Identification of Novel Palmitoyl Acyl Transferases and Characterization of The Role of Huntingtin Palmitoylation in Huntington Disease

by

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ABSTRACT

In neurons, modification by the lipid palmitate regulates trafficking and function of signaling molecules, neurotransmitter receptors and associated synaptic scaffolding proteins. HIP14 (huntingtin interacting protein 14) is the first identified and characterized mammalian palmitoyl transferase that regulates this process. I have shown that HIP14 has striking effects on modulating trafficking and function of many proteins important for synapse formation and plasticity such as PSD-95, a postsynaptic scaffolding molecule.

The importance of the finding that HIP14 is a neuronal palmitoyl transferase is further emphasized by our recent discovery that huntingtin protein folding, trafficking and function are regulated by the enzyme HIP14. Expansion of the polyglutamine tract in huntingtin as seen in Huntington Disease (HD) results in reduced association with HIP14 and decreased palmitoylation of huntingtin, which contributes to the formation of inclusion bodies and enhanced neuronal toxicity. By manipulating HIP14 levels through expression or knockdown, we can manipulate the number of huntingtin inclusion bodies and neuronal cell viability. Overall, these discoveries offer novel mechanism for HD pathogenesis and provide new approaches to therapy for HD.

The tight association of HIP14 with wild-type huntingtin, which differs from other known enzyme-substrate interactions, indicates that huntingtin serves other functions beyond being a substrate of HIP14. I have discovered that, *in vitro*, wild-type huntingtin may facilitate activity of HIP14 to palmitoylate other neuronal substrates such as SNAP25, PSD95 and GAD65. By contrast, mutant htt does not act this way, probably due to lack of interaction with HIP14. Furthermore, immunoprecipitated HIP14 from huntingtin+/- mice also exhibits less enzyme activity in palmitoylating GST-SNAP25 *in vitro,* suggesting that decreased huntingtin expression compromises HIP14 activity. *In vivo*, using Acyl Biotin Exchange assay, I have also found that palmitoylation of a number of presynaptic and postsynaptic proteins that are involved in neurotransmission are reduced in huntingtin+/- mice. This study not only ascribes an important biochemical function to wild-type huntingtin, but also suggests that defects in protein palmitoylation in general due to mutant huntingtin lack of ability to facilitate HIP14 activity may contribute to the pathogenesis of HD.

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LIST OF ABBREVIATIONS

2BP	2-bromopalmitate
ABE	acyl biotin exchange
AMPAR	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid type glutamate receptors
ANK	ankyrin repeat
APT	acyl-protein thioesterase
biotin-BMCC	1-biotinamido-4-[4'-(maleididomethyl) cyclohexanecarboxamido] butane
CaMK	Ca2+/calmodulin-dependent protein kinase
CBP	cAMP response-element binding protein (CREB)-binding protein
CRD	cysteine rich domain
CSP	cysteine string protein
DAPI	4',6-diamidino-2-phenylindole
DIV	days in vitro
DMEM	Dulbecco's Modified Eagle's Medium
ECL	Electrochemiluminescence
EM	Electron microscopic
eNOS	endothelial nitric oxide synthase
FRAP	fluorescence recovery after photobleaching
GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
GAP	Growth cone associated protein
GCP	golgi complex protein
GFP	green fluorescent protein
GKAP	guanylate kinase-associated protein
GPCR	G-protein-coupled receptor
HAM	Hydroxylamine
HRP	horseradish peroxidase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KA	Kainic acid
NEM	N-ethylmaleimide
NMDA	N-methyl-D-aspartic acid
PDZ	postsynaptic density-95 (PSD-95)/Discs large/zona occludens-1
PFA	paraformaldehyde
PPT	protein palmitoyl thioesterase
RGS	regulators of G-protein signalling
SFK	Src family of non-receptor tyrosine kinases
siRNA	small interference RNA
SNARE	soluble NSF (N-ethylmaleimide-sensitive factor) attachment receptor
SUMO	small ubiquitin-related modifier
TBP	tata-binding protein
TOR	target of rapamycin
TMD	transmembrane domain

TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
UCHL1	ubiquitin carboxy-terminal hydrolase L1
WT	wildtype
YAC	yeast artificial chromosome

ACKNOWLEDGEMENTS

CO-AUTHORSHIP STATEMENT

For Chapter 2, I performed the GST protein purification, designed and performed the *in vitro* palmitoylation assays, made the HIP14 siRNA to study the neuronal protein trafficking, and wrote the manuscript. Pamela Arstikaitis did the live imaging to look at the vesicular trafficking of HIP14 in COS cells. Anat Yanai and Rujun Kang designed and made the constructs of HIP14, SNAP25, PSD95 and synaptotagmin I. Roshni R. Singaraja initially identified HIP14 as a huntingtin interacting protein. Brendan Haigh purfied His-tagged huntingtin. Claire-Anne Gutekunst provided Electron Microscopy image of HIP14 localization in brains.

For Chapter 3, I conducted all the Btn-BMCC palmitoylation assays for full-length huntingtin, scored cells for inclusions in huntingtin-transfected COS cells and neurons, designed and characterized HIP14 siRNA, performed all experiments (and corresponding data analysis) in which the alteration of huntingtin trafficking was investigated by knocking down HIP14, conducted the virus production and infection experiments, and scored for TUNEL assay. Anat Yanai performed all DNA manipulations to generate truncated and full-length mutant huntingtin and HIP14 proteins, and performed most of the [^{3}H]palmitoylation assays and data analyses. Runjun Kang performed endogenous huntingtin [^{3}H]palmitoylation assay, scored inclusions in neurons transfected with full-length huntingtin, and performed fragment-huntingtin toxicity assay and the corresponding data analysis. Roshni Singaraja and Lu Gan performed the huntingtin and HIP14 coimmunoprecipitation experiment. Anat Yanai and I wrote the manuscript.

For Chapter 4, I designed the experiments, and performed the biotin labeling palmitoylation assays and immunostainings in this manuscript. Pamela Arstikaitis performed endogenous HIP14, synaptophysin and PSD95 staining in primary culture neurons (Figure 4.1D). Tony Cijsouw validated the acyl biotin labeling method with substrate SNAP25 (Figure 4.3B) and Lylia Nini performed western blot of Figure 4.3C. Anat Yanai assisted in making ANK-DHHC-3 construct. Anat Yanai and Roshni Singaraja provided intellectual help. I performed the data analysis for all experiments, generated the figures, and wrote the manuscript.

For Chapter 5, I designed the experiments, and performed the biotin labeling palmitoylation assays and the in vitro palmitoylation assay in this manuscript. Junmei Wan precipitated and purified all the palmitoylated proteins from wildtype and huntingtin+/- mice. I performed western blot and did the quantifications. Roshni Singaraja provided the yeast 2-hybrid result (Figure 5.2). Anat Yanai made the HIP14 ΔANK and HIP14 ΔDHHC constructs. Akio Kihara from Igarashi lab provided all the DHHC constructs. I performed the data analysis for all experiments, generated the figures, and wrote the manuscript.

1. CHAPTER 1   INTRODUCTION

1.1. PROTEIN PALMITOYLATION

1.1.1 General Introduction

Fatty acylation increases protein hydrophobicity and promotes association with the lipid bilayer, the most common being prenylation, myristoylation, and palmitoylation (reviewed in(Resh 1999)). Prenylation and myristoylation are stable modifications and occur co-translationally. In contrast, palmitoylation is a reversible posttranslational modification by which palmitate, a 16 carbon fatty acid, is covalently linked to proteins. Palmitoylation often refers to *S*-acylation, which is the covalent linkage of a 16 carbon saturated fatty acid to cysteine residues via a thioester bond(Resh 1999; Smotrys and Linder 2004). Being reversible, palmitoylation can be modulated by specific signaling pathways(Resh 1999; Qanbar and Bouvier 2003; Smotrys and Linder 2004). Thus, addition and removal of palmitate may provide an important tool for dynamically regulating responses to diverse cellular stimuli. Less frequent is N-palmitoylation, which occurs on the luminal side of secreted proteins(Chamoun *et al.* 2001).

Palmitate modifies numerous soluble and integral membrane proteins in neurons(el-Husseini Ael and Bredt 2002). In cytosolic proteins, palmitoylation occurs primarily at cysteine residues found near the amino- and carboxy- termini. Modification of cysteines located at internal sites adjacent to transmembrane domains or in conjunction with other lipid modifications has also been documented. This proximity presumably strengthens interactions with lipid membranes. In the case of dually lipidated proteins, such as *N*-myristoylation on Gly residues or prenylation at C terminal CaaX motifs, palmitoylation of these proteins usually occurs at adjacent sites, and requires myristoylation or prenylation, prior to palmitoylation. Examples of this modification

include: signal-transducing proteins, such as guanine-nucleotide-binding protein-α (G_α) subunits, non-receptor tyrosine kinases, small GTPase Ras, and the filopodia inducing protein paralemmin. However, not all prenylated proteins are palmitoylated at adjacent C-terminal cysteines. For example, the prenylated Rho GTPAse Rho3 is palmitoylated at an N-terminal cysteine (Roth *et al.* 2006a; Roth *et al.* 2006b). It is also worth noting that, in some cases, the palmitoylated cysteines are found near basic residues. The presence of positively charged basic residues facilitates interactions with the negatively charged acidic phopholipid headgroups. Thus, sequences neighboring palmitoylated cysteines contribute to the strength of protein association with membranes.

In this chapter, we will highlight approaches recently developed to detect and assess the dynamics of palmitoylation. We will also review the new emerging roles for palmitoylation in protein function, with focus on involvement of palmitoylation in controlling neuronal development and synaptic transmission. The recently discovered enzymes that regulate this lipid modification will also be discussed.

1.1.2 New approaches for studying protein palmitoylation

Classical methods used to assess the dynamics of addition and removal of palmitate have been limited due to the lack of feasible approaches to assess palmitoylation *in vivo*. This process traditionally has been studied by metabolic labeling with radioactive palmitate followed by pulse chase analysis to assess the half-life of palmitate on individual proteins. However, recently, several new approaches have been developed to measure protein palmitoylation in vivo and to monitor palmitoylation dynamics in live cells. These new methods are highlighted below and their relevance for assessing the dynamics of protein palmitoylation *in vivo* is discussed.

1.1.2.1 New methods for rapid detection of palmitoylated proteins

Methods used to quantify protein palmitoylation involve metabolic labeling of cultured cells with radiolabeled palmitate ($[^3H]$-palmitate or $[^{125}I]$-palmitate) for 3-5 hours before harvesting cells and immunoprecipitating proteins of interest (protocol reviewed in (Resh 2006b)). The use of this approach is limited because it requires the use of cultured cells as well as incubation with radiolabeled palmitate for 3-5 hours. The fraction that is available for labeling is also limited by the amount of protein synthesized and/or the rate of palmitate turnover on a particular protein during the labeling process. As a result, this method only allows detection of a small fraction of the total palmitoylated pool of a protein. A novel approach developed by Drisdel and Green overcomes these limitations by allowing detection of the total pool of palmitoylated proteins present in tissue extracts(Drisdel and Green 2004; Drisdel *et al.* 2004). This approach is known as "fatty acyl exchange labeling", which involves (i) blockade of free thiols with N-ethylmaleimide (NEM); (ii) cleavage of the Cys-palmitoyl thioester linkage with hydroxylamine (HAM); and (iii) labeling newly exposed thiols with a sulfhydryl-specific labeling compound, such as non-radioactive biotin-BMCC (1-biotinamido-4-[4'-(maleididomethyl) cyclohexanecarboxamido] butane)(Drisdel and Green 2004; Drisdel *et al.* 2004). The palmitoylated protein can then be detected by western blotting using strepdavidin or anti-biotin antibodies (Figure 1.1). This approach is highly sensitive and allows the use of a variety of probes, radiolabeled ($[^3H]$-NEM) or non-radioactive (biotin-BMCC), and for quantitative estimates of the total palmitoylated pool of a protein. This method also allows for assessment of changes in the dynamics of protein palmitoylation

after stimulation of specific signaling pathways, or after alterations in neuronal activity (Drisdel *et al.* 2006).
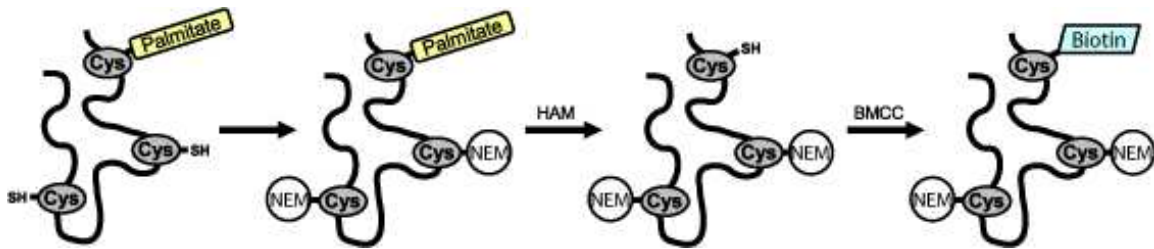
Figure 1.1. Schematic illustration of "fatty acyl exchange labeling".
This approach involves (i) immunoprecipitation of a protein with the specific antibody binded to sepharose beads; (ii) blockade of free thiols with N-ethylmaleimide (NEM); (iii) cleavage of the Cys-palmitoyl thioester linkage with hydroxylamine (HAM); and (iv) labeling newly exposed thiols with a sulfhydryl-specific labeling compound, such as non-radioactive biotin-BMCC

This approach was further modified by Davis and colleagues and elegantly used to triple the overall dimension of the yeast palmitoylome. This approach also allowed identification of specific substrates for each of the newly discovered enzymes that regulate palmitoylation(Roth *et al.* 2006a; Roth *et al.* 2006b). Starting with protein extracts, biotin tags are exchanged for protein palmitoyl-modifications, which allow the palmitoylated proteins to be specifically purified from complex protein extracts. This protocol also involves the three chemical steps described earlier in the 'fatty acyl exchange labeling' procedure developed by Drisdel and Green with some modifications (Roth *et al.* 2006a). After labeling with sulfhydryl-specific biotin, the biotinylated proteins are affinity-purified using streptavidin-agarose and identified by multi-dimensional protein identification technology (MuDPIT), a high-throughput, tandem mass spectrometry (MS/MS)-based proteomic technology (Figure 1.2) (Wan *et al.* 2007). Our group applied this proteomic technique to the neuronal proteins and identified

hundreds of new palmitoylated proteins, as summarized in Table 1.1. Proteins that are

highlighted with red are already confirmed to be palmitoylated.
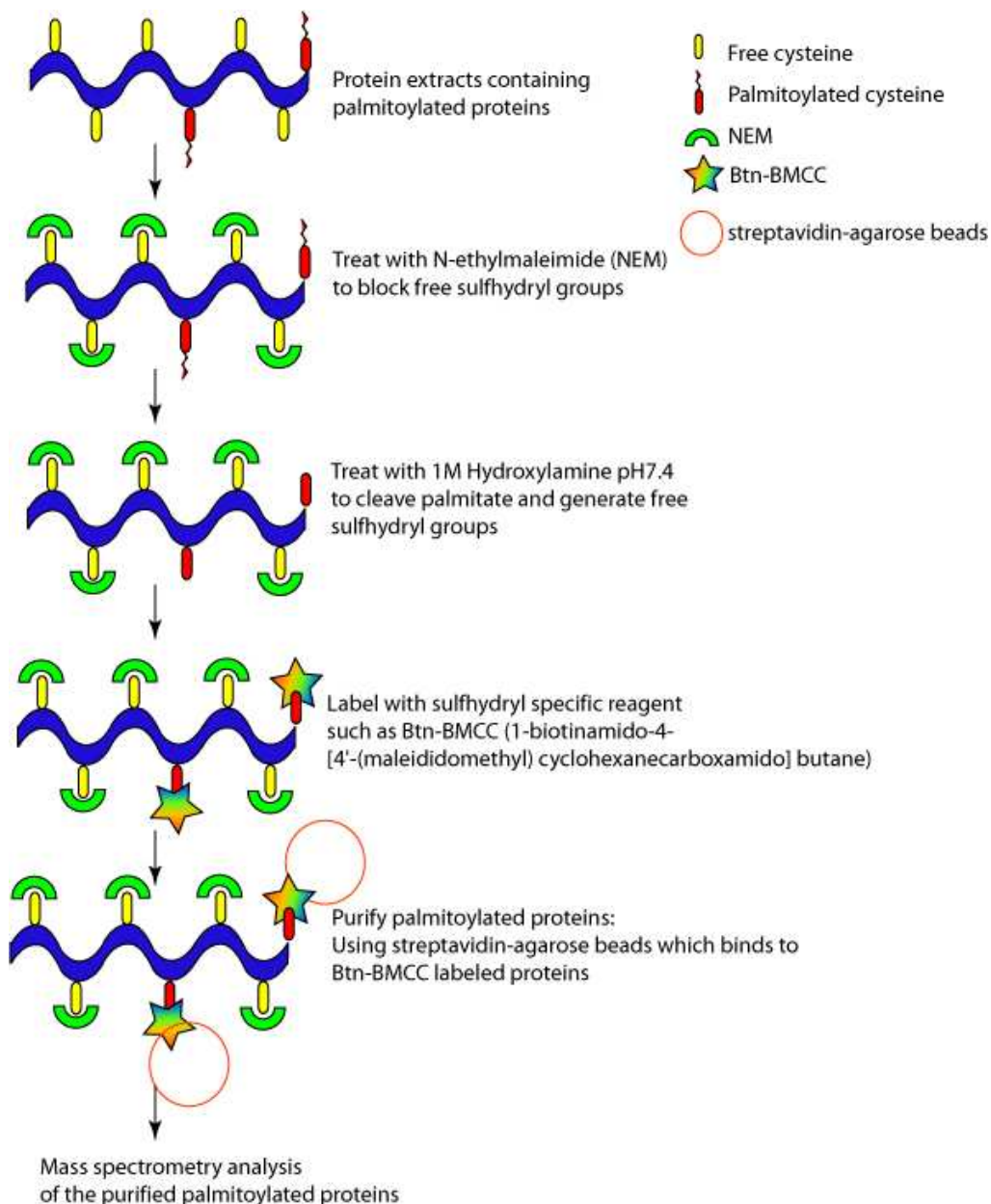
Figure 1.2. Acyl-Biotin Exchange approach for detection of palmitoylated proteins.

To identify palmitoylated proteins, denatured protein extracts are subject to three steps: (i) blockade of free thiols with N-ethylmaleimide; (ii) cleavage of the Cys-palmitoyl thioester linkage with hydroxylamine; and (iii) labeling of newly exposed thiols with sulfhydryl-specific labeling compound, such as non-radioactive biotin-BMCC (1-biotinamido-4-[4'-(maleididomethyl) cyclohexanecarboxamido] butane). After labeling, the biotinylated proteins are

affinity-purified using streptavidin-agarose beads and identified by multi-dimensional protein identification technology (MuDPIT).

Table 1.1. A table that summarizes some of the key candidate palmitoylated proteins identified by the ABE method.

The proteins that are highlighted in red are already confirmed to be palmitoylated with ^{3}H palmitate metabolic labeling.

Receptors and Channels
Grin2a(NR2A) , Grin2b(NR2B)
Scn (Voltage-gated Na channel, typeI, II, III, IX)
Ttyh, Ttyh31(tweety Cl channel)
Htr3c (ionotropic 5-HT receptor subunit)
Ptgfm (Prostaglandin receptor negative regulator)

Scaffolding proteins
Akap5(APAK79/150)
Dlgap2(PSD95 associated protein)
Gphn(Gephyrin)

Membrane trafficking
Stx1a,1b2 ,6,7,8,12,16 (syntaxin)
Rab, 2,3a,7,10,14
Scamp1,3,5
Sybl1 (synaptobrevin-like 1)
Vamp1,2,4

Cell adhesion and cytoskeleton proteins
Mcam(M-CAM)
Epha5(Eph receptorA5)   Epha3(Eph receptorA3)
Ephb2(Eph receptor B2) Efnb3(Ephrin B3; ligand)
Igsf4C (SynCAM4; Necl4)
Pcdh1,7,8,10,17 (protocadherin)
Ctnnd1,2 (δ-catenin)
Cxadr (coxsackie and adenovirus receptor)
Astn1(Astrontactin 1)
Jam3 (junctional CAM-3)
Igsf8 (PGRL; EWI-2)
Pkp4 (plakophilin4)

Axonal guidance proteins
Sema4d (Semaphorin 4D)
Lphn1 (Latrophilin 1)
Plxnb2 (Plexin B2)
Neo1 (Neogenin 1)

Myelin associated
Mbp (MBP and Golli-MBP)
Mobp (myelin-associated oligodendrocyte basic protein)
mog (myelin oligodendrocyte glycoprotein)
M6a

Transptors
Slc 1a1,2,3,4 (EAAC gluamate transptor)
Slc 6a1,32a1 (vesicular GABA transptor)
Atp2b1,b3,c1 (Ca transptor)
Slc8a1,a2(Na/Ca exchanger)
Slc3a2 (amino acid transptor)
Slc4a2 (choline transptor)

GTPases and signaling proteins
R-Ras(R-Ras) Rras2(R-Ras2)
Rala,Ralb(Ral-A, Ral-B)
Sept3(Septin 3) Sept6(Septin 6)
Gprin1, Gprin3 (G-interacting)
Arhgdia (Rho-GDIa)
Spred1, Spred2 (Sprouty related)
Adcy1,5,6,9 (Adenylyl cyclase) Sept8(Septin 8)
Pi4k2a (phosphatidylinositol 4-kinase type IIa)
Inpp5a (inositol phosphatase)
Zfyve28 (FYVE PI-binding)
Smpd3 (sphingomyelin phosphodiesterase 3)
Pde10a (cAMP/cGMP dual phosphodiesterase)

Cytoskeletal proteins
CDC42, Rac 1, Rho A
Ablim2 (Actin binding LIM protein 2)
Ckap4 (binds microtubles)
Dync1/1 (dynein intermediate chain)

Chaperonins
Canx (calnexin)
Ppib (peptidylprolyl isomerase B)

Metabolism
DHHC PATs (palmitoylation enzyme)
Agpat1 (lipid biosynthesis)
Capn5 (calpain 5)
Ggtl3 (gamma-glutamyl transpeptidase)
Cyb5r3 (Cytochrome B5 reductase)
Mpst (mercaptopyruvate sulfotransferase)

Mitochondrial
Cox6c (respiratory chain)
Vdac1,2,3 (VDAC outer membrane porins
Aldh6a1

More recently, Berthiaume and colleagues developed another method for rapid detection of palmitoylated proteins using a non-radioactive bio-orthogonal azidopalmitate analogue that can be modified with a variety of triarylphosphine-tags via the Staudinger

ligation. Azido-palmitoylated proteins are then labeled with either phosphine-associated

tags or biotin, and can be detected with tag specific antibodies or neutravidin-conjugated

HRP. Fluorescently labeled phosphine can also be used to detect palmitoylated proteins.

This method offers rapid detection of palmitoylated proteins and was recently used to

identify new palmitoylated mitochondrial proteins (Figure 1.3)(Kostiuk *et al.* 2008).
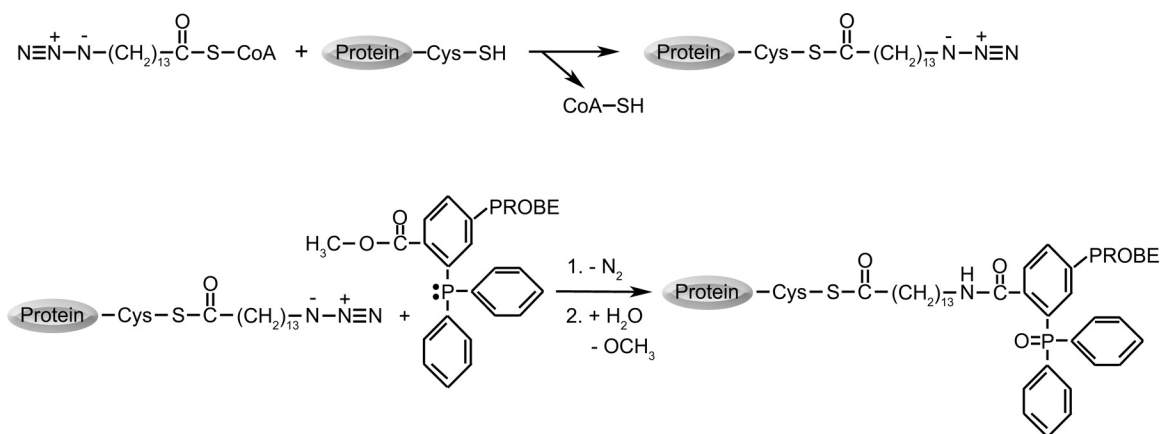
Figure 1.3. Schematic representation of the reaction for the detection of palmitoylated proteins using a fatty acid analog.

i) Azido-palmitate is transferred to a protein from azido-palmitoyl-CoA, forming a thioester bond with a cysteine residue. ii) The azide moiety of the azido-palmitate reacts with the tagged triaryl-phosphine, forming an amide bond. Probe = Myc, biotin or fluorescein. Reprint from (Kostiuk *et al.* 2008) with permission.

1.1.2.2 Approaches to study palmitate turnover

Currently, there are three methods that are widely used to inhibit protein

palmitoylation. First, application of 2-bromopalmitate (2BP), a non-metabolizable

palmitate analog that blocks palmitate incorporation into proteins (Webb *et al.* 2000).

This approach has been used to assess palmitate cycling on many proteins including

PSD-95(El-Husseini Ael *et al.* 2002), Rho family proteins(Adamson *et al.* 1992),

Ras(Chen *et al.* 2003), huntingtin(Yanai *et al.* 2006), AMPA-type glutamate

receptors(Hayashi *et al.* 2005) and nicotinic α7 receptors(Drisdel *et al.* 2004). For instance, palmitoylation of the postsynaptic density protein (PSD-95) is essential for targeting to postsynaptic sites (Figure 1.4A,B). Treatment of hippocampal neurons with 2BP that blocks the ongoing palmitoylation specifically de-clusters PSD-95 but not the presynaptic protein synaptophysin (Figure 1.4C). The advantages of this approach are twofold. Firstly, this approach allows for assessment of changes in protein trafficking by blocking protein repalmitoylation without the need to mutate the palmitoylated cysteine residues, which may indirectly influence protein folding. Second, 2BP can be used to assess the rate of palmitate turnover of endogenous palmitoylated proteins. However, since the use of 2BP globally blocks palmitoylation, this approach cannot be used to assess changes in the function of individual proteins.
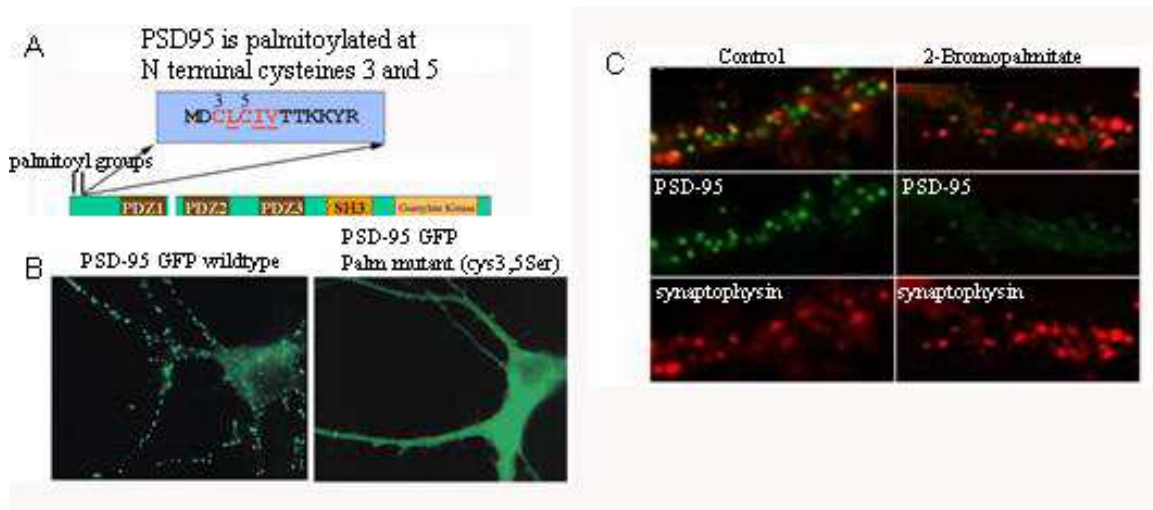
Figure 1.4 Palmitoylation is required for PSD-95 clustering at the synapse.
(A, B) Palmitoylation of the postsynaptic density protein (PSD-95) is essential for targeting to postsynaptic sites. Wild-type PSD95 clusters at synaptic sites (puncta), whereas a palmitoylation-deficient mutant form (PSD95:C3,5S) **(C)** Treatment of hippocampal neurons with 2-bromopalmitate, an agent that blocks ongoing palmitoylation, specifically de-cluster PSD-95 (loss of puncta) but not the presynaptic protein synaptophysin. Adapted from (el-Husseini Ael and Bredt 2002) with permission.

Other reagents that have been shown to function as inhibitors of protein palmitoylation include cerulenin (2,3-epoxy-4-oxo-7,10 dodecadienoylamide) and tunicamycin (Lawrence *et al.* 1999) (Patterson and Skene 1994). The inhibitory effect of cerulenin on palmitoylation has been validated in several studies with myelin proteolipid protein(DeJesus and Bizzozero 2002) and some rat adipocyte proteins(Jochen *et al.* 1995). Inhibitory effects of tunicamycin on palmitoylation have also been documented with GAP43, N-type Ca^{2+} channels, estrogen receptor α variant and myelin proteolipid protein(Patterson and Skene 1994; Jochen *et al.* 1995; Hurley *et al.* 2000; DeJesus and Bizzozero 2002; Li *et al.* 2003b).

1.1.2.3 Assessment of the dynamics of palmitoylated proteins in live cells

Imaging techniques have been significantly advanced to enable the tracking of fluorescence-tagged proteins in live cells. In particular, fluorescence recovery after photobleaching (FRAP) has gained increasing popularity as a means to monitor protein dynamics. The principle of FRAP experiments is: fluorescent molecules are irreversibly photobleached in a defined region of interest, and the exchange of bleached and unbleached molecules is then monitored over time (reviewed in (Lippincott-Schwartz *et al.* 2001; Lippincott-Schwartz *et al.* 2003)). In a similar approach but complementary mechanism, specially developed fluorescent proteins are photoactivated, enabling one to monitor their subsequent redistribution from the site of photoactivation over time (reviewed in (Politz 1999; Rocks *et al.* 2005)). These techniques have been applied to study the rapid retrograde trafficking of palmitoylated Ras isoforms from the plasma membrane to the Golgi apparatus(Rocks *et al.* 2005). In particular, Rocks et al.

selectively photobleached the entire Golgi-associated pool of protein and the fluorescence recovery was determined ratiometrically. Conversely, in the photoactivation experiment, they fused a photo-activatable variant of green fluorescent protein (paGFP) to Hras or Nras. paGFP-Hras or -Nras was selectively photoconverted at the lateral plasma membrane with 405-nm laser light, and accumulation of fluorescence was tracked at the Golgi(Rocks *et al.* 2005). Both FRAP and photoactivation methods achieved the fluorescence recovery half time on the same time scale. These approaches also revealed remarkable differences in the shuttling of mono- and dually palmitoylated Ras isoforms between the Golgi and the plasma membrane. In summary, using FRAP and photoactivation experiments, one can test the strength of binding of palmitoylated proteins to various cellular membranes, and measure the kinetics of recycling of palmitoylated proteins between the different membranous compartments.

1.1.3 Palmitoylation as a signal for protein sorting to specific membrane microdomains

Palmitoylation, being a lipid modification, facilitates protein incorporation into membranes. However, palmitate does not simply serve as a membrane anchor signal, as many different types of plasma membrane associated vesicular and transmembrane proteins are palmitoylated. There is now compelling evidence that palmitoylation plays a key role in regulating the dynamics of protein sorting within the cell (see review in (Huang and El-Husseini 2005; Greaves and Chamberlain 2007; Linder and Deschenes 2007)). In particular, palmitoylation reversibly regulates assembly and compartmentalization of many neuronal proteins to specific subcellular domains such as the presynaptic terminal and postsynaptic sites. This process influences not only the

protein sorting but also the assembly of protein complexes at the synapse that modulate synaptic transmission and neuronal function. Some of these new emerging roles are highlighted below.

1.1.3.1 Control of presynaptic protein trafficking by palmitoylation

Palmitoylation regulates trafficking and function of numerous presynaptic proteins. Some enzymes involved in neurotransmitter synthesis require palmitoylation for proper sorting. For instance, glutamic acid decarboxylase 65 kDa (GAD65), which synthesizes the inhibitory neurotransmitter γ-aminobutyric acid (GABA), depends on palmitoylation for delivery from Golgi membranes to Rab5-regulated endosomes, and eventually to presynaptic sites(Kanaani *et al.* 2004).

Several SNARE proteins and associated molecules that regulate vesicle fusion and neurotransmitter release are palmitoylated. Previous studies showed that palmitoylation of synaptosome associated protein-25 (SNAP-25) is required for efficient SNARE-complex dissociation and vesicle exocytosis(Veit 2000; Washbourne *et al.* 2001). In the case of Ykt6, another SNARE component protein, palmitoylation provides a mechanism for regulating the rate of intracellular membrane flow and vesicle fusion(Fukasawa *et al.* 2004). Palmitoylation of synaptotagmin I, a presynaptic vesicle associated protein required for Ca^{2+} regulated exocytosis, contributes to protein sorting to presynaptic terminals, and sequestration from the presynaptic plasma membrane to synaptic vesicles(Han *et al.* 2004; Kang *et al.* 2004).

More recently, the yeast palmitoylome analysis revealed that one-third of the yeast SNARE proteins are palmitoylated(Roth *et al.* 2006a). These palmitoylated yeast SNAREs are distinguished both by sequence, having cysteines positioned adjacent to

cytoplasmic C-terminal hydrophobic anchor domains and by localization, generally mediating transport steps late in the secretory pathway (e.g. Golgi, secretory vesicles, plasma membrane) or within the endosomal system.

Although the exact role of palmitoylation of individual SNARE proteins remains unclear, it is noteworthy that many of these molecules localize to raft-like domains. Thus, palmitoylation may serve to assemble these proteins at specific lipid microdoamins in anticipation of membrane fusion (Chamberlain *et al.* 2001; Salaun *et al.* 2004; Puri and Roche 2006). By directly interacting with particular lipids, palmitate may also facilitate the rate of vesicle-membrane fusion cycle and/or vesicle internalization.

In support of a vital role for palmitoylation in regulating synaptic vesicle recycling and neurotransmitter release, recent studies identified other palmitoylated presynaptic proteins. For example, cysteine string proteins (CSPs), palmitoylated on a "string" of 14 cysteine residues, are members of the cellular folding machinery family that play a role in regulated exocytosis in neurons and endocrine cells(Evans *et al.* 2003). Palmitoylation at the cysteine-string domain mediates binding of CSP to ER membranes (Greaves and Chamberlain 2006). Subsequent palmitoylation on other cysteines facilitates CSP exit from ER and for sorting to presynaptic terminals. Moreover, palmitoylation of β2A subunit of the presynaptic CaV2.2 (N type) calcium channels gates the influx of calcium ions to trigger transmitter release(Khanna *et al.* 2007) (Chan *et al.* 2007). The modification of a large number of presynaptic proteins with palmitate points to a crucial role for this modification in regulating various aspects of presynaptic signaling and neurotransmitter release (Figure 1.5).

In addition to regulating protein trafficking to synaptic sites, mounting evidence implicates a key role for palmitoylation in protein sorting to myelin-enriched membranes (Schneider *et al.* 2005). Future studies are needed to clarify whether palmitoylation is required for myelin sheath formation and assembly.
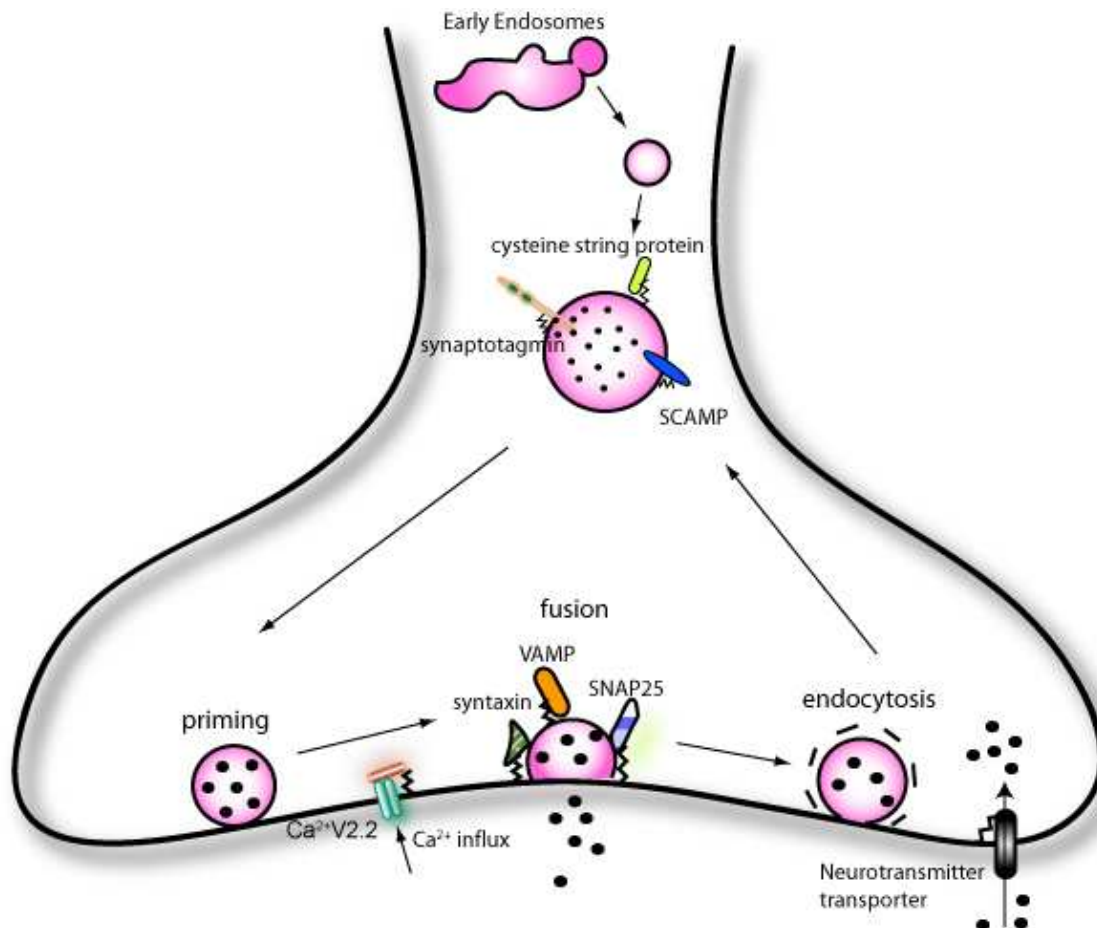
Figure 1.5. Proposed role for palmitoylation in regulation of presynaptic vesicle exocytosis and neurotransmitter release.

Palmitoylation is required for proper trafficking and function of proteins that regulate calcium dependent vesicle exocytosis such as synaptotagmin I, and others that influence neurotransmitter release such as cysteine string protein (CSP), and the SNARE proteins SNAP-25, and Ykt6. Palmitoylation also regulates presynaptic targeting of the GABA synthesizing enzyme, GAD-65. Other candidate palmitoylated proteins that influence neurotransmitter release and re-uptake include syntaxins, and several neurotransmitter transporters. Palmitoylation modulates voltage sensing and current amplitude of several voltage gated ion channels such as the β2A-subunit of the Ca^{2+} channel.

1.1.3.2   Control of postsynaptic protein trafficking by palmitoylation

Neurons assemble neurotransmitter receptors and associated proteins at postsynaptic sites opposed to presynaptic terminals that harbor the proper neurotransmitters. This perfect opposition allows for efficient synaptic transmission and rapid signal transfer between neuronal cells. The postsynaptic density-95 (PSD-95) protein is a PDZ (postsynaptic density-95 (PSD-95)/Discs large/zona occludens-1) protein which acts as a scaffold to regulate assembly of complexes containing cell adhesion molecules and neurotransmitter receptors at excitatory glutamatergic synapses(el-Husseini Ael and Bredt 2002; El-Husseini Ael *et al.* 2002). Palmitoylation of PSD-95 is required for its postsynaptic targeting and clustering of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) type glutamate receptors (Figure 1.6A) (Craven *et al.* 1999; El-Husseini *et al.* 2000b; Christopherson *et al.* 2003). Consequently, specific forms of synaptic plasticity associated with the delivery of AMPA receptor subunits to the synapse are compromised when PSD-95 palmitoylation is disrupted(El-Husseini *et al.* 2000b; Ehrlich and Malinow 2004). The ability of PSD-95 to recruit other scaffold molecules to the synapse such as Shank1B and GKAP1A, both of which are core components of the PSD, also relies on PSD-95 palmitoylation (Figure 1.6A) (Romorini *et al.* 2004). These results emphasize the vital role of palmitoylation as a postsynaptic organizing signal to regulate AMPA receptor function and synapse integrity.
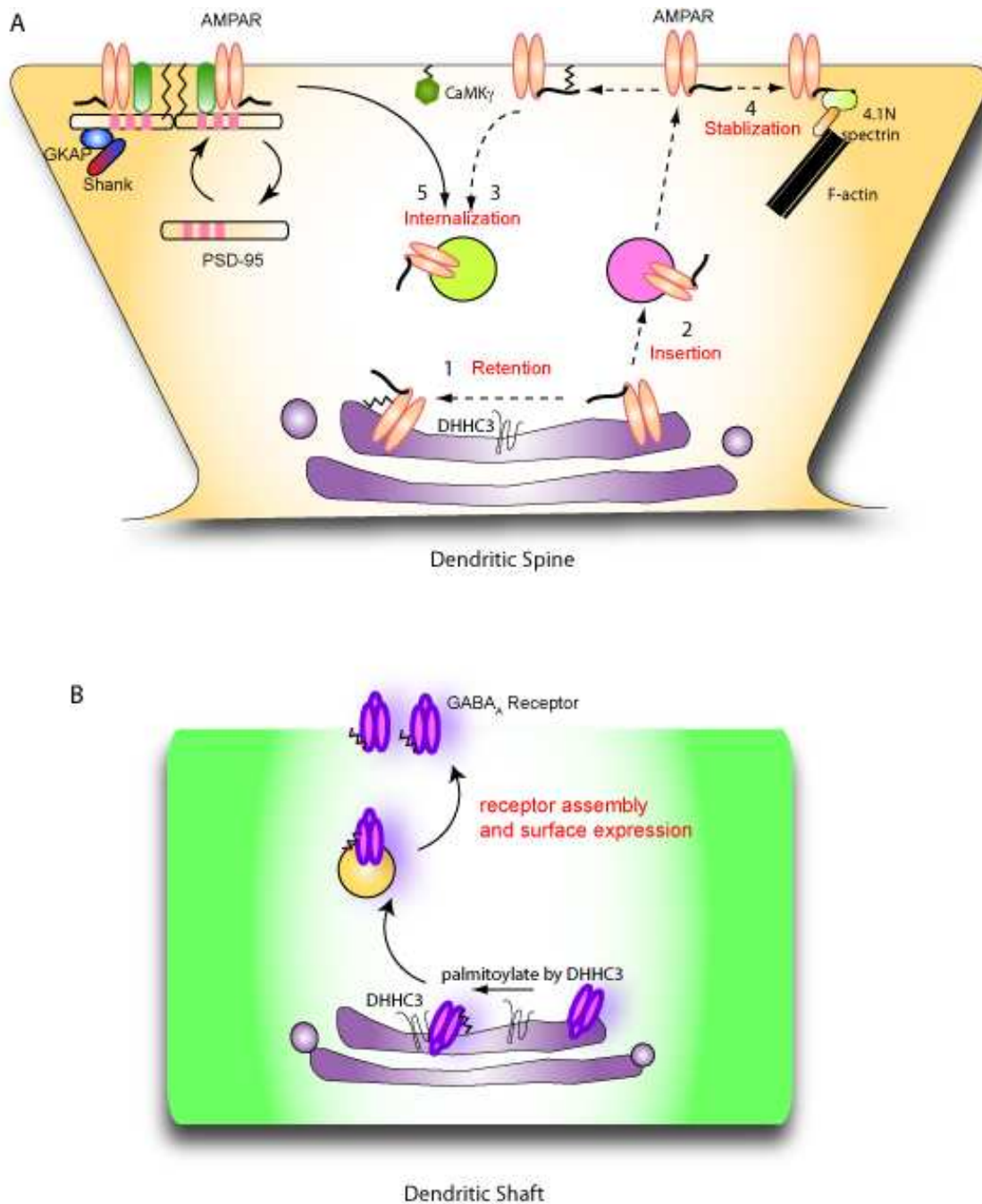
Figure 1.6. Palmitoylation acts as a signal for protein sorting to specific membrane microdomains.

(A) Palmitoylation-dependent sorting of postsynaptic receptors and scaffolding proteins at excitatory synapses. **1**. Palmitoylation of AMPA receptor (AMPAR) subunits at transmembrane 2 (TM2) regulates receptor accumulation in the Golgi apparatus, a process regulated by DHHC-3, a palmitoyltransferase enriched in the Golgi. **2**. Depalmitoylation of the TM2 site may regulate release of AMPARs from the Golgi for surface delivery. **3**. At the postsynaptic membrane, palmitoylation at the distal region of the C terminal cytoplasmic tail of AMPARs facilitates ligand-induced internalization of the receptor. **4**. Depalmitoylation of the AMPARs on the C

terminal cytoplasmic tail increases the receptors' affinity for 4.1N, which stabilizes receptors at the cell surface by the interaction of 4.1N with F-actin and spectrin. **5**. Palmitoylation regulates clustering of PSD-95, a major scaffolding molecule required for AMPAR retention at the synapse. Enhanced neuronal activity induces depalmitoylation of PSD-95 and removal from the PSD. Prolonged loss of PSD-95 from the synapse results in AMPAR removal. **(B)** DHHC-3 is also required for palmitoylation and trafficking of GABA$_A$ receptors to inhibitory synapses.

Differential palmitoylation of a subset of members of the PSD-95 family enables differential regulation of certain functions mediated by these proteins(El-Husseini *et al.* 2000c). The PSD-95 family includes PSD-93 (also known as Chapsyn-110), SAP-102 and SAP-97. Studies in heterologous cells showed that only the palmitoylated members, PSD-95 and PSD-93, are capable of clustering ion channels and associated proteins(El-Husseini Ael *et al.* 2002). Other PDZ-containing anchoring proteins for AMPA receptors have splicing variants that exhibit differential palmitoylation and subcellular localization (El-Husseini *et al.* 2000b; DeSouza *et al.* 2002; Christopherson *et al.* 2003). For example, an isoform of the AMPA receptor binding protein (ABP/GRIP2), pABP-L, is palmitoylated and concentrated at the spines, whereas the non-palmitoylated isoform ABP-L is abundant in the cell body and dendritic shafts(DeSouza *et al.* 2002). It is presumed that this provides another mechanism for these two isoforms to regulate AMPA receptor clustering at separate subcellular sites.

A similar mechanism has been proposed to control the GluRδ2, a glutamate receptor subunit predominantly expressed at postsynaptic sites of Purkinje cells of the cerebellum. This subunit plays crucial roles in synaptogenesis and synaptic plasticity by controlling the endocytosis of AMPA receptors(Matsuda *et al.* 2006). Delphilin, a binding partner of GluRδ2, is expressed in two splice forms with distinct localizations: the palmitoylated form is concentrated in dendritic spines; whereas the non-palmitoylated

form clusters in the soma and dendritic shafts. Thus, differential palmitoylation of splice variants of GRIP/ABP and delphilin regulate clustering of two separate intracellular and surface glutamate receptor pools to control glutamate signaling at different loci (Matsuda *et al.* 2006).

AMPA receptors subunits, on the other hand, are palmitoylated on two cysteine residues present in the second transmembrane domain (TM2) and the distal cytoplasmic C-terminal region (Hayashi *et al.* 2005). Palmitoylation at TM2 regulates retention of AMPA receptors in the Golgi, controls receptor surface expression, and modulates association of AMPA receptors with 4.1N, a skeletal membrane protein. In contrast, palmitoylation of the cytoplasmic C-terminal region regulates agonist induced receptor internalization (Figure 1.6).

More recently, a membrane-anchored form of Ca^{2+}/calmodulin-dependent protein kinase, CaMKIγ, has been shown to be palmitoylated (Takemoto-Kimura *et al.* 2007). In this case, palmitoylation regulates trafficking of CaMKIγ to lipid rafts, a process potentially required for controlling CaMKIγ activation at specific subcellular locations that couple changes of extrinsic neuronal activity with cytoskeletal remodeling to regulate dendritic extension and branching at particular sites. Taken together, these recent discoveries elucidate key roles for palmitoylation in mediating trafficking, assembly and function of several molecules localized at the postsynaptic side of the synapse.

