ASO-treated cortical neurons compared with untreated controls (UT) (50.9 ± 12.5%). Number of repeats was indicated in the graph. (**G**) Wild-type HTT modulates palmitoylation of HIP14 itself in a dose-dependent manner. Correlation coefficient of the linear regression $R^2 = 0.96$.

whether HIP14's activity toward other substrates is affected by wild-type HTT, we first used an *in vitro* palmitoylation assay, in which both substrate (SNAP25) and enzyme (HIP14) were fused to GST and purified using bacteria. *In vitro*, HIP14 alone significantly enhanced SNAP25 palmitoylation by ∼70%. In the presence of COS cell lysate expressing wild-type N-terminal fragment HTT (amino acids 1–548), palmitoylation of GST-SNAP25 by HIP14 was further enhanced. In contrast, mutant N-terminal fragment HTT was not capable of potentiating HIP14 activity (Fig. 5A and B). These results were reproduced with full-length HTT (Fig. 5C and D). In addition to SNAP25, palmitoylation of PSD95 (1-PDZ2) by GST-HIP14 *in vitro* could also be further enhanced by wild-type full-length HTT (Supplementary Material, Fig S1A and B). Taken together, these results point to the ability of wild-type HTT to modulate palmitoylation of HIP14 itself and thereby influencing its enzymatic activity toward other neuronal substrates.

Loss of wild-type HTT leads to palmitoylation defects of SNAP25 and GluR1 *in vivo*

If HIP14 activity is indeed enhanced by wild-type HTT, then reduced wild-type HTT levels might be expected to impair enzymatic activity of HIP14 toward other substrates *in vivo*. We first tested the activity of IPed HIP14 from *hdh*+/−

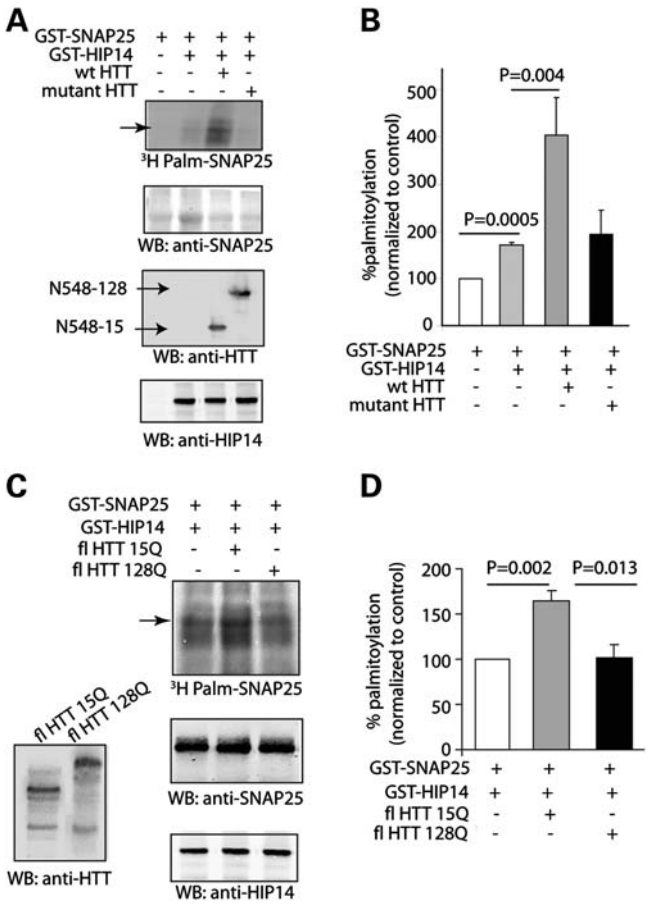

Figure 5. Wild-type HTT increases HIP14 activity to palmitoylate GST-SNAP25 *in vitro*. (**A**) GST-HIP14 enhanced palmitoylation of GST-SNAP25 in the presence of N terminal fragment wild-type HTT (1–548 amino acids), but not mutant HTT (128Q). Both substrate (SNAP25) and enzyme (HIP14) were fused to GST and purified using bacteria. COS cell lysate overexpressing wild-type or mutant HTT was added to the reaction mix containing ^{3}H-palmitoyl-CoA. Western blot shows equal amount of SNAP25, HTT or GST-HIP14 is present (second to bottom panel). (**B**) *In vitro*, HIP14 alone significantly enhanced SNAP25 palmitoylation by about 70.4 ± 6.7%. In the presence of COS cell lysate expressing wild-type HTT (amino acids 1–548), palmitoylation of GST-SNAP25 by HIP14 was further enhanced. In contrast, mutant N-terminal fragment HTT was not capable of potentiating HIP14 activity. (**C** and **D**) Similar to experiments in (A) and (B), full-length wild-type HTT and not mutant HTT also enhanced palmitoylation of GST-SNAP25 by GST-HIP14 by 34.2 ± 6.0%. All quantifications were done with three to six repeats.