1.1.3.3 Palmitoylation influences dendritic spine development

Another key process involved in compartmentalization of postsynaptic proteins and regulation of synaptic signal strength at the of the majority of excitatory synapses is the formation of spines, bulbous protrusions enriched with F-actin (Hall and Nobes 2000;

Yuste and Bonhoeffer 2004; Halpain *et al.* 2005; Matus 2005; Gerrow and El-Husseini 2006; Gerrow *et al.* 2006). Changes in spine dynamics and morphology in mature neurons, have been also associated with various learning paradigms and memory storage. Spines are thought to emerge from dendritic filopodia, and a key regulator of this process is paralemmin-1, a dually acylated protein (Burwinkel *et al.* 1998; Kutzleb *et al.* 1998; Gauthier-Campbell *et al.* 2004; Castellini *et al.* 2005; Basile *et al.* 2006; Kutzleb *et al.* 2007). Palmitoylation localizes paralemmin-1 to dendritic membranes, enables it to induce filopodia, and recruit synaptic elements such as AMPA receptors to contact sites. Interestingly, paralemmin-1 enrichment at the plasma membrane is subject to rapid changes in neuronal excitability, and this process controls protrusion size (Figure 1.7A). In addition to paralemmin-1, specific members of the Rho family of small GTPases are subject to protein palmitoylation. These molecules are well-known regulators of the actin cytoskeleton and have profound influence on spine morphogenesis(Newey *et al.* 2005) (Figure 1.7B). These findings point to a general role of palmitoylation in regulating the function of key proteins that modulate spine induction and morphology. Palmitoylation may also serve as a signal to rapidly induce changes in dendritic protrusion size and morphology, which are associated with changes in neuronal firing or those involved in learning and memory.
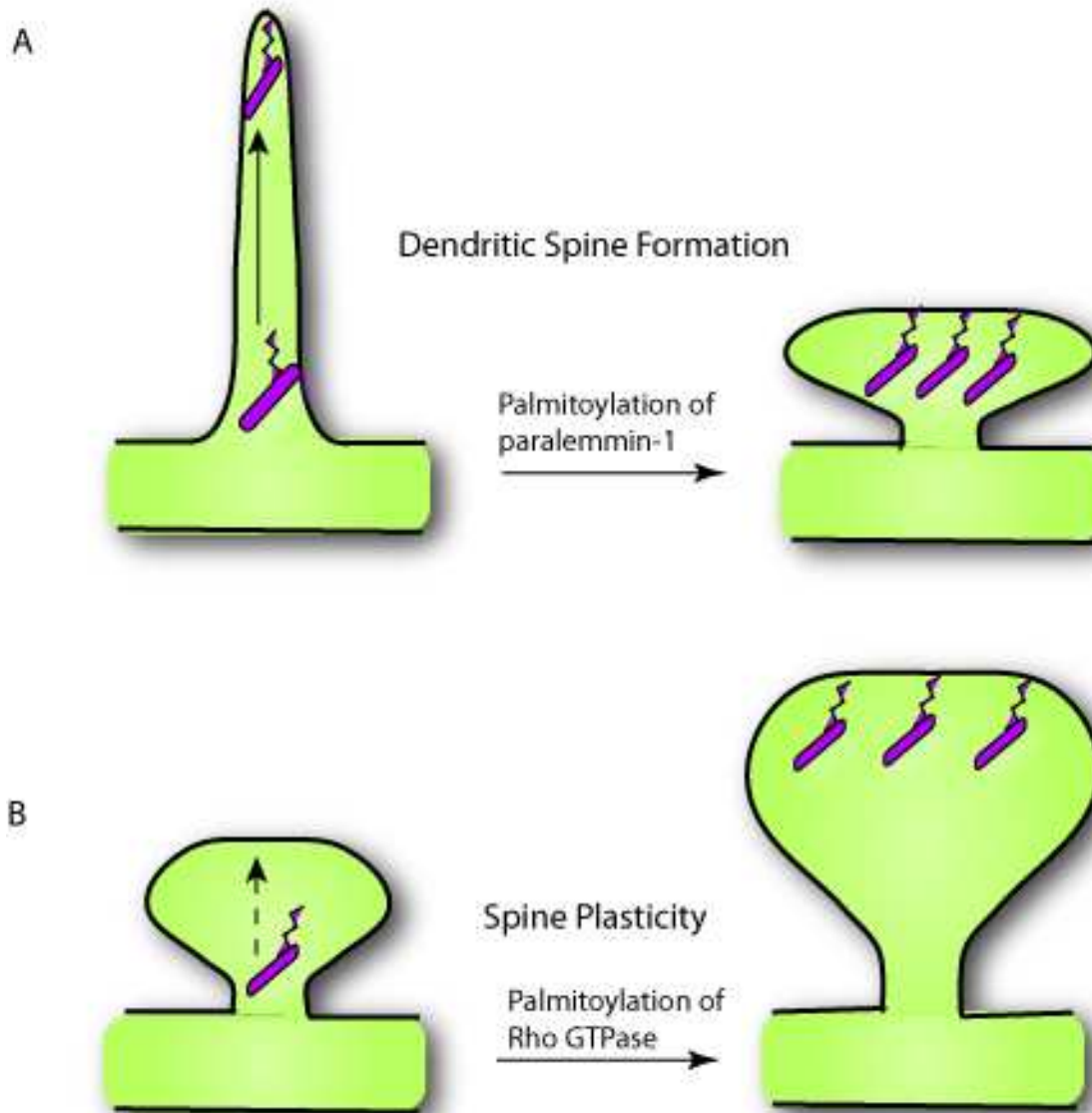
Figure 1.7. Proposed roles for palmitoylation in regulating dendritic spine development and morphology.

(A) Palmitoylation of paralemmin is required for filopodia induction and spine development. In developing neurons, palmitoylation is required for paralemmin-induced changes in membrane dynamics and actin cytoskeleton, two processes involved in filopodia induction. The increase in spine density by paralemmin is thought to be mediated through enhanced filopodia production, protrusions thought to serve as precursors of spines. **(B)** Palmitoylation of specific members of the Rho family of GTPases that influence spine morphology. In mature neurons, palmitoylation may modulate retention and activity of specific Rho GTPases at the synapse. This process may serve as a mechanism to dynamically control changes in spine morphology associated with the induction of several paradigms that control synaptic plasticity.

1.1.4 Modulation of neuronal signaling by palmitoylation

Neuronal excitability in the brain is modulated by a wide array of receptors and their downstream signaling proteins. One major group consists of neurotransmitter receptors coupled to G-proteins that control diverse signaling pathways. Many components of G-protein complexes are subject to modification by palmitate attachment. For example, palmitoylation near the amino termini of G-protein α-subunits regulates localization of G-protein signaling pathways to plasma membrane caveolae and lipid rafts(Qanbar and Bouvier 2003) (Figure 1.8). In addition, many G-protein coupled neurotransmitter receptors themselves are palmitoylated. These include metabotropic glutamate receptor mGluR1α, dopamine D1 receptor, serotonin and β-adrenergic receptors (for reviews see (Resh 1999; el-Husseini Ael and Bredt 2002; Qanbar and Bouvier 2003; Smotrys and Linder 2004; Torrecilla and Tobin 2006).
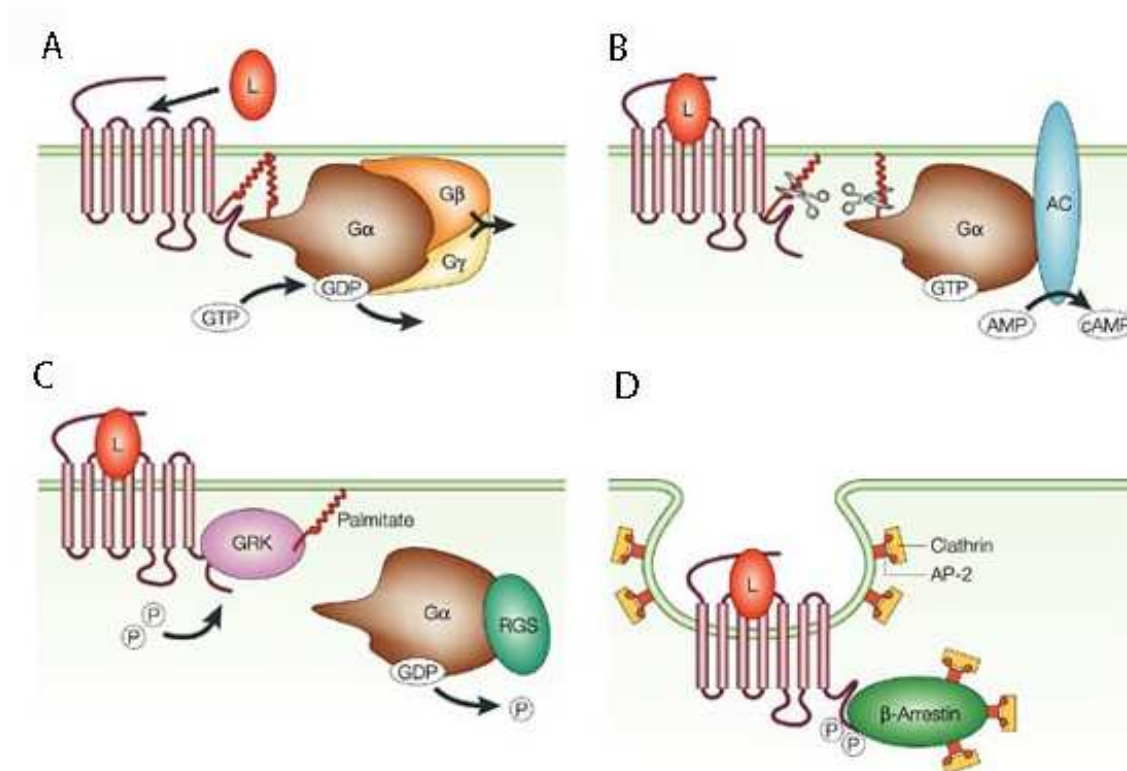
Figure 1.8. Dynamic palmitate turnover regulates signalling by GPCR pathways.

(A) Ligand (L) binding to a G-protein-coupled receptor (GPCR) induces receptor activation and interaction with the G-protein α-subunit. This association triggers GDP–GTP exchange on Gα, and the dissociation of βγ from the G-protein complex. **(B-D)** Ligand binding increases palmitate turnover, leading to depalmitoylation of the receptor and activated Gα. Depalmitoylation downregulates coupling to adenylyl cyclase (AC) by removing Gα from the membrane and enhancing its association with the RGS (regulators of G-protein signalling) GTPase. Receptor depalmitoylation facilitates GRK (GPCR kinase) phosphorylation, which recruits β-arrestin and enhances receptor internalization by the clathrin–AP-2 complex. Reprint from (el-Husseini Ael and Bredt 2002) with permission.

Specific members of the Src family of non-receptor tyrosine kinases (SFK) are also palmitoylated. Structurally, these proteins are highly homologous, and are able to perform overlapping functions. However, they are differentially activated by particular biological processes (Klinghoffer *et al.* 1999). Activation of the non-palmitoylated SFK Src requires RhoB endosome-associated actin assembly and transit to the cell periphery. In contrast, the palmitoylated Fyn is present in RhoD-positive endosomes (Sandilands *et al.* 2007). Taken together, sorting of particular kinases to specific sub-cellular compartments provides means for tightly and spatially controlling enzyme activity. Thus, palmitoylation may provide a mechanism to ensure that activation of a particular kinase occurs at the correct sub-cellular location, conferring functional specificity.

1.1.5 Palmitate cycling controls protein trafficking and function

Distinct from other lipid modification, palmitoylation is a reversible process and can be regulated by specific cellular stimuli. Binding of ligand to the β-adrenoreceptor markedly accelerates the depalmitoylation of the associated $G_{\alpha s}$ subunit, which dampens G-protein signaling(Qanbar and Bouvier 2003). Depalmitoylation of PSD-95 is regulated by neuronal activity and promotes the removal of PSD-95 from synapses(el-Husseini Ael

and Bredt 2002). This process in turn controls activity-induced internalization of the AMPA receptor subunit GluR1, and thus provides a mechanism for regulating synaptic strength(Osten *et al.* 2000; El-Husseini Ael *et al.* 2002). Likewise, AMPA receptors exhibit stimulation-dependent changes in palmitoylation status and internalization(Hayashi *et al.* 2005). These findings indicate that dynamic palmitoylation modulates directly and indirectly (via PSD-95) surface expression of AMPA receptors and hence synaptic activity.

Differential palmitate cycling on members of the Ras family of GTPases provides another potential mechanism for controlling signaling of many neurotrophic factors. Recent studies by Rocks *et al.* showed that mono-palmitoylated N-Ras displays a more pronounced Golgi localization, faster retrograde plasma membrane to Golgi trafficking, and several-fold shorter plasma membrane dwell time than dually palmitoylated H-Ras(Rocks *et al.* 2005). A high rate of palmitate turnover limits N-Ras access to endosomes resulting in compartmentalization of signaling mediated by these Ras family members (Figure 1.9).
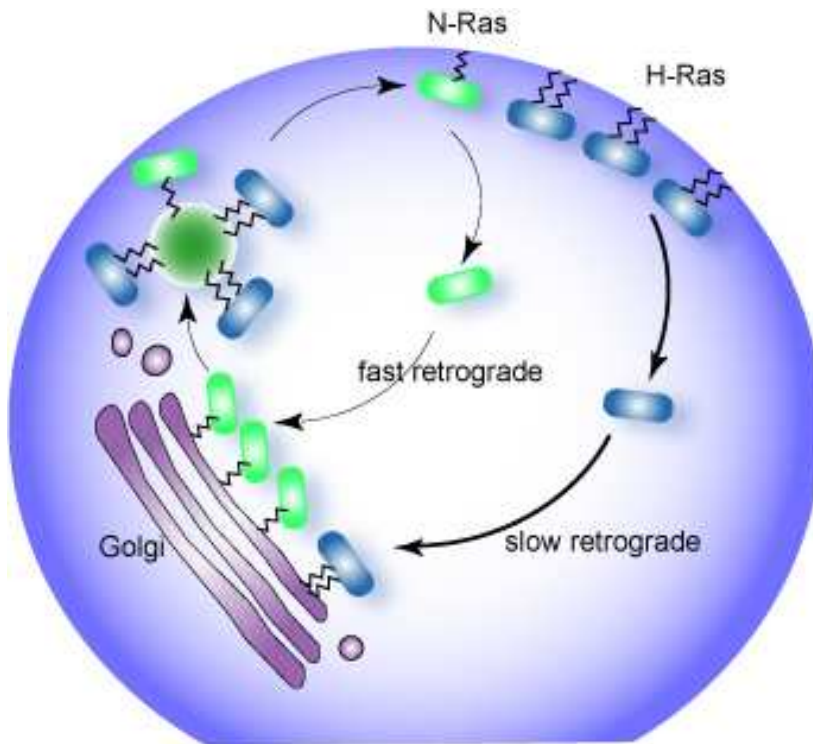
Figure 1.9. Palmitoylation-dependent cycling of Ras between the plasma membrane and the Golgi.

Constitutive cycling of palmitate on H-Ras and N-Ras regulates rapid protein shuttling between the plasma membrane and the Golgi. A differential rate of palmitate turnover regulates the rate of retrograde trafficking, resulting in a more pronounced accumulation of both N-Ras in the Golgi and H-Ras at the plasma membrane. Reprint from (Huang and El-Husseini 2005) with permission.

However, it is important to note that not all palmitoylated proteins exhibit rapid turnover of palmitate, as in the case of the presynaptic proteins, synaptotagmin I and SNAP-25, which are stably associated with palmitate. Here, palmitoylation probably functions as a structural signal for correct targeting and assembly of elements involved in neurotransmitter release(Heindel *et al.* 2003; Kang *et al.* 2004).

1.1.6 Enzymes involved in the modulation of protein palmitoylation

Although the modified sequences and enzymes involved in protein myristoylation and prenylation have been well characterized, mechanisms that control protein palmitoylation have been poorly understood due to the limited knowledge of enzymes involved in this process, the lack of consensus sequence for palmitoylation and the identity of palmitoylated proteins (Figure 1.10). The first characterization of enzymes involved in palmitoylation came from work done on flies(Chamoun *et al.* 2001; Nusse 2003; Zhai *et al.* 2004). *skinny hedgehog* and *porcupine* (*porc*) are palmitoyl acyl-transferases (PATs) that localize in the lumen of the secretory pathway to mediate palmitoylation of the secreted factors hedgehog and Wnt-1(Chamoun *et al.* 2001; Nusse 2003; Zhai *et al.* 2004). Therefore, these two enzymes are unlikely to be involved in palmitoylation of cytosolic proteins.
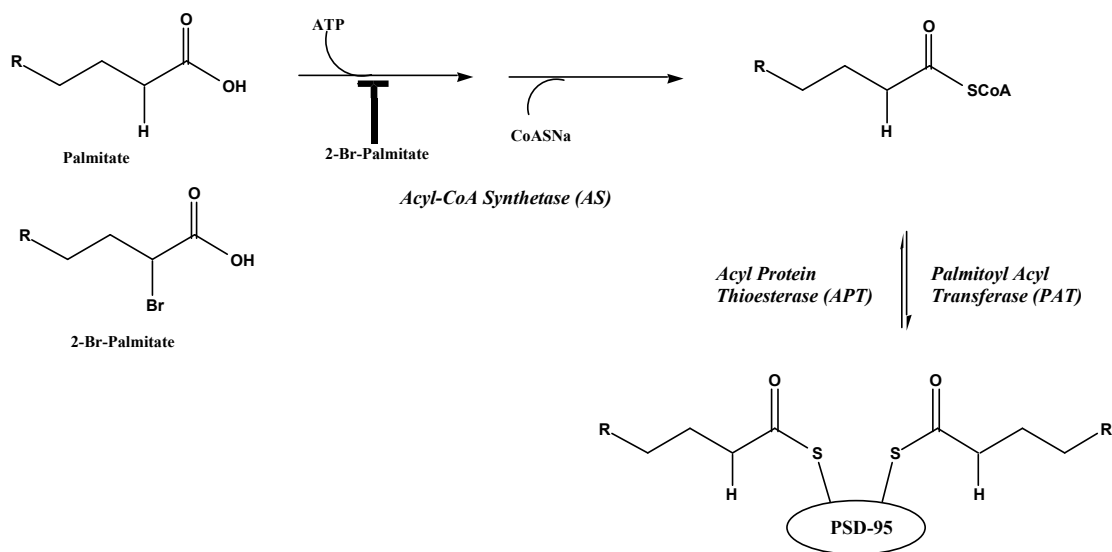
Figure 1.10. Proposed role for Palmitoyl Acyl Transferases (PATs) and Thioesterases (APTs) in regulation of palmitate addition and removal.

Palmitate addition to Co enzyme A is mediated by Acyl-CoA synthetase. The generated palmitoyl CoA is utilized by Palmitoyl acyl transferase (PAT) which catalyzes the addition of palmitate to substrates on specific cysteine residues through a thioester bond. Palmitate removal is catalyzed by acyl protein thioesterase (APT). 2-bromopalmitate, an analog of palmitate acts as an inhibitor of palmitoylation. The presence of the bromine (Br) atom interferes with the transfer of palmitate from palmitoyl CoA to substrate.

The recent discovery of some yeast PATs that mediate palmitoylation of cytoplasmic proteins uncovered a large family of enzymes essential for palmitoylation. The Erf2p–Erf4p protein complex and Akr1p were first shown to palmitoylate Ras(Lobo *et al.* 2002), and the yeast enzyme casein kinase II, respectively(Roth *et al.* 2002). Discovery of these enzymes led to the identification of a family of seven yeast PATs named DHHC proteins, all of which contain a conserved DHHC (Asp-His-His-Cys) motif present in a cysteine rich domain (CRD). These include Pfa3 and Pfa4, which palmitoylate yeast vacuole fusion factor Vac8(Hou *et al.* 2005), and yeast chitin synthase Chs3(Lam *et al.* 2006). Detailed analysis performed by the Davis group further elucidated the multiple substrates regulated by individual yeast PATs (Roth *et al.* 2006a). The identification of yeast DHHC proteins also led to the identification of 23 mammalian proteins that contain the DHHC domain as PATs (Figure 1.11 and Figure 1.12) (Fukata *et al.* 2004). Work done by Bredt and our group characterized the palmitoyl transferase activity of some of these proteins towards specific neuronal protein substrates (Fukata *et al.* 2004; Huang *et al.* 2004). Using i*n vitro* palmitoylation assays, we revealed that the huntingtin interacting protein 14 (HIP14), also known as DHHC-17, palmitoylates several proteins including huntingtin, PSD-95, SNAP-25, synaptotagmin I and GAD65(Singaraja *et al.* 2002) (Huang *et al.* 2004). DHHC-2, -3, -7 and -15 also showed the strongest specificity for palmitoylating PSD-95. Enzyme specificity towards many other substrates has been assessed by several other investigations. DHHC-2, -3, -7, -8,

and –21 have been shown to enhance incorporation of [^{3}H]-palmitate into endothelial nitric oxide synthase (eNOS). These PATs all colocalize with eNOS in the Golgi and plasma membrane and interact with eNOS(Fernandez-Hernando *et al.* 2006). DHHC-3 and -7 interact with, and palmitoylate GABA(A) receptors at a cysteine-rich 14-amino acid domain, located in the large cytoplasmic loop of the γ2 subunit(Fang *et al.* 2006). Others showed that a protein complex composed of DHHC-9 and a Golgi-localized protein designated GCP16 palmitoylates H- and N-Ras(Swarthout *et al.* 2005). Moreover, purified DHHC-9 and GCP16 exhibits substrate specificity, palmitoylating H- and N-Ras but not myristoylated G (αi1) or GAP-43, proteins with N-terminal palmitoylation motifs. These results demonstrate the requirement for specific enzymes in the regulation of protein palmitoylation.
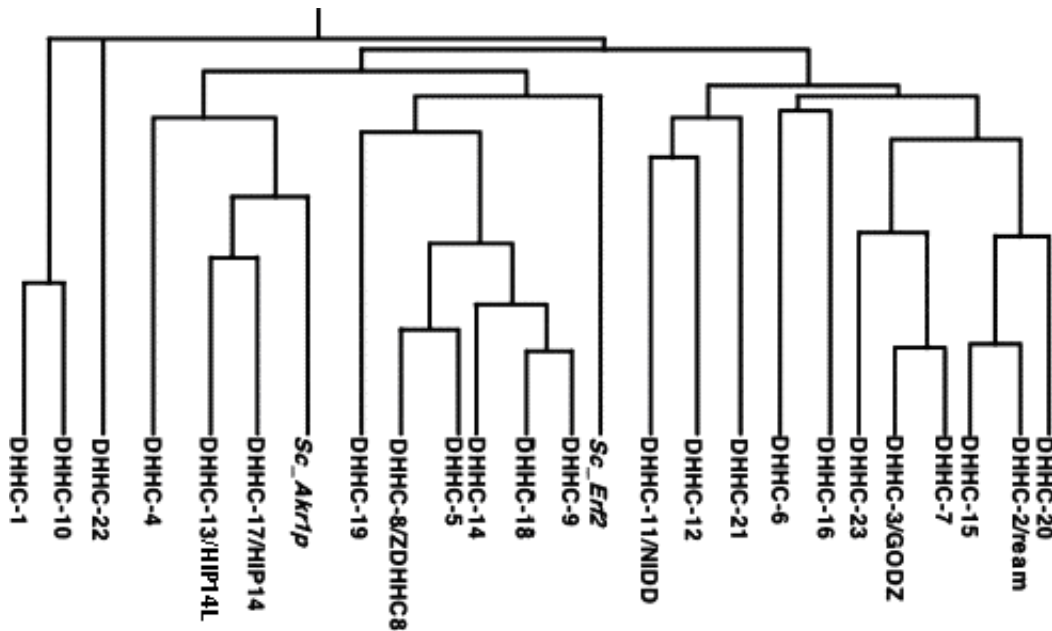
Figure 1.11. The phylogenetic tree of mouse DHHC protein family.
DHHC-3 is also known as GODZ; DHHC-17, as HIP14. Adapted from (Fukata *et al.* 2006) with permission.
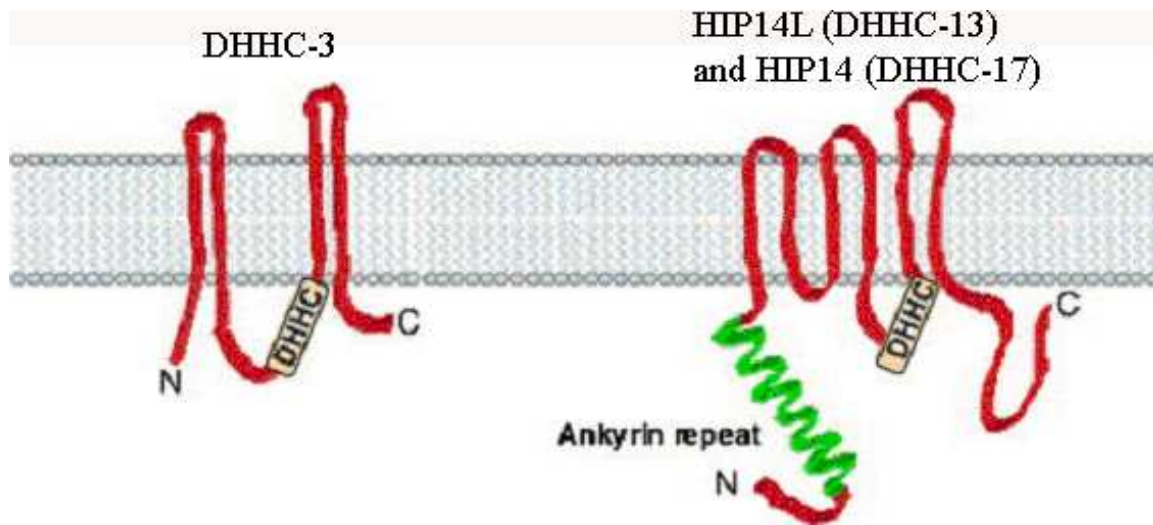
Figure 1.12. Domain structures of mammalian DHHC proteins, DHHC-3 and DHHC-17 (HIP14).

As described for yeast DHHC proteins, they have several transmembrane domains and a conserved cysteine-rich domain containing a DHHC (Asp-His-His-Cys) motif (DHHC-CRD). The DHHC sequence is essential for palmitoylating activity. DHHC-17 has six ankyrin repeat sequences in the N-terminal half. Adapted from (Fukata *et al.* 2006) with permission.

Overexpression and loss of function analyses provided further support for an important role for DHHC proteins in protein palmitoylation in mammalian cells (Fukata *et al.* 2004). For instance, knock down of HIP14 reduces synaptic clustering of PSD-95 and GAD65, and alters huntingtin distribution in the soma(Huang *et al.* 2004; Yanai *et al.* 2006). In contrast, overexpression of HIP14 in neurons increases huntingtin localization to the Golgi apparatus. Inhibition of DHHC-21 in human endothelial cells reduces eNOS palmitoylation, eNOS targeting, and nitric oxide production(Fernandez-Hernando *et al.* 2006). Furthermore, expression of a dominant-negative form of DHHC-3 (DHHC-3^{C157S}) or DHHC-3 knock down by specific short hairpin RNA (shRNA) alters γ2 subunit-containing GABA$_A$ receptors trafficking to synapses, and compromises GABAergic transmission (Figure 1.6B) (Fang *et al.* 2006).

Why have many PATs been employed by yeast and mammals to carry out the same enzymatic reaction? The palmitoyl proteome profiles of the mutant yeast strains that are deficient for single or multiple yeast DHHC proteins revealed overlapping DHHC PAT functionality yet some specificity between enzymes and substrates (Roth *et al.* 2006a). In mammalian cells, differential localization of these enzymes might enable tight regulation of protein palmitoylation at specific subcellular sites. However, recent analysis revealed that the majority of mammalian DHHC proteins are localized to the ER and/or Golgi, and few are enriched at the plasma membrane(Ohno *et al.* 2006). The enrichment of the majority of the identified DHHC proteins in the ER and/or Golgi suggests that these compartments are major sites for palmitoylation(Huang *et al.* 2004; Keller *et al.* 2004; Mukai *et al.* 2004). Studies on HIP14 revealed that this enzyme associates with tubulovesicular organelles and recycling endosomes present in dendrites, axons and spine necks(Huang *et al.* 2004). Mobile HIP14-positive vesicular structures rapidly transport from a perinuclear compartment to various subcellular locations. RNA expression analysis also revealed specificity in the expression of DHHC mRNAs to particular tissues. Taken together, these results suggest that each PAT functions to palmitoylate a limited subset of neuronal proteins at specific subcellular sites, ensuring a high level of control of protein acylation.

Although there is no consensus sequence for palmitoylation, detailed mutagenesis analysis of specific palmitoylated motifs indicates that the presence of hydrophobic residues surrounding the modified cysteines is crucial for proper palmitoylation(El-Husseini *et al.* 2000a). Postsynaptic clustering of PSD-95 in neurons is dependent on dual palmitoylation of a specific amino acid sequence, MDCLCIV(Craven *et al.* 1999). The

exclusive postsynaptic targeting of PSD-95 is disrupted if this sequence is replaced by the N terminal palmitoylation sequence from the axonally localized GAP43 protein (MLCCMRR). Consequently, a fraction of PSD-95 is redistributed to axons. Thus, specific palmitoylated motifs may direct proteins to distinct subcellular domains(El-Husseini *et al.* 2000a; Greaves and Chamberlain 2007). Different palmitoylation motifs may also be recognized by distinct PATs. In the future it will be important to determine the exact sequences required for substrate recognition by individual PATs.

Another unresolved issue is how protein depalmitoylation is regulated. It is thought that this process is controlled by palmitoyl thioesterases. However, only two enzymes that regulate depalmitoylation have been identified. One of these enzymes is acyl-protein thioesterase 1 (APT1), a cytosolic thioesterase that depalmitoylates specific signaling molecules such as Gα, Ras and endothelial nitric oxide synthase(Yeh *et al.* 1999). Another putative depalmitoylation enzyme is protein palmitoyl thioesterase-1 (PPT1), whose deficiency leads to infantile neuronal ceroid lipofuscinosis. Although PPT1 primarily localizes in lysosomes and associates with the proteosomal degradation pathway, it is also present in axons, synaptosomes and synaptic vesicles (Lehtovirta *et al.* 2001; Ahtiainen *et al.* 2003). *In vitro* palmitoylation assays revealed that PPT1 is able to hydrolyze a range of cysteinyl peptide sequences found in both neuron-specific and ubiquitous (e.g., Gα) proteins(Cho *et al.* 2000). Interestingly, PPT1 activity is also regulated by synaptic activity(Suopanki *et al.* 2002). Kainic acid (KA)-induced experimental epilepsy, a model of excitotoxicity, enhanced localization of PPT1 at presynaptic terminals. Thus translocation of PPT1 to the synapse may be required to regulate palmitoylation levels of proteins that control neurotransmitter release. However,

it remains to be confirmed whether PPT1 serves as a thioesterase toward proteins *in vivo*. The fact that PPT1 localizes primarily in the lysosomal compartment for degradation infers the existence of a larger, yet to be discovered, family of thioesterases that regulate protein depalmitoylation at other subcellular locations.

1.1.7 Concluding remarks

Recent studies indicate that palmitoylation plays a key role in controlling trafficking and function of numerous proteins. In neurons, alterations in protein palmitoylation are associated with protein mistargeting, disruption of postsynaptic protein clustering and neurotransmitter release, and altered synaptic morphology and activity. The finding that key proteins that regulate neuronal development and firing are subject to regulated cycles of palmitoylation and depalmitoylation, combined with the identification of a large family of enzymes that regulate this process suggest that palmitoylation is tightly regulated in vivo. The profound changes in the function of many neuronal proteins upon interference with palmitoylation clearly underscore the importance of palmitoylation in normal brain function. It is not surprising that defects in protein palmitoylation and loss of function of enzymes that regulate this process have been associated with neurodegenerative and psychiatric disorders. Future investigations of the mechanisms that govern protein palmitoylation will further advance our understanding of the importance of this lipid modification in health and disease. Future avenues of study include examining the endogenous localization of mammalian DHHC proteins, elucidating mechanisms for substrate specificity, and determining whether the efficacy of these enzymes is subject to changes in neuronal activity.

1.2. HUNTINGTON DISEASE

1.2.1 Huntington Disease Clinical Features

Huntington disease (HD) is an autosomal-dominant, progressive neurodegenerative disorder. Typically, onset of symptoms is in middle-age after affected individuals have had children, but the disorder can manifest at any time between infancy and senescence. Huntington disease is fatal and currently has no effective treatment or cure. The mutant protein in Huntingtin disease—huntingtin—results from an expanded CAG repeat leading to a polyglutamine strand of variable length at the N-terminus.

Clinical features of Huntington disease include cognitive, psychiatric and behavioural symptoms. Cognitive dysfunction in Huntington disease often impairs executive functions, such as organising, planning, checking, or adapting alternatives, and delays the acquisition of new motor skills(Walker 2007). These features are progressive. However, long-term memory is often spared. Motor dysfunction is characterized by the involuntary writhing movements of the face, trunk and limbs. Other motor symptoms such as abnormalities in eye movements, fine motor control, and gait (Folstein *et al.* 1986; Kremer *et al.* 1992; Di Maio *et al.* 1993; Lasker and Zee 1997) often accompany or precede(Kirkwood *et al.* 1999) chorea. Most individuals have chorea that initially progresses but then, with later onset of dystonia and rigidity, it becomes less prominent(Young *et al.* 1986; Mahant *et al.* 2003). Therefore, although useful for diagnosis, chorea is a poor marker of disease severity(Young *et al.* 1986; Mahant *et al.* 2003). Huntington disease is also associated with a wide range of psychiatric disturbances, including affective disorders(Dewhurst *et al.* 1970; Caine and Shoulson 1983; Folstein *et al.* 1983a), irritability(Bolt 1970; Pflanz *et al.* 1991; Craufurd *et al.* 2001),

apathy(Dewhurst *et al.* 1970; Caine and Shoulson 1983; Craufurd *et al.* 2001) and psychosis(Bolt 1970; Shiwach 1994; Lovestone *et al.* 1996).Both major depression(Bolt 1970; Dewhurst *et al.* 1970; Folstein *et al.* 1983a; Folstein *et al.* 1983b) and more subtle mood disturbances(Baxter *et al.* 1992) have been reported to predate clinical onset, conventionally defined by onset of motor symptoms.

1.2.2 Neuropathology

1.2.2.1   Patterns of Neuronal degeneration

Neuropathological changes in Huntington's disease are initially selective, with prominent cell loss and atrophy in the caudate and putamen of the striatum (Vonsattel *et al.* 1985). GABA-releasing medium-spiny neurons that project from the striatum are the most vulnerable. The pattern of neurodegeneration and receptor changes that occur in the basal ganglia follow a specific sequence (Glass *et al.* 2000). The initial GABAergic subpopulation of neurons to degenerate are those of the indirect striatal pathway that express enkephalin and are enriched in the dopamine receptor D2 (Reiner *et al.* 1988). It is the initial loss of neurons in this indirect pathway (and therefore loss of thalamic-cortical inhibition) that is believed to cause the choreiform movement disorder characteristic of HD (Mitchell *et al.* 1999). Later in the disease, loss of neurons in the direct pathway, composed of GABAergic neurons expressing substance P, suppresses movement and results in hypoactivity and dystonia. Interneurons are generally spared.

Other brain areas greatly affected in people with Huntington disease include the substantia nigra, cortical layers 3, 5, and 6, the CA1 region of the hippocampus(Spargo *et al.* 1993), the angular gyrus in the parietal lobe(Macdonald *et al.* 1997; Macdonald and

Halliday 2002), purkinje cells of the cerebellum(Jeste *et al.* 1984), lateral tuberal nuclei of the hypothalamus(Kremer *et al.* 1991; Kremer 1992) and the centromedialparafascicular complex of the thalamus(Heinsen *et al.* 1999).

1.2.2.2 Neuronal Inclusions
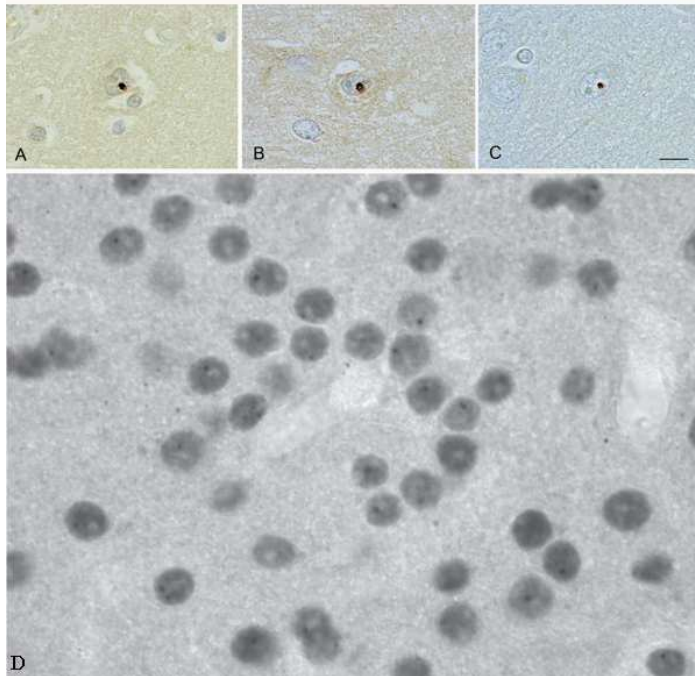
Figure 1.13. Neuronal intranuclear inclusions (NIIs) in the brains of HD mice and human HD patients.

Neuronal intranuclear inclusions **(A–C)** in the neocortex of the brains of two at-risk individuals with grade I **(A,B)** and grade 0 **(C)** HD pathology, respectively. Immunostaining was performed with an antibody against **(A)** ubiquitin and antiserum H7 **(B)** and antibody 5374 **(C)** both directed to the N-terminus of huntingtin. Scale bar **(A–C)** 10 µm. (Reprint from (Maat-Schieman *et al.* 2007) with permission) **(D)** NIIs stain as dark EM48 inclusions inside the stained nucleus of striatal neurons from a YAC128 mouse brain (R Graham).

The appearance of nuclear and cytoplasmic inclusions that contain mutant huntingtin is one of the pathological characteristics of Huntington's disease(Davies *et al.* 1997). In both transgenic mice models of HD and human HD brains, neuronal intranuclear inclusions were observed (Figure 1.13) (Davies *et al.* 1997; DiFiglia *et al.*

1997; Scherzinger *et al.* 1997; Becher *et al.* 1998). As mutant huntingtin proteins fail to fold properly, they are either refolded by chaperones or destroyed by the proteasome(Glickman and Ciechanover 2002; Young *et al.* 2004; McClellan *et al.* 2005). In neurodegenerative diseases, these quality control mechanisms are overwhelmed, possibly partly due to a natural decline in function as neurons age(Keller *et al.* 2002; Soti and Csermely 2003). Ultimately, misfolded protein accumulates and disrupts cellular function, contributing to neuronal degeneration. A classically held view of HD pathogenesis is that these nuclear inclusions cause neurodegeneration and HD symptomatology as their presence seems to correlate with pathology (Davies *et al.* 1998; Wanker 2000). However, further studies have dissociated inclusion formation and pathology, in cell culture models, animal models and even human brain samples (Klement *et al.* 1998; Saudou *et al.* 1998; Cummings *et al.* 1999; Gutekunst *et al.* 1999; Kuemmerle *et al.* 1999; Slow *et al.* 2005). Current evidence suggests that poly glutamine aggregates are not the toxic species (also summarized in**Error! Reference source not found.**).

Table 1.2. **Evidence against a relationship between inclusions or amyloid plaques and disease specific pathology**
(Adapted from (Slow *et al.* 2006) with permission)

Studies of HD	Experimental Results
Human studies	
Inclusions, plaques and NFTs do not correlate with clinical features or pathology	Inclusions do not correlate with regional specificity of neuronal degeneration(Kuemmerle *et al.* 1999)
Mouse studies	

Studies of HD	Experimental Results
Specific disease pathology or clinical features before or without inclusions or plaques	Cognitive and motor dysfunction (Menalled *et al.* 2003; Slow *et al.* 2003; Van Raamsdonk *et al.* 2005c), striatal neuronal loss (Slow *et al.* 2003)is present months before inclusions
Dissociation between treatment effects and inclusions in pathology and clinical phenotype	HDAC inhibitors (Ferrante *et al.* 2003; Hockly *et al.* 2003)or decreasing transglutaminase (Mastroberardino *et al.* 2002) improves clinical features with no improvement in or increased inclusions
Inclusions with no clinical symptoms or pathology	Shortstop mice develop widespread inclusions without motor dysfunction, brain or striatal neuron loss (Slow *et al.* 2005)
Neuroprotective role of inclusions *in vitro*	Inclusions improve survivability of neurons *in vitro*(Arrasate *et al.* 2004)and sequester mTOR, promoting autophagy of toxic huntingtin (Ravikumar *et al.* 2004)

Indeed, nuclear inclusions and the selective degeneration in striatum observed in HD are not correlated. Within the striatum, nuclear inclusions are predominantly observed in spared interneurons, whereas degenerating medium spiny neurons contain no or few inclusions. Cortex, a brain region affected later in the disease, exhibits a much greater inclusion presence (Kuemmerle *et al.* 1999). Furthermore, the shortstop mouse expresses a fragment of huntingtin (exon 1 and 2) and develops extensive nuclear inclusions throughout the brain but does not develop any other pathological features of the disease (Slow *et al.* 2005). These dissociations argue against inclusions being the toxic moiety.

Other evidence also demonstrates that it is possible to halt or reverse the pathology and symptomatology of HD including cognitive loss, early death and motor dysfunction without any effect on inclusion formation. For instance, treating the R6/2 mouse model of

HD with histone deacetylase (HDAC) inhibitors (Ferrante *et al.* 2003; Hockly *et al.* 2003) resulted in improvements in HD symptoms without decreasing huntingtin inclusion burden. Furthermore, crossing this mouse model with tissue transglutaminase knockout mice partially alleviated HD phenotype but was accompanied by a significant increase in huntingtin inclusions(Mastroberardino *et al.* 2002). Conversely, a new identified compound B2 (5-[4-(4-chlorobenzoyl)-1-piperazinyl]-8-nitroquinoline) that prevents huntingtin-mediated proteasome dysfunction actually promotes inclusion formation in cellular models of HD (Bodner *et al.* 2006).

Huntingtin protein inclusions can also sequester other cellular proteins, besides toxic huntingtin, as a neuroprotective strategy. Recording the survival of neurons that were transfected with mutant huntingtin *in vitro* revealed that, surprisingly, the neurons that formed huntingtin inclusions had an increased likelihood of survival (Arrasate *et al.* 2004).Another study applied time-lapse analysis of aggregate formation in an inducible PC12 cell model of Huntington's disease and revealed time-dependent aggregate formation that transiently delays cell death(Gong *et al.* 2008). It is suggested that inclusions may improve neuron survival, possibly by sequestering mTOR and promoting autophagy of toxic huntingtin (Ravikumar *et al.* 2004).

Rather, it is the oligomer huntingtin containing the HD mutation, or intermediate stages of poly glutamine aggregates that are the toxic species. This hypothesis is supported by the finding that translocation of soluble huntingtin into the nucleus is an early marker of HD in mouse and human studies (Menalled *et al.* 2003; Van Raamsdonk *et al.* 2005a).

1.2.3 HD Genetics

The prevalence of HD is ~7 to 10 cases per 100,000 in white populations, less in populations that are mixed white, and significantly less common in black and Asian populations (Harper 1992; Squitieri *et al.* 1994). The HD locus is located on chromosome 4p16.3 and contains 67 exons across 180kb of genomic sequence (Ambrose *et al.* 1994). Due to differential 3' polyadenylation, the human HD gene encodes two mRNA products of 10.3kb and 13.6kb, with the larger transcript predominantly expressed in the brain and the smaller transcript highly expressed in other tissues (Lin *et al.* 1993).

HD is monogenic and dominantly inherited (Harper 1993; Harper 2005). The mutation that causes HD is an expansion in the number of repeated CAG codons in exon 1 of the HD gene, which was identified in 1993 using linkage analysis (The Huntington's Disease Collaborative Research Group, 1993). If the expansion has 40 or more repeats, the disease is fully penetrant (Kremer *et al.* 1994); with 36 to 40 repeats, it is variably penetrant (Rubinsztein *et al.* 1996; Brinkman *et al.* 1997; McNeil *et al.* 1997); and with 35 repeats or less, the disease does not occur (also summarized in Table 1.3)(Rubinsztein *et al.* 1996; Langbehn *et al.* 2004). However, the instability of the expanded CAG repeat begins at repeat size of 27 or greater. In gametogenesis, these CAG repeats are prone to further expansion (Ranen *et al.* 1995), which explains that the age of onset of the disease tends to get younger with succeeding generations. This may also result in the expansion of a repeat size in the 27 to 35 range into the symptomatic range in offspring, which contributes to the ~8% of patients who have no known family history (Yamamoto *et al.* 2000; Montoya *et al.* 2006).

Table 1.3. CAG sizes for alleles in the HD gene.

	CAG SIZE
Normal Allele	7 - 26
Intermediate Allele	27 - 35
HD Allele	
Reduced Penetrance	36 - 39
Full Penetrance	40 - 60
Juvenile Onset	60 +

The age of onset of symptoms in patients correlates robustly with the number of CAG repeats, which accounts for ~50 to 60% of the variation in age of onset for any given patient(Andrew *et al.* 1993; Ranen *et al.* 1995). The majority of adult onset HD cases have between 40-55 CAG repeats (Kremer *et al.* 1994) with a typical age of onset of 30-50 years. Individuals with > 60 repeats inevitably develop juvenile-onset HD (Telenius *et al.* 1993; Trottier *et al.* 1994).

1.2.4 Mouse Models Used in Studying huntingtin and HD

1.2.4.1 Huntingtin knockout mice (huntingtin-/- and huntingtin+/-)

An important advance in the study of HD was the development of mouse models. Initially, generation of mice with a targeted disruption of the HD gene was attempted by several groups. However, mice derived from targeted embryonic stem (ES) cell lines (huntingtin-/-) do not survive to term and suffer early postimplantation embryonic lethality (Duyao *et al.* 1995; Nasir *et al.* 1995; Zeitlin *et al.* 1995).

Mice heterozygous for the *Hdh^ex5* mutation survive to adulthood, but display increased motor activity and cognitive deficits (Nasir *et al.* 1995). Detailed morphometric and stereological analyses of the basal ganglia in adult heterozygous mice demonstrated a significant neuronal apoptosis and loss from both the globus pallidus and the subthalamic nucleus. Stereological analyses in the subthalamic nucleus also revealed a significant decrease in the numerical density of symmetric synapses (43%), suggesting a relatively selective loss of inhibitory pallido-subthalamic afferents (O'Kusky *et al.* 1999). These studies show that the HD gene is essential for post-implantation development and reduction of huntingtin protein is associated with abnormal functioning of the striatum. Intriguingly, huntingtin+/- mice have behavioral deficits similar to that seen in rodents with lesions in the striatum, and they have significant reductions of neurons in the subthalamic nucleus, a nucleus which also shows obvious neuronal loss (25%) in patients with HD (Lange *et al.* 1976). Taken together, these data suggest that HD may partially result from loss of wildtype huntingtin function.

1.2.4.2   HD Transgenic Mice

The ideal HD transgenic mouse model would have a robust phenotype, rapid disease onset and progression, well-defined behavioural abnormalities that can be quantified, and neuropathological features such as selective loss of striatal projection neurons, all of which accurately replicate human HD. There are many mouse models of HD and they fall into three broad categories: (1) mice that express the full-length human HD gene (plus murine Hdh); (2) mice with pathogenic CAG repeats inserted into the existing CAG tract in murine Hdh (knock-in mice); (3) mice that express N-terminal

fragments of the human huntingtin protein containing polyglutamine mutations (in addition to both alleles of murine wild-type huntingtin).

1.2.4.2.1 <u>Full-length huntingtin transgenic mice (Human huntingtin)</u>

Yeast artificial chromosome (YAC) transgenic models of HD (YAC128) express full length mutant huntingtin with 120 CAG repeats from a YAC(Van Raamsdonk *et al.* 2007b). The YAC128 mouse contains the human HD gene endogenous promoter elements, providing appropriate developmental and tissue-specific expression of the huntingtin protein. Accordingly, they recapitulate the motor deficits, cognitive impairment and selective degeneration similar to HD in humans (Hodgson *et al.* 1999); (Slow *et al.* 2003; Van Raamsdonk *et al.* 2005a; Van Raamsdonk *et al.* 2005c). The expression level of the polyglutamine-expanded huntingtin modulates the HD phenotype in the YAC mice. Increased expression leads to an earlier age of onset, more rapid progression, increased striatal volume loss and nuclear accumulation of huntingtin (Graham *et al.* 2006).

1.2.4.2.2 <u>Knock-in transgenic mouse models (Mouse huntingtin)</u>

The knock-in mice are accurate genetic models of the disease in the murine huntingtin gene (rather than human huntingtin in the YAC and BAC lines), but in general tend to manifest milder motor or neuropathological phenotypes without overt neurodegeneration (Lin *et al.* 2001; Menalled 2005). For example, a knock-in Hdh mouse with 72–80 CAG repeats in the murine HD gene behaves aggressively and has aggregates in neuropil but shows no gliosis or cell loss(Shelbourne *et al.* 1999; Menalled *et al.* 2002). HdhQ111-knock-in mice, with 111 CAG repeats inserted into the murine HD

gene, have a progressively developing neuropathological phenotype characterized by accumulation of huntingtin in the nucleus that is specific for striatal neurons, but gait abnormalities are not apparent until 2 years of age (Wheeler *et al.* 2000).

1.2.4.2.3 <u>N-terminal huntingtin transgenic mouse models</u>

The R6/2 mouse belongs to this category. The R6/2 line is the most commonly studied model, and expresses exon 1 (67aa) of the human gene with about 150 CAG repeats (Mangiarini *et al.* 1996). The R6/2 model has many of the temporal, behavioural and neuropathological features that are observed in patients with HD, such as jerky movements and striatal atrophy (Mangiarini *et al.* 1996; Davies *et al.* 1997). Altough the efficiency and clear experimental endpoints of the R6/2 mice are advantages, these animals are not a perfect genetic and neuropathological match for human HD pathology. R6/2 mice have more extensive huntingtin aggregate distribution — including the cerebral cortex and hippocampus — than is typical of HD(Davies *et al.* 1997). Moreover, they are resistant to excitotoxicity, which might be related to an increased capacity to process calcium, and show little cell loss (Davies *et al.* 1997; Hansson *et al.* 1999; Hansson *et al.* 2001).

Other N-terminal huntingtin transgenic models include the N171 mouse (Schilling *et al.* 1999; Schilling *et al.* 2001), the conditional exon 1 model (Yamamoto *et al.* 2000), NSE mouse (Laforet *et al.* 2001) and shortstop mouse which contains the same 25kb endogenous promoter as the YAC128 and exon 1 and 2 of huntingtin. The shortstop mouse does not develop a pathological phenotype despite the widespread presence of inclusions in the brain (Slow *et al.* 2005).

1.2.5 Function of Huntingtin

Huntingtin is a completely soluble protein of 3,144 amino acids, expressed ubiquitously in humans and rodents, with the highest levels in the brain (Li *et al.* 1993; Gutekunst *et al.* 1995; Landwehrmeyer *et al.* 1995; Schilling *et al.* 1995; Sharp *et al.* 1995)and the testes(Van Raamsdonk *et al.* 2007a). Huntingtin is present in both neurons and glia (Landwehrmeyer *et al.* 1995), and the regional distribution of huntingtin in the brain, and even within cell types of the striatum, does not correlate with the selective neuropathology (Vonsattel and DiFiglia 1998).

At the subcellular level, huntingtin is found in the nucleus (Hoogeveen *et al.* 1993; De Rooij *et al.* 1996; Saudou *et al.* 1998; Dorsman *et al.* 1999; Wheeler *et al.* 2000; Tao and Tartakoff 2001; Kegel *et al.* 2002) and throughout the cytoplasm(DiFiglia *et al.* 1995; Trottier *et al.* 1995a; Jones 1999), where it is associated with various organelles, such as endoplasmic reticulum and Golgi complex(DiFiglia *et al.* 1995; Velier *et al.* 1998; Hilditch-Maguire *et al.* 2000; Hoffner *et al.* 2002; Rockabrand *et al.* 2007), and it is also found in neurites and at synapses(Li *et al.* 2003a), where it associates with vesicular structures such as clathrin-coated vesicles(DiFiglia *et al.* 1995), endosomal compartments(Velier *et al.* 1998) or caveolae(Trushina *et al.* 2004), and microtubules(Hoffner *et al.* 2002; Kegel *et al.* 2005). Taken together, the widespread regional expression and subcellular localization of huntingtin does not facilitate the definition of a single precise function in any specific compartment of the cell. Rather, current knowledge about huntingtin suggests it may have a number of functions.

1.2.5.1 Huntingtin is essential for development

Huntingtin is essential for embryonic development, as its complete inactivation of mouse homolog Hdh gene ($Hdh^{-/-}$) causes impaired gastrulation and embryonic death between Day 7.5 and Day 10.5(Duyao *et al.* 1995; Nasir *et al.* 1995; Zeitlin *et al.* 1995; Dragatsis *et al.* 1998). Interestingly, *Hdh* deletion mainly interferes with embryo viability by impairing the nutritive function of the visceral endoderm, rather than the embryo itself (Dragatsis *et al.* 1998).

While one normal huntingtin allele (50% of normal huntingtin) is sufficient in humans or mice for development and postpartum life that is largely or entirely indistinguishable from normal (Wexler *et al.* 1987; Ambrose *et al.* 1994; Duyao *et al.* 1995; Nasir *et al.* 1995; Zeitlin *et al.* 1995; Persichetti *et al.* 1996), reduction of huntingtin to levels lower than 50% of normal have been found to result in severe developmental malformations including defective neurogenesis, profound malformations of cortex and striatum, telencephalic ventricular enlargement, and agenesis of fiber tracts in late-term embryos and postpartum mice (White *et al.* 1997). The inability to produce viable chimeras containing *Hdh–/–* cells using the method of early embryo aggregation also suggests that even limited colonization of the extraembryonic membranes by *Hdh–/–* cells may impair their function so severely as to be lethal to the developing embryo.

However, several lines of evidence suggest that the CAG tract does not affect the physiological function of the protein during development, and also shows that mutant huntingtin can compensate for the loss of wild-type function in development. First, the extra-embryonic function of huntingtin (that is, in tissues such as the placenta) does not depend on the CAG tract and ancestral huntingtin has no CAG domain. Second, patients

with HD who are homozygous for the polyglutamine expansion do not express wild-type huntingtin but develop normally and are born with no apparent defects (Wexler *et al.* 1987; Myers *et al.* 1989; Gusella and MacDonald 1996). Third, the expression of mammalian huntingtin with a pathological polyglutamine expansion (even up to 128 CAG repetitions) rescues huntingtin-null mice from embryonic lethality (Hodgson *et al.* 1999; Leavitt *et al.* 2001).

1.2.5.2   Huntingtin is involved in transcriptional regulation

Huntingtin might not only act as a scaffold but could also function as a transcriptional cofactor in the nucleus. Huntingtin has been shown to interact with a large number of transcription factors including the cAMP response-element binding protein (CREB)-binding protein (CBP) (McCampbell *et al.* 2000; Steffan *et al.* 2000), p53 (McCampbell *et al.* 2000; Steffan *et al.* 2000), the co-activator CA150 (Holbert *et al.* 2001) and the transcriptional co-repressor C-terminal binding protein (CtBP) (Kegel *et al.* 2002).

Although there is no direct evidence that wildtype huntingtin regulates normal gene expression, numerous studies have revealed changes in the expression of many genes in HD brains of human patients and mice models (Cha *et al.* 1999; Cha 2000; Chan *et al.* 2002; Luthi-Carter *et al.* 2002a; Luthi-Carter *et al.* 2002b; Sipione *et al.* 2002; Spektor *et al.* 2002; Luthi-Carter *et al.* 2003; Zucker *et al.* 2005; Desplats *et al.* 2006), with the greatest number of changes occurring in the striatum (Hodges *et al.* 2006). Interestingly, expression of polyglutamine-expanded huntingtin in cell models of HD has been shown to alter the mitogen-activated protein kinase (MAPK) signalling pathway and therefore suggest this may be a viable target for therapeutic intervention (Apostol *et al.* 2006).

1.2.5.3 Neuroprotective Role of Huntingtin

Huntingtin appears to exert a neuroprotective effect against lethal stresses in cultured striatal neurons (Rigamonti *et al.* 2000; Ho *et al.* 2001; Rigamonti *et al.* 2001; Leavitt *et al.* 2006), and neurons in the mousebrain (Zhang *et al.* 2003; Leavitt *et al.* 2006), including partial protection from the toxic effects of polyglutamine-expanded huntingtin (Leavitt *et al.* 2001; Van Raamsdonk *et al.* 2005b; Van Raamsdonk *et al.* 2006). Neuroprotection is enhanced with a progressive increase in the level of wild-type huntingtin, which indicates a gene-dosage effect(Leavitt *et al.* 2006). All of these studies indicate that wild-type huntingtin can trigger molecular events that lead to increased cell survival well beyond the developmental period.

Huntingtin also appears to exert an important receptor-mediated, prosurvival effect in striatum via stimulating the production of brainderived neurotrophic factor (BDNF) (Zuccato *et al.* 2001) by corticostriatal neurons (Altar *et al.* 1997; Ivkovic and Ehrlich 1999; Schuman 1999). Wild-type huntingtin may have this effect on BDNF production by regulates the activity of the BDNF promoter (Figure 1.14) (Zuccato *et al.* 2003).
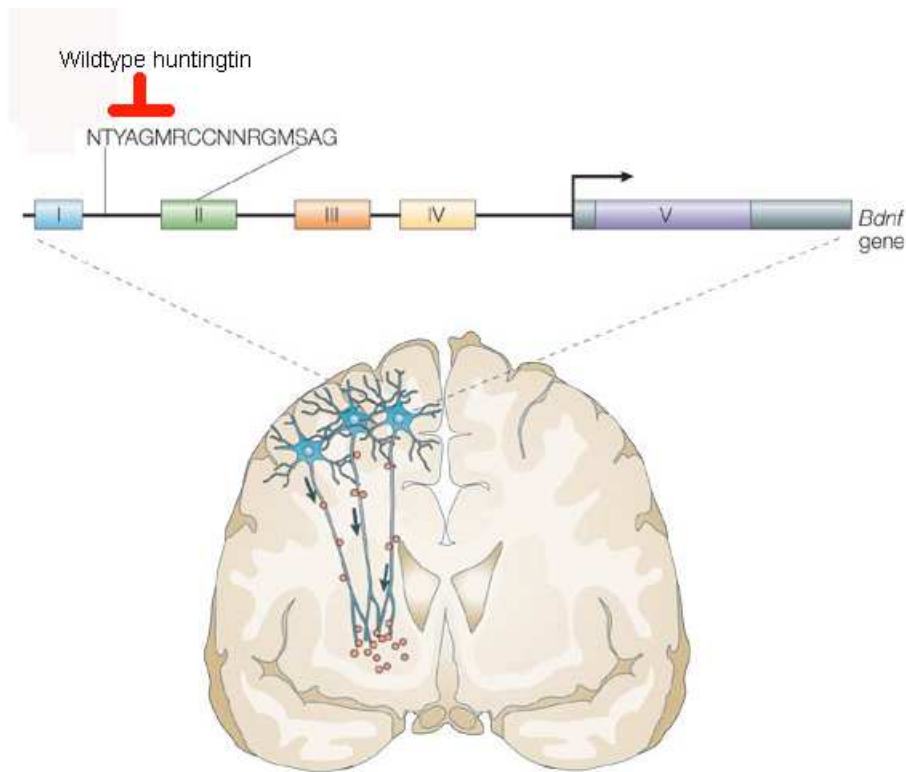
Figure 1.14. Wild-type but not mutant huntingtin facilitates cortical BDNF mRNA production.

Wild-type huntingtin contributes to brain-derived neurotrophic factor (*Bdnf*) transcription in the cortical neurons that project to the striatum by inhibiting the Repressor element 1/neuron-restrictive silencer element (RE1/NRSE) that is located in BDNF promoter exon II. I–IV indicate BDNF promoter exons in rodent *Bdnf*; V indicates the coding region. The RE1/NRSE consensus sequence is shown. Inactivation of the RE1/NRSE in *Bdnf* leads to increased mRNA transcription and protein production in the cortex. BDNF, which is also produced through translation from exons III and IV is then made available to the striatal targets via the cortico-striatal afferents. Wild-type huntingtin might also facilitate vesicular BDNF transport from the cortex to the striatum. Adapted from(Cattaneo *et al.* 2005)with permission.

1.2.5.4   Huntingtin and synaptic transmission

Besides interacting with a number of cytoskeletal and synaptic vesicle proteins that are essential for exo- and endocytosis at synaptic terminals (Smith *et al.* 2005), huntingtin also interacts with a key molecule in synaptic transmission, PSD-95, a member of the

membrane-associated guanylate kinase (MAGUK) family of proteins that binds the NMDA (*N*-methyl-D-aspartate) and kainate receptors at the postsynaptic density (Sheng and Kim 2002). Wild-type huntingtin directly binds the Src homology 3 (SH3) domains of PSD-95 (Sun *et al.* 2001). The decreased interaction of mutant huntingtin with PSD-95 may indirectly affect the activity of NMDA receptors and lead to their overactivation or sensitization, and excitotoxicity (Sun *et al.* 2001). Indeed, a shift of NMDAR subunits NR1 and NR2B from internal pools to the plasma membrane and a significantly faster rate of NMDAR insertion to the surface are reported in YAC72 medium spiny striatal neurons(Fan *et al.* 2007). By contrast, the overexpression of wild-type huntingtin seems to attenuate the neuronal toxicity induced by NMDA receptors and mutant huntingtin (Sun *et al.* 2001; Leavitt *et al.* 2006).

1.2.5.5 The Relevance of WT Huntingtin function to the Pathogenesis of HD

Is the loss of wild-type huntingtin involved in the disease process? If wild-type huntingtin has a function in mammalian neurons and this is inhibited by mutant huntingtin, the defects observed in mutant mice and cells should also be observed in the absence or reduced expression of wild-type huntingtin. Several lines of evidence indicate that at least some of the molecular dysfunctions observed in HD are a consequence of reduced wild-type huntingtin activity (Cattaneo *et al.* 2005). In several different animal or cell models with reduced wildtype huntingtin expression, neuronal cell death and neurological dysfunction were observed, similar to what were seen with HD patients and mice. Mice with <50% huntingtin level have abnormal brain development (White *et al.* 1997; Auerbach *et al.* 2001); Hdh^{+/-} mice show neuronal cell death and neurological

dysfunction in the globus pallidus and the subthalamic nucleus (Nasir *et al.* 1995; Dragatsis *et al.* 2000). Adult conditional Hdh$^{-/-}$ mice manifest a Huntington's disease-like phenotype and show apoptosis in the brain and testes(Dragatsis *et al.* 2000). On a cellular level, neurons that have less wildtype huntingtin also exhibit a similar phenotype as those expressing mutant huntingtin. Both HD mice and conditional Hdh$^{-/-}$ mice exhibit reduced vesicle and mitochondria transport(Gunawardena *et al.* 2003; Trushina *et al.* 2004). Reducing huntingtin by RNA interference in a mouse cell line also decreases BDNF vesicular transport (Gauthier *et al.* 2004).

The loss of function of wildtype huntingtin hypothesis is further supported by evidence that wildtype huntingtin can alleviate the mutant huntingtin phenotype. Increased expression of wild-type huntingtin in a mutant huntingtin background causes: i) reduced toxicity in peripheral cells overexpressing mutant huntingtin (Ho *et al.* 2001); ii) inactivation of the RE1/NRSE silencer in heterozygous mutant huntingtin knock-in cells (Zuccato *et al.* 2003); iii) reduced neuronal toxicity mediated by NMDA (N-methyl-D-aspartate) or kainate receptors in an immortalized rat hippocampal neuronal cell line (Sun *et al.* 2001). iv) improved HD phenotype in YAC128 mice (Van Raamsdonk *et al.* 2006). Conversely, depletion of wild-type huntingtin in a mutant huntingtin background causes: i) increased apoptotic cell death in the testes of YAC72$^{-/-}$ mice (in which Hdh is knocked out in both endogenous alleles, and an additional yeast-derived artificial chromosome (YAC) containing the entire human mutant huntingtin 72-CAG gene is inserted) in comparison with YAC72$^{+/+}$ mice (in which Hdh is wild-type in both endogenous alleles, and an additional YAC containing the entire human mutant huntingtin gene is inserted) (Leavitt *et al.* 2001); ii) worsening of behavioural phenotype and increased apoptosis in

the testes of YAC128$^{-/-}$ mice compared with YAC128$^{+/+}$ mice (Van Raamsdonk *et al.* 2005b).

Together these results highlight the relevant evidence that implicates wild-type huntingtin in the pathogenesis of HD.

1.2.6 Post-Translational Modification of huntingtin

Huntingtin protein is subject to a number of post-translational modifications such as palmitoylation(Huang *et al.* 2004; Yanai *et al.* 2006), phosphorylation(Humbert *et al.* 2002), ubiquination (Kalchman *et al.* 1996; Sieradzan *et al.* 1999) and SUMOlyation (Steffan *et al.* 2004) (Figure 1.15). Huntingtin is also a substrate for many enzymes that cleave proteins, including caspases, calpains and aspartyl peptidases (Wellington *et al.* 1998; Lunkes *et al.* 2002; Gafni *et al.* 2004). Various studies have shown that altered post-translational modifications are associated with mutant huntingtin and contribute to the pathogenesis of HD (Warby *et al.* 2005; Yanai *et al.* 2006; Davies *et al.* 1997; Hackam *et al.* 1998; Schilling *et al.* 1999; Cha 2000; Li *et al.* 2000; Yu *et al.* 2003).
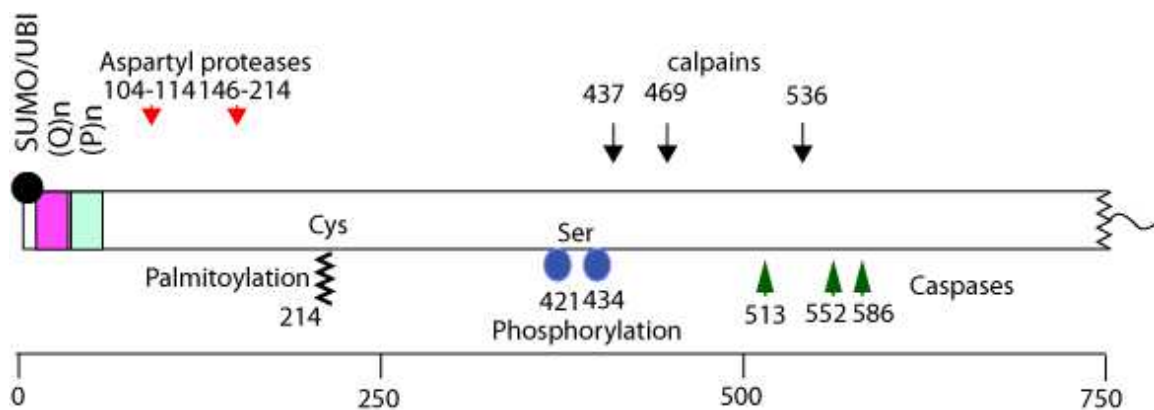
Figure 1.15. Schematic diagram of N terminal huntingtin amino acid sequence, proteolysis sites and post-translational modification sites.

(Q)*n* indicates the polyglutamine tract, which is followed by the polyproline sequence, (P)*n*. The green arrowheads indicate the caspase cleavage sites, the red arrowheads the aspartyl proteases, and the black arrows the calpain cleavage sites. The indicated post-translational modifications include: ubiquitination (UBI) and/or sumoylation (SUMO) (black circle), phosphorylation at serine 421 and serine 434 (blue circle) and palmitoylation at cysteine 214. Adapted from (Cattaneo *et al.* 2005) with permission.

1.3. PALMITOYLATION AND HUMAN DISEASE

Recent studies revealed that altered palmitoylation leads to neuronal dysfunction, and in some cases the manifestation of neurodegenerative diseases. In Alzheimer's patients, extracellular β-amyloid release is enhanced, leading to aggregate formation. β-amyloid is proteolytically cleaved from a large transmembrane glycoprotein amyloid precursor protein by a membrane-bound protease, known as β-secretase (BACE) (reviewed in (Finder and Glockshuber 2007)). Mouse BACE is palmitoylated and mutation of the palmitoylation sites changes the membrane-associated form of BACE into a shed form(Benjannet *et al.* 2001; Sidera *et al.* 2005). Notably, a decrease in the palmitoylation of BACE occurs when cells expressing it are treated with statins, a cholesterol-lowering drug(Sidera *et al.* 2005; Kivipelto and Solomon 2006). Thus, inhibition of BACE palmitoylation may reduce BACE association with cholesterol-rich rafts, and alter amyloid precursor protein processing. This in turn may favor the formation of harmful protein aggregates.

Palmitoylation of huntingtin by HIP14 (DHHC-17) is also essential to maintain normal huntingtin trafficking and function(Huang *et al.* 2004; Yanai *et al.* 2006). A defect in the association between HIP14 and huntingtin, which is triggered by poly-glutamine repeat expansion, contributes to neuronal dysfunction associated with

Huntington's disease (HD)(Gervais *et al.* 2002). Specifically, this reduced association causes a marked reduction in huntingtin palmitoylation, which further accelerates inclusion formation and increases neuronal toxicity. Down-regulation of HIP14 in neurons increases huntingtin inclusion formation, whereas overexpression of HIP14 substantially reduces inclusions(Yanai *et al.* 2006) (Figure 1.16). These results point to a possible therapeutic strategy by which mutant huntingtin function can be corrected through increasing its palmitoylation by HIP14.
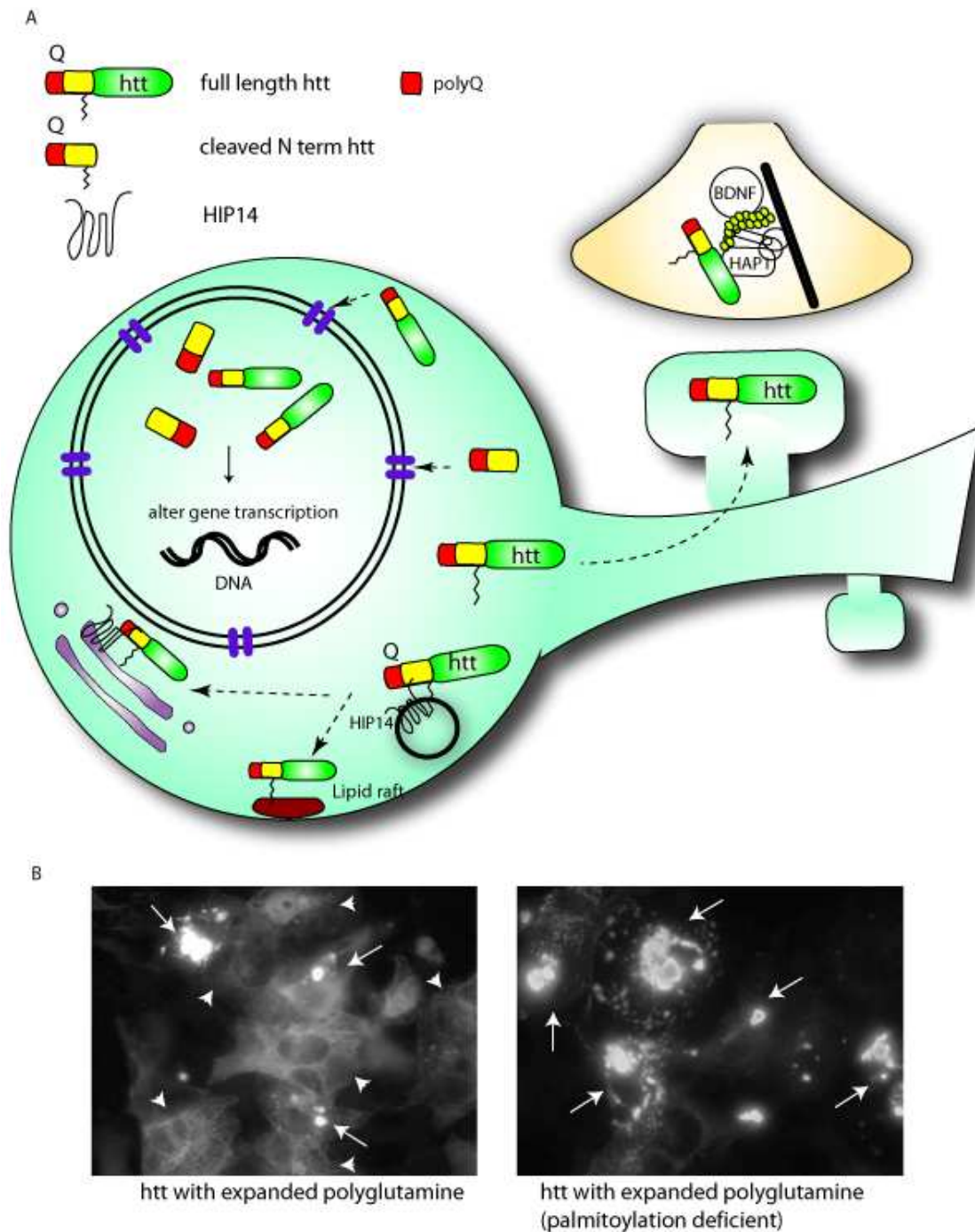
Figure 1.16. Putative roles of huntingtin palmitoylation.

(A) Palmitoylation of huntingtin (htt), may regulate protein sorting to specific lipid microdomains including lipid rafts, Golgi apparatus and post-Golgi transport vesicles, and synaptic membranes. Palmitoylation may also control huntingtin (htt) protein shuttling between cytosol and the nucleus and regulation of gene transcription. **(B)** Abolishing palmitoylation of polyglutamine expanded htt accelerates formation of inclusion bodies. Images show the enhanced number of inclusion formed in COS cells upon expression of the palmitoylation deficient form of poly-Q expanded htt.

Mutations in PPT1, causes the devastating neurodegenerative storage disorder of childhood, Infantile Neuronal Ceroid Lipofuscinosis (INCL), which is manifested by rapidly progressive brain atrophy (reviewed in(Haltia 2006; Kyttala *et al.* 2006)). Although PPT1 is predominantly expressed in lysosomes in non-neuronal cells, in neurons, studies done by Jalanko and Kopra groups revealed axonal and presynaptic localization of PPT1(Lehtovirta *et al.* 2001; Ahtiainen *et al.* 2003). Indeed, PPT-1 knockout mice show a progressive and gradual decline in presynaptic vesicle number and size, an early indicator of synapse degeneration(Virmani *et al.* 2005). Other studies also showed that PPT1 deficiency causes a defect in fluid-phase and receptor-mediated endocytosis(Ahtiainen *et al.* 2006). Although the exact molecular mechanism(s) remains unclear, the observed neurotransmission deficiency at the presynaptic terminal suggests that PPT1 may play a role in depalmitoylating proteins that facilitate presynaptic vesicle exocytosis and endocytosis. In addition to synaptic dysfunction, loss of PPT1 results in several other anomalies including altered endoplasmic reticulum structure, activation of the unfolded protein response, increased expression of chaperone proteins and activation of specific caspases and apoptosis(Zhang *et al.* 2006).

Several forms of X-linked mental retardation (XLMR) have been linked to loss of DHHC proteins (Ropers 2006). Chromosomal translocation abnormalities and mutations in ZDHHC9, which palmitoylates RAS, is associated with Marfanoid habitus(Mansouri *et al.* 2005; Swarthout *et al.* 2005). Mutations in the ZDHHC8 gene, which is located on chromosome 22q11, has been linked to bipolar disorder and schizophrenia (Chen *et al.* 2004; Mukai *et al.* 2004). Analysis of ZDHHC8-knockout mice also revealed a sexually dimorphic deficit in prepulse inhibition, a gene dosage-dependent decrease in exploratory

activity in a new environment, and decreased sensitivity to the locomotor stimulatory effects of psychomimetic drugs (Mukai *et al.* 2004). These behaviors recapitulate many aspects of schizophrenic symptoms. However, several other studies indicate that mutations in ZDHHC8 are most likely associated with a small number of individuals suffering from bipolar disorder or schizophrenia(Faul *et al.* 2005; Glaser *et al.* 2005; Otani *et al.* 2005; Saito *et al.* 2005; Demily *et al.* 2007). DHHC proteins that have been reported to be associated with human diseases are summarized in the table below.

Table 1.4. **Pathophysiological functions of DHHC proteins**
(Reprint from (Tsutsumi *et al.* 2008)with permission)

Hs, Homo sapiens; Mm, Mus musculus; Dm, Drosophila melanogaster

DHHC protein	Pathophysiological function(s)	Associated substrates	Reference(s)
DHHC-2	Colorectal cancer (Hs)		(Oyama *et al.* 2000)
DHHC-8	Schizophrenia (Hs, Mm)		(Chen *et al.* 2004; Mukai *et al.* 2004)
DHHC-9	X-linked mental retardation (Hs)		(Raymond *et al.* 2007)
	Colorectal cancer (Hs)		(Mansilla *et al.* 2007)
DHHC-11	Bladder cancer (Hs)		(Yamamoto *et al.* 2007)
DHHC-15	X-linked mental retardation (Hs)		(Mansouri *et al.* 2005)
DHHC-17/ HIP14	Huntington disease (Mm)	Huntingtin	(Yanai *et al.* 2006)
	Synaptic transmission (Dm)	CSP, SNAP-25	(Ohyama *et al.* 2007; Stowers and Isacoff 2007)

1.4. THESIS HYPOTHESIS AND OBJECTIVES

In chapter 1, we have discussed that palmitoylation regulates the subcellular trafficking and function of numerous proteins in neurons (el-Husseini Ael and Bredt 2002; Smotrys and Linder 2004; Huang and El-Husseini 2005; Resh 2006a). Palmitoyl acyl-transferases (PATs) are the class of enzymes that transfer palmitate to a substrate, while palmitoyl thioesterases catalyze the reverse reaction. At the beginning of this

graduate study in 2003, little was known about the identity of the enzymes that regulate the process of palmitoylation. In 2002, Singaraja et al found that a new huntingtin interacting protein named HIP14 was a mammalian homolog of Akr1p, a PAT in the yeast. This initial finding raised two questions: whether HIP14 is a mammalian PAT and whether huntingtin is palmitoylated. These two questions promoted the pursuit of the following specific goals in this thesis:

1. **Identify HIP14 as a neuronal palmitoyl acyl transferase (PAT)**

HIP14 is the first identified mammalian protein that is homologous to the yeast PAT Akr1p. Expression of human HIP14 results in rescue of the temperature-sensitive lethality in akr1Δ yeast cells and, furthermore, restores their defect in endocytosis, demonstrating a role for HIP14 in intracellular trafficking in mammals. *Is HIP14 the neuronal PAT? What proteins are substrates of HIP14? Is the trafficking of these proteins influenced by HIP14?*

2. **Determine the effect of huntingtin palmitoylation at C214 by HIP14**

Huntingtin interacts with HIP14, therefore could potentially be a substrate of HIP14 enzyme. HIP14's interaction with htt is inversely correlated to the polyglutamine expansion length in htt. *Is huntingtin palmitoylated by HIP14? Which cysteines are the palmioylation sites? Does polyglutamine expansion influence the palmitoylation of huntingtin? How does huntingtin palmitoylation contribute to our understanding of HD pathogenesis?*

3. **Analyze four neuronal DHHC palmitoyl acyl transferases and compare their substrate specificity**

HIP14 is one of the 23 existing DHHC proteins, all of which are putative PATs.

Why mammals need so many proteins to exert the same enzymatic process? We focus on four neuronal expressed DHHCs and explore the following questions: *Do they have substrate specificity? Do enzymes and their respective substrates colocalize? Do they interact in vitro and in vivo? Do regions other than DHHC domain contribute to the specificity between enzymes and their substrates?*

4. Determine the role of wildtype huntingtin in regulating HIP14 enzymatic activity

Huntingtin associates with HIP14 both in vitro and in vivo. Unlike the relationship between other DHHC enzymes and their substrates, interaction of huntingtin with HIP14 does not require the presence of palmitoylated cysteines. *Does huntingtin function beyond only being a substrate of HIP14? Does wildtype huntingtin influence enzymatic activity of HIP14? If true, what role does wildtype huntingtin play in HD pathogenesis?*

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2. CHAPTER 2   Huntingtin Interacting Protein HIP14 Is A Palmitoyl Transferase Involved in Palmitoylation and Trafficking of Multiple Neuronal Proteins

A version of this chapter has been published.

2.1. INTRODUCTION

Trafficking of proteins to specialized subcellular and plasma membrane domains is vital for normal cell development and function (Lee and Sheng 2000; da Silva and Dotti 2002). Modification by the lipid palmitate, a 16-carbon fatty acid chain, influences sorting and function of several proteins (El-Husseini Ael *et al.* 2002; Linder and Deschenes 2004; Smotrys and Linder 2004). In particular, palmitoylation has recently emerged as a critical modification in neurons where it controls axon pathfinding, filopodia formation, polarized protein targeting and clustering of scaffolding and signaling proteins (Strittmatter *et al.* 1995; Ueno 2000; El-Husseini Ael *et al.* 2001; El-Husseini Ael *et al.* 2002; Gauthier-Campbell *et al.* 2004).

Postsynaptic targeting and clustering of the postsynaptic density (PSD) protein PSD-95 requires palmitoylation and this process regulates glutamate receptor retention at the synapse (Craven *et al.* 1999; El-Husseini Ael *et al.* 2002). Palmitoylation also regulates SNAP25-mediated SNARE complex disassembly, presynaptic targeting of the GABA synthesizing enzyme GAD-65, multimerization and presynaptic trafficking of the calcium sensor synaptotagmin I (Chapman *et al.* 1996; Fukuda *et al.* 2001; Washbourne *et al.* 2001; Kanaani *et al.* 2002; Kang *et al.* 2004; Rathenberg *et al.* 2004). In addition, palmitoylation of secreted proteins regulates neuronal differentiation (Chamoun *et al.* 2001; Linder and Deschenes 2004). Remarkably, physiological stimuli and neuronal activity dynamically alter protein palmitoylation levels, and this provides an important mechanism for the regulation of neuronal cell development and activity (Resh 1999; El-Husseini Ael *et al.* 2002; Smotrys and Linder 2004).

While the importance of palmitoylation in protein trafficking is well documented, the palmitoyl transferases involved in this process remain unknown (Smotrys and Linder 2004). The diversity of sequences modified by palmitate hindered the identification of these enzymes using classical protein purification approaches. A significant advance in the field came from genetic analysis in yeast which revealed a family of PATs that contains a DHHC domain, a cysteine rich region that harbors a conserved tetra-peptide motif. These include Erf2p/Erf4p protein complex, which palmitoylate H-Ras proteins, and Akr1p which is involved in the palmitoylation of the yeast casein kinase Yck2p (Roth *et al.* 2002; Linder and Deschenes 2004). However, it remained unknown whether related proteins serve a similar function in mammalian cells. Here, we use biochemical assays to show that huntingtin interacting protein, HIP14, is a neuronal palmitoyl transferase involved in palmitoylation of specific substrates. We also show that HIP14 modulates palmitoylation-dependent vesicular trafficking and clustering of a subset of synaptic proteins.

2.2. MATERIALS AND METHODS

2.2.1 Constructs, cDNA Cloning and Mutagenesis

Full length HIP14 was subcloned into pCI-neo as described earlier (Singaraja *et al.* 2002). HIP14 GFP was generated by inserting EGFP (Clontech, CA) at the C-terminus, and HIP14-Flag was generated by PCR using a reverse primer containing FLAG sequence. HIP14ΔDHHC was generated by deletion of nucleotides encoding amino acids 440 to 487. Construction of GFP fusion proteins of wild type and mutant forms of synaptotagmin I, SNAP25, GAD-65, and PSD-95 were generated by PCR and subcloned

into the Hind III and EcoR I sites in frame with GFP in pEGFP-N3 (Clontech). Wild type and palmitoylation mutant GST-SNAP25 fusion proteins were PCR amplified and subcloned into EcoR I/Sal I of pGEX-6P3 (Amersham Biosciences, UK). Synaptotagmin I GST fusion constructs were subcloned into BamH I/Sal I of pGEX-6P3. HIP14-GST was generated by insertion of full length HIP14 cDNA in frame with GST into pGEX-6P3 at Xma I site. For production of 6×His-huntingtin, sequences corresponding to amino acids 1-548 of huntingtin were subcloned into EcoR I and Hind III sites of pTrcB (Gibco-Invitrogen, Calsbad, CA). For the generation of GST fusion proteins of other acylated proteins, nucleotide sequences corresponding to the acylated motifs of paralemmin (amino acids 364-383), lck (amino acids 1-20aa), synaptotagmin VII (amino acids 25-44) and H-Ras (amino acids 169-188) were subcloned into BamH I and Xho I sites of pGEX-4T1 (Amersham). PSD95 (1-PDZ3) in pGEX-2T was a gift from Dr. David Bredt (University of California at San Francisco, CA). All constructs were verified by DNA sequencing.

2.2.2 Cell Culture, Transfection and Time Lapse Imaging

COS cells, hippocampal and cortical neurons from embroynic day 18 rat brains were cultured as described earlier (Craven *et al.* 1999; El-Husseini *et al.* 2000a). Neuronal cultures were maintained in neurobasal media (Gibco-Invitrogen) supplemented with B27, penicillin, streptomycin, and L-glutamine. For transient expression, both COS cells and 4 days *in vitro* (DIV) neurons were transfected with Lipofectamine 2000 (Gibco-Invitrogen) according to manufacturer's protocol and stained 8-14 h later. For time-lapse Imaging, COS cells were transfected and images were collected 10 h post-transfection using a 63x oil objective affixed to a Zeiss inverted light microscope and

Axiovision software. While images were acquired, cells were kept in a 37ºC chamber, supplemented with 5% carbon dioxide. Images were collected every 3 seconds for a total time of 3 minutes.

2.2.3 Immunofluorescence

Cells were washed in phosphate-buffered saline (PBS) (20 mM NaH_2PO_4, 0.9% NaCl, pH7.4), fixed in 4% paraformaldehyde in PBS, and permeabilized in 0.3% Triton X-100, 1% paraformaldehyde. For immunofluorescence, primary antibodies were incubated in 2% NGS in PBS for 1 h at room temperature. Cells were washed in PBS between each incubation followed by incubation with appropriate secondary antibodies conjugated to Alexa 488 and Alexa 568. Primary antibodies used include mouse monoclonal antibodies recognizing α-adaptin, Rab5 and Rab8 (Transduction Laboratories), GM-130 (BD Biosciences, Palo Alto, CA), GST and actin (Santa Cruz Biotechnology, Inc. CA), PSD-95 (Affinity Bioreagents. Inc., CO), huntingtin antibody 2166 (Chemicon, CA), and goat polyclonal antibodies against Rab7 (Santa Cruz). Rabbit polyclonal antibodies against GFP (Clontech Laboratories Inc., CA) were also used. Rabbit polyclonal antibodies against HIP14 have been described previously (Singaraja *et al.* 2002). Guinea pig antibody against SAP102 was a gift from Dr. David Bredt (University of California at San Francisco, CA).

2.2.4 Electron microscopy

Electron microscopy on HIP14 in the brain was performed as previously described (Singaraja *et al.* 2002). Ultra-small colloidal gold conjugated secondary antibody (Aurion, Wageningen, The Netherlands) was used. Following a post-fixation with 2.5% glutaraldehyde, gold particles were intensified using the R-gent SE-EM silver

enhancement kit (Aurion). Sections were further fixed with 0.5% OsO_4 in 0.1 M PB, dehydrated in ethanol and propylene oxide (1:1) and flat-embedded in Eponate 12 (Ted Pella, Redding, CA). Ultrathin sections (90 nm) were cut using a Leica Ultracut S ultramicrotome, and counterstained with 5% aqueous uranyl acetate for 5 min followed by lead citrate for 5 min. Thin sections were examined using a HITACHI H-7500 electron microscope.

2.2.5 Cell Radiolabeling and Immunoprecipitation

COS cells were labeled with 1 mCi/ml [^{3}H]palmitate (57 Ci/mmol; Perkin Elmer Life Sciences, Inc.) for 3 h while expressing the transfected proteins. Labeled cells were washed with ice-cold PBS and re-suspended in 0.1 ml of lysis buffer containing TEE (50 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1 mM EGTA), 150 mM NaCl, and 1% SDS. Triton X-100 was added to 1% to neutralize the SDS in a final volume of 0.5 ml. Insoluble material was removed by centrifugation at 14,000 rpm for 10 min at 4^0C. For immunoprecipitation, samples were incubated with SNAP25 monoclonal antibodies for 1 h at 4^0C. After addition of 20 μl protein G sepharose beads (Pharmacia), samples were incubated for 1 h at 4^0C. Immunoprecipitates were washed three times with buffer containing TEE, 150 mM NaCl, and 1% Triton X-100, boiled in SDS-PAGE sample buffer with 1 mM DTT for 2 min, and separated by SDS-PAGE and dried under vacuum. Gels were exposed to Hyper Film (Amersham) with intensifying screens at -80^0C for 3 to 20 days.

2.2.6 Expression and Purification of Fusion Proteins

GST fusion proteins were produced in E. coli using the pGEX 6p3 expression system (Amersham) and isolated using Glutathione Sepharose^{TM} 4B (Amersham) from

clarified cell lysates. The substrates were dialyzed and then eluted with 20mM Glutathione. GST-HIP14 was purified in the presence of bovine liver lipids with no detergent to maintain proper protein folding. To generate a free N-terminus of PSD-95, GST-PSD-95 protein was cleaved using thrombin (50mM Tris-HCl pH 8.0, 150mM NaCl, 2.5mM CaCl2, 0.1% 2-mercapto-ethanol). His-tagged huntingtin protein was purified from DH5α with Ni-NTA magnetic agarose beads (Qiagen).

2.2.7 Immunoprecipitation of HIP14 GFP

Transfected COS cells were sonicated for 15 seconds in TEEN buffer (50 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1 mM EGTA, 150 mM NaCl) and insoluble material was removed by centrifugation at 14000rpm for 10 min at 4^0C. Supernatants were incubated with anti-GFP antibody (10μl) for 1 hr at 4^0C. 50 μl Protein A Sepharose 4 Fast Flow beads (Amersham) were added and samples were incubated for 1 h at 4^0C. Immunoprecipitates were washed 2X with buffer containing TEEN, 150 mM NaCl and then subjected to *in vitro* palmitoylation assays described below.

2.2.8 Synthesis of Palmitoyl CoA

[^{3}H]palmitoyl-CoA, [^{3}H]myristoyl-CoA and palmitoyl-CoA were synthesized enzymatically from [9,10-^{3}H(N)]palmitic acid (5 mCi/ml; Perkin Elmer Life Sciences), [9,10-^{3}H(N)]myristic acid (1mCi/ml; Perkin Elmer Life Sciences) and palmitic acid (Sigma-Aldrich), respectively. Synthesis components also included co-enzyme A (CoA), ATP and acyl-CoA synthase (Sigma-Aldrich). Synthesized products were purified as previously described (Dunphy *et al.* 1996). Synthesized [^{3}H]palmitoyl-CoA and [^{3}H]myristoyl-CoA were subjected to TLC (TLC aluminium sheet, silica gel, EM Science, Germany) to determine the efficiency. Ninhydrin Staining was used for the detection of

synthesized non-radiolabeled palmitoyl-CoA. The synthesis was highly efficient, with >95% conversion of acid to fatty acid-CoA. Specific activity for [^{3}H]palmitoyl-CoA was 60 Ci/mmol and for [^{3}H]myristoyl-CoA was 30Ci/mmol.

2.2.9 Palmitoylation Assay

Palmitoylation reaction (60 µl) contained 5 µCi of [^{3}H]palmitoyl-CoA, 0.33 µg/µl substrate protein, 1 mM ATP, 50 mM MES, pH 6.4, 0.2 mg/ml bovine liver lipids, and either 20µl of COS cell lysates expressing HIP14, immunoprecipitated HIP14 GFP, or 0.5µg of purified HIP14-GST as described in results. After 15min incubation at 37°C, sample buffer was added with final concentration of 5mM DTT was added and samples were subjected to SDS-PAGE analysis.

2.2.10 HIP14 siRNA

HIP14 siRNA primers were annealed and inserted into the *Hin*dIII/*Bgl*II sites of pSUPER vector (Oligo Engine). Primers used to generate HIP14 siRNA: complementary oligonucleotides: 5'-
GATCCCCGGAGATACAAGCACTTTAATTCAAGAGATTAAAGTGCTTGTATCTC
CTTTTTGGAAA-3', and 5'-
AGCTTTTCCAAAAAGGAGATACAAGCACTTTAATCTCTTGAATTAAAGTGCTT
GTATCTCCGGG-3' (corresponding to nucleotides 2039–2057 of rat HIP14 mRNA). Primers used to generate scrambled siRNA (control siRNA): 5'-
GATCCCCGATAAGAACAGCGGCTATATTCAAGAGATATAGCCGCTGTTCTTA
TCTTTTTA-3' and 5'-
AGCTTAAAAAGATAAGAACAGCGGCTATATCTCTTGAATATAGCCGCTGTTC
TTATCGGGGATCGGG -3'. The specificity HIP14 siRNA was tested both against

exogenously expressed HIP14 in COS cells and endogenous HIP14 in cultured hippocampal and cortical neurons.

2.2.11   Imaging and Analysis

Images were acquired on a Zeiss Axiovert M200 motorized microscope by using a monochrome 14-bit Zeiss Axiocam HR charge-coupled device camera at 1,300×1,030 pixels. Exposure times were individually adjusted to yield an optimum immunofluorescent brightness without saturation. Images were analyzed in Northern Eclipse (Empix Imaging, Missasauga, Canada). Briefly, images were processed at a constant threshold level (of 32,000 pixel values) to create a binary image, which was multiplied with the original image by using Boolean image arithmetics. An observer blinded to the identity of proteins examined performed analysis in protein clustering upon expression of siRNA. For analysis of protein clustering, dendrites of the cell of interest were outlined using a combination of the GFP fluorescence signal and differential interference contrast bright-field images. Puncta were defined as sites of intensities at least twice the dendritic background. The number of dendritic puncta per unit dendritic length was measured. The two-tailed two sample unequal variance Student T test was used to compare the average number of puncta per unit length between experimental groups. For analysis of clustering of GAD-65 GFP, the intensity of axonal clusters was determined. This was done by tracing 3-4 representative sections of axons. For each cell, the average intensity of axonal puncta versus axonal background was used to calculate the degree of clustering of GAD-65. Results were analyzed by a student t test using a two-tailed distribution and two-sample unequal variance.

2.3. RESULTS

2.3.1 HIP14 is a neuronal palmitoyl transferase

HIP14, a mammalian ortholog of Akr1p, was previously identified as a huntingtin interacting protein involved in regulating protein trafficking (Singaraja *et al.* 2002). This protein is predominantly expressed in neurons in the brain and localizes to Golgi membranes and cytoplasmic vesicles (Singaraja *et al.* 2002). Structurally, HIP14 contains 5 predicted transmembrane domains and a DHHC domain similar to that seen in yeast PATs (Figure 2.1A). These observations prompted us to test whether HIP14 is involved in the palmitoylation of several neuronal proteins.

First we co-expressed HIP14 with SNAP25, a well characterized palmitoylated neuronal protein(Hess *et al.* 1992; Gonzalo and Linder 1998), followed by metabolic labeling with [^{3}H]palmitate. These experiments demonstrated that SNAP25 palmitoylation was potentiated in the presence of HIP14, suggesting that SNAP25 may serve as a substrate for this putative enzyme (Figure 2.1B). Next, several *in vitro* assays were performed to determine if the enzymatic reaction was directly mediated by HIP14. Purified GST-SNAP25 was incubated with extracts obtained from COS cells transfected with wild type HIP14 in the presence of [^{3}H]palmitoyl-CoA and this induced palmitoylation of SNAP25 (Figure 2.1C). In contrast, lysates containing HIP14 failed to palmitoylate a mutant form of SNAP25 lacking the cysteines normally modified by palmitate.

Consistent with the postulated role for the DHHC domain in PAT activity, we also found that a mutant form of HIP14 lacking this domain failed to catalyze palmitoylation of SNAP25. To further characterize the role of HIP14, GST-SNAP25 was incubated with

HIP14 GFP immunoprecipitates (IP) obtained from transfected cells to determine whether HIP14 is sufficient for SNAP25 palmitoylation. *In vitro* labeling assays showed that SNAP25 palmitoylation occurs only in the presence of wild type but not a mutant form of HIP14 lacking the DHHC domain. (Figure 2.1D and E). To determine whether purified HIP14 is sufficient to induce protein palmitoylation, a HIP14-GST fusion protein was generated in bacteria and then purified using glutathione beads. The eluted protein was used in an *in vitro* assay in the presence or absence of [^{3}H]palmitoyl-CoA (Figure 2.1F-H). Indeed, purified HIP14-GST was sufficient to palmitoylate GST-SNAP25 in the presence of [^{3}H]palmitoyl-CoA but not [^{3}H]palmitate, and this reaction was competed by addition of excess non-radiolabeled palmitoyl-CoA (Figure 2.1H). To further characterize the specificity of this enzyme in this reaction, we next evaluated whether purified HIP14-GST may mediates the transfer of other lipids such as myristate. However, in the presence of [^{3}H]myristoyl-CoA, HIP14 failed to modify GST-SNAP25 with myristate (Figure 2.1H). These findings establish that HIP14 utilizes palmitoyl-CoA to specifically modify SNAP25 with palmitate on cysteine residues normally modified by this lipid *in vivo*.
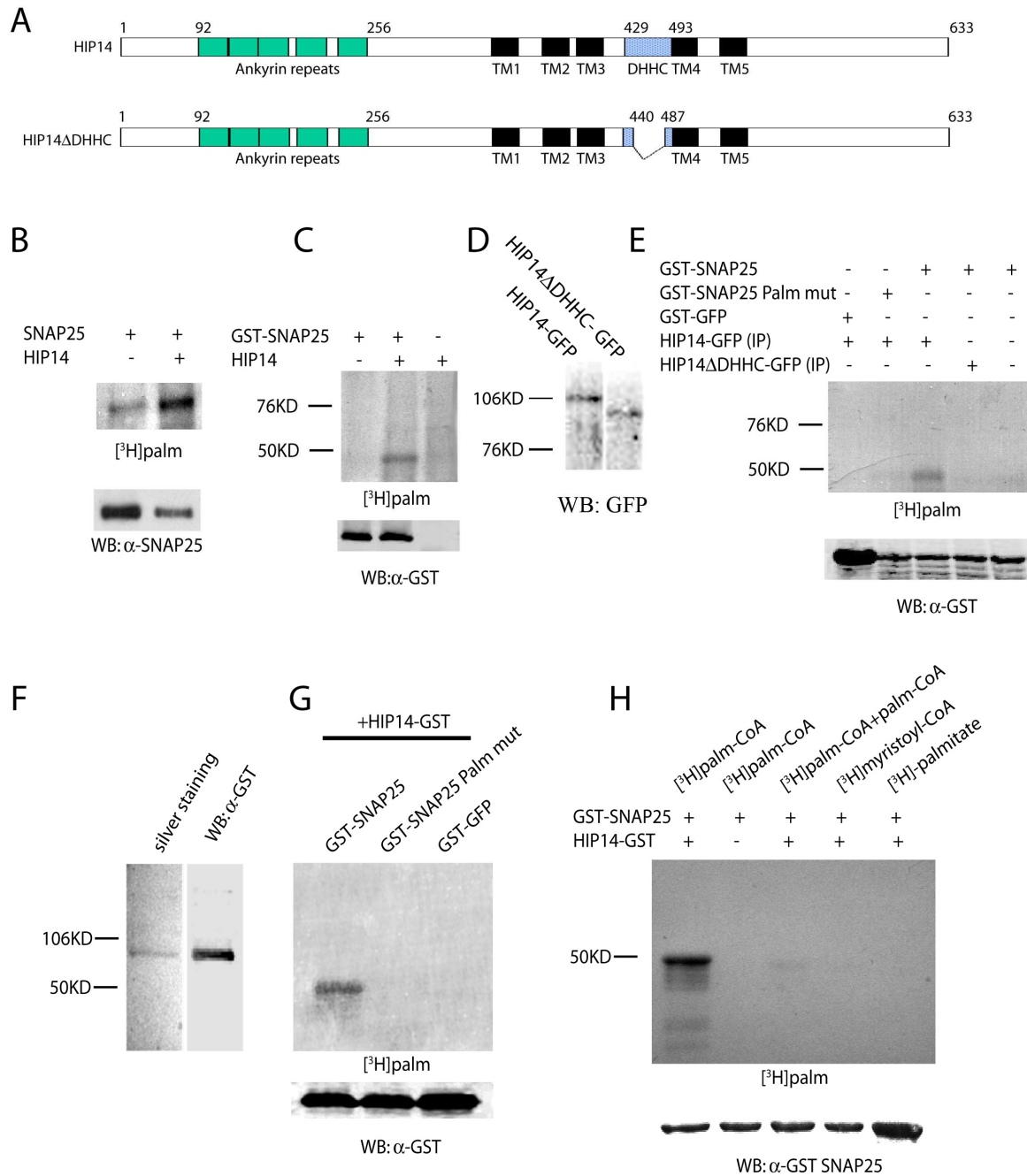
Figure 2.1. HIP14 is a Neuronal Palmitoyl Transferase.