mice toward GST-SNAP25. Palmitoylation of SNAP25 by IPed HIP14 from brains of *hdh*+/− mice was significantly less than that of wild-type mice (Fig. 6A and B). Next we applied the ABE method to examine whether there is an alteration in palmitoylation of endogenous HIP14 substrates when HTT expression level is reduced. We showed that palmitoylation of endogenous SNAP25 and AMPA receptor subunit, GluR1, was reduced in brains of *hdh*+/− mice (Fig. 6C and D). Furthermore, in the HTT-ASO-treated cortical neuronal system, further reduction in palmitoylation of SNAP25 and GluR1 was detected (Fig. 6E and F). These results point to the key role of HTT directly modulating palmitoylation of HIP14 substrates *in vivo*.

DISCUSSION

It is well established that wild-type HTT is neuroprotective (22–25) and that the loss of the normal function of HTT may contribute to the HD phenotype (26–28). Nonetheless, the normal function of HTT remains poorly defined. Previous work suggests that HTT plays distinct roles in several biological processes, including synaptic transmission, intracellular transport and neuronal transcription (reviewed in 12,29,30).

In this study, we discovered a novel function of wild-type HTT. In addition to serving as a palmitoylation substrate, wild-type HTT also modulates the palmitoylation of HIP14 itself. Using brains of *hdh*+/− mouse and knockdown of HTT in cultured cortical neurons, we were able to demonstrate that palmitoylation of HIP14 itself is affected by the level of wild-type HTT in a dose-dependent fashion. Reduced HIP14 palmitoylation results in its defective enzymatic activity. *In vitro*, wild-type HTT and not mutant HTT can potentiate HIP14's enzymatic activity towards SNAP25. *In vivo*, palmitoylation of SNAP25 and GluR1 [endogenous HIP14 substrates (8)] is significantly reduced in both brains of *hdh*+/− mice and neurons treated with HTT-ASO. This study describes an important biochemical function for wild-type HTT modulation of HIP14 palmitoylation and enzymatic activity.

There are several mechanisms by which HTT may modulate HIP14 palmitoylation and enzymatic activity.

First, HTT may act as an allosteric activator of HIP14. By binding to HIP14 at the ankyrin repeats site, HTT can regulate the function, structure and/or flexibility of HIP14. It is not uncommon that a substrate of an enzyme can also be an allosteric activator. For instance, full activity of 6-phosphogluconate dehydrogenase is only achieved when carrying its substrate 6-phosphogluconate (31). It is possible that, by influencing the tertiary structure of HIP14, HTT makes the catalytic DHHC domain of HIP14 more readily accessible to substrates. As a result, HTT allows HIP14 to catalyze the palmitoylation of neuronal substrates more effectively.

Secondly, HIP14 may gain access to its neuronal substrates through HTT which binds to a large array of intracellular proteins. Overall, the predicted structure of HTT is consistent with a cellular role as a scaffold protein (32). There are predicted to be up to 36 HEAT repeats which are α helix–loop–α helix motifs that mediate protein–protein interactions, spanning most of the HTT polypeptide (33). By physically bringing substrates to HIP14, HTT may facilitate the palmitoylation of these neuronal proteins by HIP14. Indeed, SNAP25 was identified to interact with HTT in a mass spectrometry analysis of affinity pull-down products of HTT (34). Thus far, no study has shown a direct interaction between GluR1 and HTT. However, GluR1 subunit may interact with HTT indirectly through postsynaptic scaffold proteins Shank and PSD95 (35).

Thirdly, HTT may be crucial for the trafficking of HIP14-containing vesicles to subcelluar sites where HIP14 executes its enzymatic function. Several studies have implicated HTT in the control of intracellular dynamic processes, including secretion and trafficking of vesicles from the Golgi apparatus, vesicular transfer between the actin and microtubule cytoskeleton, transport along microtubules in axons and synaptic