(A) Schematic illustration of HIP14 primary structure. HIP14 lacking the DHHC domain (HIP14ΔDHHC) was generated by deletion of amino acids 440-487 containing the cysteine rich zinc finger domain. **(B)** Enhancement of SNAP25 palmitoylation *in vivo* by HIP14. COS cells were transfected either with SNAP25 alone or with HIP14. 8 h post-transfection, cells were metabolically labeled with [³H]palmitate, and SNAP25 was immunoprecipitated and analyzed by

western blotting (WB) and autoradiography ([^{3}H]palm). SNAP25 palmitoylation levels were enhanced in cells co-expressing HIP14. **(C)** Extracts of COS cells transfected with HIP14 induce palmitoylation of purified GST-SNAP25 in the presence of [^{3}H]palmitoyl-CoA. **(D,E)** COS cells were transfected with GFP fusion of either wild type HIP14 (HIP14 GFP) or the mutant form lacking the DHHC domain (HIP14ΔDHHC GFP). GFP fusion proteins were then immunoprecipitated (IP) using GFP antibodies. HIP14 GFP (IP) induced palmitoylation of wild type (GST-SNAP25) but not the palmitoylation mutant form of SNAP25 (GST-SNAP25 Palm mut) or GST-GFP. In contrast, HIP14ΔDHHC failed to palmitoylate GST-SNAP25. **(F,G)** Purified HIP14-GST is sufficient to induce palmitoylation of GST-SNAP25. HIP14-GST produced in bacteria was purified and samples were subjected to western blotting and silver staining. No palmitoylated products were detected when GST-SNAP25 Palm mut or GST-GFP were incubated with HIP14-GST. **(H)** Acylation mediated by HIP14 requires palmitoyl-CoA. HIP14 requires [^{3}H]palmitoyl-CoA but not [^{3}H]palmitate for SNAP25 palmitoylation. This reaction was competed with excess cold palmitoyl-CoA. HIP14 did not induce transfer of the lipid myristate from [^{3}H]myristoyl-CoA.

2.3.2 Multiple Neuronal Proteins Serve as Substrates for HIP14

Previous work established that the postsynaptic density protein, PSD-95, is palmitoylated at two N-terminal cysteines present at positions 3 and 5 and that this lipid modification is required for trafficking of PSD-95 to the synapse (Craven *et al.* 1999; El-Husseini *et al.* 2000a). To test whether HIP14 may also palmitoylate PSD-95, we first generated a GST fusion protein of PSD-95 containing the N-terminal amino acids 1-385, which contains the palmitoylation motif and PDZ domains 1-3 (PSD-95 (1-PDZ3)). Because PSD-95 palmitoylation requires a free N-terminus, GST was cleaved and a purified PSD-95 (1-PDZ3) was obtained. Both extracts from COS cells transfected with HIP14 as well as purified HIP14-GST induced PSD-95 palmitoylation in vitro (Figure 2.2A andFigure 2.3).

To further analyze the role of HIP14 in the acylation of other neuronal proteins, we preformed in vitro assays on GST fusion proteins of synaptotagmin I, and acylated sequences of GAD-65, H-Ras, lck, paralemmin and synaptotagmin VII. This analysis showed that HIP14-GST can also palmitoylate GST-synaptotagmin I, a presynaptic

vesicle protein palmitoylated on 5 cysteine residues (Cys-74, Cys-75, Cys-77, Cys-79, and Cys-82) present in the junction between the transmembrane and cytoplasmic region of synaptotagmin I (Heindel *et al.* 2003) (Figure 2.2B). HIP14 also modified sequences of GAD-65 containing the N-terminal palmitoylation motif (Figure 2.3C). However, HIP14 did not palmitoylate the C-terminal motif of the dually palmitoylated and prenylated neuronal protein paralemmin (Kutzleb *et al.* 1998) (Figure 2.2A). Moreover, HIP14 did not induce palmitoylation of acylated sequences of H-Ras, lck and synaptotagmin VII (Figure 2.3C). These results indicate that HIP14 is involved in the palmitoylation of a specific subset of neuronal proteins.

The association of N-terminal sequences of huntingtin (amino acids 1-548) with HIP14 suggested that huntingtin is also a substrate for HIP14. Metabolic labeling analysis showed that the N-terminal region of huntingtin is subject to palmitoylation (Figure 2.2C). Using a similar in vitro assay to the one described for SNAP25, we also find that extracts of COS cells expressing HIP14 induced the palmitoylation of purified His-tagged huntingtin (Figure 2.2D). These results demonstrate that huntingtin is another substrate subject to palmitoylation by HIP14.
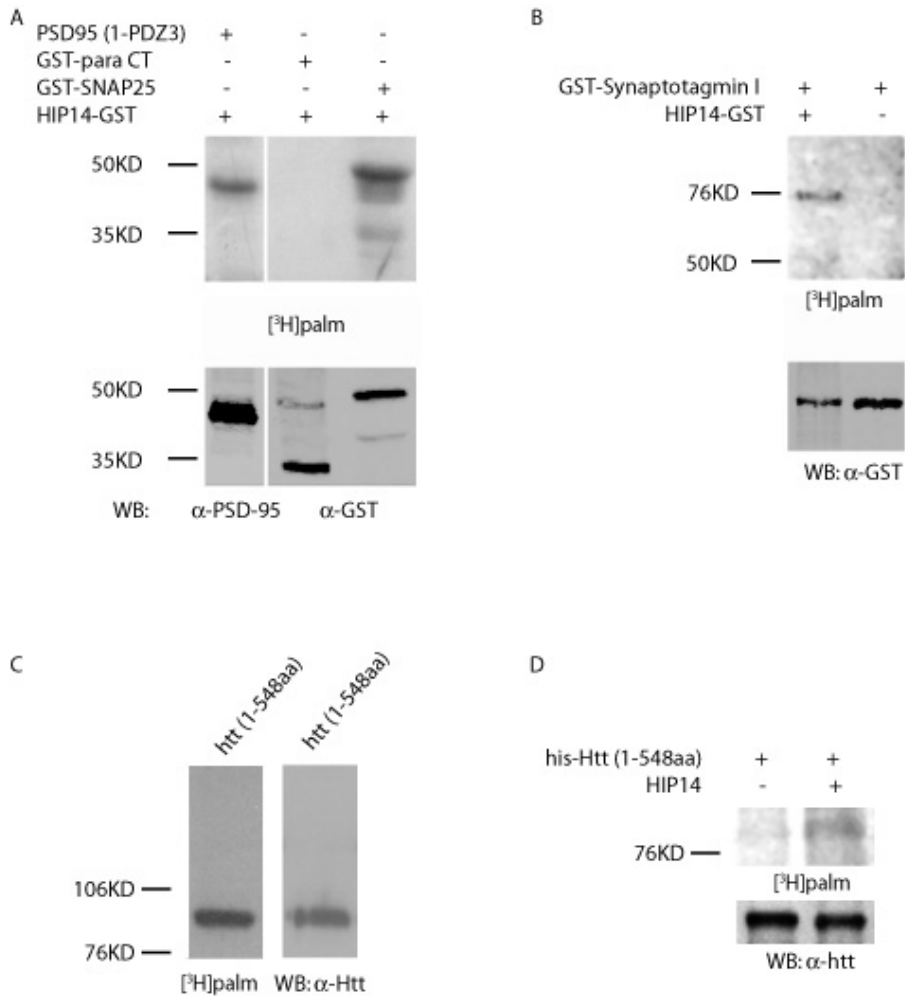
Figure 2.2. HIP14 Palmitoylates Multiple Substrates.

(A) HIP14-GST induces palmitoylation of purified PSD-95 (1-PDZ3) and GST-SNAP25, but not the dually acylated motif of paralemmin (GST-para CT). HIP14-GST purification efficiency is low. Since only 1/10 of the reaction is loaded for western blot, the amount of HIP14-GST is very little compared to GST-substrate concentration. Therefore, HIP14-GST band could only be visualized with long exposure. **(B)** Palmitoylation of GST fusion protein of synaptotagmin I (GST-synaptotagmin I) by purified HIP14-GST. **(C)** Huntingtin is palmitoylated. COS cells transfected with the N-terminal fragment of huntingtin containing amino acids 1-548 (huntingtin (1-548aa)) were metabolically labeled with [³H]palmitate and palmitoylation of immunoprecipitated Huntingtin (1-548aa) was analyzed by western blotting (WB) and autoradiography ([³H]palm). **(D)** Huntingtin is a substrate for HIP14. Purified His-Huntingtin (1-548aa) was palmitoylated in the presence of COS cell extracts expressing HIP14.
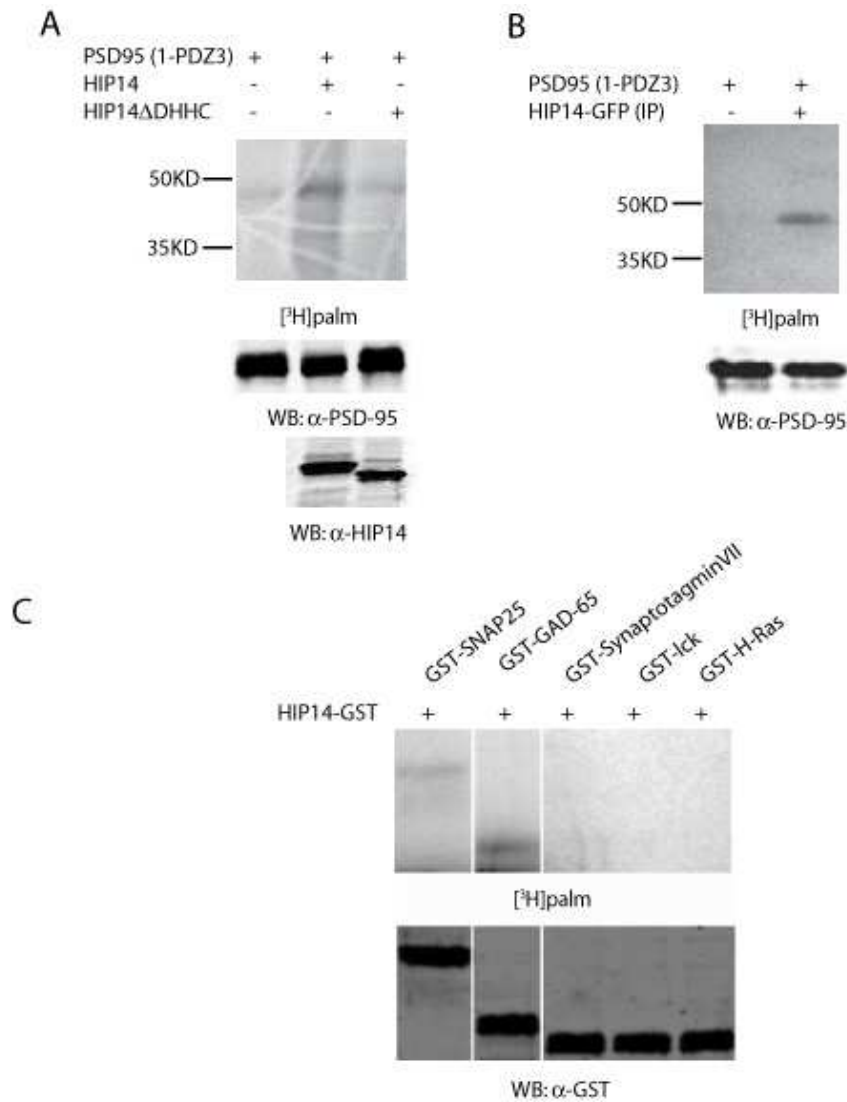
Figure 2.3. HIP14 palmitoylates specific neuronal proteins.

(A) Palmitoylation of purified PSD-95 (1-PDZ3) was enhanced in the presence of COS cell extracts expressing wild type HIP14 but not with extracts obtained from either mock transfected cells or cells expressing a mutant form of HIP14 lacking the DHHC domain (HIP14ΔDHHC). Number of repeats n=2. **(B)** Immunoprecipitated (IP) HIP14 GFP induces palmitoylation of purified PSD-95 (1-PDZ3). Number of repeats n=2. **(C)** HIP14 palmitoylates SNAP25 and N-terminal sequences of GAD-65 but not the acylated motifs of H-Ras, lck and synaptotagmin VII. GST fusion proteins containing the acylated motifs of GAD-65, H-Ras, lck, synaptotagmin VII or full length SNAP25 were incubated with purified HIP14 GST and with [^{3}H]palmitoyl-CoA. Products were analyzed by western blotting (WB) and autoradiography ([^{3}H]palm). Number of repeats n=1.

In addition to modifying several substrates with palmitate, metabolic labeling studies showed that HIP14 itself is palmitoylated and that this process relies on the presence of its DHHC domain (Figure 2.4A). These findings indicate that akin to Akr1p, acylation of the DHHC domain within HIP14 may also be required for the enzyme activity. Interestingly, deletion of the DHHC domain disrupted accumulation of HIP14 in the Golgi and resulted in a diffuse localization of this protein in the soma and dendrites (Figure 2.4B). Thus, the DHHC domain also appears to contribute to proper folding and/or trafficking of HIP14.
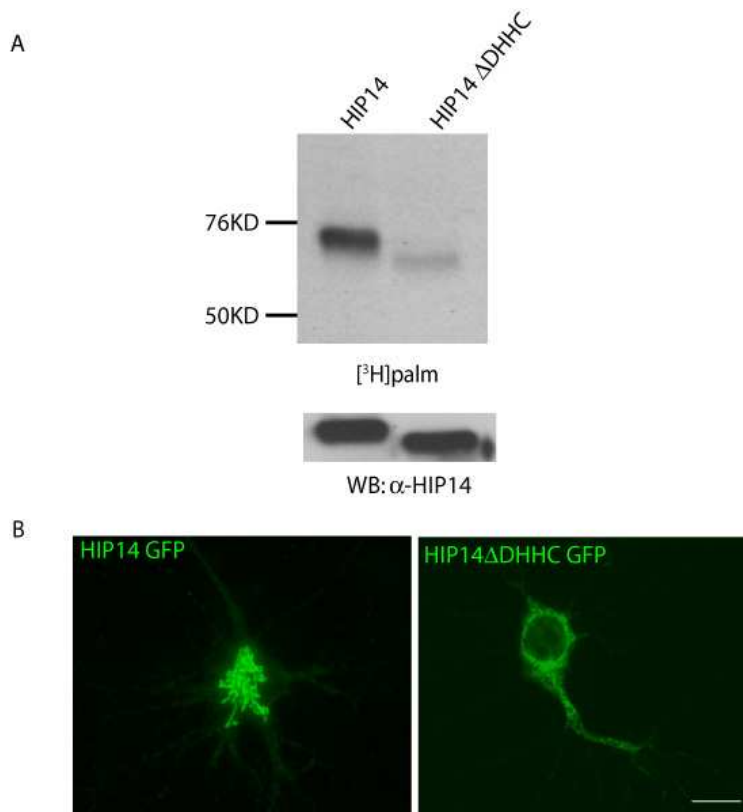
Figure 2.4. HIP14 Palmitoylation and Golgi Localization Requires the DHHC Domain.

(A) HIP14 is palmitoylated *in vivo*. COS cells transfected with either wild type HIP14 or a mutant form lacking the DHHC domain (HIP14ΔDHHC). Cells were metabolically labeled with [^{3}H]palmitate and palmitoylation of immunoprecipitated proteins was analyzed by western blotting (WB) and autoradiography ([^{3}H]palm). **(B)** Hippocampal neurons were transfected with GFP tagged wild type HIP14 or HIP14ΔDHHC. Distribution of HIP14ΔDHHC in neurons is altered.

2.3.3 HIP14 associates with multiple vesicular compartments.

Previous studies showed that endogenous HIP14 is localized to Golgi and in vesicles located in the cytoplasm (Singaraja *et al.* 2002). Double labeling analysis performed in NT2 cells showed that HIP14 is partially co-localized with the recycling and late endosomal markers Rab7 and Rab8. In contrast, HIP14 positive puncta did not significantly overlap with the early endosomal marker Rab5 or the adaptor protein AP2 (Figure 2.5). Electron microscopic (EM) analysis revealed that HIP14 is associated with the cytosolic side of diverse vesicular structures present in the soma and dendrites (Figure 2.6A-G). In addition, immunogold particles were observed in dendritic spines and at the plasma membrane (Figure 2.6). Taken together, these results indicate that HIP14 associates with several vesicular structures including the Golgi as well as sorting/recycling and late endosomal structures.
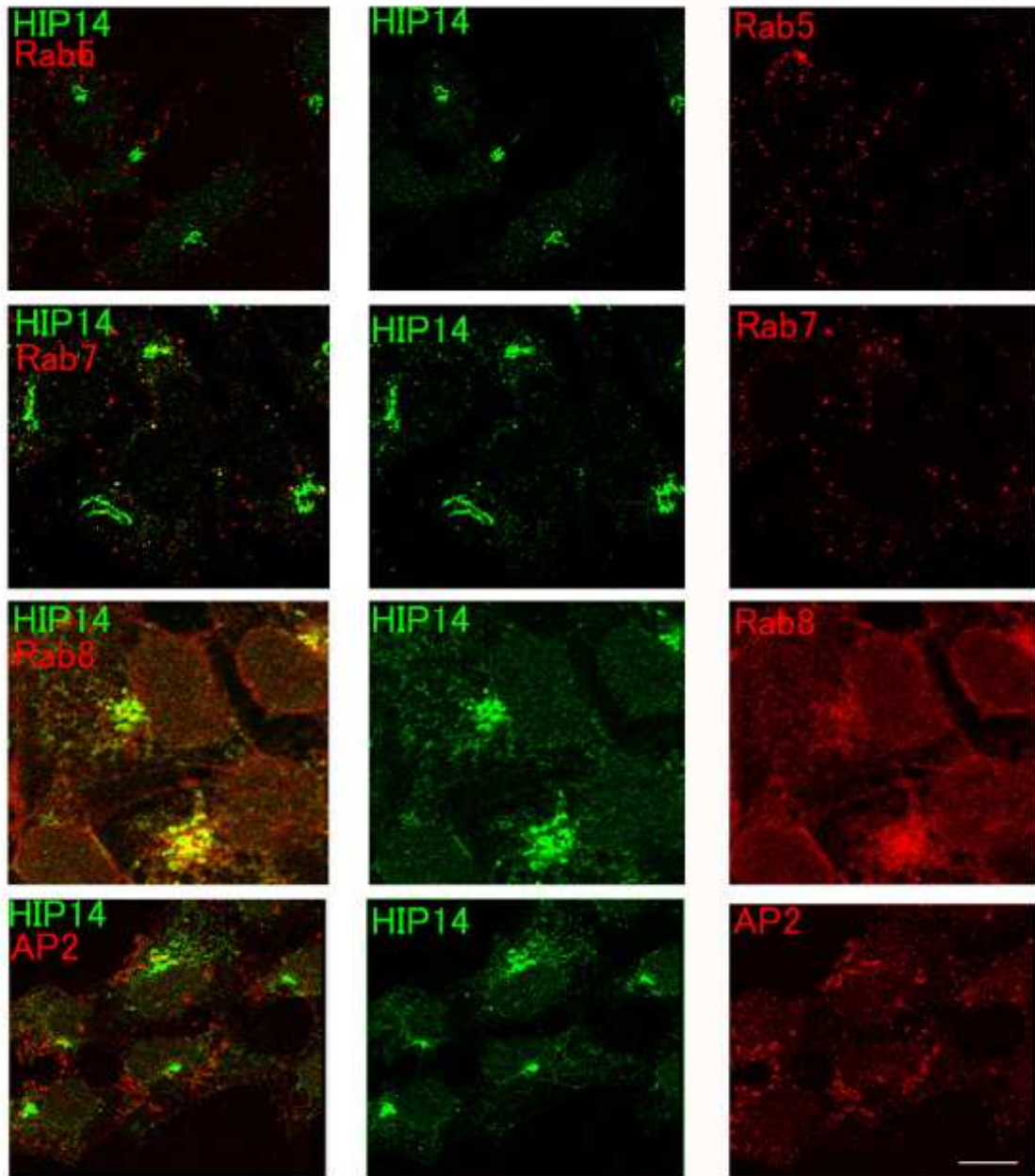
Figure 2.5. Localization of HIP14 With Endosomal Markers in NT2 Cells.

NT2 cells were double labeled with antibodies against HIP14 and either with Rab5, Rab7, Rab8 or AP2. Results show partial co-localization of HIP14 with Rab7 and Rab8. space bar= 10μm.

In cultured hippocampal neurons, HIP14 showed prominent staining in a perinuclear compartment that significantly co-localizes with the Golgi marker GM-130

(Figure 2.6H). Similar distribution was also observed in COS cells transfected with HIP14 GFP (Figure 2.6I). Time lapse imaging of COS cells transfected with HIP14 GFP, showed that HIP14 traffics in tubulovesicular structures budding from the perinuclear compartment (Figure 2.6J). Moreover, vesicular structures containing SNAP25 GFP and HIP14 DsRed were seen undergoing rapid transport in the cytoplasm (Figure 2.6K). These results indicate that HIP14 is involved in the cytosolic modification of both cytoplasmic and integral membrane proteins. Moreover, HIP14 in all likelihood modifies substrates at several subcellular locations.
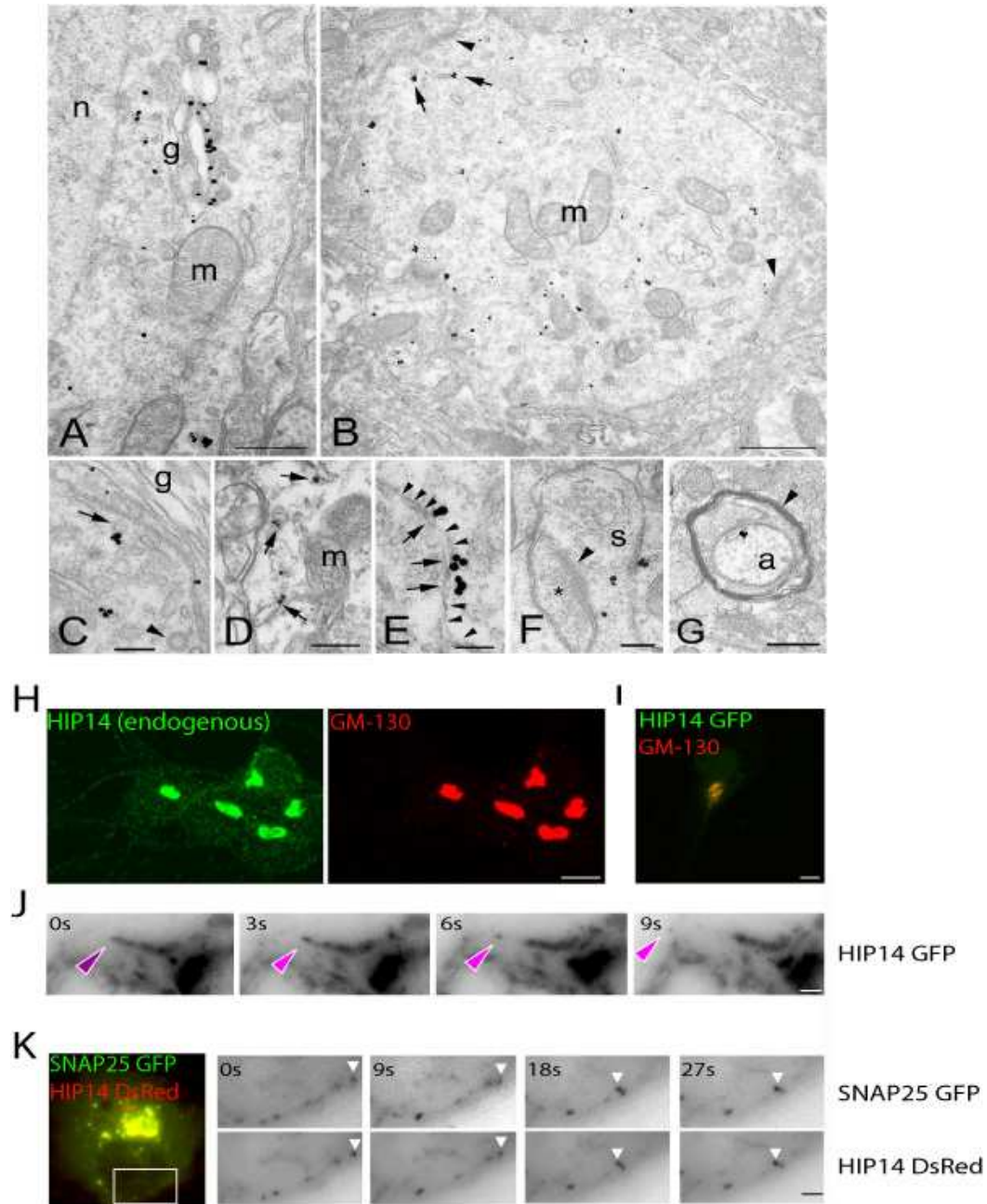
Figure 2.6. Association of HIP14 with Golgi and Cytoplasmic Vesicles.

(A-G) Electron micrographs showing the subcellular localization of HIP14. **(A)** Micrograph showing a portion of neuronal perikarya. HIP14 immunogold particles are mostly decorating the cytoplasmic side of the Golgi stacks. **(B)** Micrograph showing HIP14 immunogold particles in a large caliber dendrite receiving synaptic contacts (arrowheads). Immunogold particles are either free in the cytoplasm, or associated with tubulo-vesicular elements (arrows). **(C)** Micrograh showing HIP14 immuogold particles associated with a coated vesicle (arrow) near the Golgi apparatus. A non-labeled vesicle is also visible (arrowhead). **(D)** Micrograph of HIP14 immunogold particles associated with the cytoplasmic side of tubulovesicular elements (arrows) near the plasma membrane in the perikarya of a neuron. **(E)** HIP14 immunogold particles (arrows)

associated with the plasma membrane (outlined by the arrowheads). **(F)** Micrograph showing HIP14 immunogold particles in the neck of a dendritic spine (s) receiving a synaptic contact (arrowhead) from an axon terminal (star). **(G)** HIP14 immunogold in an axon (a) surrounded by myelin (arrowhead). **(H)** HIP14 is mainly localized to the Golgi and cytoplasmic vesicles in hippocampal neurons. Hippocampal neurons (DIV 8) were stained for HIP14 and the Golgi marker GM-130. HIP14 accumulates in a perinuclear region positive for GM-130 and in small puncta throughout the cytoplasm. **(I)** Co-localization of HIP14 GFP and GM-130 in COS cells. **(J)** Tubulovesicular-like structures (arrowheads) containing HIP14 GFP budding from the perinuclear region in COS cells. Inverse color time lapse images are shown. **(K)** HIP14 DsRed and SNAP25 GFP co-localize in the perinuclear region and cytoplasmic vesicles. Time lapse images show trafficking of both HIP14 DsRed and SNAP25 GFP in vesicle-like structures (arrowheads) present in the cytoplasm. Inverse color time lapse images are shown. Abbreviations: a: axon; g: Golgi apparatus; m: mitochondria, n: nucleus; s: dendritic spine. Scale bars: A = 500 nm; B = 833 nm; C and D = 250 nm; E = 150 nm; F = 200 nm; G =500 nm; H and I = 10 μm, Magnified panels in J and K = 1 μm.

2.3.4 Palmitoylation Dependent Trafficking is Regulated by HIP14

Palmitoylation is essential for vesicular trafficking of several proteins including PSD-95, GAP-43 and SNAP25 and usually results in the accumulation of these proteins in a compartment located in a perinuclear region (El-Husseini *et al.* 2000a). Hence, we used this approach to assess the effects of HIP14 on the trafficking of these putative substrates in both heterologous cells and neurons. This analysis revealed that exogenous HIP14 enhanced perinuclear accumulation of PSD-95 and SNAP25 (Figure 2.7 and Table 2.1 and Figure 2.8). Enhanced perinuclear accumulation was also observed for some of the other proteins examined including GAP-43, GAD-65 and synaptotagmin I. In contrast, HIP14 did not alter the distribution of paralemmin and synaptotagmin VII or the palmitoylation-deficient forms of SNAP-25 and PSD-95(Table 2.1). Consistent with these findings, metabolic labeling studies in heterologous cells showed that expression of HIP14 enhanced palmitoylation of synaptotagmin I, GAP-43 and SNAP25 by 30-50%. However, no change in the palmitoylation of synaptotagmin VII was observed (data not

shown). The strong correlation between altered protein trafficking in vivo and protein acylation in both heterologous cells and in vitro assays indicate an important role for HIP14 in the acylation of several proteins in neurons.
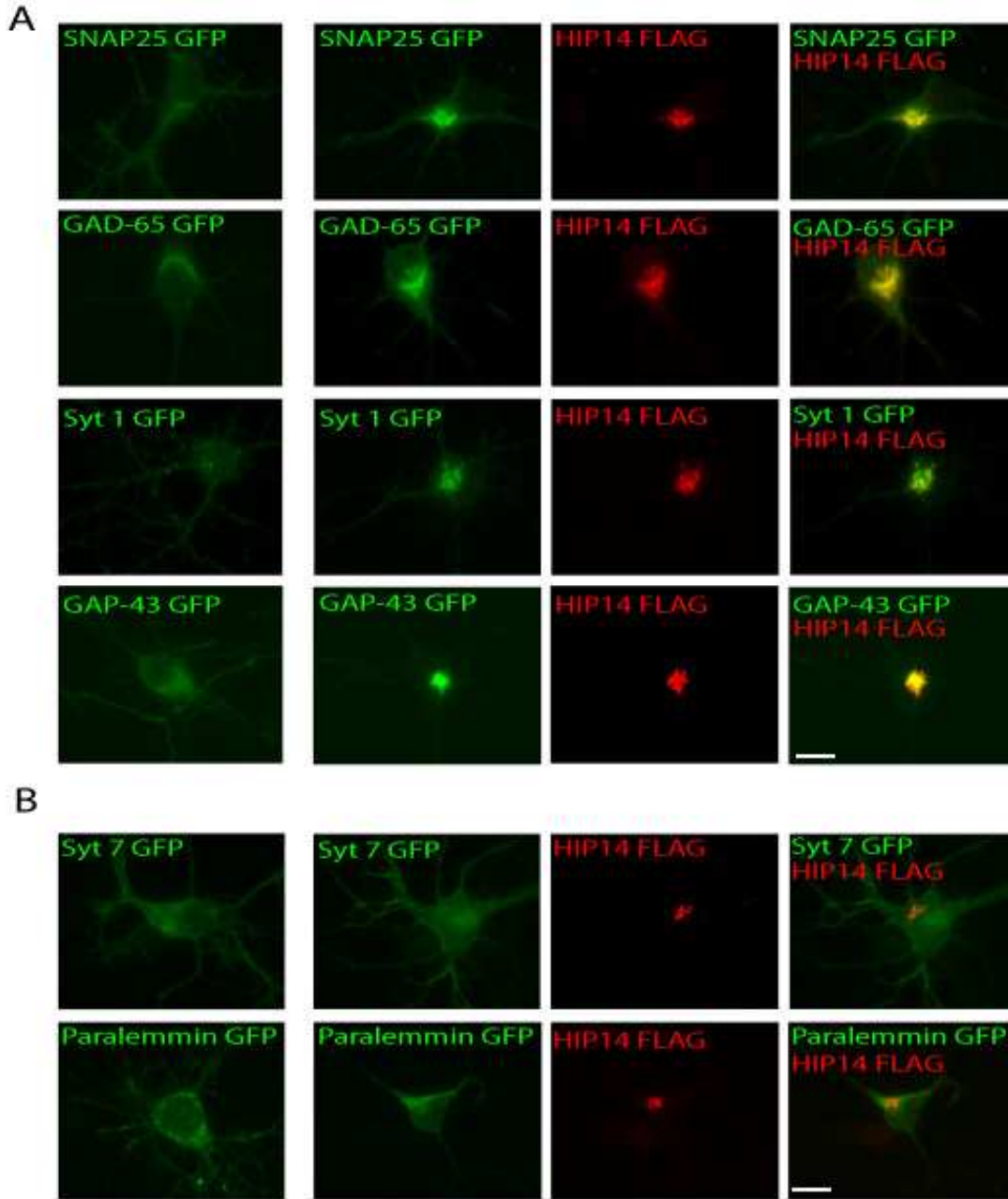
Figure 2.7. HIP14 Modulates Trafficking of Palmitoylated Proteins in Neurons.

(A,B) GFP fusion proteins of SNAP25, GAD-65, synaptotagmin I (Syt 1), GAP-43, paralemmin and HA-tagged synaptotagmin VII (Syt 7 HA) were transfected into cultured neurons (DIV 4) alone (left panels) or with a FLAG-tagged HIP14 (HIP14-FLAG; right panels). Overexpression

of HIP14 enhances perinuclear accumulation of SNAP25 GFP, GAD-65 GFP, Syt 1 GFP, and GAP-43 GFP. In contrast, HIP14 did not alter the distribution of paralemmin GFP and Syt 7 HA. Scale bars = 10 μm.

Table 2.1. Summary of Changes in the Localization of Palmitoylated Proteins in the Presence of HIP14 in Both COS Cells and Neurons.

(A) HIP14 induces redistribution of a subset of palmitoylated proteins. The effects on palmitoylated proteins require the presence of cysteines modified by palmitate. **(B)** Sequences of the palmitoylated motifs of proteins analyzed.

A. Altered localization of palmitoylated proteins in the presence of HIP14 in COS cells and neurons

	COS cells Enhanced perinuclear accumulation	Co-localization with HIP14	Neurons Enhanced perinuclear accumulation	Co-localization with HIP14
PSD-95-GFP	+++	C	+++	C
PSD-95-C3,5S-GFP	-	N	-	N
SNAP25-GFP	+++	C	+++	C
SNAP25-Palm mut GFP	-	N	-	N
GAP-43-GFP	+++	C	+++	P
Synaptotagmin I-GFP	++	C	+++	C
GAD-65-GFP	++	C	+++	C
Synaptotagmin VII-HA	-	N	-	N
Paralemmin-GFP	-	P	-	P
PSD95-prenyl-GFP	-	P	-	P

C: co-localized; N: not co-localized; P: partially co-localized

B. Sequences of palmitoylated motifs analyzed

Protein	Palmitoylation motif	Location
SNAP25	LGKFCGLCVCPCNKLKSSDA	Internal
PSD-95	MDCLCIVTTKKY	Amino terminus
GAP-43	MLCCMRRTKQV	Amino terminus
SynaptotagminI	VVTCCFCVCKKCL	Internal
GAD-65	ARAWCQVAQKFTGGIGNKLCALLYG	Internal
Synaptotagmin VII	VTIVLCGLCHWCQRKLG	Internal
lck	MGCVCSSNPEDDWME	Amino terminus
HRas	GPGCMSCKCVLS	Carboxyl terminus
Paralemmin	DMKKHRCKCCSIM	Carboxyl terminus
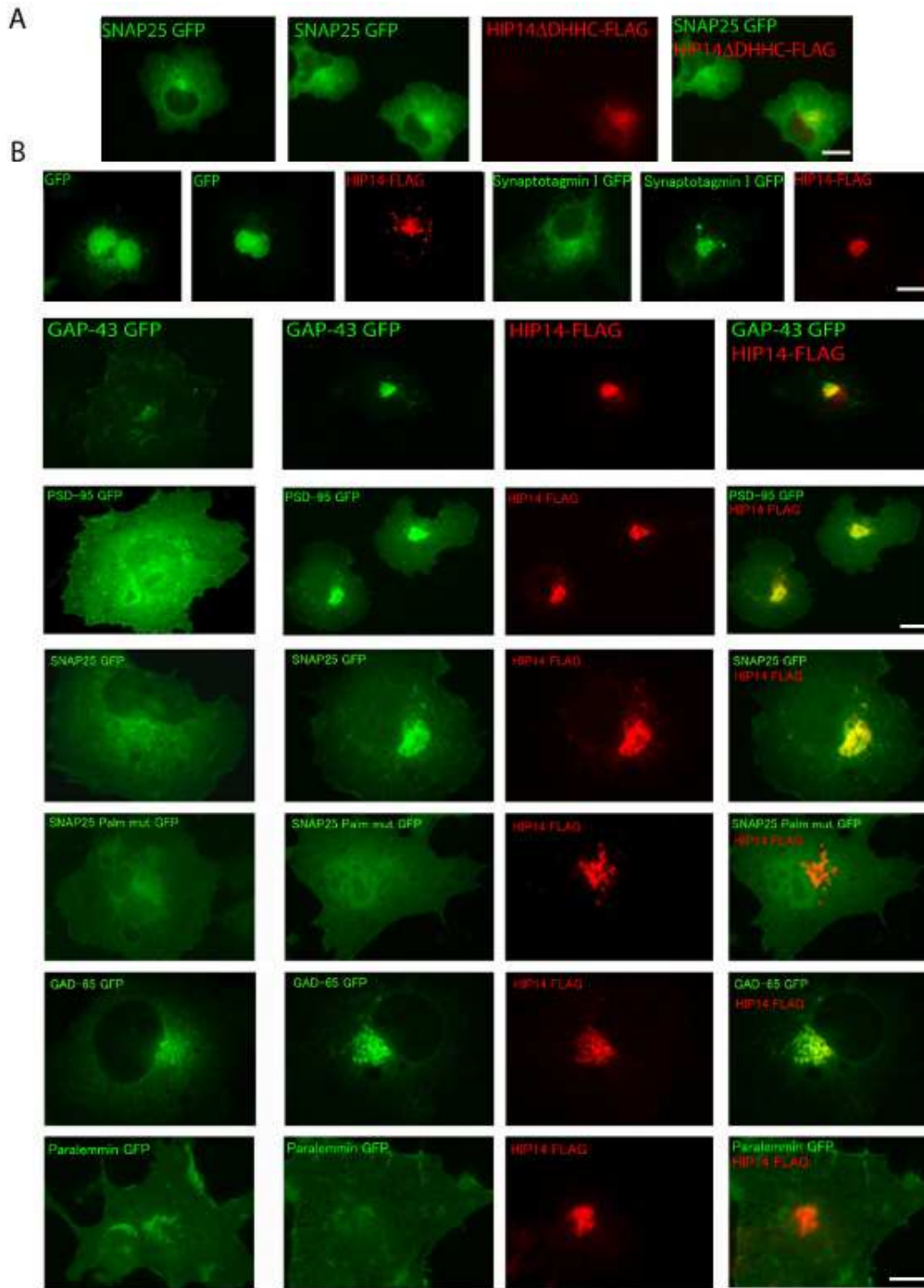
Figure 2.8. Differential Distribution of Acylated Proteins in Cells Expressing HIP14.

(A) FLAG tagged HIP14 lacking the DHHC domain (HIP14 DHHC-FLAG) does not induce redistribution of SNAP25 GFP. **(B)** Distribution of transfected GFP alone or GFP fused to several acylated proteins as indicated in various panels. HIP14 induces perinuclear accumulation of SNAP25, GAD-65, GAP-43, synaptotagmin I and PSD-95. In contrast, HIP14 did not alter the

distribution of paralemmin, synaptotagmin VII or a mutant form of SNAP-25 lacking the palmitoylated cysteines (SNAP25 Palm mut-GFP). Scale bars = 10 μm.

Previous work has established that palmitoylation is important for trafficking of neuronal proteins to either the presynaptic or postsynaptic compartment. Specifically, palmitoylation is essential for postsynaptic targeting of PSD-95 (El-Husseini *et al.* 2000b). When expressed in developing neurons, PSD-95 forms clusters that can be detected 12 h post-transfection (El-Husseini *et al.* 2000c). The number of clusters is gradually increased in the following 2-3 days. To assess whether HIP14 can modulate palmitoylation dependent PSD-95 trafficking, we compared changes in PSD-95 GFP clustering 12-14 h post-transfection when expressed alone or with HIP14. Figure 2.9A shows that expression of HIP14 specifically enhanced clustering of wild type PSD-95, but did not alter the distribution of the palmitoylation deficient form of PSD-95 or PSD-95 fused to a prenylated motif. These results are consistent with the proposed role for HIP14 in palmitoylation and trafficking of PSD-95 in neurons.

To further address whether endogenous HIP14 is important for palmitoylation-dependent trafficking of neuronal proteins, we generated a small interference RNA (siRNA) that specifically disrupts HIP14 expression (Figure 2.9B and C). Next, we examined whether disruption of HIP14 expression alters trafficking of PSD-95 and GAD-65, two neuronal proteins that rely on palmitoylation for targeting to post- and presynaptic sites, respectively (Craven *et al.* 1999; Kanaani *et al.* 2002). Remarkably, neurons transfected with HIP14 siRNA showed significant reduction in clustering of endogenous PSD-95 (Figure 2.9D-F). In contrast, clustering of SAP-102, a non-palmitoylated synaptic protein closely related to PSD-95, was not affected by HIP14 siRNA expression. In addition to altered localization of PSD-95, HIP14 siRNA

significantly reduced axonal clustering of GFP-tagged GAD-65 (Figure 2.10). These results establish that HIP14 is critical for trafficking of acylated proteins targeted to both pre- and postsynaptic sites.
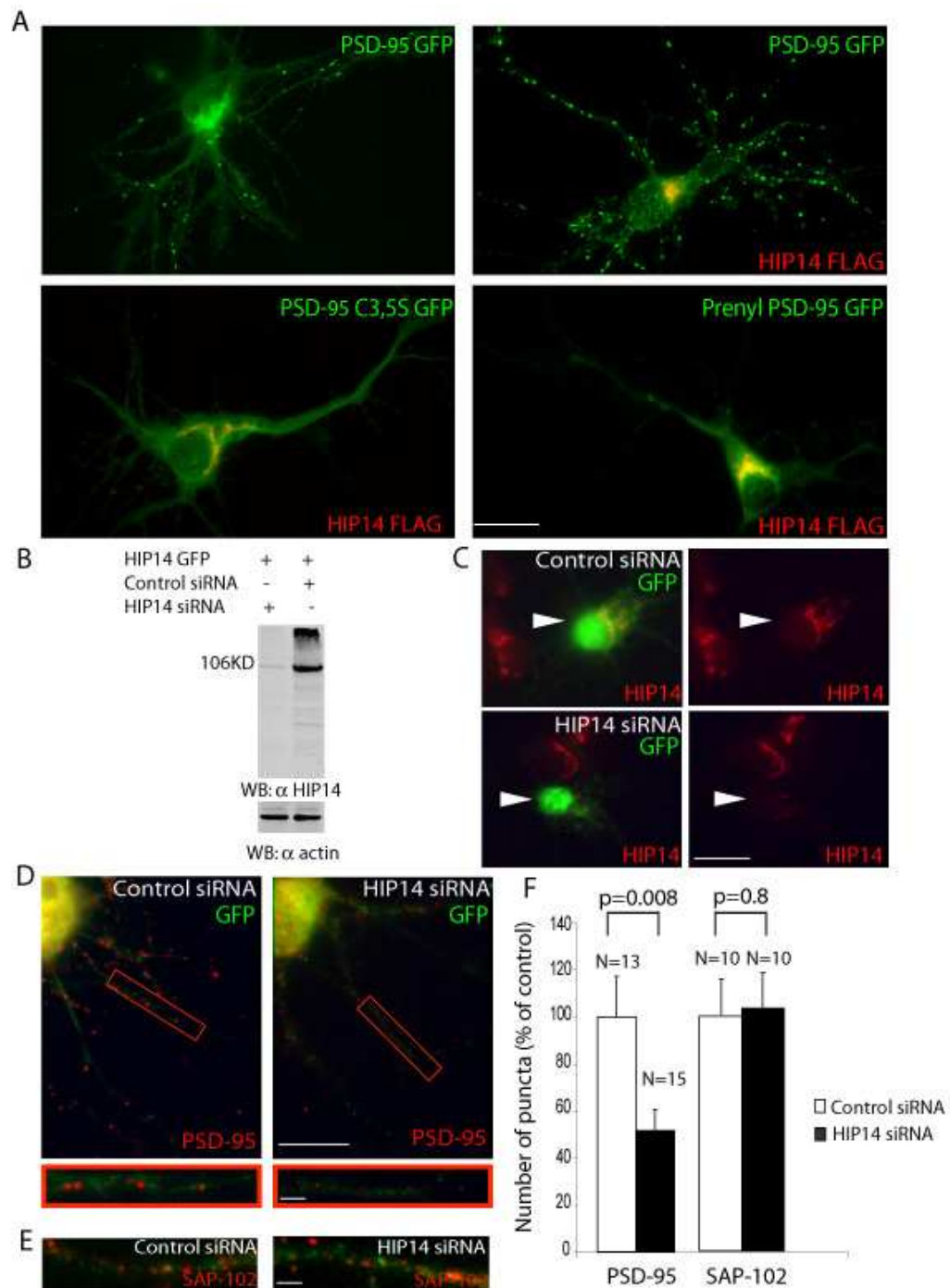
Figure 2.9. Manipulation of Endogenous Expression of HIP14 Alters Clustering of PSD-95 in Neurons.

(A) HIP14 enhances palmitoylation dependent clustering of PSD-95. Cultured hippocampal neurons (DIV 4) were transfected with the indicated constructs. 12 h post-transfection, clusters of wild type PSD-95 GFP can be observed. In the presence of HIP14-FLAG, PSD-95 GFP clustering was enhanced. HIP14-FLAG did not alter trafficking of either the palmitoylation deficient form (PSD-95 GFP C3,5S) or a mutant form of PSD-95 containing a prenyl motif (PSD-95 GFP Prenyl). **(B-F)** Blocking endogenous expression of HIP14 reduces clustering of PSD-95. **(B)** Western blot analysis of COS cells transfected with HIP14 GFP and either with HIP14 specific small interference RNA (HIP14 siRNA) or scrambled siRNA (Control siRNA) and then probed with antibodies against GFP (Top panel) and actin (Bottom panel). Analysis shows that HIP14 siRNA blocks expression of HIP14 GFP. **(C)** Hippocampal neurons (DIV 7) were transfected with GFP and either with HIP14 siRNA or control siRNA. At DIV 10, neurons were fixed and stained with antibodies against HIP14. Loss of HIP14 staining in neurons transfected with HIP14 siRNA (Bottom panels; arrowheads) but not control siRNA (Top panels; arrowheads). **(D,E)** HIP14 siRNA reduces clustering of **(D)** PSD-95 but not **(E)** SAP-102. **(F)** Summary of changes in the number of puncta of PSD-95 and SAP-102 in the dendrites of neurons expressing HIP14 siRNA compared to neurons expressing control siRNA. Scale bars in A, C and D = 10 μm, enlarged panels in D and E = 1 μm.
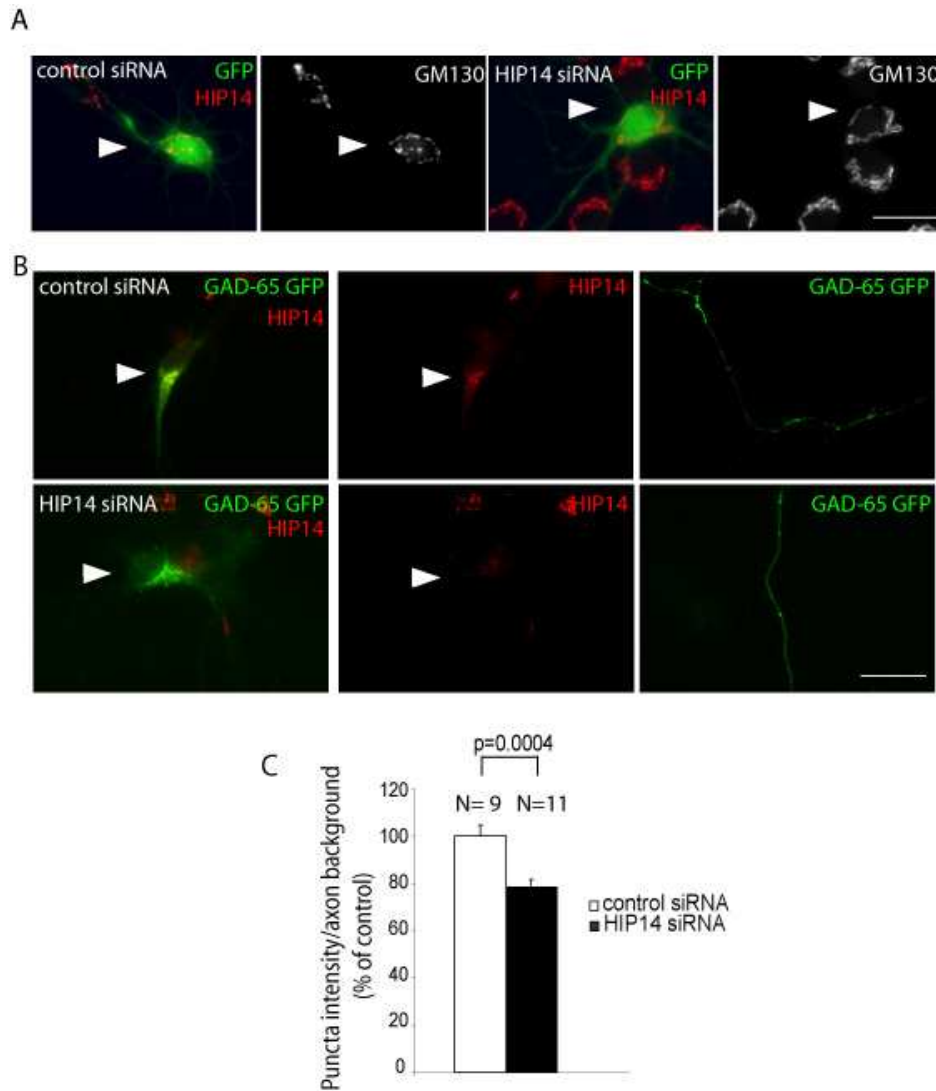
Figure 2.10. Interference with Endogenous Expression of HIP14 reduces axonal clustering of GAD-65 in Neurons.

(A) HIP14 specific small interference RNA (HIP14 siRNA) blocks expression of HIP14 but does not alter the expression or localization of the Golgi marker GM-130. Neurons were transfected at DIV 7 with GFP and either with HIP14 siRNA or scrambled siRNA (Control siRNA) and then stained at DIV 10 with antibodies against HIP14 and GM-130. Loss of HIP14 staining in neurons transfected with HIP14 siRNA but not with control siRNA. **(B)** Neurons were transfected with GAD-65 GFP and either with HIP14 siRNA or control siRNA. HIP14 siRNA reduces axonal clustering of GAD-65. **(C)** Summary of changes in the intensity of puncta of GAD-65 in axons of neurons expressing HIP14 siRNA compared to neurons expressing control siRNA. Scale bars = 10 μm.

2.4. DISCUSSION

In this study, we have identified HIP14 as a mammalian palmitoyl transferase that confers multiple substrate specificity. We demonstrate that HIP14 influences palmitoylation-dependent sorting and localization of acylated proteins in heterologous cells and neurons. Remarkably, HIP14 induced palmitoylation of diverse neuronal proteins sorted to both pre- and postsynaptic sites. The altered clustering of PSD-95 and GAD-65 in neurons lacking HIP14 supports an *in vivo* role for this enzyme in protein palmitoylation. Another important finding of this study is that huntingtin is also subject to palmitoylation by HIP14.

Although characterization of the kinetics of this enzyme have not been established, the ability of purified HIP14 to catalyze the transfer of palmitate on specific cysteine residues palmitoylated *in vivo* strongly supports its action as a palmitoyl transferase. Palmitoylated sequences modified by HIP14 lack a conserved consensus sequence and can be found at the N-terminus or at internal sites. This finding is in contrast to enzymes involved in other lipid modifications such as myristoylation and prenylation that require a more defined consensus sequence located either at the N-terminus or C-terminus of modified proteins, respectively (El-Husseini Ael *et al.* 2002; Linder and Deschenes 2004; Smotrys and Linder 2004).

Previous investigations indicate that palmitoylation may occur at many cellular sites, including the cytosol, plasma membrane, Golgi/ER, and synaptic membranes (Resh 1999; Smotrys and Linder 2004). In this study we show that HIP14 is a palmitoyl transferase that is mainly localized to the Golgi. Enrichment of HIP14 and other putative palmitoyl transferases such as GODZ and ZDHHC8 in the Golgi (Keller *et al.* 2004;

Mukai *et al.* 2004) suggests that this compartment is a major site for palmitoylation. Importantly, several proteins examined in this study, including SNAP25, synaptotagmin I, GAD-65 and GAP-43 traffic through the Golgi (Gonzalo and Linder 1998; Resh 1999; el-Husseini Ael and Bredt 2002; Kanaani *et al.* 2002; Smotrys and Linder 2004). Thus, acylation of some of the analyzed proteins may occur in this compartment.

Our EM analysis revealed that HIP14 is mainly found associated with the cytosolic side of vesicles present in the cytoplasm. HIP14 immunogold particles were also detected at the plasma membrane and dendritic spines. Further evidence for the enrichment of HIP14 in vesicular structures trafficking in the cytosol was revealed by time lapse imaging of cells expressing HIP14 GFP. The association of HIP14 with several vesicular compartments that may include recycling and late endosomes indicates that this enzyme acts at multiple subcellular locations. However, the ability of HIP14 to modulate acylation of cytosolic proteins such as PSD-95 is intriguing. Previous studies showed that PSD-95 and huntingtin associate with vesicular membranes (DiFiglia *et al.* 1995; Velier *et al.* 1998; El-Husseini *et al.* 2000a). In addition, vesicular trafficking of PSD-95 to a perinuclear compartment is palmitoylation dependent and can be blocked by agents that disrupt the Golgi (El-Husseini *et al.* 2000a; El-Husseini *et al.* 2000b; El-Husseini *et al.* 2000c). The detection of HIP14 on the cytosolic side of vesicular structures suggests that HIP14 may directly modify PSD-95 in the cytoplasm or on vesicles containing HIP14.

A recent report identified ski as an acyl-transferase involved in palmitoylation of proteins within the lumen of the secretory pathway (Chamoun *et al.* 2001). In contrast, HIP14 appears to mediate palmitoylation of several intracellular proteins trafficking through a Golgi-dependent vesicular pathway, most likely at the cytosolic side of the membrane.

These findings suggest the existence of at least two families of PATs that regulate acylation of intracellular and secreted proteins.

It is intriguing that HIP14 induced palmitoylation and altered trafficking of several unrelated neuronal proteins. These results suggest that HIP14 is a major PAT in neurons. However, the DHHC domain of HIP14 is conserved in 22 other DHHC containing proteins that are present in both the mouse and the human genome (Smotrys and Linder 2004). It is as yet unknown whether these proteins are functionally redundant palmitoyl transferases, or whether they are differentially localized and specialized in the palmitoylation of a subset of proteins.

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3. CHAPTER 3   Palmitoylation Of Huntingtin By HIP14

Is Essential For Its Trafficking And Function

A version of this chapter has been published.

3.1. INTRODUCTION

Post-translational modification of cysteine residues with palmitate has recently emerged as an important and reversible modification, which is involved in trafficking and functional modulation of different membrane proteins and their signalling pathways (El-Husseini *et al.* 2000a; el-Husseini Ael and Bredt 2002; Huang and El-Husseini 2005). We have recently demonstrated, by *in vitro* labelling assays, that HIP14 is a palmitoyl transferase that palmitoylates numerous neuronal substrates, including huntingtin (Huang *et al.* 2004). Expansion of the polyglutamine tract of huntingtin is the mutation underlying HD (The Huntington's Disease Collaborative Research Group, 1993).

A characteristic feature of HD (The Huntington's Disease Collaborative Research Group, 1993) is neuronal toxicity markedly affecting medium spiny neurons of the striatum associated with formation of intranuclear and cytoplasmic inclusions (Hackam *et al.* 1998). An early feature of the pathogenesis of HD is the translocation of mutant huntingtin into the nucleus (Wheeler *et al.* 2002; Van Raamsdonk *et al.* 2005a). Huntingtin is normally located on plasma and intracellular membranes and associates with vesicles and different organelles such as the Golgi (DiFiglia *et al.* 1995; Velier *et al.* 1998). Disturbed trafficking of mutant huntingtin appears to be an early and consistent feature of HD. However, the factors influencing the trafficking of huntingtin are unknown.

Different post-translational modifications of huntingtin alter its cellular function. Ubiquitination targets huntingtin for degradation (Kegel *et al.* 2005) and SUMOylation promotes its capacity to repress transcription (Steffan *et al.* 2004). Phosphorylation of

mutant huntingtin is reduced and is associated with increased toxicity (Humbert *et al.* 2002; Warby *et al.* 2005).

Here we show that huntingtin is palmitoylated *in vivo* and that alteration in palmitoylation of huntingtin affects its normal distribution and function. We provide evidence that the palmitoylation of huntingtin *in vivo* is regulated by HIP14 and is crucial for its normal trafficking to the Golgi. Furthermore, palmitoylation of mutant huntingtin is dramatically reduced *in vivo*, as a result of its lower interaction with HIP14, leading to increased inclusion formation and enhanced toxicity.

3.2. MATERIALS AND METHODS

3.2.1 Antibodies

The generation of huntingtin-specific antibodies BKP1 and HD650 are described elsewhere (Kalchman *et al.* 1996; Slow *et al.* 2003). Antibodies used in this study: BKP1 (1:500 dilution), HD650 (1:85), huntingtin antibody 2166 (Chemicon;1:250 for immunoprecipitation, 1:2000 for Western blotting and 1:1000 for immunofluorescent assays), monoclonal GFP, EEA1 and GM130 (BD Biosciences; 1:5000, 1:500 and 1:200, respectively), anti Hsp70 (Neomarker; 1:200), monoclonal transferrin receptor (Zymed; 1:1000), polyclonal caveolin antibody (Santa Cruz Biotechnology; 1:500), goat and rabbit anti-mouse HRP conjugates (BioRad; 1:5000), Phalloidin, Lysotracker, Alexa Fluor 488 and 568 (Molecular Probes; 1:50, 1:7000, 1:1000, respectively), Cy3 donkey anti-mouse (Jackson ImmunoResearch laboratories; 1:200), monoclonal anti-γ-tubulin (Sigma; 1:100), anti-proteasome Lmp2 (Abcam; 1:400 dilution) and polyclonal anti-ubiquitin (DakoCytomation; 1:500).

3.2.2 DNA mutagenesis and cloning

Truncated   huntingtin   constructs   N548   and   N224   were   previously
described(Wellington *et al.* 1998). Cysteine substitutions were generated by PCR based
site-directed mutagenesis as described earlier (Wellington *et al.* 1998). Generation of C-
terminal GFP tagged proteins was performed using a similar strategy, with the stop codon
of the tagged proteins replaced by the initiation codon of EGFP. All mutated DNA
constructs were sequence confirmed.

3.2.3 Cell culture and transfections

All reagents for cell cultures were purchased from Invitrogen Corporation
(Carlsbad, CA). COS cells were cultured as previously described (Huang *et al.* 2004).
For live cell imaging, DMEM without Phenol Red was used, to eliminate
autofluorescence. COS cells were transiently transfected with FuGene 6 transfection
reagent (Roche) or Lipofectamine 2000 (Invitrogen) as indicated by the manufacturers.
24 to 48 hours post transfection, cells were processed as described for each experiment.
Cultured cortical and striatal neurons were prepared as previously described (Singaraja *et al.* 2002) and experiments were performed at 5-12 days *in vitro* (DIV). Neurons were
transfected with a Nucleofector (Amaxa Inc) at day 0.

3.2.4 [3H]-palmitoylation assay and immunoprecipitation

Transfected COS cells were labeled with 1 mCi/ml [^{3}H]-palmitic acid (57 Ci/mmol;
Perkin Elmer Life Sciences, Inc.) for 3 hrs and processed as previously decribed(Huang
et al. 2004). For pulse-chase experiments, transfected cells were labeled for 3 hrs and
then chased for 0, 1, 3 and 6 hr with cold palmitate.

3.2.5 Huntingtin co-immunoprecipitation with HIP14

Brains from YAC18 and YAC128 transgenic mice were homogenized in PBS, containing protease inhibitors, and precleared in the presence of 0.2% SDS and 0.8% Triton X-100 for 1.5 hour at 4ºC, followed by centrifugation at 2700 g for 5 min. Normal mouse IgG, or anti-HIP14 mouse monoclonal antibody were incubated with 3mg precleared lysate at 4ºC for 1 hour. 30µl of equilibrated Protein(A+G) Sepharose 4 Fast Flow beads were added and samples were further incubated at 4ºC overnight. Beads were washed with PBS, containing 1% Triton X-100, boiled at 95ºC for 5 min and run on NuPAGE Novex 3-8% Tris-Acetate Gel (Invitrogen). Western Blots were probed with anti huntingtin monoclonal antibody HD650 which recognizes the transgene.

3.2.6 Immunofluorescence and time-lapse imaging

Transfected cells growing on coverslips were processed as previously decribed (Huang *et al.* 2004) with the indicated antibodies. For time lapse, images were collected 28 hours post-transfection using a 63x oil objective affixed to a Zeiss inverted light microscope, and AxioVision software. While images were acquired, cells were kept in a 37ºC chamber, supplemented with 5% CO_2. Images were collected every 15 minutes for a total time of 2 hours.

3.2.7 Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

Day 0 Rat cortical neurons were transfected with different N-terminal truncations of huntingtin. DMEM was replaced with neurobasal medium 1 hr posttransfection. Neurons (5-11 DIV) were exposed to 500 µM NMDA for 10min and further incubated with neurobasal medium for 24hr, fixed for 10 min in 4% paraformaldehyde in PBS (pH

7.4) and then 5 min in 100% methanol at -20^0C. Cells were stained with GFP antibody for 1 hr followed by incubation with a secondary antibody conjugated to Alexa 488 fluorophore for 1 hr at RT. Neurons were stained using the ApopTag Red *In Situ* Apoptosis Detection Kit (Chemicon) according to the manufacturer's instructions. Nuclei were counterstained with 0.5 µg/ml DAPI (Molecular Probes). Neurons transfected with various huntingtin constructs were counted under the microscope, and the number of TUNEL-positive (red) neurons was determined as a fraction of DAPI-positive (blue) neuronal nuclei in each transfected cell (green) by an observer blinded to the identity of the samples. Experiments were repeated 5 times and 100-200 cells were counted in each experiment. The fractions of TUNEL-positive nuclei determined for each experiment were averaged, and the results are presented as means ± SD (*n*= number cells counted).

3.2.8 Cell death assay

Cell death/toxicity was monitored by scoring the proportion of transfected COS-7 cells with apoptotic nuclear morphology as previously described (Wyttenbach *et al.* 2002).

3.2.9 Imaging and Anaylsis

Images were acquired on a Zeiss Axiovert M200 motorized microscope by using a monochrome 14-bit Zeiss Axiocam HR charge-coupled device camera at 1,300×1,030 pixels. Image analysis was performed as previously described (Huang *et al.* 2004).

3.2.10 Flotation assay

Brains from wild type and YAC128 mice were homogenized, sonicated and then centrifuged at 9500 *g* for 10 min in a SW55 Ti rotor (Beckman Coulter) to remove debris. Following further centrifugation at 100.000 *g* for 90 min, the plasma membrane fraction

was resuspended in 2ml solubilization buffer. 5mg total protein was brought up to 2ml and further diluted with an equal volume of 80% (w/v) sucrose in MBS, and loaded at the bottom of thin wall ultracentrifuge tubes. 4ml of 30% sucrose was overlaid on the membrane fraction and 4 ml of 5% sucrose made the top layer. Gradients were centrifuged at 118,000 g for 16hr at 4º in a SW41 Ti rotor to isolate detergent resistant membranes. Fractions of 1 ml were collected from the top and analyzed on SDS-PAGE.

3.2.11 Labeling with biotin-conjugated 1-biotinamido-4-[4-(maleimidomethyl)cyclohexanecarboxamido] butane (Btn-BMCC)

Brains from YAC 18Q and YAC128Q mice were homogenized and processed for immunoprecipitation as described above. The beads were then washed with wash buffer (PBS, containing 1% Triton X-100) supplemented with 10 mM N-ethylmaleimide (NEM), followed by treatment with 1 M hydroxylamine (NH_2OH pH 7.4) for 1 h at 25°C. Samples were then processed as previously described (Drisdel and Green 2004).

3.2.12 Viral infection

Control siRNA and HIP14 siRNA were cloned into human immunodeficiency virus type 1 (HIV-1)-based lentiviral vectors pLL3.7. Production of lentiviral supernatants and infection of dissociated primary cortical neurons were done as previously described (Janas *et al.* 2006). Briefly, rat cortical neurons (8 DIV) were infected with viruses expressing siRNA for 4 d, followed by exposure to 500 M NMDA for 10 min; they were then further incubated with neurobasal medium. After 24 h, cells were processed for the TUNEL assay as described above.

3.3. RESULTS

3.3.1 Palmitoylation of huntingtin at Cys214 is modulated by CAG size

The observation that palmitoylation is critical for the distribution of proteins to particular membrane locations, combined with the presence of huntingtin in detergent-resistant membranes (Figure 3.1), raised the possibility that the palmitoylation of huntingtin may be crucial for regulating its trafficking and function. Immunoprecipitation of huntingtin from cortical neurons revealed that full-length huntingtin is indeed palmitoylated (Figure 3.2A). Treatment of cells with NMDA, which induces cleavage of huntingtin, revealed that the N-terminal region is modified by palmitate (Figure 3.2A). Deletion mapping with N-terminal truncations of huntingtin showed that palmitoylation occurred in the first 224 amino acids (Figure 3.2B; N224). Palmitoylation was abolished by treatment with hydroxylamine (NH_2OH; Figure 3.2C), indicating that the incorporation of palmitate is due to the modification of cysteines through a thioester bond. Further deletion mapping localized the site of palmitoylation to a fragment containing six cysteine residues (Figure 3.2D and Figure 3.1; Huntingtin 79–224). Subsequent mutagenesis localized the palmitoylated residue to three cysteines in the C terminus of this fragment (Figure 3.2D and Figure 3.1; Huntingtin 141–224). Protein sequence alignment (The Huntington's Disease Collaborative Research Group, 1993) suggested that C214 was a likely candidate for palmitoylation by virtue of it being the only site in this region that was conserved in all the species we analyzed, including *Drosophila* (Figure 3.1). Indeed, C214 was identified as the site at which the N-terminal fragment of huntingtin (Figure 3.2D) and full-length huntingtin (Figure 3.2E) are

palmitoylated, consistent with the existence of a single major site (C214) for palmitoylation within huntingtin.

Protein palmitoylation is regulated by flanking sequences that can markedly alter the levels of protein palmitoylation(el-Husseini Ael and Bredt 2002). As the major site of palmitoylation is in the N terminus of huntingtin, close to the polyglutamine tract, this immediately raised the possibility that palmitoylation of huntingtin was modulated by CAG size. Indeed, palmitoylation of the N-terminal fragment of huntingtin in COS cells (N548) was significantly reduced in the presence of a disease-associated expansion of the polyglutamine tract (Figure 3.2F,G; $P = 0.001$). Palmitoylation of other proteins such as SNAP25 was not altered in the presence of the mutation for Huntington disease (Figure 3.1). The fact that polyglutamine expansion markedly decreases huntingtin palmitoylation raised the question of whether some of the pathogenic effects of mutant huntingtin might be operating through this mechanism.

A

Fraction: 1 2 3 4 5 6 7 8 9 10 11 12
(DRM)

Huntingtin

Caveolin

Transferrin Receptor

B

polyQ C105,109 C137 C152 C204 C214

Htt 1-224

Htt 79-224 GFP

Htt 79-149 GFP

Htt 141-224 GFP

polyQ C152 C204

Htt 1-548

C

	C105 C109	C137	C152	C204	C214
Human	RVNHCLTICENIVA	AMELFLLCSDDAESDVRMVADECLNKVIKA		VRPQKCRPYLVNLLPCLTRTSKR	
Pig	RVNHCLTICENIVA	AMELFLLCSDDAESDVRMVADECLNKVIKA		VRPQKCRPYLVNLLPCLTRTSKR	
Mouse	RVNHCLTICENIVA	AMELFLLCSDDAESDVRMVADECLNKVIKA		VRPQKCRPYLVNLLPCLTRTSKR	
Rat	RVNHCLTICENIVA	AMELFLLCSDDAS-RRRMVADECLNKVIKA		VRPQKCRPYLVNLLPCLTRTSKR	
Denio rerio	RVTHCLTICENIVA	AMEMFLLCSDDKESDVRMVADECLNKIIKA		VRPQKCRPYLVNLLPCLTRITKR	
Fugu	RVSHCLTICENIVA	AMEMFLLCSDDSESDVRMVADECLNRIIKA		IRPQKCRPYLVNLLPCLTRITKR	
Drosophila	KITCFQQIAECIMS	ATNVLLLFCEDVDSVVRMSAEENLNKILRS		IKEKHIKWYAVRLLQCMTTISQR	

Figure 3.1. Huntingtin is associated with detergent resistant membranes and Cysteine 214 is conserved in all species analyzed.

(A) Huntingtin is associated with detergent resistant membranes (DRM). Membranes from mouse brains were solubilized in 0.5% Triton X-100 lysis buffer and subjected to a flotation assay. Some huntingtin localizes to the fraction containing detergent resistant membranes (fraction 5), similar to caveolin but in contrast to the transferrin receptor, which is solubilized under these conditions. **(B)** A diagram representing huntingtin truncations and amino acid substitutions used to determine the site of palmitoylation within huntingtin. **(C)** Huntingtin protein sequence alignment demonstrates that C214 is conserved in all species analyzed.

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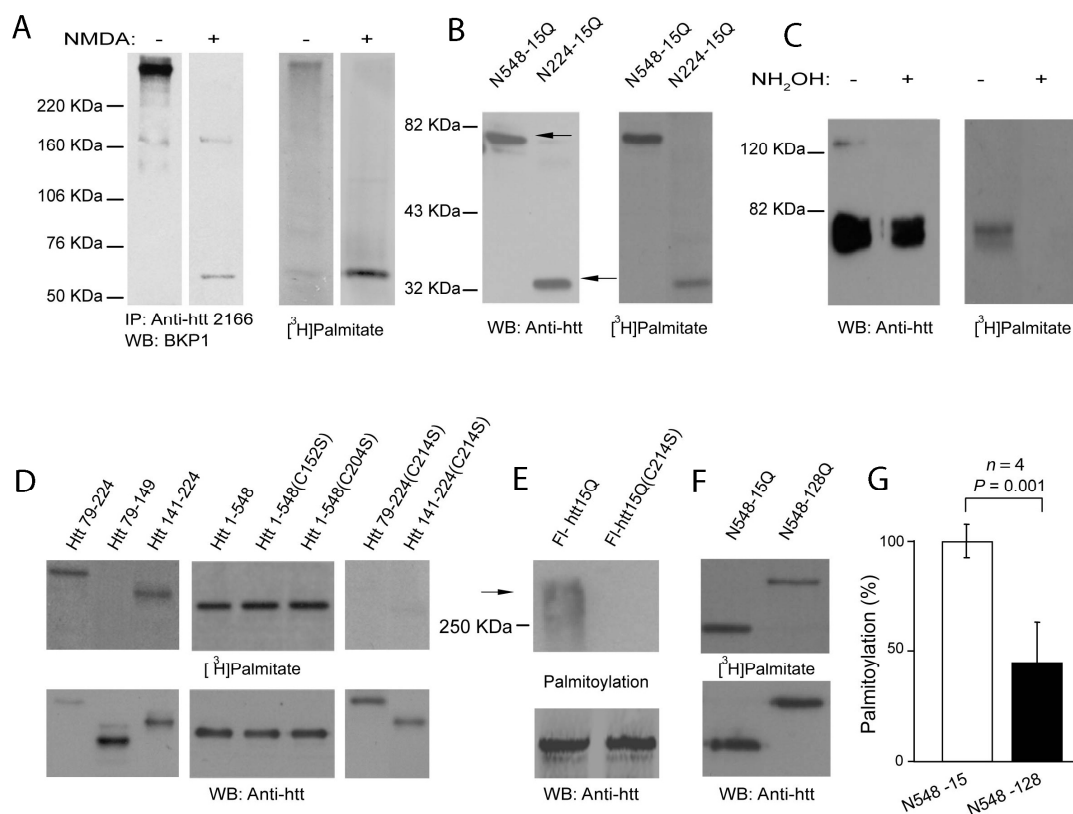
Figure 3.2. Huntingtin is palmitoylated in neurons and COS cells. Palmitoylation is modulated by CAG size.

(A) Rat cortical neurons were treated (+) with 100μM NMDA for 10 min, followed by [^{3}H]-palmitate metabolic labeling. Huntingtin (huntingtin) was immunoprecipitated (IP) and visualized by Western blotting and fluorography. A [^{3}H]-palmitate band is detected in untreated cells, whereas following NMDA treatment a palmitoylated fragment of huntingtin, detected by N-terminus specific antibody BKP1, is generated (right panel). **(B)** COS cells transfected with two truncated fragments of huntingtin (N548, N224) were metabolically labeled and processed as described above. The smallest palmitoylated huntingtin fragment contains six cysteine residues. **(C)** COS cells were transfected with N548-15 and metabolically labeled. Immunoprecipitates were treated with or without 1M NH$_2$OH and processed as previously described. Palmitate is cleaved from huntingtin following NH$_2$OH treatment (right), indicating that it is coupled to huntingtin through a thioester bond. **(D,E)** Palmitoylation of huntingtin occurs on cysteine 214. COS cells were transfected with plasmids encoding full length huntingtin or containing six, three N- or three C-terminal cysteines, tagged with GFP, or eight cysteines for C152S and C204S substitutions (diagram in **Figure 3.1B**). Huntingtin from labeled cells was analyzed as described above. Substitution of C214 to serine (C214S) abolished the palmitoylation of Huntingtin 79-224, Huntingtin 141-224 **(D)** and full length huntingtin **(E)**. **(F)** COS cells were transfected with N548, containing 15 or 128Q, and metabolically labeled. Huntingtin from labeled cells was analyzed as described above. **(G)** Results of four independent experiments were quantified using Image J software, adjusted for protein levels and demonstrated a reduction of ~50% in the palmitoylation of mutant huntingtin (p=0.001; Student's t-test).

3.3.2 Palmitoylation regulates distribution and function of huntingtin

We investigated whether the palmitoylation of huntingtin influences its subcellular distribution, frequency of inclusion body formation and toxicity. Palmitoylation-resistant full-length wild-type huntingtin (huntingtin-15(C214S)), mutant huntingtin (huntingtin-128(C214S)) and the N-terminal fragment N548-128(C214S) showed a marked redistribution of the protein in both COS and HEK-293 cells (Figure 3.3A,B arrows, Figure 3.4 and data not shown). Expression of N548-128(C214S) resulted in a threefold increase in the number of cells containing huntingtin inclusions (Figure 3.3B). This tendency for increased inclusion formation appeared specific to the C214S change, as mutation of an adjacent cysteine residue (C204S) did not increase the formation of inclusions (Figure 3.3B). Consistent with this, time-lapse imaging in COS cells revealed that inclusions formed three times faster in cells expressing palmitoylation-resistant huntingtin (N548-128(C214S); Figure 3.3C,D).
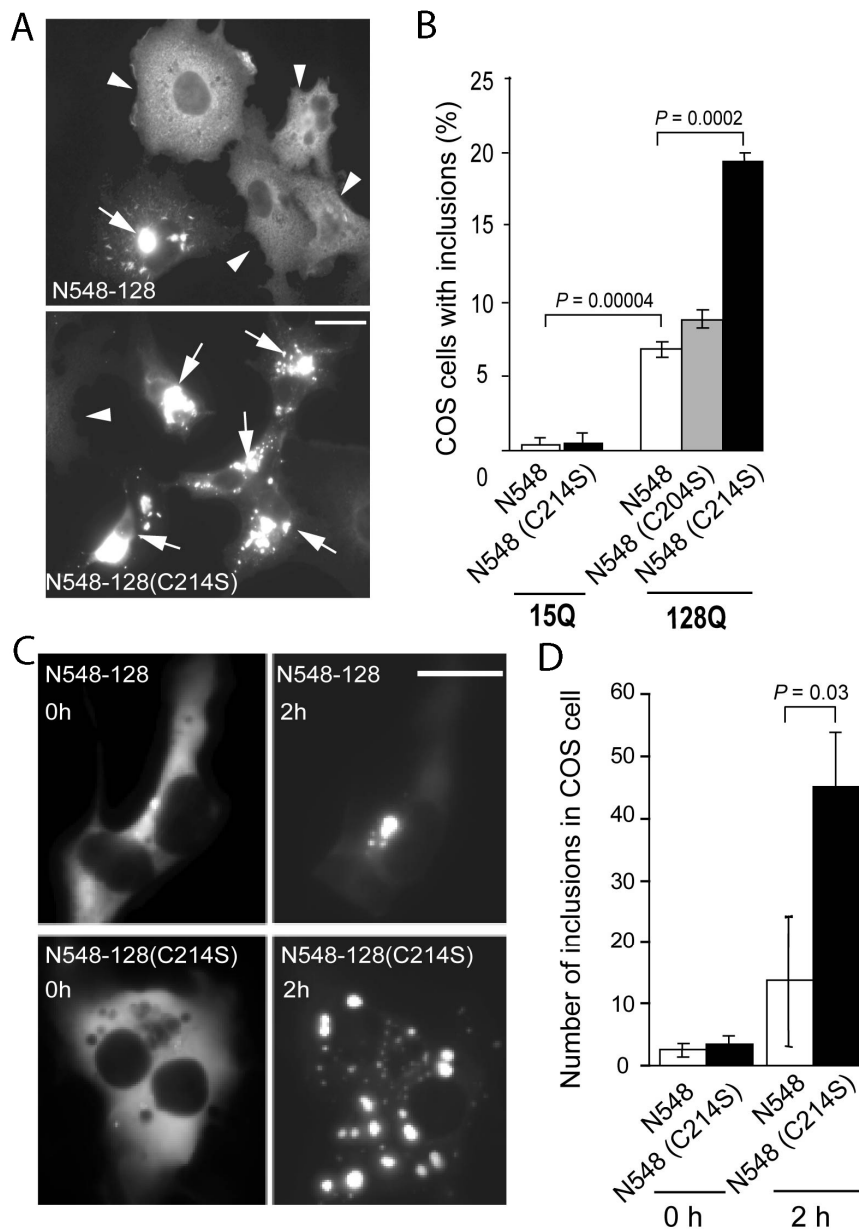
Figure 3.3. Increased inclusions of palmitoylation-resistant mutant htt in COS cells.
(A-D) COS cells were transfected with N548-128, with or without the C214S substitution, and stained with htt antibody. **(A)** Inclusion bodies (arrows) are occasionally seen with expression of N548-128. However, a C214S substitution altered mutant htt distribution and enhanced the formation of inclusions. Scale bar = 10 μm. **(B)** The percentage of cells containing inclusions increases in COS cells expressing N548-128 (6.7% ± 0.46) and is maximal in cells expressing N548-128(C214S) (19.4% ± 0.58). This effect was not observed with N548-128(C204S) (8% ± 0.68%). **(C)** Time lapse images captured over 2 hours revealed an accelerated rate of development of inclusions in the presence of the C214S substitution in mutant htt (lower panels). Scale bar = 10 μm. **(D)** The rate of inclusion formation in N548-128(C214S) transfected cells was significantly faster than in N548-128 transfected cells.
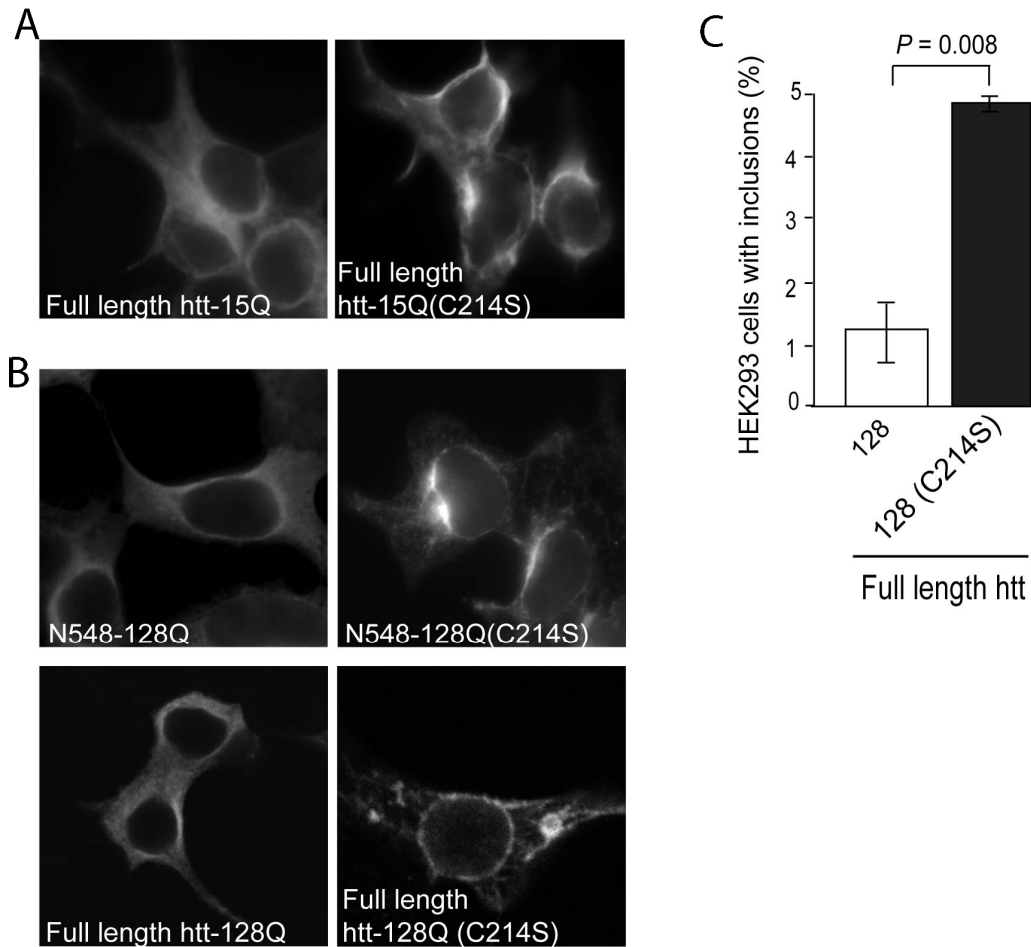
Figure 3.4. Altered distribution of palmitoylation resistant huntingtin in HEK-293 cells.

Cells were transfected with full-length huntingtin constructs and an N-terminal fragment of mutant huntingtin, with or without the C214S substitution, and stained with huntingtin antibody. The C214S substitution resulted in altered distribution of full length wt huntingtin **(A)** and of full length as well as a truncated form (N548-128) of mutant huntingtin **(B)** to a perinuclear compartment indicating a defect in protein sorting. **(C)** The percentage of cells containing full length mutant huntingtin inclusions was 1.2%, and significantly increased upon expression of mutant huntingtin with the C214S mutation (4.8%, p=0.008).

An important question is whether palmitoylation directly influences the trafficking of huntingtin or whether this effect could be operating via another mechanism. Mutation of the palmitoylated cysteine may have indirectly disrupted other protein-protein

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interactions or resulted in an altered conformation of huntingtin that promoted inclusion body formation. To exclude this possibility, we assessed whether treatment with drugs that specifically block protein palmitoylation alter huntingtin trafficking. The palmitate modification of huntingtin is transient, with a half-life of 2.5 h (Figure 3.5). This rapid turnover allowed us to use the inhibitor 2-bromopalmitate to block palmitoylation and investigate the trafficking of nonpalmitoylated huntingtin in cells. Indeed, 14 h of treatment with 2-bromopalmitate resulted in a significant increase in huntingtin inclusion bodies in cells expressing N548-128 (Figure 3.5; P =0.006). Taken together, these findings indicate that a defect in palmitoylation specifically alters huntingtin distribution in COS cells. In fact, inclusions in cells expressing palmitoylation-resistant huntingtin colocalized with γ-tubulin, ubiquitin, Lmp2 and Hsp70, indicating that C214S huntingtin is misfolded (Kopito 2000; Waelter *et al.* 2001) in both COS cells and neurons (Figure 3.6).
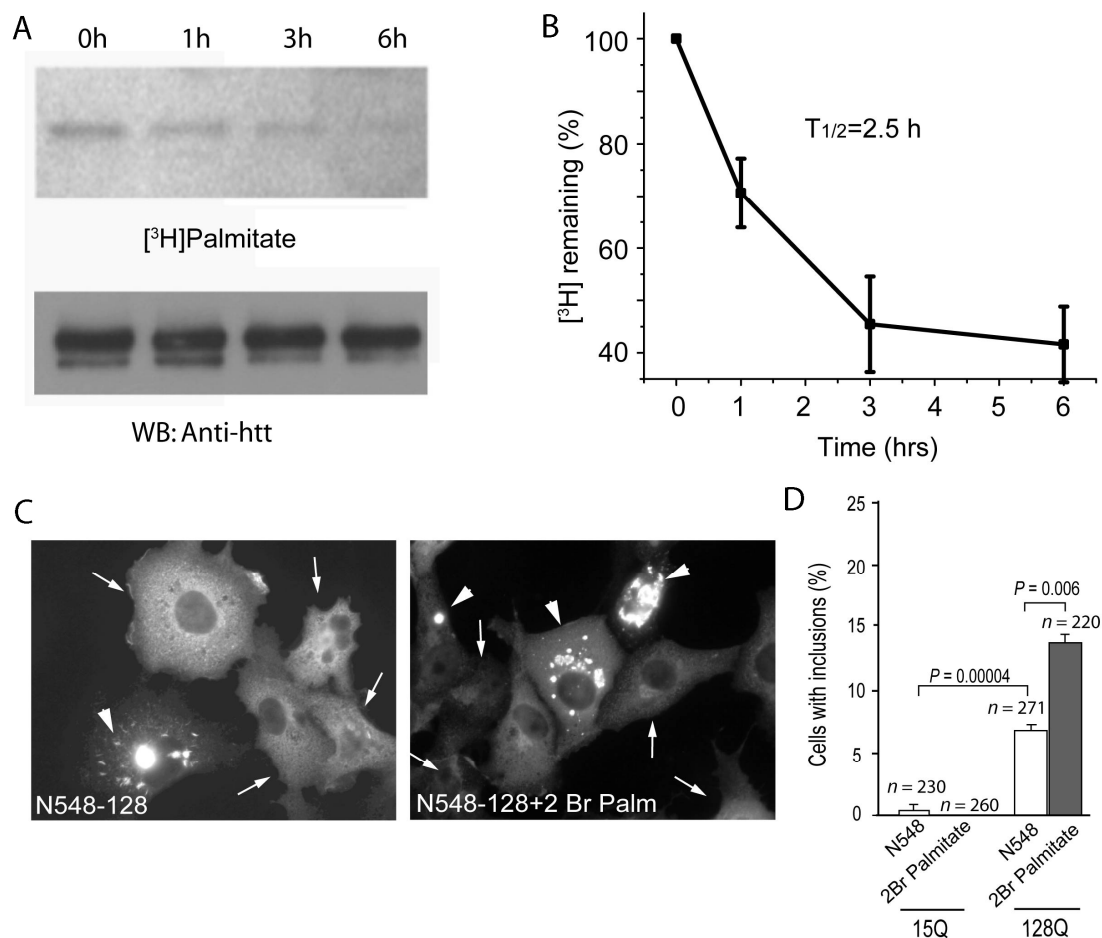
Figure 3.5. Plamitate on huntingtin turns over and inhibition of palmitoylation increases the inclusion formation.

(A,B) Palmitate turns over on huntingtin. **(A)** COS cells transfected with wild type huntingtin (N548-15) were metabolically labeled with [³H]-palmitate for 3 hours and then chased with unlabeled palmitate for 0,1,3 and 6 hours. Huntingtin was immunoprecipitated and subjected to SDS-PAGE and autoradiography. **(B)** The pulse-chase experiment demonstrated that palmitate turnover on wildtype huntingtin has a half-life of 2.5 hours. The half-life of palmitate reported here is the calculated time that would be needed to remove 50% of the original [³H]palmitate. **(C)** COS cells transfected with mutant huntingtin were treated with or without the palmitoylation inhibitor 2 bromopalmitate (2 Br Palm). Cells were stained with huntingtin antibody. 2 Br Palm treatment markedly altered mutant huntingtin distribution and significantly increased the number of inclusions. **(D)** The percentage of cells containing huntingtin inclusions in cells expressing mutant huntingtin was 6.7% (n=271), and significantly increased following 2 Br Palm treatment (13.7%; n=220, p=0.006).
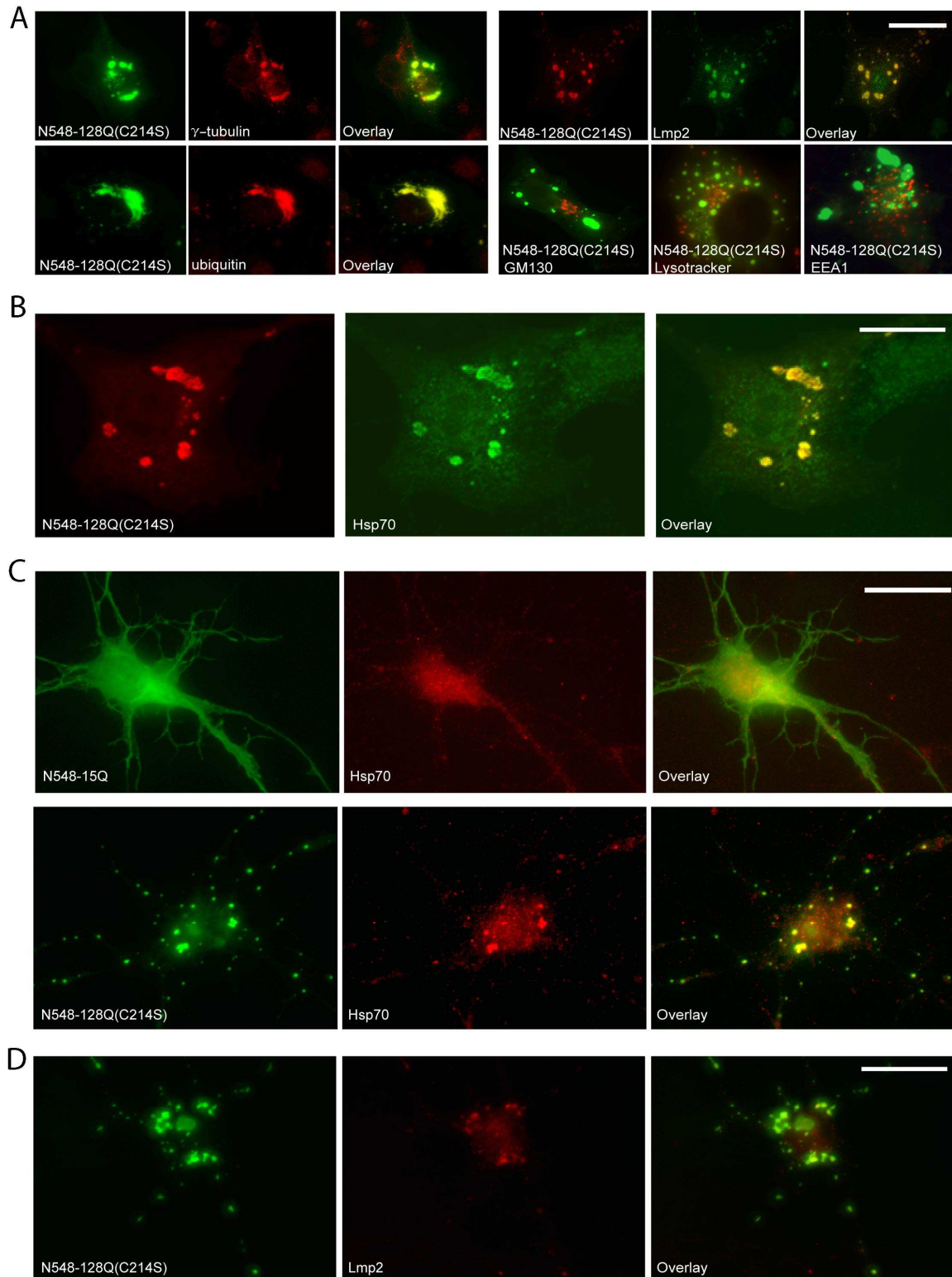
Figure 3.6. Altered distribution of palmitoylation-resistant mutant huntingtin in COS cells and neurons.

(A,B) COS cells transfected with N548-128(C214S) were immunolabeled with antibodies detecting huntingtin and several markers. Huntingtin inclusions colocalized with the centrosome marker γ-tubulin, the proteasome markers ubiquitin and Lmp2, and misfolded protein marker Hsp70, but not with the Golgi marker GM-130, the lysosomal marker Lysotracker or the early endosome marker EEA1. Scale bar: 10 μm in a, 5 μm in b. **(C)** Rat cortical neurons transfected with N548-15-GFP or N548-128(C214S)-GFP were immunolabeled with antibodies detecting Hsp70 (red). Mutant huntingtin with the C214S substitution accelerated the formation of inclusions that colabeled with Hsp70. Scale bar, 10 μm. **(D)** Representative images showing that the inclusions formed in cortical neurons expressing N548-128(C214S) also colocalized with Lmp2. Scale bar, 10 μm.

In an independent assay, we examined what effect the loss of huntingtin palmitoylation has on COS cell survival by analyzing nuclei of transfected cells for fragmentation or Pyknosis (Wyttenbach *et al.* 2002). Cell death in the presence of palmitoylation-resistant mutant huntingtin (N548-128(C214S)) was significantly higher than in the presence of huntingtin with polyglutamine expansion alone (Figure 3.7A,B; $P = 10^{-5}$). To examine whether these effects are seen in a cell type relevant to Huntington disease, we next investigated the functional effects of a loss of huntingtin palmitoylation on inclusion formation and cell survival in neurons. For both truncated (N548) and full-length huntingtin, inclusion formation was more frequent in the presence of polyglutamine expansion (Figure 3.7C-E) and was significantly increased when both wild-type ($P = 0.02$) and mutant huntingtin (N548: $P = 0.01$; full-length: $P = 10^{-5}$) were made palmitoylation resistant (C214S; Figure 3.7C-E). We then examined the effects of a loss of huntingtin palmitoylation on the localization of huntingtin in neurons. After transfection of wild-type and mutant full-length huntingtin, huntingtin was predominantly expressed in the cytosol and inclusions were seen only in the presence of mutant huntingtin (Figure 3.7D). In contrast, after transfection of palmitoylation-resistant huntingtin, inclusions were occasionally seen even in the presence of a normal polyglutamine tract (Figure 3.7D,E). Notably, in the presence of palmitoylation-resistant

huntingtin with polyglutamine expansion, the frequency of nuclear inclusions was increased (Figure 3.7D,E). Localization of mutant huntingtin in the nucleus is an early reproducible marker of huntingtin toxicity *in vivo* in animal models for Huntington disease(Van Raamsdonk *et al.* 2005a) and in humans(Sapp *et al.* 1997). This suggests that one mechanism for the localization of mutant huntingtin in the nucleus may be the decreased palmitoylation consequent to the expansion of the polyglutamine tract.

We made use of the fact that neurons expressing mutant huntingtin are more vulnerable to NMDA-induced toxicity(Zeron *et al.* 2002) by transfecting cortical neurons with N-terminal fragments of wild-type and mutant huntingtin, including palmitoylation-resistant huntingtin (C214S), treating the cells with 500 μM NMDA and assessing changes in neuronal viability. Notably, both wild-type and mutant huntingtin became significantly more toxic in the presence of the C214S mutation (Figure 3.7F; $P = 0.01$ and $P = 0.03$, respectively).
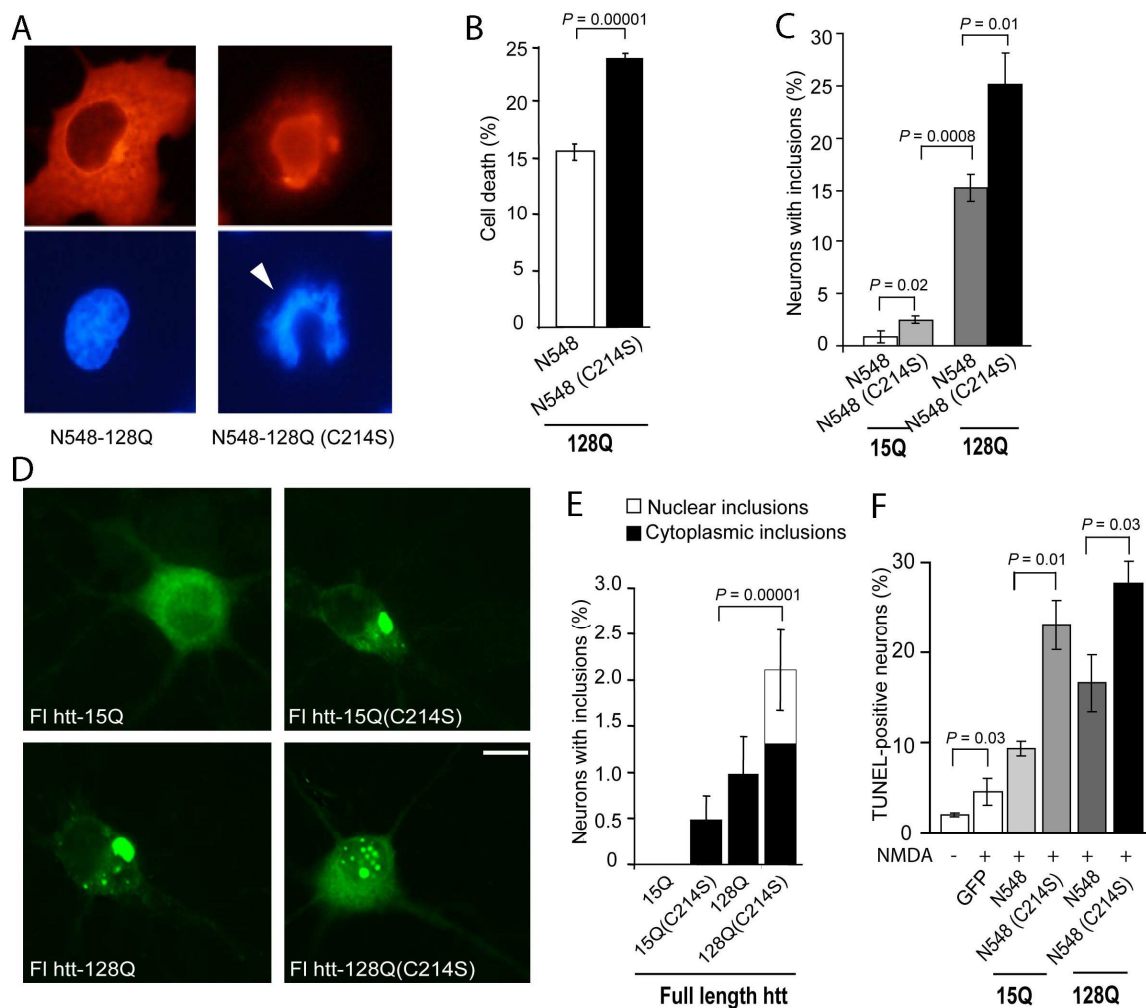
Figure 3.7. Enhanced toxicity of palmitoylation-resistant mutant huntingtin.
(A,B) COS cells transfected with N548-128 and N548-128(C214S) were scored for toxicity by analyzing nuclei for changes associated with cell death. Cell death was significantly ($P = 10^{-5}$) induced by the expression of N548-128(C214S). Representative images show a normal nucleus and an apoptotic nucleus (arrowhead). **(C–E)** Rat cortical neurons were transfected with truncated huntingin (N548) or full-length huntingtin (fl htt) constructs as indicated, and stained with huntingtin antibody. **(C)** The percentage of neurons containing inclusions increased in neurons expressing N548-15(C214S) (2.5 ±0.35%), increased still further in neurons expressing N548-128 (15.17 ±1.3%) and was maximal in neurons expressing N548-128(C214S) (25.08 ±3.1%). **(D)** Representative images showing neurons expressing the indicated fl htt constructs. Scale bar, 5 m. **(E)** The percentage of neurons containing inclusions increased in neurons expressing flhuntingtin-15Q(C214S) (0.5 ±0.27%), increased still further in neurons expressing fl htt-128Q (1 ±0.41%) and was maximal in neurons expressing fl htt-128Q(C214S) (2.1 ±0.44%). There was a significant ($P = 10^{-5}$) increase in the percentage of cells with nuclear inclusions in neurons expressing fl htt-128Q(C214S). **(F)** Rat cortical neurons transfected with the indicated huntingtin constructs were treated with NMDA for 10 min and processed for the TUNEL assay. The percentage of cell death was significantly greater in transfected cells expressing N548-15Q

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(C214S) (23.16 ±5.2; P = 0.01) and N548-128Q(C214S) (27.75% ±4.7; P = 0.03), demonstrating that the loss of palmitoylation significantly reduces cellular viability.