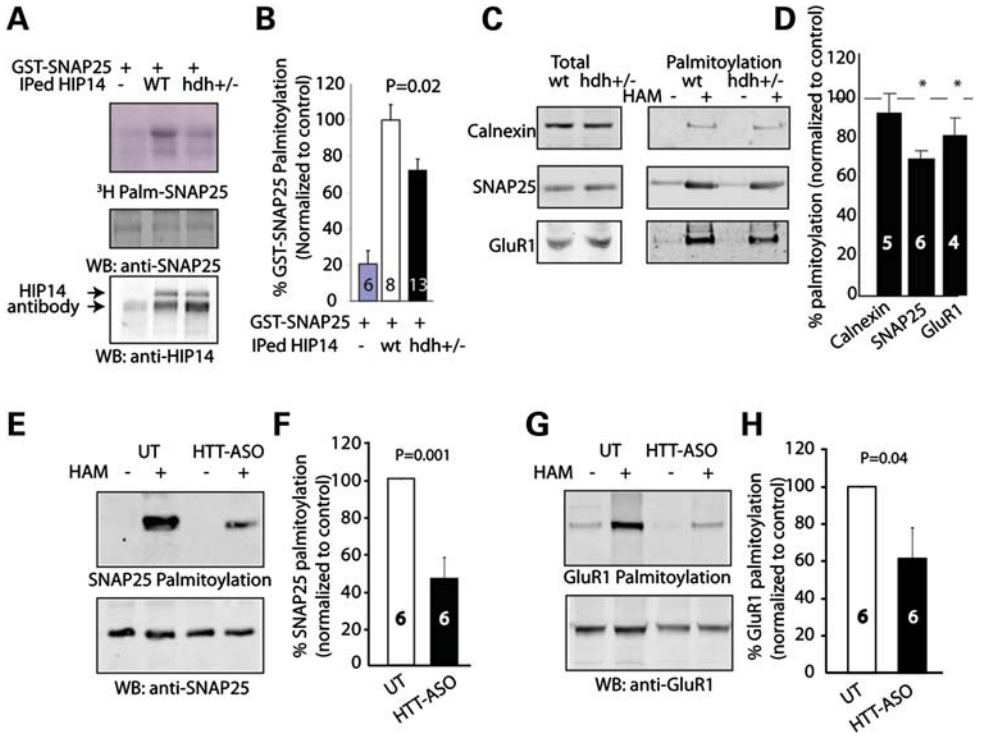

Figure 6. Loss of wild-type HTT leads to palmitoylation defects of SNAP25 and GluR1 *in vivo*. (**A**) Representative image shows IPed HIP14 of *hdh*+/− mice brain exhibited less enzymatic activity to palmitoylate GST-SNAP25, compared with that of wild-type mice. HIP14 antibody bound to sepharose beads was used to IP HIP14 from whole brain lysate of wild-type or *hdh*+/− mice. Then the beads containing IgG control or IPed HIP14 were added to reaction mix containing ^{3}H-palmitoyl-CoA to palmitoylate GST-SNAP25 *in vitro*. (**B**) Quantification revealed that IPed HIP14 of *hdh*+/− mice brain had ∼30% reduction in its enzymatic activity. (**C** and **D**) ABE palmitoylation assay was performed on wild-type and *hdh*+/− mice brain to determine palmitoylation level of neuronal proteins. After ABE palmitoyl-protein purifications of wild-type and *hdh*+/− mice, extracted palmitoylated proteins were subject to protein electrophoresis and western blotting with calnexin, SNAP25 or GluR1 antibodies. Quantification and representative blots showed alteration in palmitoylation of SNAP25 (80.6 ± 8.4%) and GluR1 (79.4 ± 3.8%) in *hdh*+/− brain compared with the wild-type (100%). (**E**–**H**) In HTT-ASO-treated mouse cortical neurons, palmitoylation of SNAP25 (46.4 ± 11.8%) and GluR1 (61.6 ± 16.7%) was further reduced as determined by ABE palmitoylation assay described in (C) and (D). Number of repeats was indicated in the graph.

endo/exocytic events (36,37). In particular, HTT protein associates with the molecular motor complex that transports organelles along microtubules in axons (38–40). Although we cannot exclude such a mechanism, our *in vitro* results that HTT can increase the palmitoylation of GST-SNAP25 by GST-HIP14 (Fig. 5) suggest that this mechanism is the less likely.

Our finding that the ability of HTT to modulate enzymatic activity of HIP14 is inversely related to the length of the polyglutamine tract provides direct association between the mutation in HD and HIP14 enzymatic activity. The interaction of mutant HTT with HIP14 in the brain is much less compared with that of wild-type HTT (9). Therefore, it is possible that mutant HTT is less capable of presenting its interacting proteins to HIP14. Alternatively, the mutation may inhibit the allosteric property of HTT. Mutant HTT also forms inclusions and loses the ability to facilitate vesicular transportation along the cytoskeleton. Thus, it is also possible that the trafficking of HIP14-containing vesicles is disturbed. Instead, HIP14 may be aggregated into mutant HTT-containing proteasome degrading machinery. Nonetheless, it will be critical to test whether palmitoylation of HIP14 itself and its neuronal substrates are affected in transgenic mouse models of HD (Singaraja R and Hayden MR, manuscript in preparation).