3.3.3 HIP14 regulates the palmitoylation and trafficking of huntingtin

HIP14, a protein that interacts with huntingtin *in vitro* (Singaraja *et al.* 2002), is a conserved mammalian palmitoyl transferase for different neuronal substrates, including huntingtin(Huang *et al.* 2004). HIP14 is mainly localized in the Golgi(Singaraja *et al.* 2002). Sorting of some neuronal proteins, such as Ras, requires palmitoylation for trafficking to Golgi membranes and for delivery to transport vesicles. Palmitoylated Ras shows a pronounced Golgi localization and faster retrograde trafficking from the plasma membrane to the Golgi(Huang and El-Husseini 2005). HIP14 alters the distribution of a subset of palmitoylated proteins in a palmitoylation-dependent manner(Huang *et al.* 2004). We found that HIP14 overexpression resulted in the redistribution of endogenous huntingtin (Figure 3.8A), and to a lesser extent of mutant huntingtin (Figure 3.8B), to the Golgi. This redistribution was not observed with palmitoylation-resistant huntingtin (Figure 3.8C), suggesting that huntingtin trafficking to the Golgi is at least partially regulated by palmitoylation.

HIP14's known interaction with huntingtin, combined with the fact that huntingtin is palmitoylated and its trafficking is regulated at least in part by palmitoylation, raised the possibility that HIP14 is involved in huntingtin palmitoylation *in vivo*. Overexpression of HIP14 with wild-type and mutant huntingtin significantly (P = 0.03 and P = 0.02, respectively) increased their palmitoylation (Figure 3.8D,E), demonstrating

that HIP14 catalyzes huntingtin palmitoylation. We obtained further evidence for the role of palmitoylation in the normal distribution of huntingtin by examining the effect of HIP14 on the formation of inclusions in neurons. Overexpression of HIP14 significantly ($P = 0.005$) reduced the number of inclusions seen in the presence of polyglutamine expansion (Figure 3.8F) but had no effect on that seen with palmitoylation-resistant mutant huntingtin (Figure 3.8F), highlighting the importance of HIP14 palmitoylation of huntingtin in the intracellular distribution and trafficking of huntingtin.
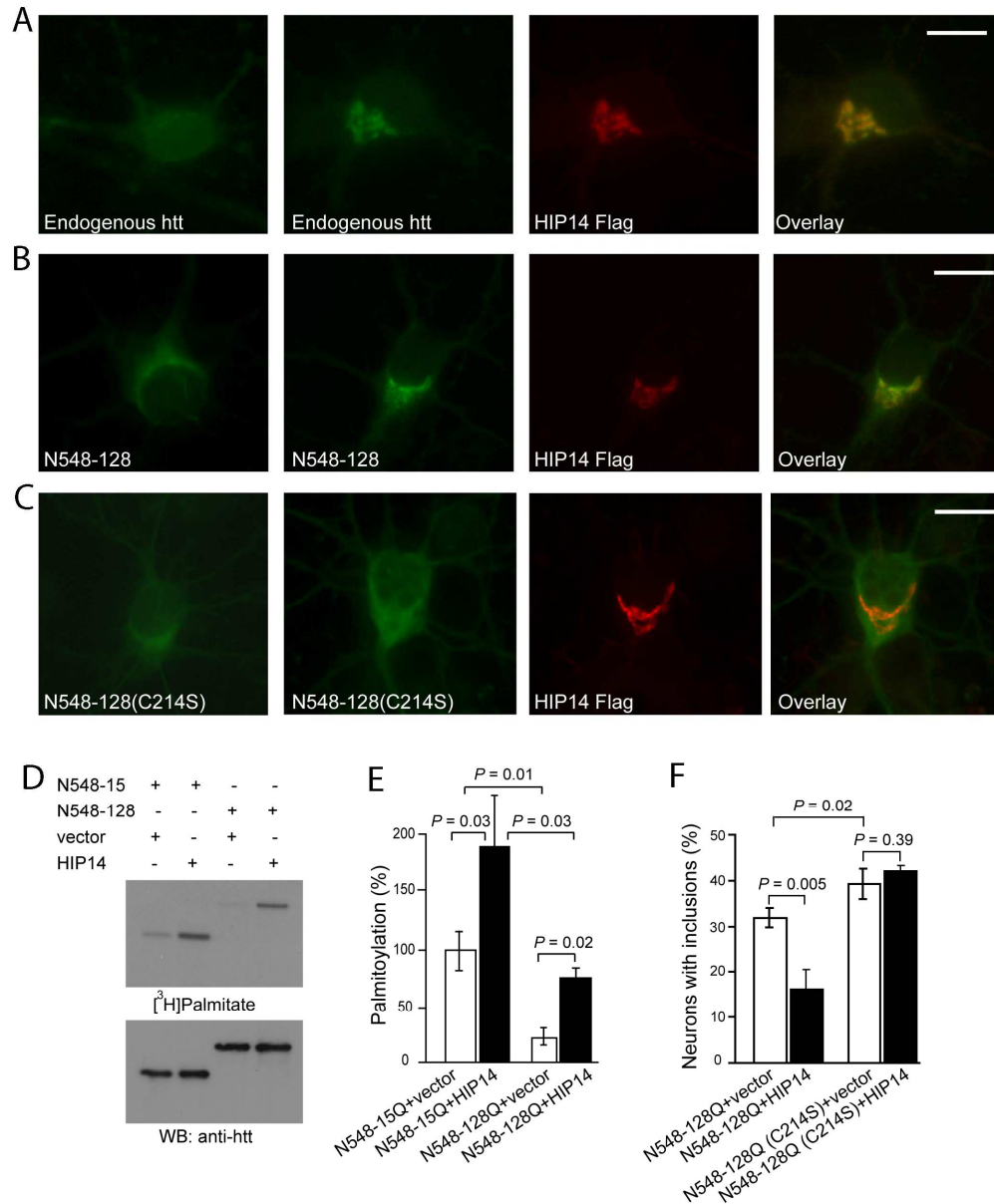
Figure 3.8. HIP14 influences the distribution and catalyzes the palmitoylation of htt in neurons.

(A) Cortical neurons transfected with control vector (left) or with FLAG-tagged HIP14 were labeled with the appropriate antibodies. The overexpression of HIP14 resulted in marked redistribution of endogenous htt to the Golgi. Scale bar, 5 μm. **(B,C)** Cortical neurons transfected with N548-128Q-GFP or N548-128Q(C214S)-GFP alone (left) or with HIP14-FLAG were labeled with the appropriate antibodies. Scale bar, 5 μm. **(B)** Partial redistribution of N548-128Q-GFP was observed in the presence of HIP14. **(C)** No effect in the distribution of palmitoylation-resistant htt (N548-128Q(C214S)-GFP) was observed in the presence of HIP14. **(D)** COS cells transfected with N548-15Q or N548-128Q, and with a control vector or HIP14, were metabolically labeled. Immunoprecipitated htt was analyzed as described above. **(E)** Palmitoylation was significantly ($P = 0.03$) increased in cells coexpressing wild-type htt and

HIP14. In addition, HIP14 significantly ($P = 0.02$) increased palmitoylation of mutant htt. However, in the presence of HIP14, mutant htt was still significantly ($P = 0.03$) less palmitoylated than wild-type htt. **(F)** Cortical neurons transfected with GFP-tagged N548-128Q or N548-128Q(C214S), and with a control vector or FLAG-tagged HIP14, were labeled with the appropriate antibodies. The percentage of cells containing htt inclusions was significantly ($P = 0.005$) lower in the presence of HIP14 whereas no change was detected in the number of C214S inclusions, emphasizing the importance of HIP14 palmitoylation of htt in its distribution.

The reduced palmitoylation of mutant huntingtin (Figure 3.2F) suggested that a defect in huntingtin palmitoylation may have resulted from an altered association with HIP14. Our analysis showed that the *in vivo* interaction of HIP14 with huntingtin was markedly reduced in the presence of mutant huntingtin in brains of YAC128 mice(Slow *et al.* 2003) (Figure 3.9A), indicating that reduced palmitoylation of mutant huntingtin results, in all likelihood, from a decreased association with HIP14. To further test this, we compared palmitoylation(Drisdel and Green 2004) of full-length wild-type huntingtin and mutant huntingtin obtained from the brains of YAC18 and YAC128 mice, respectively. Similar to our findings with the N-terminal fragment of mutant huntingtin in heterologous cells (Figure 3.2F), palmitoylation of full-length mutant huntingtin was significantly ($P = 0.01$) diminished in YAC128 brain extracts (Figure 3.9B,C).
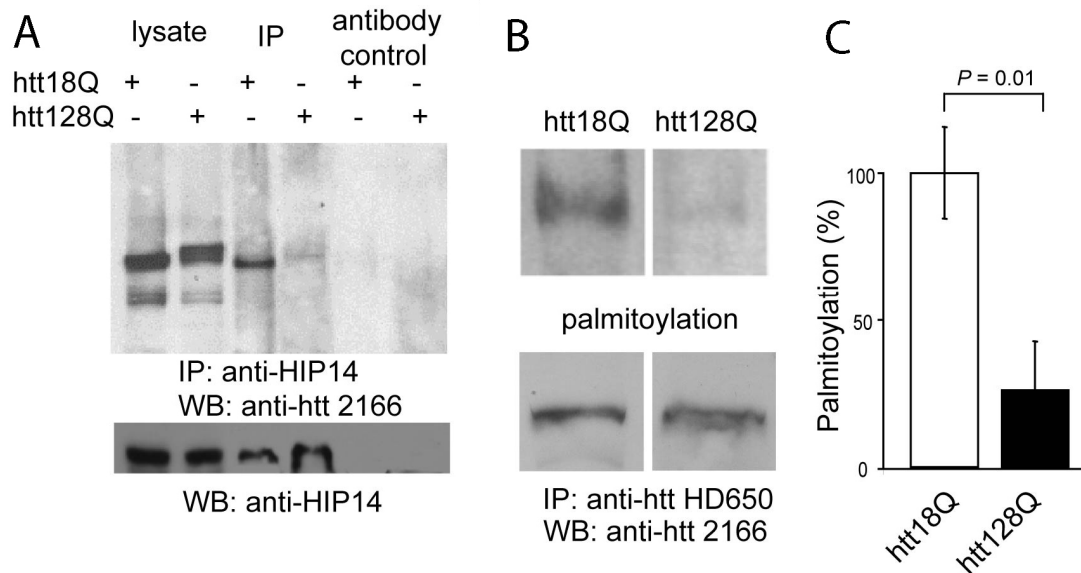
Figure 3.9. HIP14 associates less with mutant htt, and mutant htt is less palmitoylated *in vivo*.
(A) Coimmunoprecipitation of HIP14 and htt from brains of YAC18 and YAC128 mice demonstrated a weaker interaction between mutant htt and HIP14. **(B)** Immunoprecipitated htt from YAC18 and YAC128 mouse brain lysate was incubated in the presence or absence of 1 M NH_2OH and labeled with Btn-BMCC sulfhydryl-specific reagent. Western blots were probed with streptavidin-conjugated HRP (top) to detect biotin-labeled htt or probed with htt antibody (bottom) to detect total immunoprecipitated htt. **(C)** Palmitoylation was significantly ($P = 0.01$) reduced in htt with an expanded polyglutamine tract.

To further investigate the alteration in the palmitoylation of mutant huntingtin and its decreased association with HIP14, and their role in the disturbed trafficking and increased inclusion formation seen in Huntington disease, we used a small interfering RNA (siRNA) that disrupts HIP14 expression as previously described(Huang *et al.* 2004) (Figure 3.10). Neurons derived from YAC18 and YAC128 brains and transfected with HIP14 siRNA showed significant redistribution of both wild-type and mutant huntingtin (Figure 3.11A-F; $P = 3 \times 10^{-4}$ and $P = 8 \times 10^{-5}$, respectively). In YAC128Q neuronal

cultures, HIP14 downregulation resulted in the increased formation of huntingtin inclusions (Figure 3.11B, arrowhead). Accordingly, we observed a significant increase in inclusions in cortical neurons transfected with N548-128 and HIP14 siRNA (Figure 3.11G; $P = 0.025$). Moreover, reduced levels of HIP14 significantly increased perinuclear distribution of the proteasome marker Lmp2 (Figure 3.11C,F; $P = 0.02$). In contrast, we observed no increase in the perinuclear accumulation of several other proteins examined, including GM130, the NMDAR receptor subunit NR1 and the postsynaptic proteins PSD-95 and SAP-102, upon downregulation of HIP14; this indicated that the altered trafficking of huntingtin is not due to a disruption of Golgi function or a generalized change in protein sorting (Figure 3.10 and data not shown). We also found that decreasing HIP14 expression in neurons increased their susceptibility to NMDA treatment (Figure 3.11H), indicating that reduced palmitoylation is detrimental to neuronal viability. These results establish that HIP14 is critical for the normal targeting and folding of huntingtin *in vivo*. Our discoveries that HIP14 palmitoylates huntingtin (Figure 3.8D) and that polyglutamine expansion markedly decreases the interaction of huntingtin with HIP14 *in vivo* (Figure 3.9A) provide an explanation for the decreased palmitoylation of mutant huntingtin.
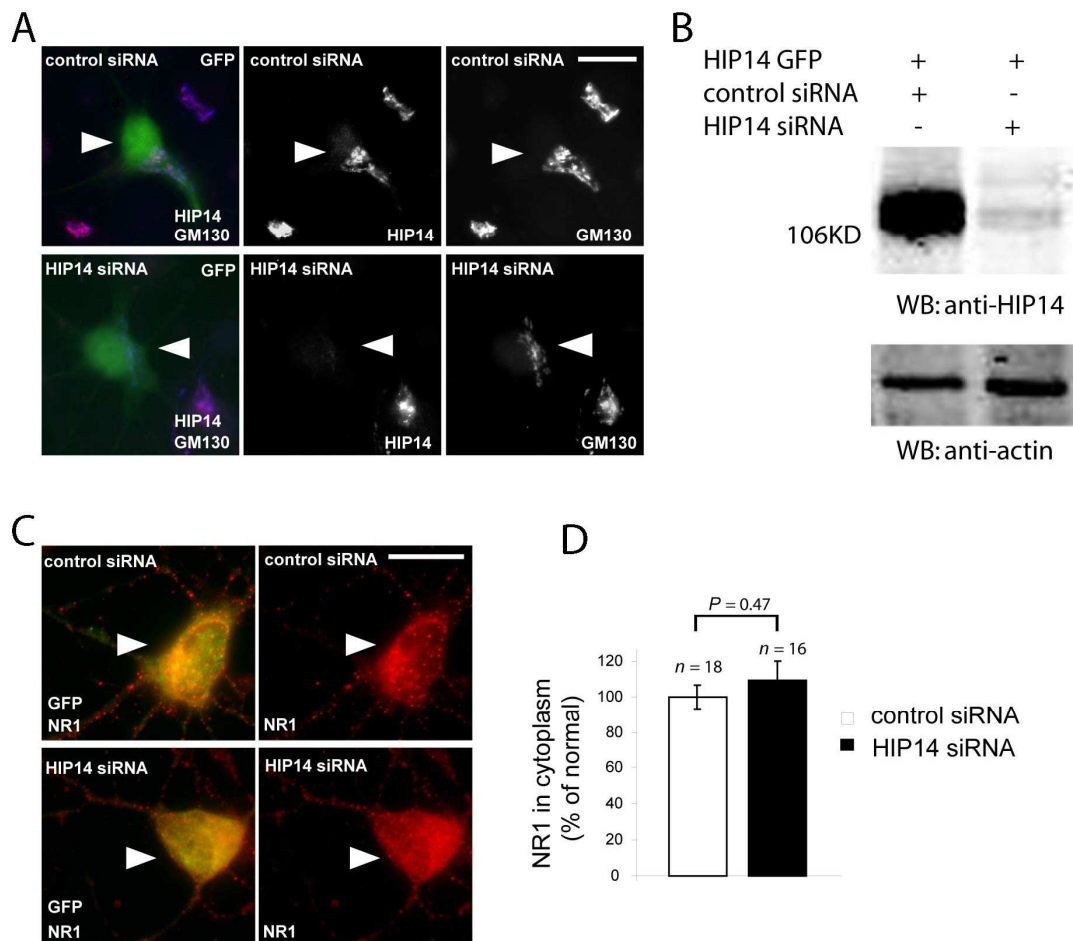
Figure 3.10. Knockdown of HIP14 in neurons by HIP14 specific siRNA.

(A) Wild type mouse cortical neurons (DIV 7) were transfected with either HIP14 or control siRNA. At DIV 10, neurons were fixed and stained with antibodies against HIP14. Loss of HIP14 staining is evident in neurons transfected with HIP14 siRNA (bottom panels) but not with control siRNA (top panels). As a control, GM130 staining shows that the Golgi structure is intact. **(B)** Western blot analysis of COS cells transfected with HIP14-GFP and with either HIP14 or control siRNA were probed with HIP14 antibodies (top panel) and actin (bottom panel) demonstrating that HIP14 siRNA markedly decreases expression of HIP14 GFP. **(C,D)** As a control, cytoplasmic NR1 was not affected by HIP14 knockdown.
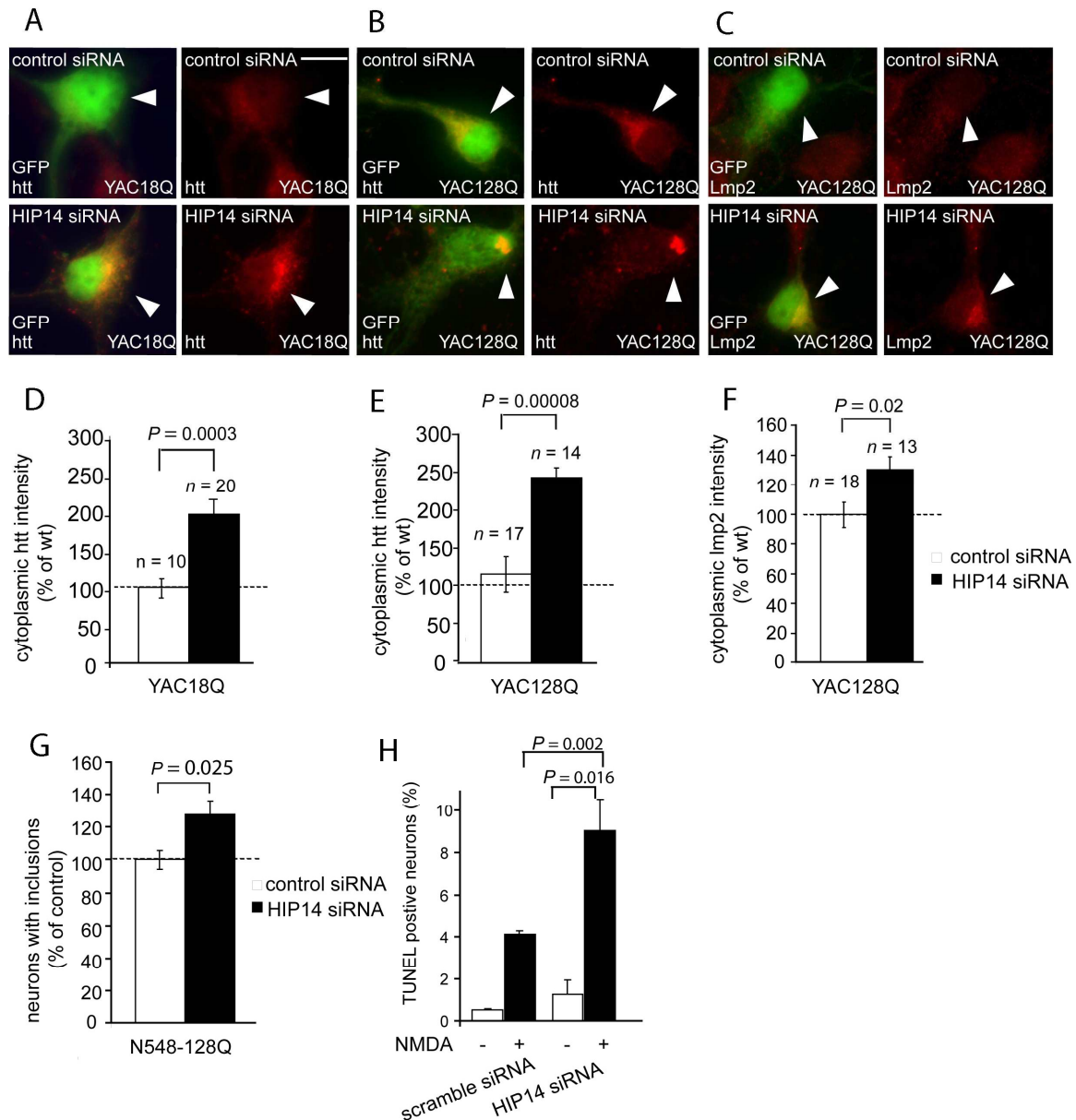
Figure 3.11. HIP14 regulates the palmitoylation and distribution of huntingtin *in vivo*.

(A–F) Downregulation of HIP14 expression altered the trafficking of wild-type and mutant htt and increased perinuclear accumulation of Lmp2. Cortical neurons (6 DIV) from YAC18 and YAC128 mice were transfected with GFP and either control siRNA or HIP14 siRNA. Arrowheads in panels a,b and c indicate transfected cells. Scale bar, 5 μm. Representative images show an increase in the cytoplasmic accumulation of wild-type htt in YAC18 neurons **(A)**, and mutant htt **(B)** and Lmp2 **(C)** in neurons from YAC128 mice expressing HIP14 siRNA. Quantification of htt **(D,E)** and Lmp2 **(F)** staining intensity in the cytoplasm of neurons expressing control or HIP14 siRNA demonstrated that htt and Lmp2 intensities were substantially higher in cells expressing HIP14 siRNA. **(G)** Downregulation of HIP14 in neurons transfected

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with N548-128 significantly ($P = 0.025$) increased the percentage of neurons with inclusions. **(H)** Downregulation of HIP14 in neurons decreased cellular viability following NMDA-induced cell death. Cortical neurons (8 DIV) were infected with lentiviruses expressing either control or HIP14 siRNA for 3 d, followed by NMDA treatment (Methods). The percentage of cell death in neurons infected with HIP14 siRNA virus was significantly ($P = 0.002$) higher, demonstrating that decreased expression of HIP14 significantly increases cell death.

3.4. DISCUSSION

In this study, we demonstrated a critical role for palmitoylation in regulating the trafficking and folding of huntingtin. Palmitoylation may contribute to the sorting of huntingtin to cytosolic transport vesicles or it may serve as a structural signal for proper protein folding and for the association of huntingtin with other molecules required for its proper trafficking. A protein domain in the N terminus of huntingtin, spanning amino acids 172–372, is essential for the membrane association of huntingtin and for targeting wild-type huntingtin to the plasma membrane(Kegel *et al.* 2005). Here we showed that the palmitoylation of huntingtin at cysteine 214 might be contributing to this finding. The importance of palmitoylation for the correct folding and assembly of the α7 nicotinic receptor, for PSD-95–regulated clustering and for the function of AMPA-type glutamate receptors at the synapse has recently been demonstrated(El-Husseini Ael *et al.* 2002; Drisdel *et al.* 2004). Notably, the reduced palmitoylation of nicotinic receptors results in their aggregation(Rakhilin *et al.* 1999), similar to that seen in palmitoylation-deficient mutant huntingtin. Also, the reversible nature of palmitoylation makes it subject to regulation by several stimuli that modulate neuronal protein function and synaptic strength. For instance, cycles of palmitoylation and depalmitoylation regulate the localization and function of specific Ras isoforms(Rocks *et al.* 2005) and of R7BP (Drenan *et al.* 2005), a membrane anchor for the RGS7 family. Furthermore, the cycling

of palmitate on PSD-95 at the synapse is also regulated by neuronal activity, and this modulates the retention of both PSD-95 and specific glutamate receptor subunits at the synapse(El-Husseini Ael *et al.* 2002). How the disturbance of huntingtin palmitoylation may be contributing to previously described disturbances in synaptic transmission in Huntington disease(Zeron *et al.* 2002) remains to be determined. The palmitoylation of huntingtin normally occurs as a result of its interaction with HIP14. The presence of polyglutamine expansion disturbed the interaction of huntingtin with HIP14 (Figure 3.9A), resulting in both decreased palmitoylation (Figure 3.2F and Figure 3.9B) and altered distribution of huntingtin (Figure 3.3, Figure 3.6, Figure 3.7, Figure 3.8); consequently, huntingtin accumulated in inclusions and failed to reach its appropriate cellular destinations. Palmitoylation thus is an important regulator of huntingtin trafficking *in vivo*. When huntingtin is less palmitoylated, as seen in the YAC model for Huntington disease, disturbances in its trafficking are evident; this is associated with enhanced toxicity and increased cell death in neurons. The presence of insoluble huntingtin inclusions in the brains of individuals with Huntington disease has led to the hypothesis that these inclusions contribute to the neuronal dysfunction and ultimate cell death that are characteristics of the disease. Much research has focused on these inclusions and on the discovery of aggregation inhibitors as possible therapeutic interventions. However, an increasing body of data suggests that these inclusions are not the disease-causing agents. For example, in YAC128 mouse models of Huntington disease, huntingtin inclusions are first observed months after the initial onset of motor and cognitive dysfunction(Slow *et al.* 2003). In addition, experimental manipulation of mouse models of the disease has revealed the dissociation between insoluble inclusions

and neuronal dysfunction and loss(Mastroberardino *et al.* 2002; Ferrante *et al.* 2003). Furthermore, in a transgenic mouse model expressing a short fragment of huntingtin whose CAG size, tissue distribution and level of expression are identical to those in the full-length YAC128 model, inclusions form earlier and are more prevalent, but this model does not manifest the neuronal dysfunction or degeneration present in the YAC mouse(Slow *et al.* 2005). Results from recent *in vitro* studies(Arrasate *et al.* 2004) indicate that although the insoluble form of huntingtin may not be toxic, it is likely that a soluble, diffuse form of huntingtin is toxic to neurons. In this study, we showed that inclusion formation, resulting from the decreased palmitoylation of huntingtin, serves as a biomarker for the altered trafficking of huntingtin but does not necessarily directly cause cell death or cellular toxicity.

Removal of palmitate from proteins is thought to be mediated by thioesterases, which cleave cysteine linkages(Verkruyse and Hofmann 1996; Soyombo and Hofmann 1997). We have shown that the increased expression of HIP14 with increased palmitoylation of mutant huntingtin partially restores the normal trafficking and distribution of huntingtin. Once the enzymes involved in the depalmitoylation of huntingtin are identified, inhibiting their function could result in increased palmitoylation of mutant huntingtin; potentially, this could restore normal trafficking and alleviate the cellular defects induced by polyglutamine expansion in this protein.

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4. CHAPTER 4   Neuronal Palmitoyl Acyl Transferases Exhibit Distinct Substrate Specificity

A version of this chapter will be submitted for publication.

4.1. INTRODUCTION

Protein palmitoylation represents a common lipid modification of neuronal proteins. This posttranslational change involves addition of the saturated 16 carbon palmitate lipid in a thioester linkage to specific cysteine residues. In neurons, at least 32 proteins have been shown to be palmitoylated(el-Husseini Ael and Bredt 2002). These include channels, cell adhesion molecules, scaffolding molecules, neurotransmitter release machinery and signaling proteins (Huang and El-Husseini 2005; Resh 2006a; Roth *et al.* 2006a; Linder and Deschenes 2007) as well as huntingtin(Huang *et al.* 2004; Yanai *et al.* 2006), a protein that when mutated causes Huntington disease (HD)(The Huntington's Disease Collaborative Research Group. 1993).

Importantly, palmitoylation occurs in a reversible fashion, which allows palmitoylation to dynamically regulate protein function and to participate in diverse aspects of neuronal signaling (reviewed in(el-Husseini Ael and Bredt 2002; Huang and El-Husseini 2005)). For example, glutamate receptor activity regulates palmitoylation of PSD95, where the regulated addition and removal of palmitate on this postsynaptic scaffolding protein in turn regulates the synaptic retention/removal of glutamate receptors(El-Husseini Ael *et al.* 2002). This process is thought to be fundamental for synaptic plasticity, the dynamic changes in the content and morphology of synapses associated with learning and memory.

The identity of the protein fatty acyltransferases (PATs) that enzymatically modify palmitoylated proteins has recently been discovered. The defining feature of this family of PATs is the presence of a cysteine-rich domain (CRD) with a core Asp-His-His-Cys

(DHHC) motif, which is essential for PAT activity both *in vitro* and *in vivo*(Roth *et al.* 2002; Fukata *et al.* 2004; Huang *et al.* 2004). Proteins with DHHC-CRD are conserved from yeast to mammals. Genetic and biochemical studies have identified substrates for several DHHC proteins in *S. cerevisiae* (Lobo *et al.* 2002; Roth *et al.* 2002), and proteomic analyses have expanded the repertoire of substrates for this family of enzymes significantly (Roth *et al.* 2006a). Mammals contain 23 DHHC proteins(Fukata *et al.* 2004). Their tissue distribution and subcellular localization has recently been documented (Ohno *et al.* 2006). When overexpressed in HEK cells, a majority of these DHHC proteins localize to the ER, Golgi and endosomal vesicles and some also localize to the plasma membrane(Ohno *et al.* 2006). However, whether the localization of an enzyme directly correlates with its potential substrate is not yet known. An unanswered question is whether different PATs exhibit distinct substrate specificity and what factors may influence this selectivity. In this study, we chose four brain enriched DHHC containing enzymes, namely DHHC-3, DHHC-8, DHHC-13 (HIP14L) and DHHC-17 (HIP14), to examine these questions.

The methods used to quantify protein palmitoylation involve metabolic labeling of cultured cells with radiolabeled palmitate ($[^3H]$-palmitate or $[^{125}I]$-palmitate) for 3-5 hours before harvesting cells and immunoprecipitating the protein(s) of interest (protocol reviewed in (Resh 2006b). The use of this approach is limited to cultured live cells as well as incubation with expensive radiolabeled palmitate for 3-5 hours. The fraction that is available for labeling is also limited by the rate of palmitate turnover on a particular protein during the labeling process. As a result, this method, not only requires weeks to months to expose the readiolabeling signal, but also only allows detection of a small

fraction of the total palmitoylated pool of a protein. A novel approach developed by Drisdel and Green overcomes these limitations by allowing detection of the total pool of palmitoylated proteins present in tissue extracts(Drisdel and Green 2004; Drisdel *et al.* 2004). This approach is known as "fatty acyl exchange labeling", which involves (i) blockade of free thiols with N-ethylmaleimide; (ii) cleavage of the Cys-palmitoyl thioester linkage with hydroxylamine; and (iii) labeling newly exposed thiols with a sulfhydryl-specific labeling compound, such as non-radioactive biotin-BMCC (1-biotinamido-4-[4'-(maleididomethyl) cyclohexanecarboxamido] butane)(Drisdel and Green 2004; Drisdel *et al.* 2004). The palmitoylated protein can then be detected by western blotting using strepdavidin or anti-biotin antibodies. This approach is highly sensitive and allows the use of a variety of probes, radiolabeled ($[^3H]$-NEM) or non-radioactive (biotin-BMCC), and facilitates quantitative estimates of the total palmitoylated pool of a specific protein. This method also allows for rapid assessment of changes in the dynamics of protein palmitoylation after stimulation of specific signaling pathways, or upon alterations in neuronal activity (Drisdel *et al.* 2006).

Using this approach, we examined the enzymatic activity of four neuronal DHHC proteins, DHHC-3, DHHC-8, DHHC-13 and HIP14, toward several proteins. We determined that these DHHC proteins have substrate specificity/preference, a finding that is in agreement with the loss-of-function analysis in yeast (Roth *et al.* 2006a). Our study also suggests that domains within the protein, other than the catalytic DHHC domain, may influence the specificity for the interaction between the enzyme and its substrate.

4.2. MATERIALS AND METHODS

4.2.1 Plasmid Constructions

The following cDNAs were used for COS cell transfection: DHHC-3 cDNAs (Flag tagged and GFP tagged) (provided by Dr. Bernhard Lüscher, Penn State university), DHHC-8 in pEF-Bos-HA (provided by Dr. Masaki Fukata, National Institute for Physiological Sciences Japan and Dr. David Bredt, Eli Lilly), GluR1 and GluR2 GFP in pRK5 (provided by Dr. Takashi Hayashi, and Dr. Richard L. Huganir, Johns Hopkins University), DHHC-13 Flag and HIP14 Flag in pCIneo vector, HIP14ΔDHHC (Flag tagged and GFP tagged) lacking amino acids 440 to 487 in pCIneo vector, HIP14ΔANK-GFP lacking amino acids 89 to 257 in pCIneo vector, SNAP25 in pEGFP-C1 vector, PSD95-GFP in pGW1 vector, PSD95 Flag in pcDNA3 vector, huntingtin 1-548aa in pCIneo vector, paralemmin in pEGFP-C1 vector. ANK-DHHC3 was generated by two step cloning: first, DHHC-3 cDNA was subcloned into Hind III and EcoR I sites of pEGFP N3; Then, HIP14 amino acid 1-294 was further subcloned into the BglII and HindIII sites of the previous construct.

4.2.2 Antibodies and chemicals

The following primary antibodies were used for immunocytochemistry and immunoblot (immunoreactivity and dilution as indicated): huntingtin 2166 (mouse, 1:1000, Chemicon), HA (mouse, 1:1000, BABCO), Flag M2 (mouse, 1:1000, Sigma), GFP (rabbit, 1:1000, homemade), GFP (rabbit, 1:2000, synaptic system), GM130 (mouse, 1:200, BD Biosciences), Calnexin (rabbit, 1:500, Sigma), EEA1 (mouse, 1:200, Abcam), PSD-95 (rabbit, 1:1000, homemade), and synaptophysin (mouse, 1:1000, Abcam).

Appropriate fluorescently conjugated secondary antibodies for immunocytochemistry were used as previously described (Prange *et al.* 2004). Fluorescently conjugated secondary antibodies for immunoblotting were IRDye 800 antibody (mouse, 1:10000, Rockland) and Alexa fluor 680 antibody (rabbit, 1:10000, Invitrogen).

4.2.3 Cell Culture and Transfections

All reagents for cell cultures were purchased from Invitrogen Corporation (Carlsbad, CA). COS cells were cultured as previously described(Huang *et al.* 2004). COS cells were transiently transfected with Lipofectamine 2000 (Invitrogen) as indicated by the manufacturers. 24 to 48 hours post-transfection, cells were processed as described for each experiment.

4.2.4 Immunocytochemistry and Imaging

The coverslips were removed from culture wells and fixed in 2% PFA. The cells were washed three times with phosphate-buffered saline containing 0.3% Triton-X-100 before each antibody incubation. All of the antibody reactions were performed in blocking solution (2% normal goat or horse serum) for 1 h at room temperature or overnight at 4 °C. The coverslips were then mounted on slides (Frost Plus; Fisher) with

Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL). Images were acquired on a Zeiss Axiovert M200 motorized microscope by using a monochrome 14-bit Zeiss Axiocam HR charge-coupled device camera at 1,300×1,030 pixels.

4.2.5 Immunoprecipitation and Immunoblotting

COS-7 cells were lysed in ice-cold buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton, 1 mg/ml protease inhibitor cocktail (Roche), and 0.25 mg/ml

PMSF. Cell lysates were rotated at 4°C for 30 min before the insoluble material was

removed by centrifugation at 14,000 rpm for 15 min. Lysates were precleared by

incubation with protein A+G agarose (GE health) for 45 min at 4°C with rocking.

Precleared samples were then incubated with anti-GFP (3ug, rabbit, homemade) antibody

at 4°C for an hour, followed by incubation with 30ul of protein A agarose (GE health) for

another hour. Proteins in both the cell lysates and immunoprecipitates were heated in

SDS sample buffer before separation by SDS-PAGE. After overnight transfer of the

proteins onto nitrocellulose membranes, western blots were performed using the

antibodies described above (see Antibodies and chemicals).

4.2.6 Btn-BMCC Labeling

After the immunoprecipitation, the beads were then washed with wash buffer

(HEPES, containing 1%Triton X-100) supplemented with 50 mM NEM for an hour at

4°C, followed by treatment with 1 M hydroxylamine pH 7.4 for 1 hour at room

temperature. Samples were then processed as previously described (Drisdel and Green

2004).

4.2.7 Statistical Analysis

The results are expressed as mean ± SD. Statistical comparisons between groups

were done by the *t* test, using the Statgraphics Plus 5.0 program (Statistical Graphics

Corp.).

4.3. RESULTS

4.3.1 DHHC-3, -8, -13 (HIP14L) and –17 (HIP14) all reside in the golgi compartment

23 DHHC containing proteins have recently been cloned. We chose four that are enriched in the brain. Previous studies have established the subcellular localizations of some of these DHHC proteins. DHHC-3 (also named GODZ) localizes to the Golgi apparatus(Uemura *et al.* 2002). HIP14 is primarily concentrated in Golgi apparatus but also associated with endosomal vesicles in soma and processes(Singaraja *et al.* 2002; Huang *et al.* 2004). However, in drosophila motor neurons, exogenous HIP14 localized in presynaptic terminals(Ohyama *et al.* 2007; Stowers and Isacoff 2007). Overall, the homology among these four DHHC proteins is restricted to the cysteine rich domain (Figure 4.1A, B), as is shown in the schematic illustration. For example, the ankyrin repeat domain, which is common in HIP14L and HIP14, is not found in either DHHC-3 or DHHC-8. Despite their distinct difference in molecular identity, when expressed in COS cells, these four proteins all colocalized with the Golgi marker GM130 (Figure 4.1C), but not with EEA1, an early endosomal marker, nor with Calnexin, an ER marker (Figure 4.2). In rat cultured *Day in vitro*18 hippocampal neurons, endogenous HIP14 mainly associates with the Golgi apparatus, and sparsely associates with vesicles (Huang *et al.* 2004). Co-immunostaining of endogenous HIP14 with a presynaptic marker synaptophysin or a postsynaptic marker PSD95 shows that HIP14 is is not obviously present in synapses (Figure 4.1D).
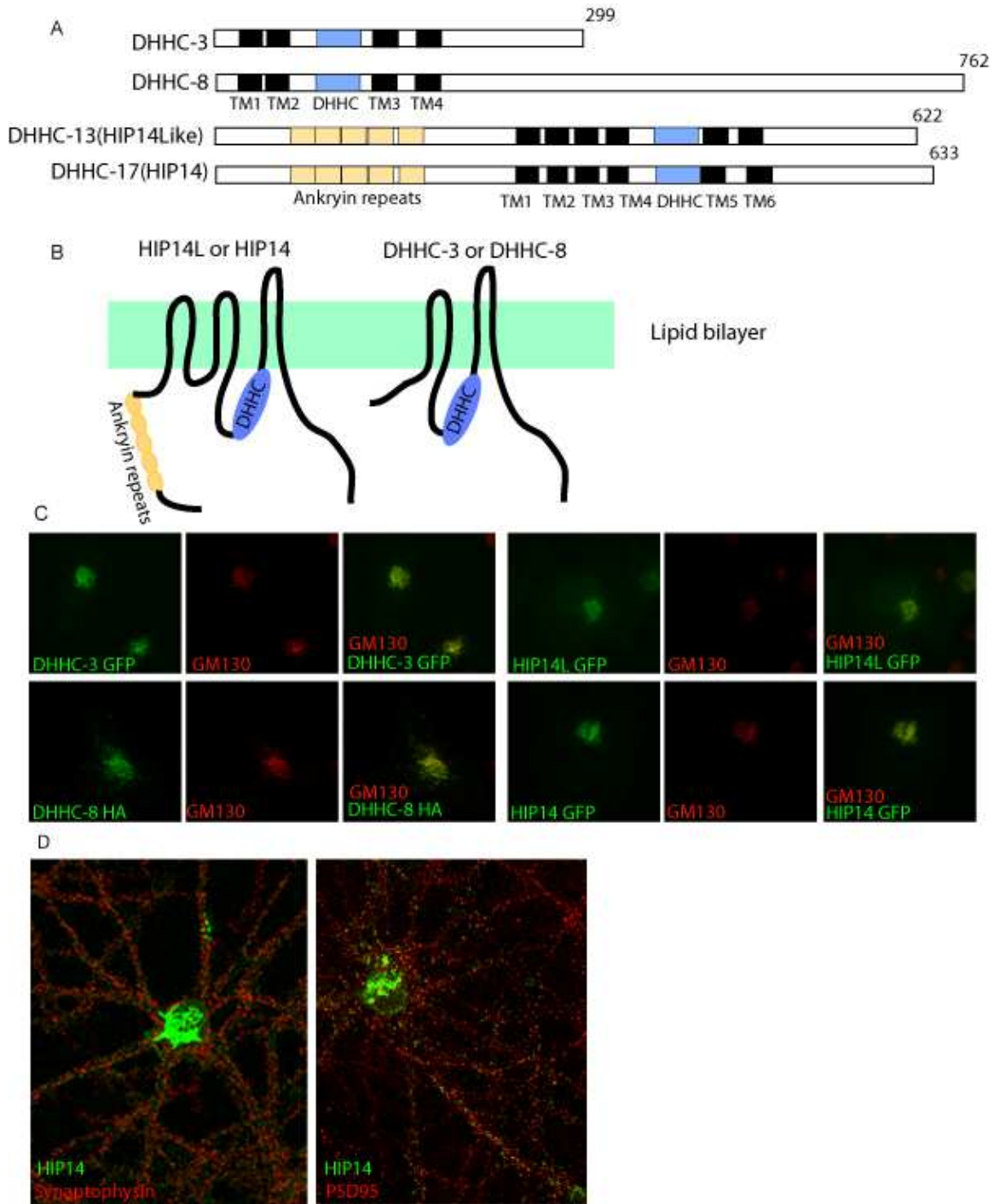
Figure 4.1. Structures and Localizations of four neuronal DHHC proteins.

(A) Schematic illustration of the primary structures of the four DHHC proteins. **(B)** 3D schematic illustration of the primary structures of the four DHHC proteins. **(C)** Localization of four DHHC proteins in COS cells. Exogenous DHHC-3, DHHC-8, HIP14L and HIP14 (green) are localized in the Golgi compartment which is positive for GM130 (red), a cis-Golgi marker. **(D)** Localization of HIP14 in *DIV* 18 hippocampal neurons. Endogenous HIP14 in neurons do not

colocalize with either a presynaptic protein marker synaptophysin or a postsynaptic protein marker PSD95.
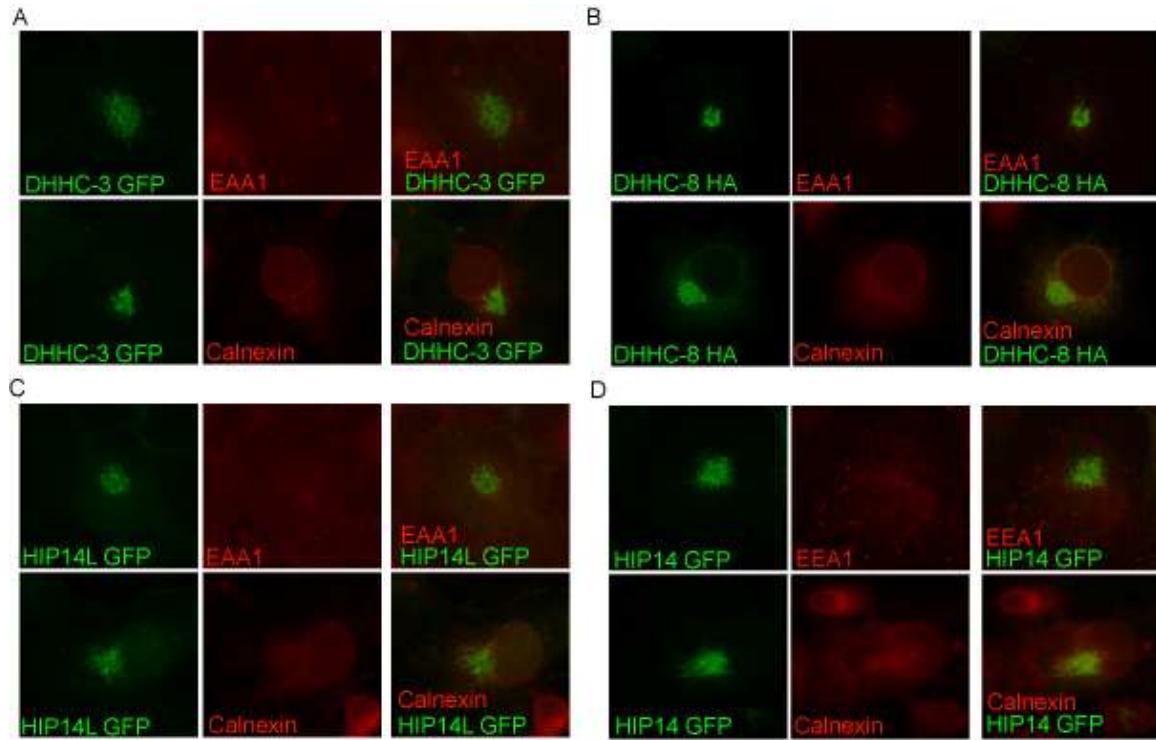
Figure 4.2. DHHC-3, -8, HIP14L (DHHC-13) and HIP14 do not colocalize with early endosomal marker or with ER marker.

(A,B,C,D) Green Fluorescent Protein (GFP) tagged DHHC proteins were overexpressed in COS cells and stained with EAA1 (red), an early endosomal marker, or with Calnexin (red), an ER marker.

4.3.2 DHHC-3, -8, -13 (HIP14L) and –17 (HIP14) exhibit distinct substrate preferences

To determine the level of protein palmitoylation, we used the acyl biotin exchange (ABE) assay. Unlike radioactive palmitate labeling, the ABE palmitoylation assay relies on hydroxylamine cleavage of the thioester bond between the fatty acid and the cysteine side chain, followed by reaction of the newly generated free sulfhydryl with sulfhydryl-specific reagents, such as Biotin-BMCC or ^{3}H-NEM (Figure 4.3A). These techniques are

significantly more sensitive than metabolic labeling, and can be used quantitatively to measure levels of protein palmitoylation(Drisdel and Green 2004; Drisdel *et al.* 2006). Previously, this method was applied to examine the palmitoylation of SNAP25 and Nicotinic α7 subunits in mammals(Drisdel and Green 2004; Drisdel *et al.* 2004), and then to many palmitoylated yeast proteins through proteomic analysis (Lam *et al.* 2006; Roth *et al.* 2006a). To validate this method in detecting an increase in palmitoylation of a protein by an enzyme, we used SNAP25 as a substrate upon co-expression with each of the four DHHC enzymes (Figure 4.3B). After blocking the free thiols on SNAP25 with 50mM N-ethylmaleimide, half of the sample was treated with 1M hydroxylamine to cleave the Cys-palmitoyl thioester linkage; the other half (no hydroxylamine) served as our control. Next, the newly exposed thiols were labeled with a 10μM biotin-BMCC. After protein gel electrophoresis, nitrocellulose membranes containing SNAP25 protein were probed with strepdavidin-HRP, and palmitoylation level of SNAP25 was detected by standard ECL. Provided that the expression of DHHC-3, DHHC-8, HIP14L and HIP14 was about equal (Figure 4.3C), DHHC-3, DHHC-8 and HIP14 significantly enhanced the palmitoylation of SNAP25 while DHHC-13 did not. Samples that were not treated with hydroxylamine, and do not have free cysteines to be re-labeled by biotin, therefore are not detected by strepdavidin (Figure 4.3B).
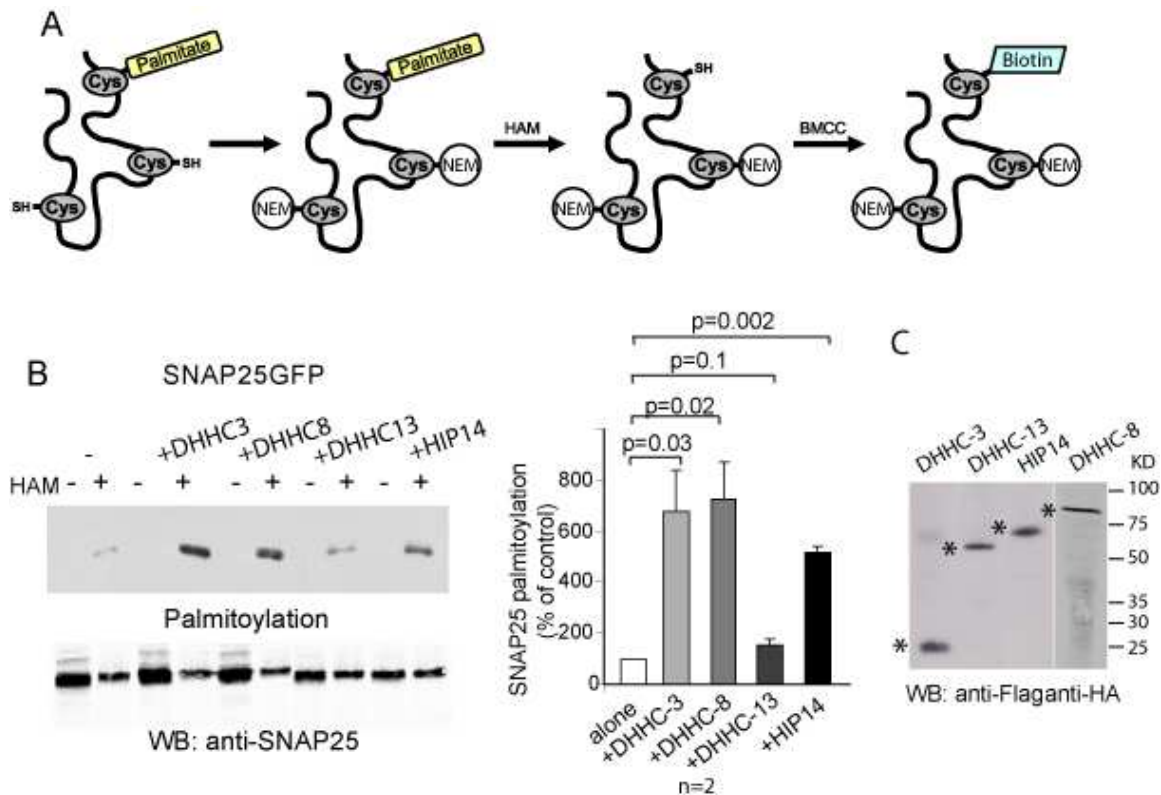
Figure 4.3. Validation of ABE-Acyl Biotin Exchange-method with substrate SNAP25.

(A) A diagram of ABE procedure. First, GFP tagged SNAP25 was immunoprecipitated by GFP antibody. Next, the Protein A sepharose that contains SNAP25 was incubated with 50mM NEM at 4°C for an hour to block the free thiols. Then, 1M Hydroxylamine was used to cleave the Cys-palmitoyl thioester linkage. At last, the newly exposed free thiols were labeled with a sulfhydryl-specific labeling compound, biotin-BMCC (1-biotinamido-4-[4'-(maleididomethyl) cyclohexanecarboxamido] butane). **(B)** Application of ABE method in examining the enhancement of SNAP25 palmitoylation by four DHHC proteins. Palmitoylation level is detected by strepdavidin-HRP on nitrocellular membrane. Untreated samples (-HAM) were used to estimate residual, non-specific binding of the biotin-BMCC with antibodies and sepharoses (negative control). In treated samples (+HAM), SNAP25-GFP substrate protein loading were even, yet its palmitoylation level was enhanced to different extent by DHHC-3, DHHC-8 and HIP14. DHHC-13, in contrast, did not increase SNAP25 palmitoylation. **(C)** Expression of DHHC-3, DHHC-8, DHHC-13 and HIP14 in COS cells were even. The stars point to the protein bands.

We next used the same approach to determine the potential substrates for these

DHHC enzymes. Our group has previously demonstrated that huntingtin, in which a

poly-glutamine tract expansion causes Huntington disease, is a substrate of HIP14(Huang

et al. 2004; Yanai *et al.* 2006). Using the ABE method, we now show that huntingtin can also be palmitoylated by DHHC-13, a protein that shares 57% similarity with HIP14 (Figure 4.4A). Interestingly, DHHC-13 also interacts with huntingtin when co-expressed in COS cells (data not shown). Unlike huntingtin, palmitoylation of paralemmin, a protein implicated in spine formation (Arstikaitis *et al.* 2008), is specifically enhanced by DHHC-8 (Figure 4.4B). It has shown by metabolic labeling approach that DHHC-3 enhances palmitoylation of AMPA receptor GluR2 (Hayashi *et al.* 2005). The ABE method reveals that the AMPA receptor GluR2 is a substrate of both DHHC3 and HIP14 (Figure 4.4C). In addition to huntingtin, paralemmin and AMPA receptors, we also examined the effects of these DHHC proteins on palmitoylation of other neuronal proteins including GABAA receptor γ2 subunit, GAD65, and synaptotagmin VII (Figure 4.5). In accordance with published ^{3}H palmitate labeling results, palmitoylation of GABAA receptor γ2 subunit is enhanced specifically by DHHC-3 (Fang *et al.* 2006). All four DHHC proteins can enhance the palmitoylation of GAD65 while none of them seem to regulate the palmitoylaiton of Synaptotagmin VII. In conclusion, individual palmitoyl transferase DHHC enzymes exhibit distinct substrate preference. However, whether each DHHC enzyme recognizes its respective substrates via specific consensus motifs is not determined.
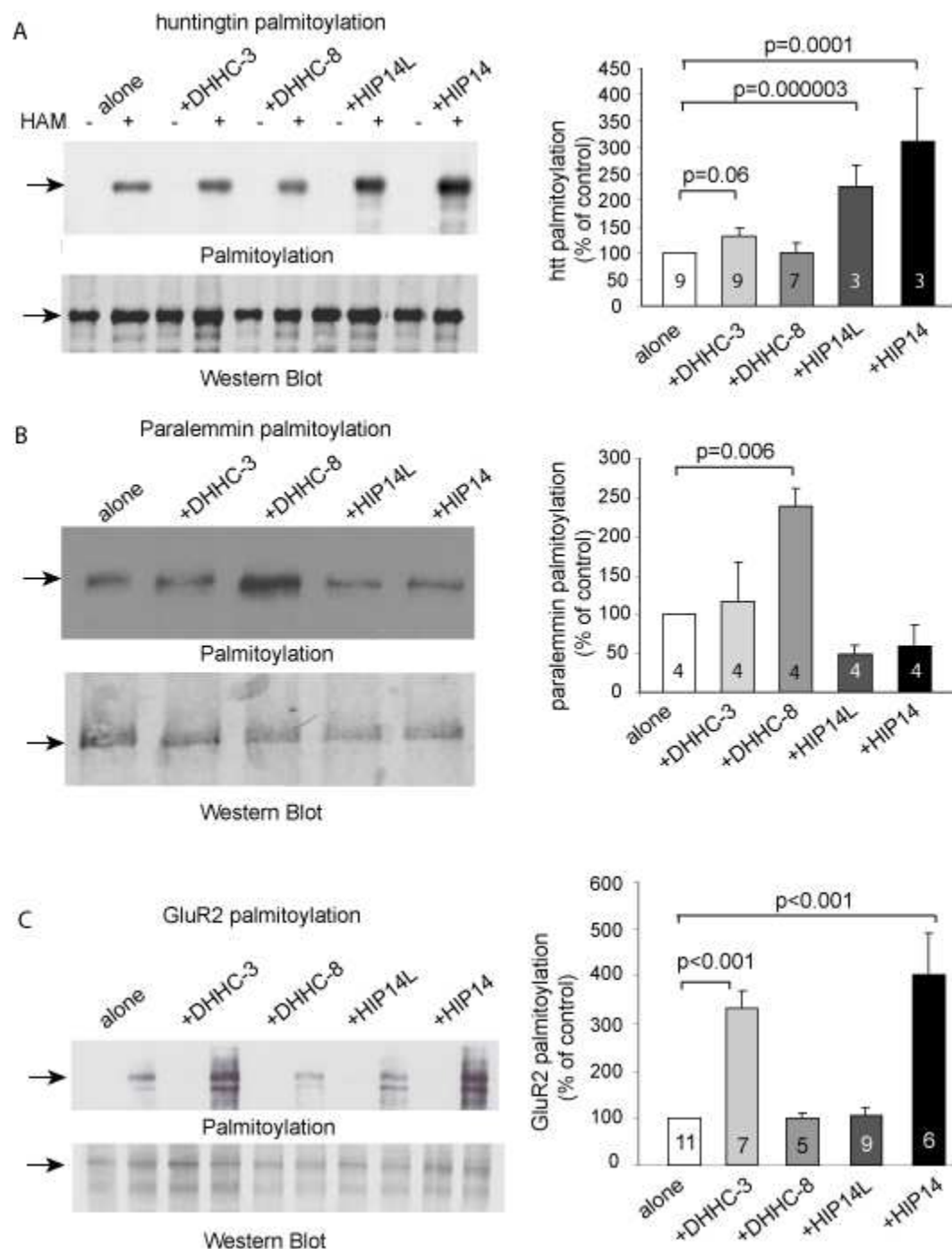
Figure 4.4. Application of ABE method to identify substrate and DHHC enzyme specificity.

(**A**) palmitoylation of huntingtin is specifically enhanced by HIP14 and HIP14L. (**B**) palmitoylation of paralemmin, a protein involved in inducing spines, is specifically enhanced by DHHC-8. (**C**) palmitoylation of AMPA receptor GluR2 subunit is specifically enhanced by DHHC-3 and HIP14. Quantification in A, B and C were controlled for the protein expression. The number of repeat was indicated in each column. The arrows point to the protein bands.
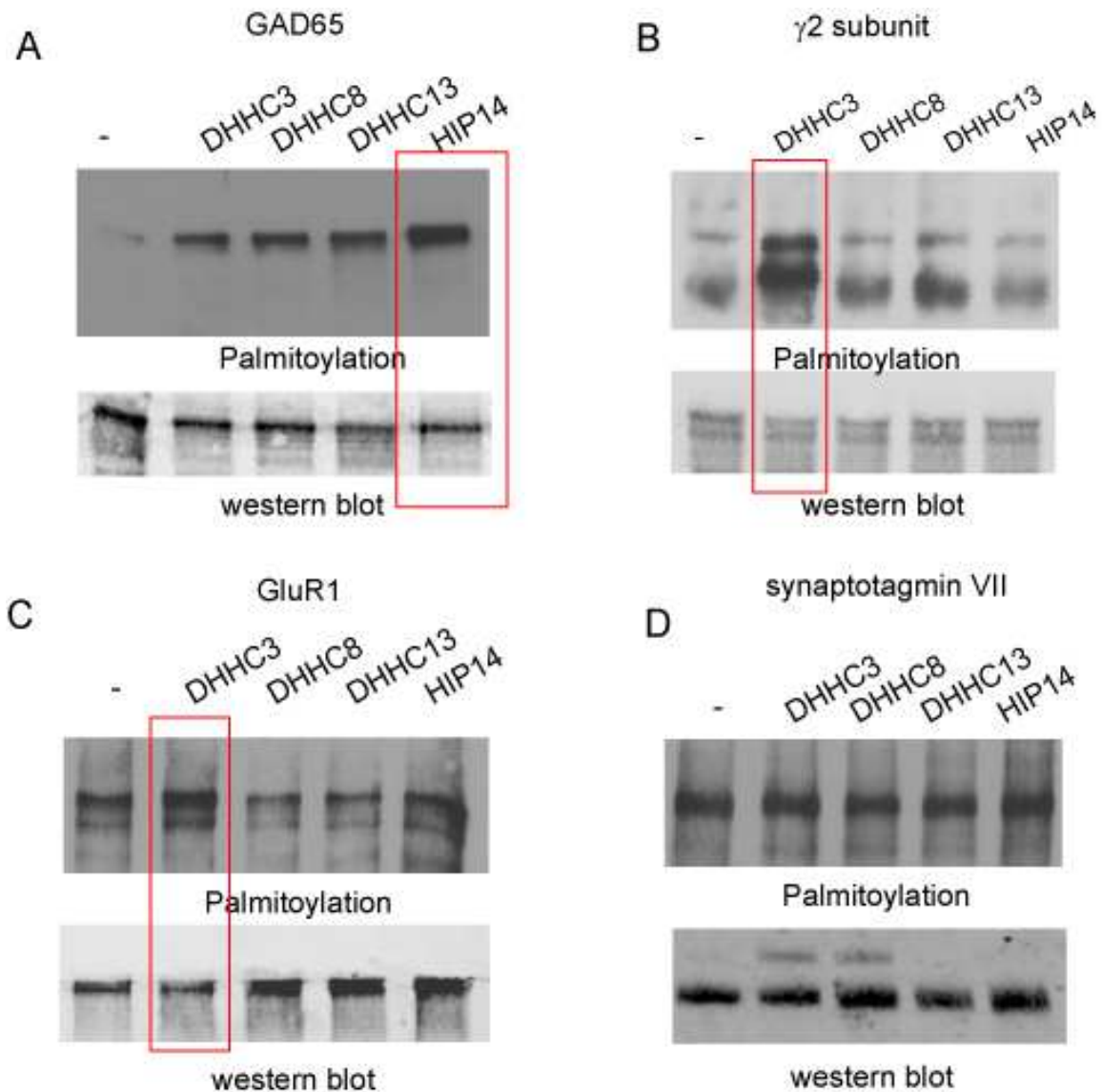
Figure 4.5. Palmitoylation of GAD65, GABAA receptor γ2 subunit, AMPA receptor GluR1 subunit and synaptotagmin VII by DHHC-3, -8, -13 and HIP14.

For each panel, a representative image of at least 2 experiments is shown. **(A)** DHHC-3, -8, -13 and HIP14 all increases GAD65 palmitoylation in COS cells, with HIP14 being the most efficient. **(B, C)** DHHC-3 increases palmitoylation of GABAA receptor γ2 subunit and AMPA receptor GluR1 subunit in COS cells. **(D)** None of these four DHHC proteins influence palmitoylation of synaptotagmin VII.

4.3.3 Expression of DHHC proteins alters the distribution and trafficking of their respective substrates

Previous studies showed that palmitoylation targets PSD-95 to a perinuclear domain (El-Husseini *et al.* 2000a). In agreement with this finding, we previously showed that HIP14 enhances palmitoylation dependent vesicular trafficking of huntingtin to the perinuclear region in both COS cells and neurons (Huang *et al.* 2004). In addition, Fukata et al demonstrated that DHHC-15, a PSD95 PAT, increased the segregation of PSD-95 to a perinuclear region (Fukata *et al.* 2004). In this study, we also found that DHHC-13 enhanced perinuclear accumulation of huntingtin, while DHHC-3 and DHHC-8 did not (Figure 4.6A,B). Paralemmin specifically accumulated in a perinuclear domain as a result of DHHC-8, but not DHHC-3, -13 or HIP14 overexpression (Figure 4.6A,C). Accumulation of GluR2 Receptor in the perinuclear domain was specifically mediated by DHHC-3, but not by DHHC-8, DHHC-13 or HIP14 (Figure 4.6A,D). Taken together, our results indicate that these enzymes play two closely related roles, including regulation of palmitoylation and modulation of vesicular trafficking of their respective substrate.
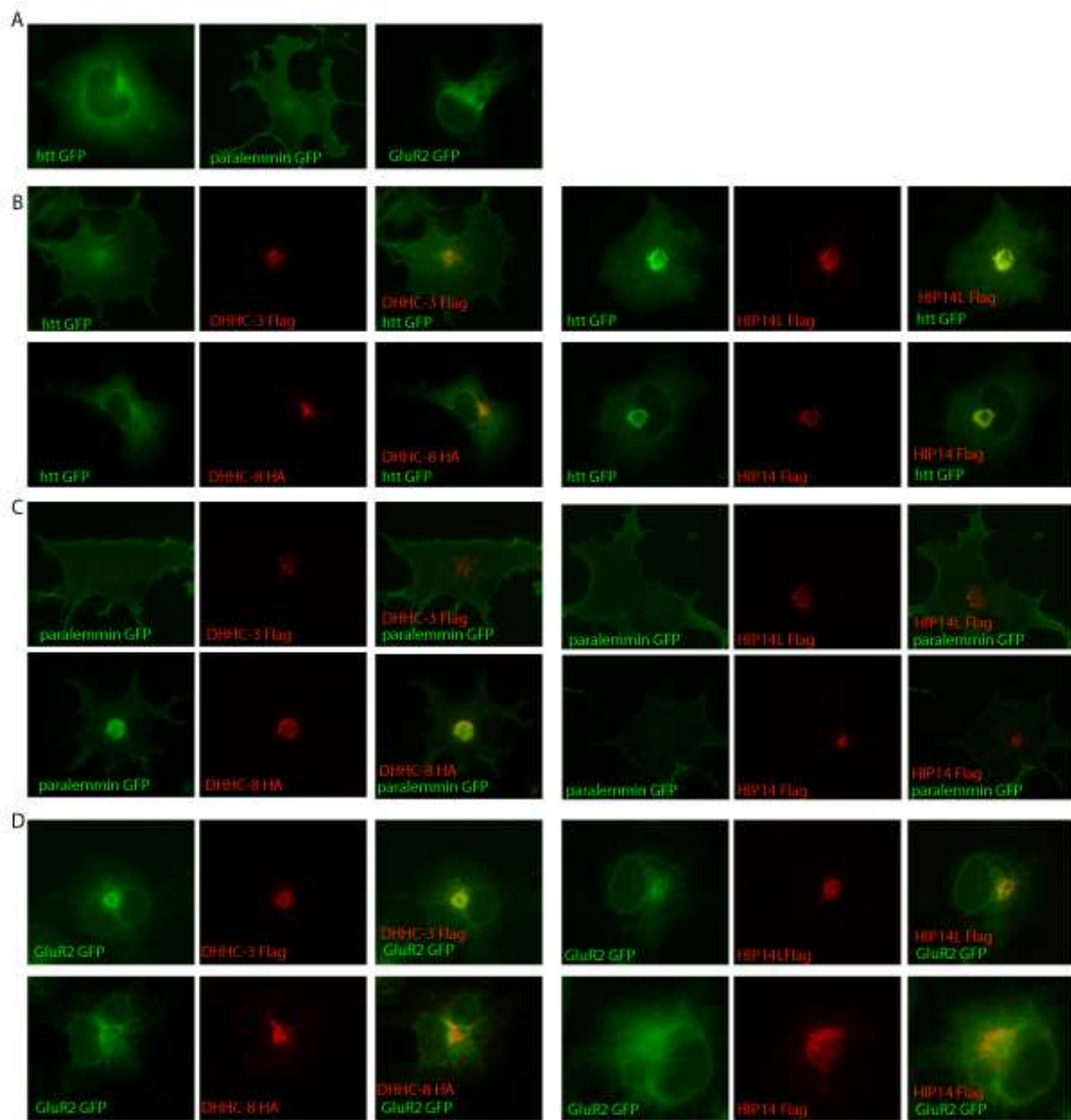
Figure 4.6. DHHC enzymes Modulates Trafficking of their respective Palmitoylated substrates.

(A,B) huntingtin alone localizes to the cytoplasm in a diffuse pattern. Exogenous HIP14 and HIP14L enhance the trafficking of huntingtin into a perinuclear region, where enzyme and huntingtin colocalize. **(A,C)** paralemmin alone predominantly localizes to plasma membranes and slightly at the Golgi. Exogenous DHHC-8 enhances the trafficking of paralemmin into the perinuclear region where both colocalize. **(A,D)** DHHC-3, PAT of GluR2, also enhances the trafficking of GluR2 into the perinuclear region where the two proteins partially colocalize.

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4.3.4 DHHC enzymes interact with their respective substrates

Mounting evidence suggests that an interaction exists between a substrate and its potential palmitoylating enzyme. The interaction may be transient and mediated by palmitate when the enzyme transfers palmitate to the substrate. Alternatively it could be a long-term protein-protein interaction, as in the case of huntingtin and HIP14, which is mediated by the ankryin repeats domain (Singaraja *et al.* 2002). Previous results from several groups have shown that DHHC-3 interacts with its substrates GABAA receptor γ2 subunit and AMPA receptors when overexpressed in heterologous cell lines (Uemura *et al.* 2002; Keller *et al.* 2004). In another study, putative enzymes that palmitoylate endothelial nitric oxide synthase (eNOS) including DHHC-2, DHHC-3, DHHC-7, DHHC-8 and DHHC-21 all interact with eNOS (Fernandez-Hernando *et al.* 2006).

We therefore examined whether the respective enzymes specifically interact with their substrates and whether this can be used as a new approach for identifying putative enzyme-substrate interactions. As is seen in Figure 4.7A, when we overexpressed paralemmin-GFP or huntingtin-GFP with HIP14 and immunoprecipitated GFP tagged substrates using a GFP polyclonal antibody, HIP14 was only found in an association with huntingtin, not with paralemmin. Likewise, when we overexpressed paralemmin-GFP or huntingtin-GFP with DHHC-8, DHHC-8 was only found in an association with paralemmin, not with huntingtin (Figure 4.7B). These results indicate that the interaction is rather specific to the enzyme and its respective substrates. In addition to paralemmin and huntingtin, SNAP-25 and PSD95, which are palmitoylated by both DHHC-3 and HIP14, also interact with these two enzymes (Figure 4.7C).

Next, we examined whether palmitoylation of a specific substrate is essential for its interaction with the respective enzyme. Our previous work demonstrated that palmitoylation of PSD-95 is mediated by a short NH_2-terminal consensus sequence that critically relies on five consecutive hydrophobic amino acids (Cys-Leu-Cys-Ile-Val) (El-Husseini *et al.* 2000a). Mutations of both cysteines abolished PSD95 palmitoylation. Mutating amino acids 4-Leu or 6-Ile to histidine and serine respectively (L4D, I6S) also significantly reduced palmitoylation (El-Husseini *et al.* 2000a). Interestingly, the interactions between these PSD95 mutants and HIP14 are also altered, corresponding to their level of palmitoylation (El-Husseini *et al.* 2000a) (Figure 4.7D). This result suggested to us that the amino acids flanking the palmitoylated cyteines are likely to be critical for the interaction between enzyme and substrate.

Next, we asked whether the palmitoylated motif of a substrate is sufficient for the interaction with the enzyme. Previous work identified that the first 13 amino acids of PSD95 are essential for its palmitoylation and correct vesicular sorting(El-Husseini *et al.* 2000a). Consistent with this, we also found that this short N-terminal motif of PSD95 is sufficient for the interaction with HIP14 when exogenously expressed in COS cells (Figure 4.7E). To further confirm that the DHHC enzyme recognizes the palmitoylation motif, we made a chimeric protein containing the full length PSD95 C3,5S (palm deficient mutant) and 12 amino acids of the paralemmin Carboxyl terminus that contain the palmitoylated cysteines. A coimmunoprecipitation experiment shows that this new protein, containing only the palmitoylation motif of paralemmin, is sufficient to interact with DHHC-8 (Figure 4.7F).
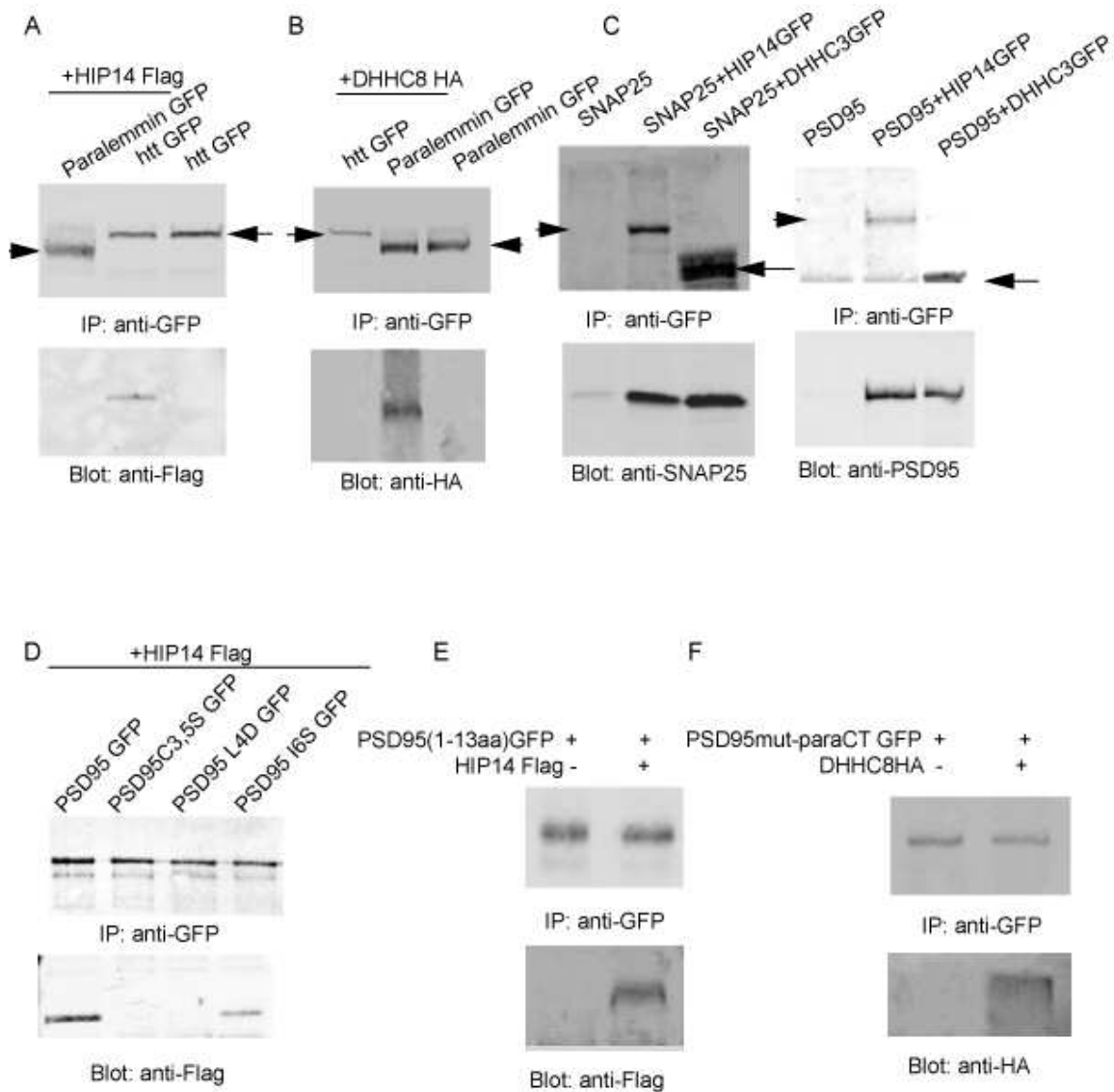
Figure 4.7. DHHC enzymes specifically interact with their respective palmitoylated substrates.

(A) huntingtin, but not paralemmin, interact with HIP14. COS cells overexpressing huntingtinGFP alone, huntingtinGFP with HIP14Flag and paralemminGFP with HIP14Flag were lysed and subjected to immunoprecipitation by GFP antibody. Western blot was probed with Flag antibody to detect whether HIP14 Flag was associated with the substrates. Arrow: httGFP; arrowhead: paralemmine GFP. **(B)** Conversely, paralemmin, but not huntingtin, interact with DHHC-8. Arrow: httGFP; arrowhead: paralemmine GFP. Coimmunoprecipitation procedure was the same as described in **(A)**. **(C)** Both SNAP25 and PSD95 coimmunoprecipitates with their palmitoylating enzymes DHHC-3 and HIP14. Arrow: DHHC3 GFP; arrowhead: HIP14 GFP. **(D)** Interaction of HIP14 and PSD95 requires the dual palmitoylated cysteines and the nearby amino acids. **(E)** PSD95 palmitoylation motif, containing amino acid 1 to 13, is sufficient to interact with HIP14. **(F)** paralemmin pamitoylation motif, containing the last 12 amino acid at its C terminus, is sufficient to interact with DHHC-8.

4.3.5 Regions distinct from the DHHC catalytic domain determines the specificity between substrates and enzymes

Unlike DHHC-3 and DHHC-8, huntingtin palmitoylation enzymes, HIP14, and HIP14L, contain an ankryin repeats domain that interacts with a N-terminal fragment of huntingtin (Singaraja *et al.* 2002). To determine whether the ankyrin repeats domain within HIP14 plays a role in palmitoylating huntingtin, we made a new chimeric protein, which contains ankryin repeats fused to the full length DHHC-3. This chimeric protein, which mimics the HIP14 structure, is now able to interact with huntingtin and redistributes htt into a perinuclear region via a palmitoylation dependent vesicular trafficking (Figure 4.8A, B). Remarkably, this chimera is capable of enhancing the palmitoylation of huntingtin in COS cells to the same extent as HIP14 (Figure 4.8C). This result suggested a possibility that regions other than the DHHC domain of the enzyme determine the specificity between substrates and enzymes.
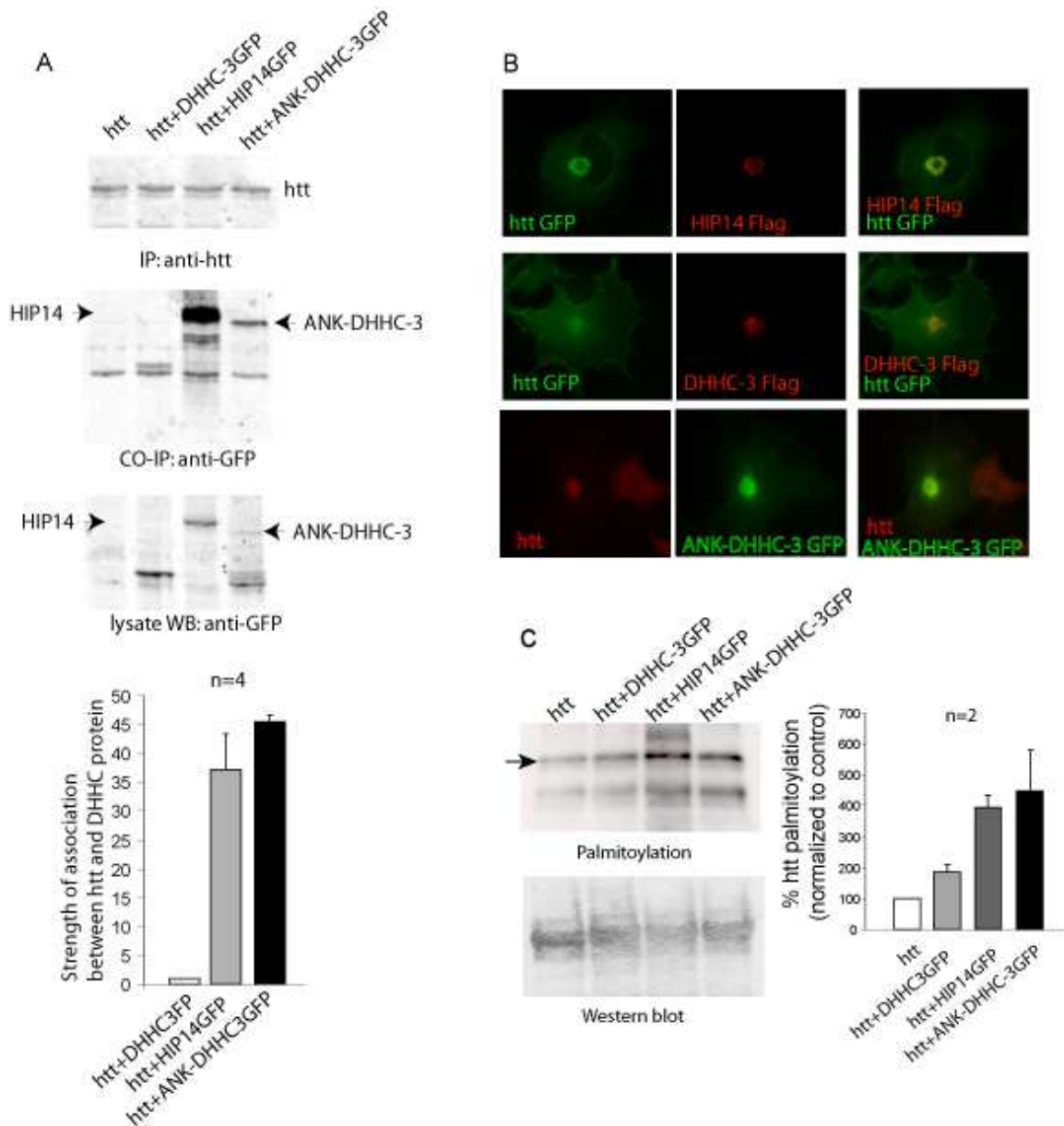
Figure 4.8. The substrate specificity is determined by specific domains of the DHHC enzymes.

(A) A chimeric protein, composed of Ankyrin repeats and full length DHHC-3, is now able to interact with huntingtin. The strength of the interaction between ANK-DHHC-3 with huntingtin is comparable to that of HIP14 with huntingtin. **(B)** The new chimera protein ANK-DHHC-3 is now also able to accumulate huntingtin into a perinuclear region. **(C)** The new chimeric protein ANK-DHHC-3 is also able to palmitoylate huntingtin in COS cells. All quantifications were normalized to the protein expression.

4.4. DISCUSSION

The number of DHHC PATs suggested that substrate specificity would exist for each PAT. In yeast, loss-of-function analysis mapped the potential substrates of each DHHC enzyme (Roth *et al.* 2006a). Our study, along with others, indicates that mammalian DHHC enzymes also have substrate specificity(Tsutsumi *et al.* 2008). Compelling evidence has revealed that DHHC3 and the closely related DHHC7 seem to have broad substrate specificity(Tsutsumi *et al.* 2008). These two DHHC enzymes enhance the palmitoylation of various substrates, including PSD95, eNOS, the GABAA receptor γ2 subunit, SNAP25, Gαs, and GAP43 (Fukata *et al.* 2004; Fang *et al.* 2006; Fernandez-Hernando *et al.* 2006). DHHC3 also enhances the palmitoylation of GluR receptors (Hayashi *et al.* 2005). In contrast, DHHC2 and DHHC15 are more specific to PSD95 and GAP43 (Fukata *et al.* 2004). HIP14 (DHHC-17) palmitoylates huntingtin, but also increases palmitoylation of SNAP25, PSD95, GAD65 and synaptotagmin1 *in vitro* (Huang *et al.* 2004). DHHC-8 is specific to paralemmin, and DHHC-9 and DHHC-18 are specific to H-Ras (Fukata *et al.* 2004; Swarthout *et al.* 2005). Taken together, these combined data clearly supports distinct substrate specificity of individual PATs.

However, the determinants of the specific interaction between PATs and their substrates remain elusive. One possibility is that subcellular localization of each DHHC protein may play a role in palmitoylating substrates that share the same compartments during substrate posttranslational modification and transport. Indeed, 23 DHHC enzymes showed various expression patterns in HEK cells (Ohno *et al.* 2006). However, the four DHHC proteins examined in this study all localize to Golgi compartment, yet exhibit

distinct substrate preference, suggesting other factors must be contributing to the enzyme-substrate specificity.

In this study, we utilize the biotin labeling palmitoylation method to examine the level of protein palmitoylation. The disadvantage of traditional metabolic labeling method is that ^{3}H palmitate only labels a pool of protein that turns over rapidly during the 3-4 hour of labeling process. Therefore, if the half-life of palmitate turnover on a substrate is longer than the labeling hours, then only a proportion of the total palmitoylated protein pool is accessible with ^{3}H palmitate. This limitation also applies when a DHHC enzyme is coexpressed. Thus, the ^{3}H palmitate signal detected on the film does not reflect the total palmitoylated protein, and it may not be linearly correlated to the total palmitoylated protein. Therefore, we postulated that it is possible that two different methods may show different or even opposing results due to the limitation of ^{3}H palmitate labeling. Additional tools, such as generation of mice with targeted disruption of DHHC proteins or the use of specific small interference RNAs will help to definitely determine the *in vivo* specificity of palmitoylation substrate for each enzyme.

Previous work has shown that PSD-95 transiently associates with a perinuclear membranous compartment and traffics with vesiculotubular structures(El-Husseini *et al.* 2000a). Trafficking of PSD-95 with these vesiculotubular structures requires dual palmitoylation. In many examples, palmitoylation of a substrate by an enzyme correlates with the ability of the enzyme to enhance the association of the substrate with perinuclear membraneous compartment and vesiculotubular structures(Fukata *et al.* 2004; Huang *et al.* 2004). This is exemplified by HIP14 with huntingtin, SNAP25 and PSD95, DHHC8 with paralemmin, DHHC3 with GluR receptors and GABAA receptor γ2 subunit, and

DHHC-15 with PSD95. Therefore, the strong recruitment of a substrate into the perinculear compartment by an enzyme can be used to predict the putative enzyme-substrate interaction.

We also revealed the specificity of the interaction between an enzyme and a substrate by coimmunoprecipitation. The stable interaction of an enzyme and its substrate is palmitoylation dependent, as mutations on palmitoylated cysteines abolish the interaction. It is intriguing that the palmitoylation motif is sufficient to be recognized by the respective enzyme, which suggests that a consensus sequence may exist. However, whereas these DHHC enzymes readily form a stable complex on overexpression with their respective substrates in heterologous cells, no such complex could be detected in brain extracts ((Uemura *et al.* 2002; Keller *et al.* 2004) and data not shown). Therefore, such stable complexes detected in overexpression experiments might represent long-lived enzymatic reaction intermediates resulting from low catalytic processing of enzymes in heterologous cells (also in (Fang *et al.* 2006)). Nevertheless, the stable interaction between the DHHC proteins and substrates is useful for predicting the putative enzyme-substrate relationship.

What determines the specificity and whether palmitoylation consensus sequences exist are not yet known. Previous analyses have prompted the classification of the 23 DHHC proteins into several subfamilies. There may be some structural correlations to enzyme specificity. Besides the catalytically critical DHHC domain, an individual DHHC protein may have regulatory regions such as the SH3 domain in DHHC6, the ankyrin repeat domain in HIP14 and HIP14L, and type II PDZ-binding motif in DHHC-3 (Tsutsumi *et al.* 2008)(similarly, protein kinases also share a core catalytic region and

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differ in regulatory domains that afford differential control systems). It is conceivable that these regions may recruit specific substrates or regulators to DHHC proteins. This is exemplified in our result showing that fusing the ankyrin repeat domain to DHHC3 now nables this new protein to behave like HIP14, interacting with and palmitoylating huntingtin.

It is noteworthy that DHHC protein enzyme may require binding partners to be functionally active. This was first shown in yeast that, Erf2, a yeast DHHC protein, functions as a PAT for yeast Ras2 in a complex with Erf4(Zhao *et al.* 2002). Recently, DHHC9 has been shown to require GCP16, a mammalian functional ortholog of Erf4, for its PAT activity toward H/N-Ras and protein stability(Swarthout *et al.* 2005). Binding partners of DHHC proteins may also contribute to the substrate specificity, enzymatic kinetics, subcellular localization, and stability of some DHHC proteins as an auxiliary subunit.

Catalysis of protein palmitoylation by a DHHC enzyme also seems to require the autopalmitoylation of the enzyme itself. For example, deletion of HIP14 DHHC domain, which abolishes HIP14 autopalmitoylation, also abrogates its ability to palmitoylate SNAP25 {Huang, 2004 #63}. PSD-95 PAT DHHC-15 with mutations or deletions such as AAHC-15, DHHC-15-C159S, or DHHC-15-ΔC (aa 1–238), all of which abolish autopalmitoylation, loses its ability to enhance PSD-95 palmitoylation {Fukata, 2004 #108}. These examples support the notion that the PAT activity of a DHHC enzyme correlates with its autopalmitoylation.

To summarize this work, we addressed the hypothesis that neuronal PATs have distinct substrate specificity. We showed that one factor that contributes to this specificity

is the regulatory domains beside from catalytic DHHC domain. We also identified a consistent correlation among palmitoylation, altered distribution of a substrate by its enzyme, and the interaction between sustrate and enzyme. These detailed analysis provide further understanding of the mechanisms of DHHC enzymes.

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5. CHAPTER 5 Huntingtin Regulates the Enzymatic Activity of the Neuronal Palmitoyl Transferase HIP14

A version of this chapter will be submitted for publication.

5.1. INTRODUCTION

Protein palmitoylation represents a common lipid modification of neuronal proteins. This posttranslational change involves addition of the saturated 16 carbon palmitate lipid in a thioester linkage to specific cysteine residues(Resh 1999). In neurons, at least 32 proteins have been shown to be palmitoylated. These include channels, cell adhesion molecules, scaffolding molecules, neurotransmitter release machinery and signaling proteins(el-Husseini Ael and Bredt 2002; Huang and El-Husseini 2005; Resh 2006a). The molecular identity of the protein fatty acyltransferases (PATs) that modify palmitoylated proteins has recently been determined(Lobo *et al.* 2002; Roth *et al.* 2002; Fukata *et al.* 2004; Huang *et al.* 2004). The defining feature of this family of enzymes is the presence of a cysteine-rich domain (CRD) with an Asp-His-His-Cys (DHHC) core motif, which is essential for PAT activity both *in vitro* and *in vivo*. HIP14, the huntingtin interacting protein 14, is one of the 23 mammalian DHHC proteins in this family(Huang *et al.* 2004).

Huntington disease (HD) is a neurodegenerative disorder caused by an expansion of a polyglutamine tract in the neuronal protein huntingtin (The Huntington's Disease Collaborative Research Group,1993). Recent studies by our group reveal that defective palmitoylation of mutant huntingtin contributes to the neuronal dysfunction associated with HD. In these studies we also demonstrate that the polyglutamine expansion in huntingtin results in a reduced association with HIP14. This defect causes a marked reduction in huntingtin palmitoylation both *in vitro* and in whole mouse brain, which further accelerates inclusion formation and enhances neuronal toxicity and cell death. Downregulation of HIP14 in mouse neurons expressing wildtype and mutant huntingtin

increases inclusion formation, whereas overexpression of HIP14 substantially reduces inclusions. Taken together, these results indicate that loss of palmitoylation exacerbates huntingtin protein aggregation and contributes to the molecular pathology of HD.

In vitro, HIP14 palmitoylates other neuronal substrates, including PSD95 and SNAP25 (Huang *et al.* 2004). However, in contrast to the stable association of HIP14 and huntingtin, there is no detectable interaction between HIP14 and PSD95 or SNAP25 examined by coimmunoprecipitation experiments from brain lysate (data not shown). This suggested to us that huntingtin may function beyond only being a substrate of HIP14. Although polyglutamine expansion of mutant huntingtin has been shown to disrupt various cellular events such as gene transcription and intracellular transport, little is known about the function of wildtype huntingtin. In this study we investigated how normal huntingtin may be involved in regulating the enzymatic activity of HIP14.

5.2.   MATERIALS AND METHODS

5.2.1 Plasmids

All DHHC constructs were previously described and were generously provided by Dr. Yasuyuki Igarashi (Ohno *et al.* 2006). Full length HIP14 was subcloned into pCI-neo as described earlier (Singaraja *et al.* 2002). HIP14 GFP was generated by fusing EGFP (Clontech, CA) at its C-terminus, and HIP14-Flag was generated by PCR using primers containing the FLAG sequence. HIP14ΔDHHC was generated by deletion of nucleotides encoding amino acids 440 to 487. HIP14ΔANK was generated by deletion of the Ankyrin repeats, nucleotides encoding amino acids 89 to 257. GST-SNAP25 fusion proteins were PCR amplified and subcloned into EcoR I/Sal I of pGEX-6P3 (Amersham Biosciences,

UK). HIP14-GST was generated by insertion of full length HIP14 cDNA in frame with GST into pGEX-6P3 at Xma I site. For the generation of GST-GAD65 (1-80aa), nucleotide sequences corresponding to GAD65 amino acids 364-383 were PCR amplified and subcloned into BamH I and Xho I sites of pGEX-4T1 (Amersham). PSD95 (1-PDZ3) in pGEX-2T was a gift from Dr. David Bredt (University of California at San Francisco, CA). Truncated huntingtin constructs N548 were previously described(Singaraja *et al.* 2002). Cysteine substitutions were generated by PCR based site-directed mutagenesis as described earlier (Singaraja *et al.* 2002). All mutated DNA constructs were sequence confirmed.

5.2.2 Antibodies and Chemicals

htt antibody 2166 (Chemicon;1:250 for immunoprecipitation, 1:2000 for western blotting), HIP14 anti-rabbit antibody (homemade; 1:50 for immunoprecipitation, 1:250 for western blotting), GFP anti-rabbit antibody (homemade, 1:100 for immunoprecipitation), GFP anti-rabbit antibody(Synaptic System, 1:2000 for western blotting), paralemmin (provided by Dr. Manfred Kilimann), Ras, PSD95 (homemade, 1:500 for western blotting), SNAP25 (Stressgen, 1:1000 for western blotting and 1:500 for immunoprecipitation), GAD65 (Synaptic System, 1:1000 for western blotting), SYT1 (Synaptic System, 1:1000 for western blotting and 1:500 for immunoprecipitation), AKAP150, GluR1 (provided by Dr. Yutian Wang), GluR2 (provided by Dr. Yutian Wang), SCAMP (Upstate Biotech, 1:1000 for western blotting), Syntaxin 1B (Synaptic system, 1:1000 for western blotting), Glutamate Transporter 1 (EAAC1) (Chemicon, 1:1000 for western blotting).

5.2.3 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, Anti-Mouse Alexa Fluor 680) (Rockland) were used in a ratio of 1:10000. The signal was scanned using Odyssey Infrared Imaging System (Li-COR Bioscience), and quantified by means of Image J software. A distribution profile was calculated with Excel software (Microsoft Office) as the average value ± SD from at least 3 independent experiments. Data were analyzed by Student T test.

5.2.4 Cell culture and transfections

All reagents for cell cultures were purchased from Invitrogen Corporation (Carlsbad, CA). COS cells were cultured as previously described (Huang *et al.* 2004) and were transiently transfected with Lipofectamine 2000 (Invitrogen) as indicated by the manufacturers. 24 to 48 hours post transfection, cells were processed as described for each experiment. Cultured cortical neurons were prepared as previously described (Huang *et al.* 2004) and experiments were performed at 14 days *in vitro* (DIV).

5.2.5 Synthesis of Palmitoyl CoA

[^{3}H]palmitoyl-CoA, [^{3}H]myristoyl-CoA and palmitoyl-CoA were synthesized enzymatically from [9,10-^{3}H(N)]palmitic acid (5 mCi/ml; Perkin Elmer Life Sciences), [9,10-^{3}H(N)]myristic acid (1mCi/ml; Perkin Elmer Life Sciences) and palmitic acid (Sigma-Aldrich), respectively. Synthesis components also included co-enzyme A (CoA), ATP and acyl-CoA synthase (Sigma-Aldrich). Synthesized products were purified as

previously described (Dunphy *et al.* 1996). Synthesized [³H]palmitoyl-CoA and [³H]myristoyl-CoA were subjected to TLC (TLC aluminium sheet, silica gel, EM Science, Germany) to determine the efficiency. Ninhydrin Staining was used for the detection of synthesized non-radiolabeled palmitoyl-CoA. The synthesis was highly efficient, with >95% conversion of acid to fatty acid-CoA. Specific activity for [³H]palmitoyl-CoA was 60 Ci/mmol and for [³H]myristoyl-CoA was 30Ci/mmol.

5.2.6 Immunoprecipitation

Brains from wildtype and huntingtin-/- mice were homogenized in 50mM Tris and 150mM NaCl, containing 1% Igpal, protease inhibitors (Roche), and ZVAD. Tissue debris were removed by centrifuging the brain lysate at 45000rpm, 4ºC for 30min. Normal rabbit IgG, or anti-HIP14 rabbit polyclonal antibody were incubated with 3mg precleared lysate at 4ºC for 1 hour. 30µl of equilibrated Protein A Sepharose 4 Fast Flow beads were added and samples were further incubated at 4ºC for 2 hours. Beads were washed 3-6 times with buffer containing 50mM Tris and 150mM NaCl. Residual buffer in the beads was taken away by thin gel loading tips.

5.2.7 Palmitoylation Assay

Palmitoylation reaction (60 µl) contained 5 µCi of [³H]palmitoyl-CoA, 0.33 µg/µl substrate protein, 1 mM ATP, 50 mM MES, pH 6.4, 0.2 mg/ml bovine liver lipids, and 20µl of the Protein A Sepharose beads containing HIP14 immunoprecipitates from the brain. After 15min incubation at 37°C, sample buffer was added with final concentration of 5mM DTT was added and samples were subjected to SDS-PAGE analysis.

5.2.8 Btn-BMCC labeling

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 hour at room temperature. Samples were then processed as previously described(Drisdel and Green 2004).

5.2.9 Yeast 2 hybrid analysis

The series of truncated huntingtin constructs (Hackam *et al.* 1998) were inserted into the yeast expression vector pGBT and sequences were verified. Yeast transformations with pGADHIP14 and each pGBT9 huntingtin constructs were performed using the lithium acetate method (Gietz *et al.* 1995). To detect the intensity of interaction, liquid β-galactosidase assays were performed by inoculating 3 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_2HPO4, 0.04M NaH_2PO4, 10mM KCl), and frozen and thawed three times. Z buffer supplemented with 20% SDS, 1M DTT, β-mercaptoethanol and O-Nirophenyl β-D-galactopyranoside were added to the tubes and reactions continued until a yellow colour appeared. Reactions were stopped by adding 1M Na_2CO3 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).

5.3. RESULTS

5.3.1 Huntingtin and HIP14 form a tight complex that has enzymatic activity

We previously showed that HIP14 is a palmitoyl transferase for SNAP25 (Huang *et al.* 2004). Consistent with this, immunoprecipitated HIP14 from cortical neurons (Figure 5.1A left) and brain (Figure 5.1B left) exhibited palmitoyl transferase activity toward GST-SNAP25 *in vitro*. When we immunoprecipitated (IP) huntingtin from cortical neurons (Figure 5.1A right) and brain (Figure 5.1B right), the resulting immunoprecipitate also increased palmitoylation of GST-SNAP25. This suggested to us that huntingtin's binding partner, HIP14, was co-IPed and resulted in PAT activity. Western blots (Figure 5.1A, B bottom panels) confirmed that, in all IPs, the respective binding partner was co-IPed. . These results suggest that huntingtin and HIP14 form a tight complex that contains enzymatic activity.
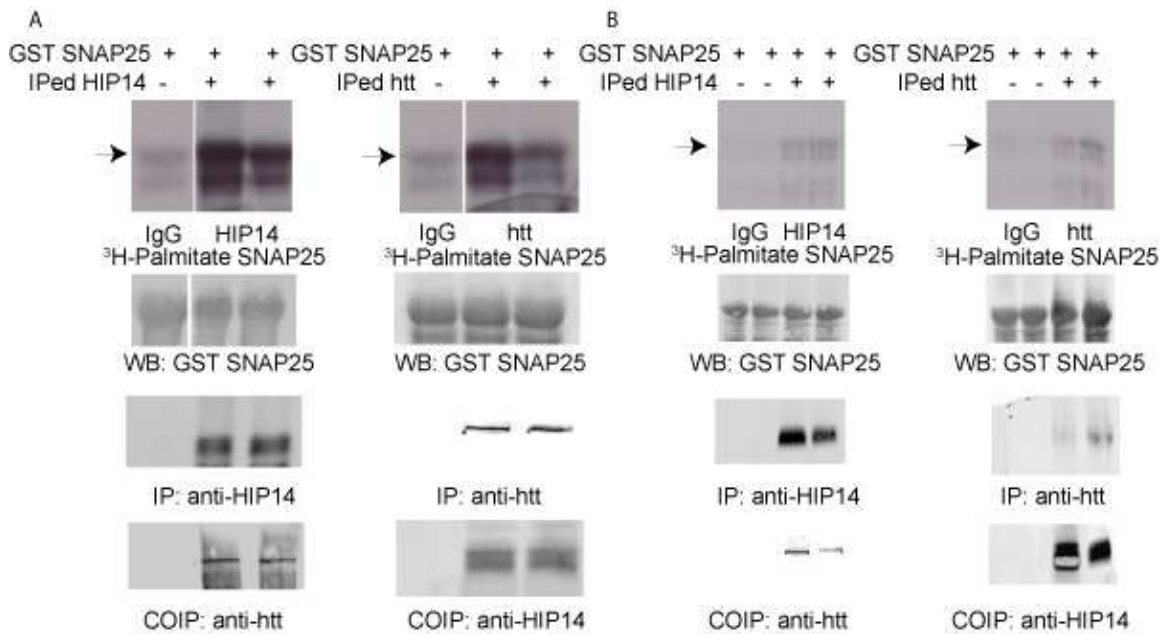
Figure 5.1. huntingtin and HIP14 form a complex in the brain.