Another interesting finding is that first 548 amino acids of wild-type HTT are required for the interaction of HTT and HIP14 (Fig. 2). Shorter fragments, such as exon 1 and N171, do not interact with HIP14. Therefore, proteolytic cleavage of mutant HTT *in vivo* at multiple sites generate fragments that may lose interaction with HIP14 either partially or completely, further compromising the ability of mutant HTT to modulate HIP14 activity. This result suggests that the loss of normal HTT function plays a role in the pathogenesis of HD. However, this proposed mechanism does not contradict current evidence that HD arises predominantly from gain of a toxic function from these mutant HTT fragments. Smaller fragments detected in human post-mortem tissue and in mouse models (41–44) can be highly toxic *in vivo*, as seen in the R6/2, N171-82Q and YAC128 mouse models. Conversely, transgenic mice expressing mutant HTT resistant to caspase 6 cleavage have strikingly less pathology (45). Our result that mutant HTT lacks the ability to modulate HIP14 activity due to polyglutamine expansion and aberrant proteolysis does not contradict current gain of toxic function hypothesis and suggests that HD pathology may in fact arise from both a gain of toxic function and a loss of wild-type HTT function.

It has been previously shown that some DHHC enzymes must form complexes with other proteins in order to be

functional PATs. The yeast DHHC protein Erf2 has to form a complex with Erf4 in order to palmitoylate the yeast Ras (6). In mammals, DHHC9 and GCP16 display the properties of a functional human ortholog of the yeast Ras palmitoyltransferase (46). This raised the question as to whether wild-type HTT is indispensible for HIP14 activity. HIP14 can palmitoylate a number of neuronal proteins including SNAP25 and GluR1 exogenously expressed in COS cells without wild-type HTT, suggesting that HIP14 alone is still functional (8). In our study, even when >95% of endogenous wild-type HTT expression was decreased, a significant portion of HIP14 itself was still palmitoylated. This residual palmitoylation may be sufficient for HIP14 to carry on some basal level of enzymatic activity *in vivo*. Indeed, ~50% of SNAP25 and GluR1 palmitoylation was retained. It is also possible that other DHHC PATs, such as HIP14L, may compensate for the defect of HIP14 activity as DHHC PATs exhibit some overlap in their substrate specificity (8). Another significance of our finding is that binding partners of DHHC proteins may determine the substrate specificity, enzymatic kinetics, subcellular localization and stability of these DHHC proteins.

Our study also revealed that, HIP14L (DHHC13), a homolog of HIP14, also increases palmitoylation of HTT strongly in COS cells. The similarity in structures of HIP14 and HIP14L promotes further experiments to determine whether HTT interacts with HIP14L also in poly-glutamine length-dependent manner and whether HTT can modulate palmitoylation and enzymatic activity of HIP14L.

In summary, our study describes an important biochemical function for wild-type HTT and suggests that the loss of the wild-type HTT capacity to modulate palmitoylation of HIP14 neuronal substrates may contribute to the pathogenesis of HD.

MATERIALS AND METHODS

Plasmids

All DHHC enzymes were obtained as previously described (47) (generous gift by Dr Akio Kihara, Hokkaido University). Full-length HIP14 was subcloned into pCI-neo as described earlier (48). HIP14 GFP was generated by fusing enhanced green fluorescent protein (Clontech, CA, USA) at its C-terminus. HIP14 ΔDHHC GFP was generated by deletion of nucleotides encoding amino acids 440–487. HIP14 ΔANK GFP was generated by deletion of the ankyrin repeats, nucleotides encoding amino acids 89–257. Generation of GST-SNAP25 and HIP14-GST was described previously (3). All mutated DNA constructs were sequence confirmed.

Antibodies and chemicals

The antibodies used in this study were: HTT antibody 2166 (Millipore, 1:250 for immunoprecipitation, 1:1000 for western blotting), HIP14 anti-rabbit antibody (Sigma H7414, 1:200 for western blotting), GFP anti-rabbit antibody (developed in-house, 1:100 for immunoprecipitation), GFP anti-rabbit antibody (Synaptic System, 1:2000 for western blotting), SNAP25 (Covance, 1:1000 for western blotting and 1:500 for immunoprecipitation), GluR1 (provided by Dr Yutian Wang, the University of British Columbia) and Calnexin (Sigma C4731 1:5000 for western blotting).

Western blotting analysis

Primary antibodies were applied for an hour at room temperature in Odyssey blocking buffer (Li-COR Bioscience); the secondary antibodies (anti-rabbit IRD800 Rockland, Anti-Mouse Alexa Fluor 680, Invitrogen) were used at a ratio of 1:10000. The fluorescence was scanned and quantified using Odyssey Infrared Imaging System (Li-COR Bioscience). A distribution profile was calculated with Excel software (Microsoft Office) as the average value ± standard error from at least three independent experiments. Data were analyzed by Student's *t*-test, analysis of variance or nonparametric test.

Primary neuronal culture and htt-ASO treatment

Embryos were dissected from E15.5–E17.5 wild-type FVB/N pregnant. Cortical tissue was isolated and cultured, essentially as described (3). Pan-HTT ASO (Htt-387916; ISIS Pharmaceuticals) was resuspended in sterilized phosphate buffered saline (PBS) to a concentration of 1–5 mM. Neurons were fed with 200 μl neurobasal media with B27 on DIV3. For antisense oligonucleotide treatment, ASO stock was added to supplementary media to create a final concentration of 0.5 μM. Neurons were harvested on DIV18.

Palmitoylation assay

[^{3}H]palmitoyl-CoA was synthesized enzymatically from [9,10-^{3}H(N)]palmitic acid (5 mCi/ml; Perkin Elmer Life Sciences) as described previously (3). GST fusion proteins were produced in *E. coli* using the pGEX 6p3 expression system (Amersham) and isolated using Glutathione Sepharose 4B (Amersham). To generate a free N terminus of PSD-95, GST-PSD-95 protein was cleaved using thrombin [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 2.5 mM $CaCl_2$, 0.1% 2-mercapto-ethanol). Palmitoylation reaction (60 μl) contained 5 μCi of [^{3}H]palmitoyl-CoA, 0.33 μg/μl substrate protein, 1 mM adenosine triphosphate, 50 mM 2-(N-morpholino)ethanesulfonic acid, pH 6.4, 0.2 mg/ml bovine liver lipids and 20 μl of PAT enzyme. After 15 min incubation at 37°C, sample buffer was added with final concentration of 5 mM DTT was added and samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

ABE palmitoyl-protein purifications

Brains or cell pellets were harvested and immediately snap-frozen in liquid nitrogen and then stored in −80°C. In brief, frozen brains or cell pellets were homogenized into 500 μl lysis buffer (150 mM NaCl, 50 mM Tris, 5 mM ethylenediaminetetraacetic acid, pH 7.4) with 10 mM N-ethylmaleimide (NEM). Following the initial Triton X-100 detergent solubilization and NEM blockade steps, each sample was divided into two portions for treatment with, or as a control, without hydroxylamine (i.e. + or − HAM), which then is processed in parallel through the subsequent biotin-N-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)-propionamide biotinylation and streptavidin

agarose steps (49,50). Total palmitoyl proteins, purified from whole brain or cell pellets either in the presence (+) or absence (−) of HAM, were subjected to western analysis with antibodies specific to the different candidate proteins being tested, as described previously (49,50).

IP-ABE palmitoylation assay

COS cells overexpressing HTT alone or HTT with individual DHHC proteins were lysed and processed for immunoprecipitation as described above. The beads were then washed with wash buffer (PBS, containing 1% Triton X-100) supplemented with 50 mM NEM, followed by treatment with 1 M hydroxylamine pH 7.4 for 1 h at room temperature. Samples were then processed as previously described (8,51).

Yeast two-hybrid analysis

HIP14 was identified in the pGAD yeast expression vector during a library screen with HTT (20). The series of truncated HTT constructs were inserted into the yeast expression vector pGBT and sequence verified. Yeast transformations with pGADHIP14 and each pGBT9 HTT constructs were performed using the lithium acetate method (52). To detect the intensity of interaction, liquid β-galactosidase assays were performed by inoculating three individual colonies from each transformation in liquid leu^-trp^- media. After overnight growth at 30°C, cells were pelleted, washed and resuspended in Z buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 10 mM KCl) and freeze-thawed three times. Z buffer supplemented with 20% SDS, 1 M DTT, β-mercaptoethanol and O-Nirophenyl β-D-galactopyranoside were added to the tubes and reactions continued until a yellow color appeared. Reactions were stopped by adding 1 M $NaCO_3$ and spun to remove cell debris. The intensity of the reaction was quantified at OD420 and relative units calculated using the formula Relative units = (1000*OD420)/(time*concentration factor*volume of lysate*yeast cell density).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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