(A, B) In cultured rat cortical neurons and the whole rat brain, immunoprecipitated (IP) HIP14 or huntingtin forms a tight complex, exhibiting palmitoyl transferase activity toward GST-SNAP25 in vitro.

5.3.2 Only HIP14 and HIP14L palmitoylate huntingtin

We have previously shown that palmitoylation of huntingtin by HIP14 is essential for its trafficking and function (Yanai *et al.* 2006). The identification of 22 other DHHC proteins in this PAT family raised the questions as to whether other DHHC proteins may palmitoylate huntingtin and whether the tight association of huntingtin and its PAT HIP14 is unique. We therefore, applied the ABE (Acyl Biotin Exchange) labeling method to examine the palmitoylation level of huntingtin upon expression of individual DHHC proteins in COS cells. Both HIP14 (DHHC-17) and HIP14L (DHHC-13), a homolog of HIP14, are the only two PATs that significantly increase palmitoylation of huntingtin (Figure 5.2A,B). Examining the internal structures of the 23 DHHC proteins reveals that only HIP14 and HIP14L contain an ankryin repeats domain, which appears to mediate

these enzymes binding to huntingtin(Fukata *et al.* 2004; Ohno *et al.* 2006; Tsutsumi *et al.* 2008).
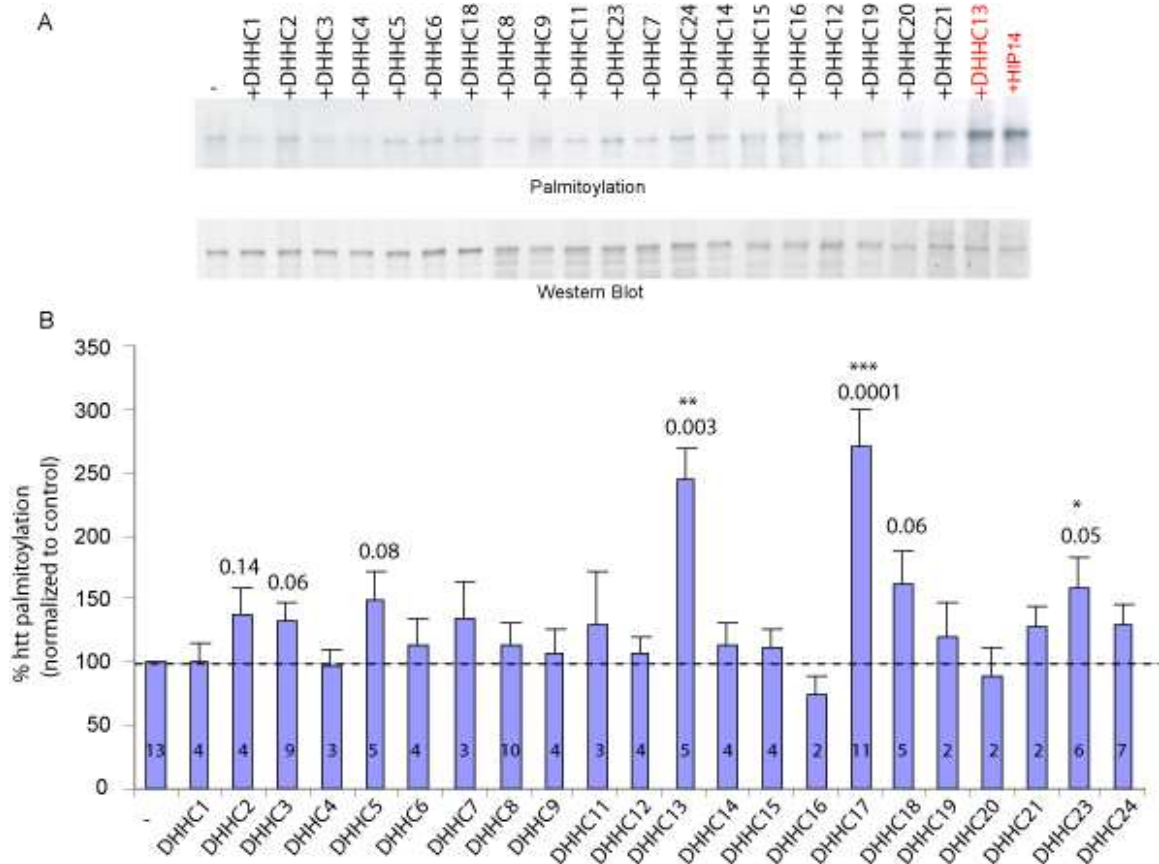
Figure 5.2. Among DHHC proteins, only HIP14 and HIP14L palmitoylate huntingtin.
(A) COS cells overexpressing either huntingtin alone or huntingtin with individual DHHC protein were subject to the biotin palmitoylaiton labeling assay. Among the indicated DHHC proteins, only HIP14L (DHHC-13) and HIP14 (DHHC-17) palmitoylate huntingtin. **(B)** Quantification of huntingtin palmitoylation increase by individual DHHC protein. HIP14L and HIP14 increase huntingtin palmitoylaiton about 2.5 folds. The numbers in the columns of the graph indicate the repeat times.

5.3.3 Interaction of HIP14 and huntingtin is distinct from other palmitoylation enzyme-substrate interaction

We previously showed that the interaction of a PAT with its substrate requires the presence of the palmitoylated cysteines and its surrounding amino acids. For instance,

mutations of PSD95 C3,5S, L4D and I6S which abolish or reduce palmitoylation significantly (El-Husseini *et al.* 2000a) also abrogate or decrease the interaction of PSD95 with HIP14 (see Figure 4.7D). Similarly, other groups reported that mutations of eNOS such as C15/26S eNOS and L2S eNOS (both N-myristoylated but not palmitoylated) disrupted the interaction with its palmitoylation enzymes DHHC-3 and DHHC-21 (Fernandez-Hernando *et al.* 2006). In contrast to PSD95 and eNOS, huntingtin C214S mutation, despite a compromise in palmitoylation, remained strongly associated with its PAT HIP14 (Figure 5.3A). This result suggests that, unlike other substrate and enzyme interaction that might be transient, the association between huntingtin and HIP14 is very stable, and does not require the palmitoylated cysteine.

To determine the interacting domains between huntingtin and HIP14, we generated HIP14 constructs lacking either the ankryin repeats domain or the catalytic DHHC domain. We found that deletion of the ankyrin repeats greatly reduced the interaction between HIP14 and the N-terminal fragment of huntingtin, whereas deletion of HIP14's catalytic DHHC domain preserved the interaction (Figure 5.3B). Therefore, this particular interaction does not require the catalytic domain, which also differs from other substrate- PAT association such as eNOS and DHHC-21. Mutations in the DHHC catalytic domain of DHHC-21 abolishes interaction of eNOS and DHHC-21(Fernandez-Hernando *et al.* 2006). Furthermore, using yeast-2-hybrid, we mapped the region that is required for the interaction to be downstream of amino acid 427 in huntingtin, a region not including huntingtin's palmitoylation site (Cysteine 214; Figure 5.3C,D). The tight association of HIP14 with wild type huntingtin, which differs from other known PAT-substrate interactions, suggests that huntingtin may play a role in regulating HIP14's

palmitoyl transferase activity.
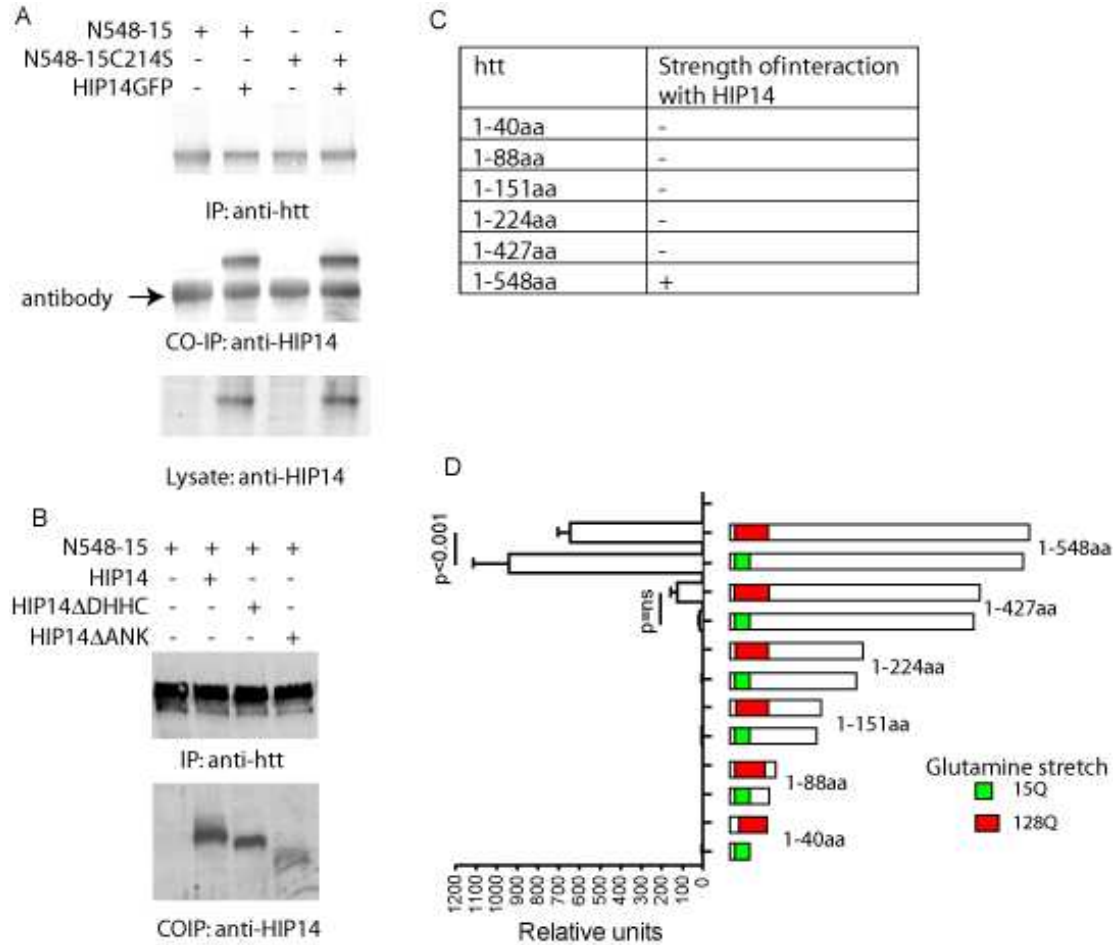
Figure 5.3. The interaction of HIP14 and htt requires ankyrin repeats domain and is palmitoylation independent.

(A) In COS cells, exogenous wt htt (1-548aa) interacts with HIP14. Mutation of the palmitoylated cysteine does not affect the interaction with HIP14, suggesting that palmitoylation of htt is not required for the interaction. **(B)** Deletion of Ankyrin repeats significantly reduces the association of HIP14 and huntingtin. In contrast, deletion of DHHC catalytic domain does not affect the interaction. **(C, D)** Yeast 2 hybrid semiquantitative β-galactosidase assays to map the region of htt to which HIP14 binds. **(C)** 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 aa427 to 548. **(D)** HIP14 showed strong interaction with wt htt (18CAG) containing aminoacids 1 to 548. The interaction of HIP14 with mutant htt (128CAG) on the 1-548aa fragment was significantly reduced, indicating that the efficiency of the interaction between HIP14 and mutant htt is reduced. The interaction of HIP14 with the 1-427aa htt fragment containing both 18 and 128 CAG was significantly reduced compared to its interaction with the 548aa fragment. No interaction between HIP14 and the further truncated 224, 151, 88 and 40 aa htt fragments were observed. Thus HIP14 interacts specifically with a region of htt encompassing aa427 to 548. 3 independent experiments were performed to obtain the statistical analysis.

5.3.4 Palmitoylation of GST-SNAP25 by GST-HIP14 is potentiated in vitro in the presence of wildtype but not mutant huntingtin

To examine whether huntingtin can modulate HIP14's activity toward other substrates, we 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 enhanced GST-SNAP25 palmitoylation by about 70%. In the presence of COS cell lysate that expressed wildtype N-terminal fragment huntingtin (amino acids 1-548), palmitoylation of GST-SNAP25 by HIP14 was further significantly enhanced. In contrast, mutant N-terminal fragment huntingtin was not capable of potentiating HIP14 activity (Figure 5.4A,B). These results were reproduced with full-length huntingtin (Figure 5.4C,D).
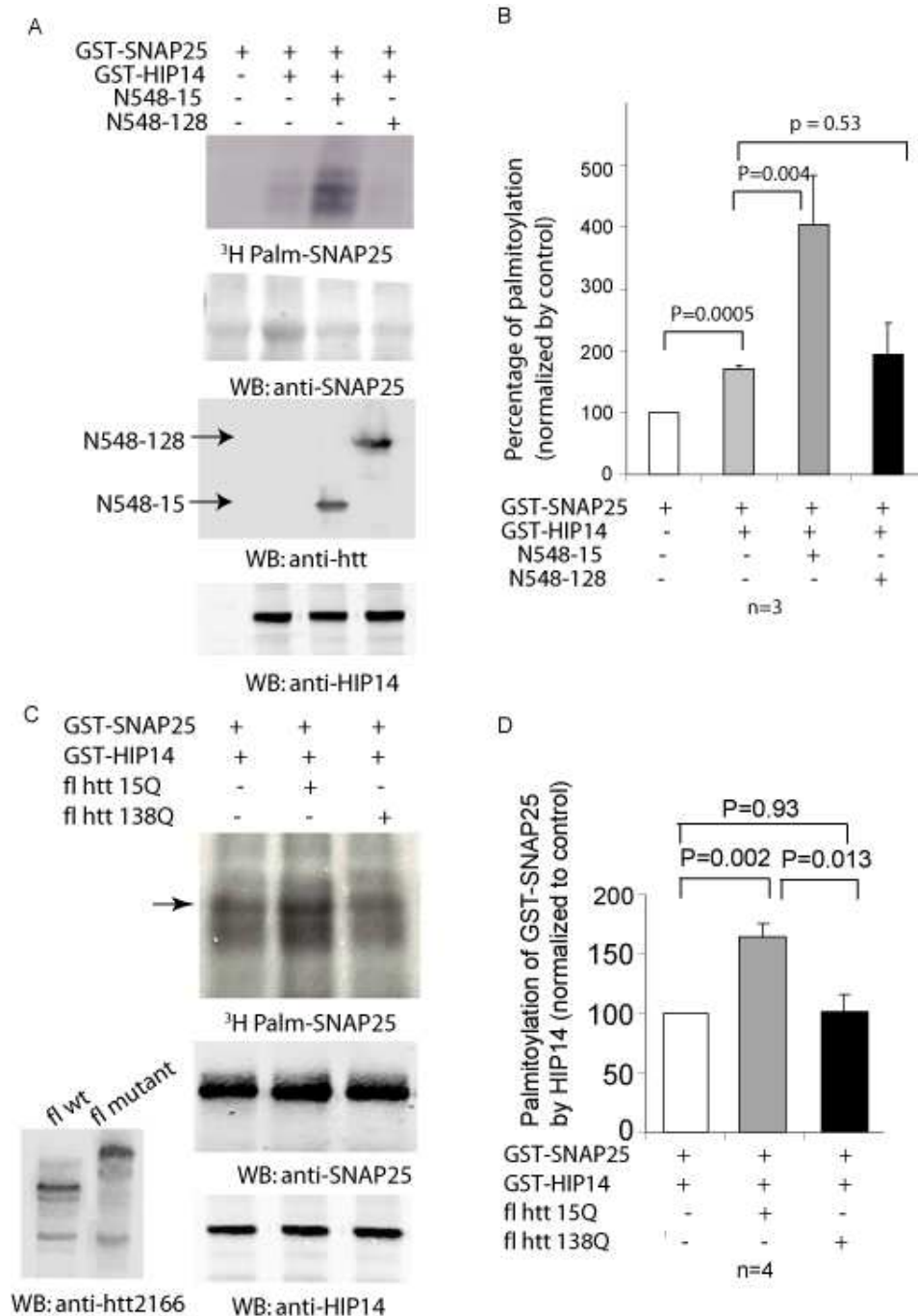
Figure 5.4. Huntingtin (htt) increases HIP14 activity to palmitoylate GST-SNAP25 *in vitro*.

(A) Representative blot showing that GST-HIP14 could enhance palmitoylation of GST-SNAP25 in the presence of N terminal fragment wt htt (1-548aa), but not mutant htt. **(B)** Quantification showing that GST-HIP14 could enhance palmitoylation of GST-SNAP25 by 70%. In the presence of wt htt, HIP14 activity was further potentiated by about 2 folds. **(C)** Representative blot showing that GST-HIP14 could enhance palmitoylation of GST-SNAP25 in the presence of full length wt htt (1-548aa), but not mutant htt. **(D)** Quantification showing that HIP14 activity was further potentiated by 2 folds with full-length wt htt, but not mutant htt.

Similarly, palmitoylation of PSD-95(1-PDZ2) and GST-GAD65(1-80aa) by GST-HIP14 was also potentiated *in vitro* in the presence of wildtype huntingtin but not mutant huntingtin (Figure 5.5). To validate these *in vitro* palmitoylation assays, we reproduced the results in two similar paradigms. First, we used immunoprecipitated huntingtin from COS cell lysate expressing huntingtin (Figure 5.5). Second, HIP14 and huntingtin were expressed in COS cells and immunoprecipitated as a complex (Figure 5.5). In both paradigms, HIP14 enhanced the palmitoylation of PSD95 only in the presence of wildtype huntingtin (Figure 5.5). Next, we expressed DHHC-3 and huntingtin in COS cells and immunoprecipitated DHHC-3 as an enzyme to palmitoylate GST-SNAP25. Huntingtin was unable to further enhance the activity of DHHC-3 (Figure 5.6A). Furthermore, GAD-65, a substrate of HIP14, was not able to enhance the activity of HIP14 (Figure 5.6B). These results indicate that the ability of huntingtin to regulate HIP14 enzymatic activity is a unique feature of the two proteins. Next, we expressed GAD65, HIP14 and normal huntingtin in COS cells and palmitoylation of GAD65 was measured by the ABE assay. Palmitoylation of GAD65 by HIP14 was also further potentiated by wildtype huntingtin (Figure 5.7).
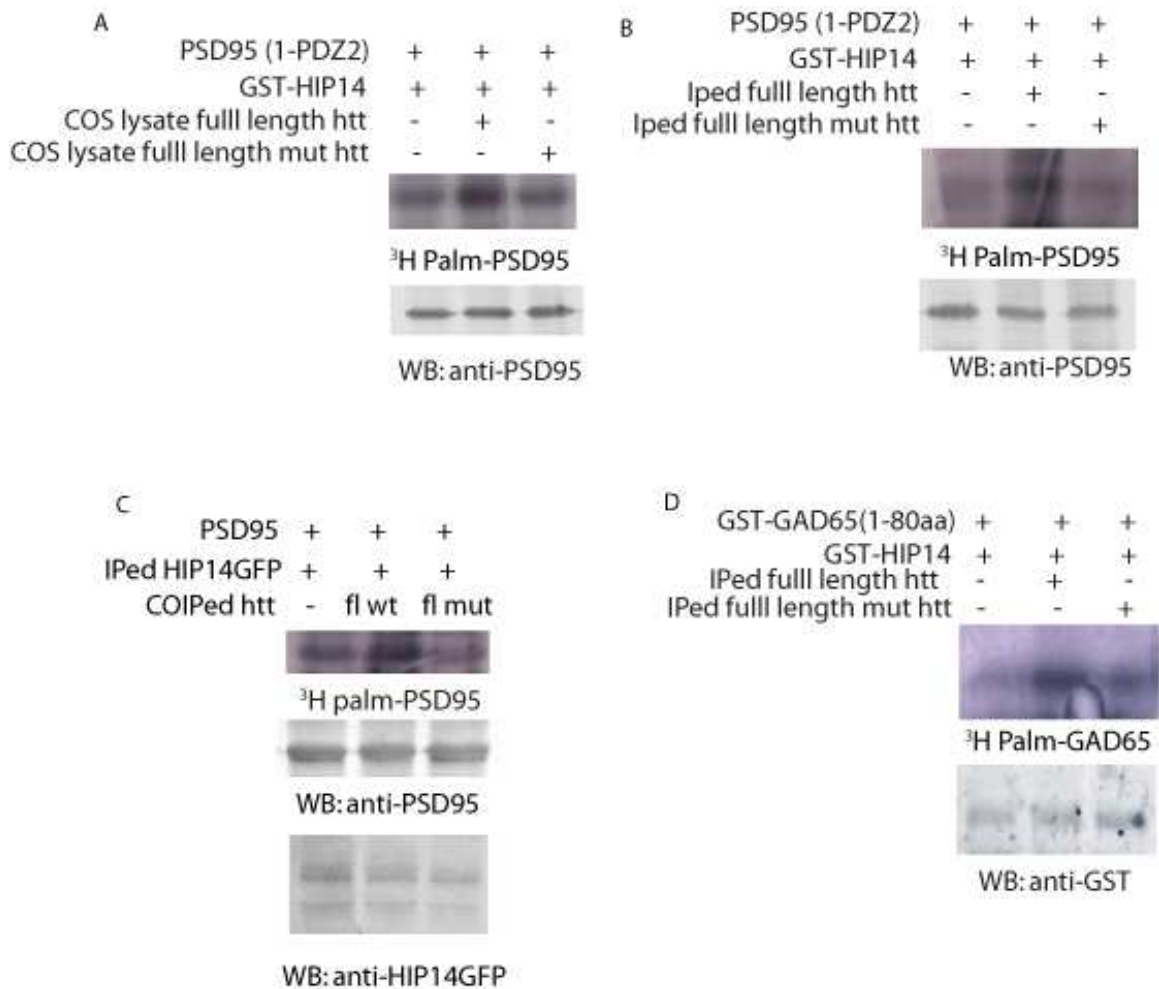
Figure 5.5. Palmitoylation of PSD95 and GAD65 by HIP14 in vitro can also be enhanced by huntingtin.

We showed that huntingtin could potentiate HIP14 activity to palmitoylate PSD95 in vitro with three different approaches. **(A)** purified GST-HIP14 and COS cell lysate overexpressing huntingtin. **(B)** purified GST-HIP14 with IPed huntingtin from COS cell lysate overexpressing huntingtin. **(C)** IPed HIP14 and huntingtin complex from COS cell lysate overexpressing both. **(D)** in vitro palmitoylation of GST-GAD65 (1-80aa) by GST-HIP14 is also enhanced in the presence of IPed huntingtin from COS cell lysate overexpressing huntingtin.
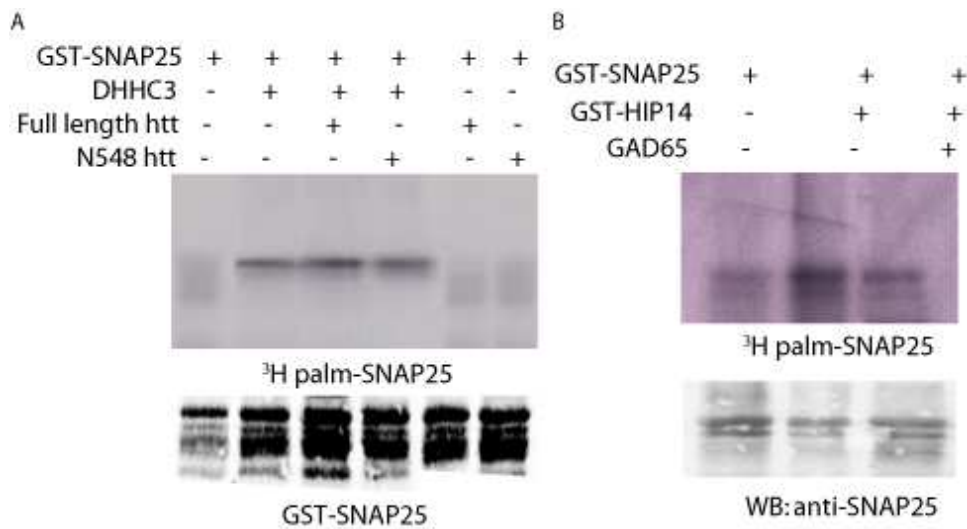
Figure 5.6. Potentiation of enzymatic activity by huntingtin is unique to HIP14.

(A) DHHC-3 enzymatic activity to palmitoylate GST-SNAP25 is not affected by either wt htt or mutant htt. Moreover, full length or N terminus truncated htt itself does not exhibit palmitoylation enzymatic activity. **(B)** The ability to potentiate HIP14 enzymatic activity is specific to huntingtin. GAD65, which is also a palmitoylation substrate of HIP14, does not increase HIP14 activity to palmitoylate GST-SNAP25 in vitro.
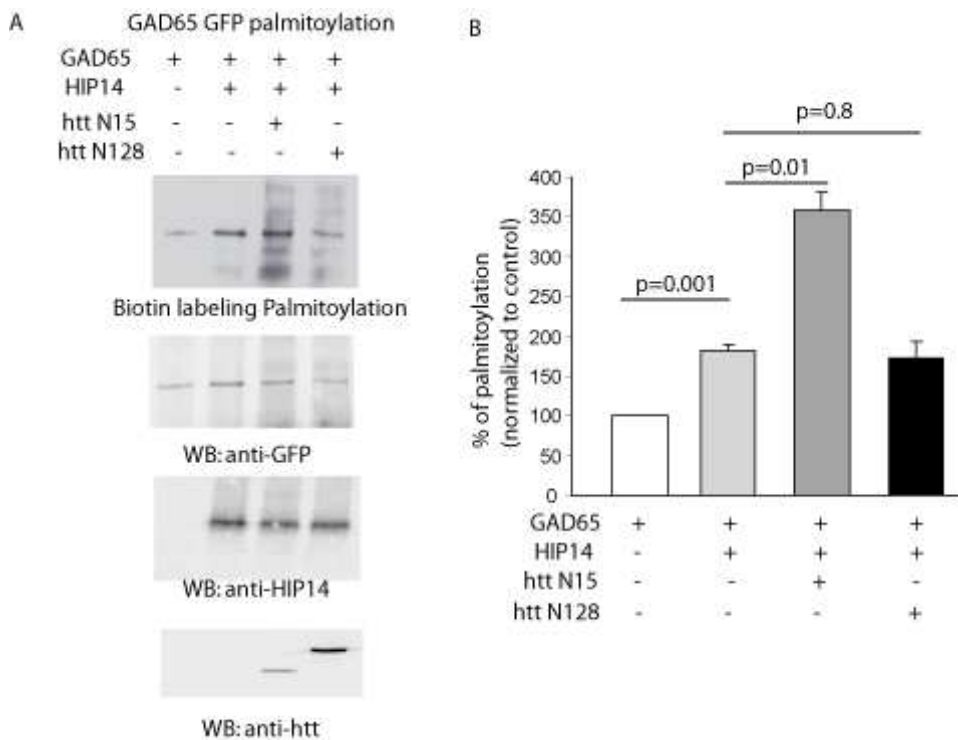
Figure 5.7. Palmitoylation of GAD65 by HIP14 in COS cells can also be enhanced when wt huntingtin is co-expressed.

(A) COS cells overexpressing GAD65-GFP alone or with HIP14 and huntingtin were subject to ABE assay to access the palmitoylation level of GAD65. **(B)** Quantification showed that HIP14 normally enhanced palmitoylation of GAD65 by 75%, whereas presence of wildtype huntingtin further potentiated HIP14 activity by another fold.

5.3.5 HIP14 activity in mice lacking wildtype huntingtin (huntingtin+/-) is significantly reduced

If HIP14 activity were indeed enhanced by wildtype huntingtin *in vivo*, then reduced huntingtin level would be expected to impair HIP14's enzymatic activity toward other substrates. To test this hypothesis, we immunoprecipitated HIP14 from the brain lysate of wildtype mice and heterozygous mice with targeted deletion of huntingtin (huntingtin+/-), and performed the *in vitro* palmitoylation assay on GST-SNAP25. Indeed, HIP14 from these mice exhibited less enzymatic activity toward GST-SNAP25 (Figure 5.8).

Next, we applied the ABE method to examine the global palmitoylation changes in huntingtin +/- mice and determined palmitoylation of a number of presynaptic and postsynaptic proteins was reduced. These include SNAP25, SynaptotagminI, GAD65, SCAMP, the glutamate transporter and PSD95 (Figure 5.9). To validate these results, immunoprecipitation of individal substrates, including SNAP25 and PSD95, were performed and the palmitoylation level of these proteins in wildtype and huntingtin+/- mice brains was examined (Figure 5.10). Indeed, similar reduction of SNAP25 and PSD95 palmitoylation was observed in mice expressing reduced level of huntingtin. In agreement with the *in vitro* palmitoylation data, these results suggest that, *in vivo*, huntingtin plays a role in maintaining the normal HIP14 enzymatic activity.
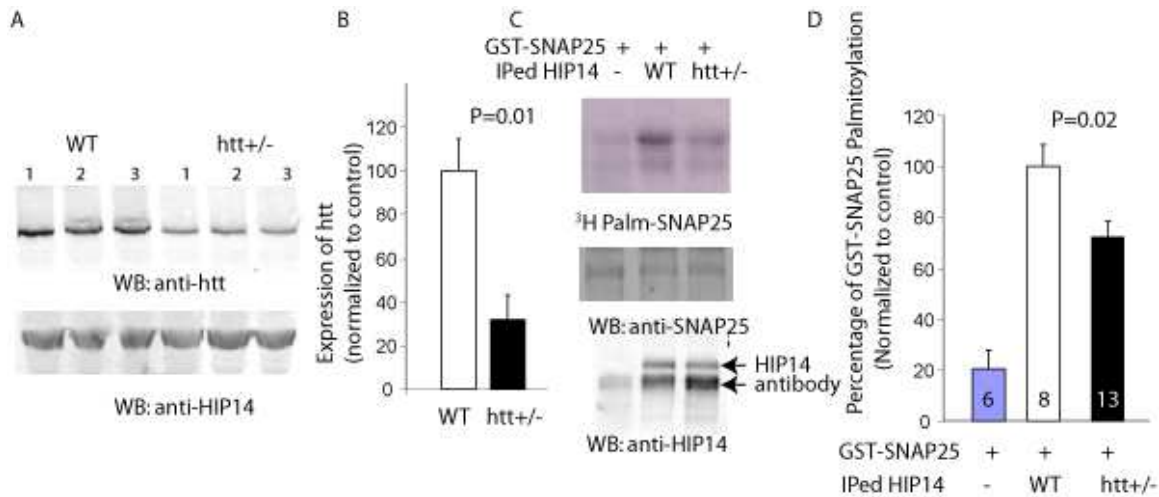
Figure 5.8. HIP14 from huntingtin+/- mice exhibits less palmitoylation enzyme activity toward GST-SNAP25.

(A) Western blot of the brain lysate from huntingtin+/- mice showed reduced huntingtin expression but normal HIP14 expression, compared to wt mice. **(B)** Quantification revealing approximately 60% reduction of huntingtin expression level in huntingtin+/- mice brain. N=3. **(C)** Representative image showing immunoprecipitated HIP14 of huntingtin+/- mice brain lysate exhibited less enzyme activity to palmitoylate GST-SNAP25, compared to that of wt mice. **(D)** Quantification revealing that purified HIP14 from huntingtin+/- mice brain showed approximately 30% reduction in activity. The numbers in the columns of the graph indicate the repeat times.
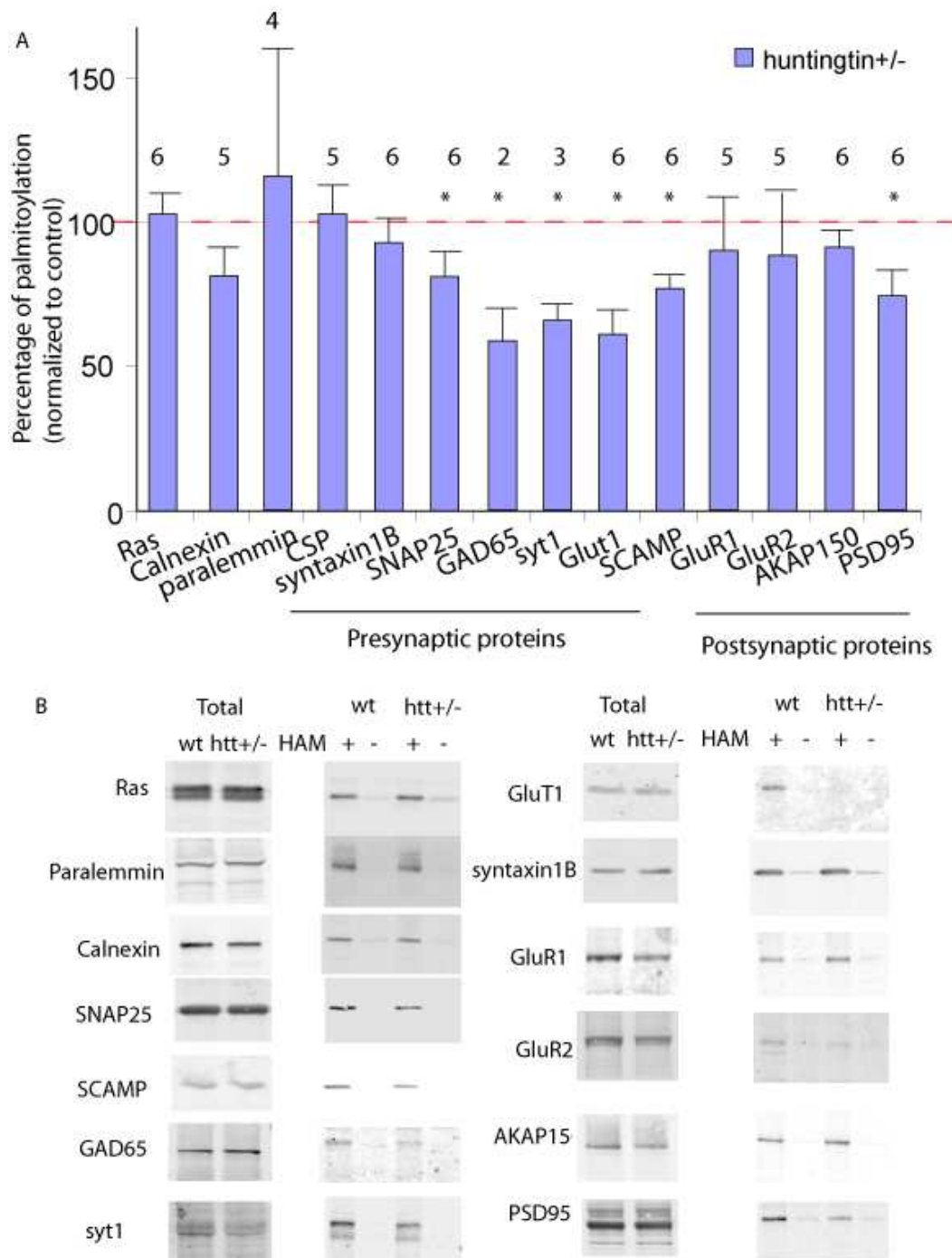
Figure 5.9. Global analysis of palmitoylation reveals palmitoylation defect in a number of neuronal proteins in huntingtin+/- mice.

(A,B) ABE assay was performed on wt and huntingtin+/- mice brain to precipitate all the palmitoylated proteins. Samples were then subject to electrophoresis and western blotting analysis. Quantification and representative blots showed alteration in palmitoylation of a number of neuronal proteins, predominantly presynaptic.
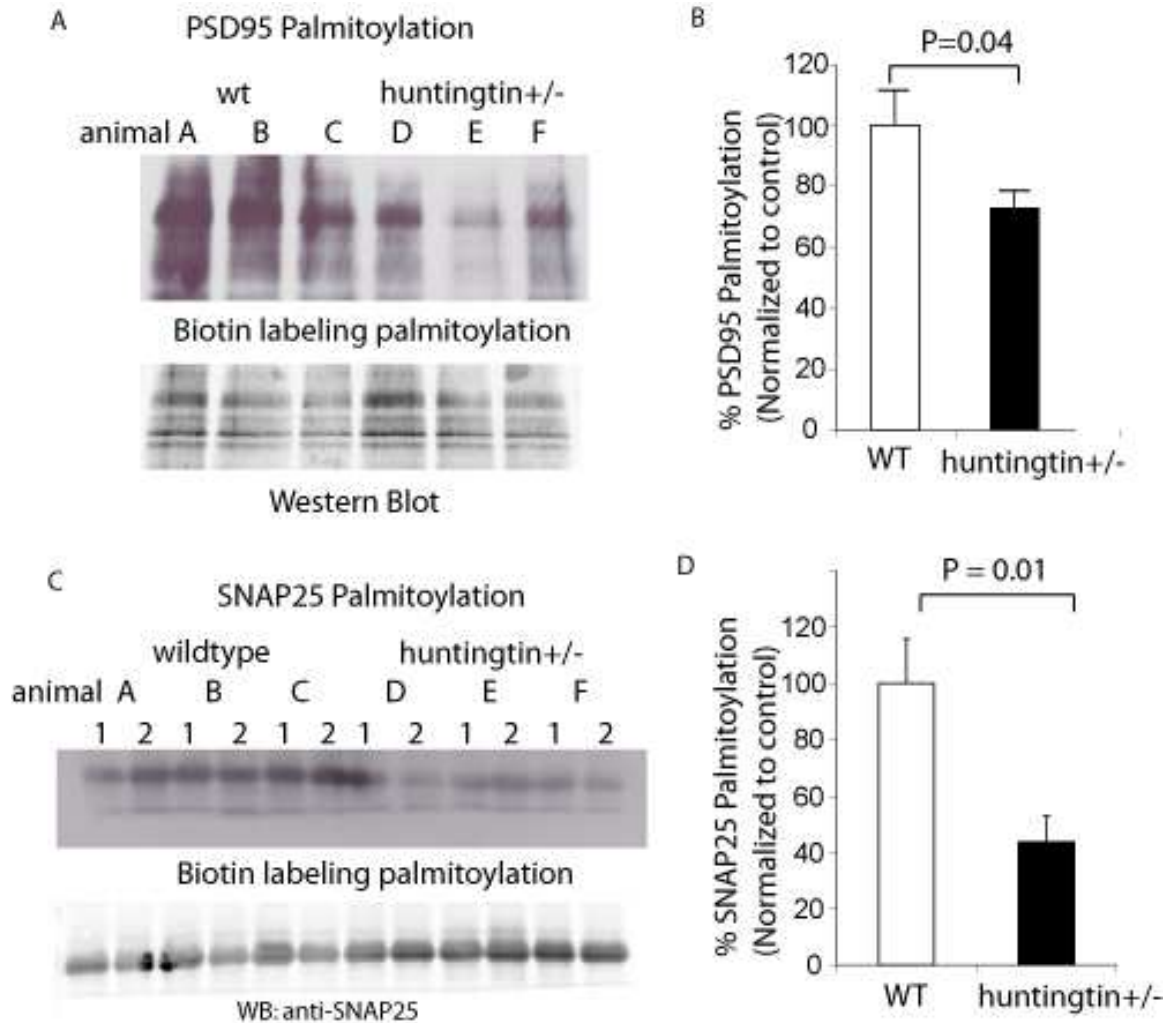
Figure 5.10. Confirmation of PSD95 and SNAP25 palmitoylation defect in huntingtin+/- mice by ABE assay.

Brain lysate from 3 wt and 3 huntingtin+/- mice were subject to PSD95 or SNAP25 immunoprecipitation followed by ABE assay. Quantification showed a 25% reduction in PSD95 palmitoylation and a 40% reduction in SNAP25 palmitoylation in huntingtin+/- mice.

5.4. DISCUSSION

Huntingtin is associated with membranes (Kim *et al.* 2001; Kegel *et al.* 2005; Suopanki *et al.* 2006)including caveolae(Trushina *et al.* 2006), clathrin coated vesicles(DiFiglia *et al.* 1995), and other endosomal compartments (Velier *et al.* 1998). Huntingtin also associates with microtubules (Hoffner *et al.* 2002) and other organelles such as the endoplasmic reticulum (ER) and Golgi (Rockabrand *et al.* 2007). The widespread regional distribution and subcellular localization of huntingtin does not help define a precise function in any specific compartment of the cell. However, a number of different functions have been proposed for wildtype huntingtin (reviewed in (Cattaneo *et al.* 2005)): i) It is a scaffolding molecule which binds to many proteins; ii) It is directly and indirectly involved in regulating gene expression; iii) It is also responsible for intracellular transport of some proteins; iv) It is required for normal embryonic development.

Our study shows that huntingtin, beyond these proposed functions, can regulate the enzymatic activity of one of its binding partners, the neuronal palmitoyl transferase HIP14. The association of huntingtin with HIP14 in the Golgi and other endosomal compartments suggests that these may be the subcellular locations where huntingtin regulates HIP14 activity.

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. In mammals, DHHC9 and GCP16 display the properties of a functional human ortholog of the yeast Ras palmitoyltransferase. Therefore, binding partners of some DHHC proteins may determine

the substrate specificity, enzymatic kinetics, subcellular localization, and stability of these DHHC proteins as an auxiliary subunit.

Our finding that huntingtin plays an auxiliary subunit role in regulating HIP14's enzymatic activity is very intriguing because of the relative large three-dimensinal structure of huntingtin protein. Future studies are needed to examine the crystal structures of huntingtin and HIP14.   Such studies may help determine how this interaction facilitates the palmitoylation of other proteins by HIP14. It is also worth noting that examination of global palmitoylation level changes reveal palmitoylation deficiency of a number of neuronal proteins, most of which are presynaptic proteins involved in neurotransmitter release and uptake. These proteins could directly be the *in vivo* substrates of HIP14, or indirectly affected by lack of palmitoylation of other proteins. It is still to be determined how the predominant palmitoylation deficiency in these presynaptic proteins correlates with the phenotype observed in huntingtin+/- mice. However, our results do complement two recent Drosophila HIP14 knockout studies, both of which identify a role of the Drosophila HIP14 in neurotransmitter release. The drosophila studies also reveal that two exocytic components-cysteine string protein (CSP) and SNAP25-are severely mislocalized at *hip14* mutant synapses, suggesting these are the two *in vivo* substrate of drosophila HIP14. In huntingtin+/- mice, however, palmitoylation of CSP is not affected. This may be due to the fact that DHHC proteins have different substrate preference in different organisms. Therefore, generation and characterization of HIP14-/- mice will provide a better knowledge of the *in vivo* substrates of HIP14 in mammals.

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6. CHAPTER 6   Conclusions

6.1. SUMMARY OF FINDINGS

The objective of this thesis was to conduct molecular and cell biological studies to identify HIP14 as a neuronal palmitoyl transferase, determine whether huntingtin palmitoylation was regulated by HIP14, and examine what role huntingtin might play in HIP14 enzymatic activity and whether this role is implicated in the disease.

The first specific goal was to identify HIP14 as a neuronal palmitoyl acyl transferase (PAT). To do this, we adapted the *in vitro* palmitoylation assay from yeast to the mammalian system. In this *in vitro* palmitoylation, we showed that GST-HIP14 was able to transfer ^{3}H Palmitoyl from synthesized ^{3}H Palmitoyl-CoA onto the cysteines of GST-SNAP25, synaptotagmin I, GAD65 and PSD95. We also found that, in both COS cells and neurons, palmitoylation dependent trafficking of these neuronal proteins were altered by overexpression or knockdown of HIP14, in agreement with the *in vitro* palmitoylation result. From this study, we also believed that huntingtin, through binding to HIP14, could be palmitoylated by HIP14 *in vivo*. This study led to the second specific goal of my thesis.

The second specific goal was to determine whether huntingtin is palmitoylated by HIP14, which cysteines are the palmitoylation sites, whether huntingtin palmitoylation is implicated in pathogenesis of HD and what role HIP14 plays in huntingtin trafficking and function. We demonstrate that huntingtin is palmitoylated by HIP14 primarily at Cysteine 214. Expansion of the polyglutamine tract in mutant huntingtin reduces its association with HIP14 and results in decreased palmitoylation of huntingtin, which contributes to the formation of inclusion bodies and enhanced neuronal toxicity. By manipulating

HIP14 level through overexpression or knockdown, we can manipulate the number of huntingtin inclusion bodies and neuronal cell viability.

The existence of 23 DHHC proteins as palmitoyl transferases which all contain a cysteine rich catalytic domain led to the third specific goal of this thesis. Why are so many PATs required in mammals? Is there substrate specificity? What determines the substrate preference of these DHHCs? We were able to apply the new technique, acyl biotin exchange palmitoylation assay to examine the substrate specificity of four brain expressed DHHCs, namely DHHC-3, DHHC-8, DHHC-13 (HIP14L) and DHHC-17 (HIP14). They exhibit distinct substrate preference, with DHHC-3 showing the least substrate specificity. These DHHCs interact with their respective substrates in a manner that requires the presence of the palmitoylated cysteines and proper surrounding amino acids. By adding the ankryin repeat domain of HIP14 to DHHC-3, we reveal that a region other than the catalytic DHHC domain probably determines substrate preference.

The fourth specific goal of this thesis was to examine whether wildtype huntingtin functions in regulating HIP14 enzymatic activity. The tight association between huntingtin and HIP14 distinguishes them from other known substrate and PAT pairs, which led us to hypothesize that huntingtin may function beyond being a substrate of HIP14. Determining a specific function for wildtype huntingtin proves to be a challenging task considering the complex and multiple functions of huntingtin, and lack of a defined functional assay. However, we were able to show that, both *in vitro* and *in vivo,* wildtype huntingtin can modulate HIP14 activity toward palmitoylating other neuronal substrates, including a number of presynaptic proteins and PSD-95. *In vitro*, mutant huntingtin loses the capacity to enhance HIP14 PAT activity, suggesting that

deficient protein palmitoylation may contribute to pathogenesis of HD.

The significance of this thesis lies in several aspects. This work identifies the first neuronal palmitoyl transferase HIP14 that regulates the trafficking of multiple neuronal proteins. Then we demonstrate how HIP14 regulates the trafficking and function of huntingtin protein, and how palmitoylation deficiency in mutant huntingtin is implicated in HD pathology. The fact that we are able to manipulate inclusion formation and neuron viability through changing HIP14 expression levels promises a potential in therapeutic strategy. In addition, this work provides some pioneer studies in comparing and contrasting substrate specificity among different DHHC PATs. Although the exact elements that determine the specificity between enzymes and substrates are not clear, this work reveals that regions other than the catalytic domain may be critical for the recognition of a substrate. Finally, this work discovers a new function for wildtype huntingtin. Besides being a scaffold, neuroprotective and facilitating vesicle transporting, wildtype huntingtin can modulate HIP14 PAT activity toward other neuronal substrates. The fact that mutant huntingtin lacks this function indicates that one mechanism underlies the pathogenesis of HD is a defect of protein palmitoylation.

6.2. ROLE OF PALMITOYLATION IN THE MOLECULAR FUNCTION OF HUNTINGTIN

How does C214 palmitoylation fit in with the current understanding of huntingtin function?

6.2.1 Palmitoylation associates huntingtin with the membranes

Palmitoylation, in many cases, increases the hydrophobicity of a protein and directs it to membranous structures. Recently, using imaging, integrative proteomics, and cell biology, huntingtin has been defined as a membrane-associated protein, with activities related to axonal trafficking of vesicles and mitochondria. The discovery of huntingtin palmitoylation at C214 reinforces the fact that huntingtin is membrane associated. In addtion, huntingtin is also reported to contain a membrane association domain (1-18aa) that reversibly targets to vesicles and the endoplasmic reticulum (ER) (Atwal *et al.* 2007; Atwal and Truant 2008).

6.2.2 Palmitoylation may modify huntingtin folding

The framework of the huntingtin protein is flexible. Differential detection of huntingtin by various antibodies, depending on the polyglutamine size (ie 1C2 antibody) and subcellular location (ie EM48 antibody), indicates that this protein's intrinsic structure changes in such circumstances(Trottier *et al.* 1995b; Trettel *et al.* 2000; Ko *et al.* 2001). Therefore, palmitoylation, which increases the hydrophobicity of huntingtin, may also have an impact on the folding of the protein itself. Indeed, we have shown that palmitoylation-deficient forms of even wildtype polyglutamine huntingtin exhibit significant more neuronal inclusions, indicating palmitoylation contributes to huntingtin

proper folding.

Similarly, the importance of palmitoylation in proper folding of several other proteins has been demonstrated. The stability of the coronavirus envelope protein depends on its palmitoylation(Lopez *et al.* 2008). Drisdel et al also showed that palmitoylation is critical for proper protein folding and ER processing of the Bgt Receptor (Drisdel *et al.* 2004). Early α7 subunit precursors, which exist in the form of aggregates, and unassembled subunits or assembling subunits that have not yet formed Bgt binding sites, are not or poorly palmitoylated. In contrast, intracellular BgtRs are highly palmitoylated, and inhibition of α7 subunit palmitoylation in PC12 cells blocks formation of new Bgt binding sites. In another example, palmitoylation influences the oligomerization state of Gα(o) to regulate the GDP/GTP exchange of Gα (o) and thereby modulate the on-off switch of the G protein in G protein-coupled signal transduction(Yang *et al.* 2008). However, palmitoylation is not always a favorable default. In the case of the mammalian water channel aquaporin-4, formation of aquaporin-4 arrays is inhibited by palmitoylation of N-terminal cysteine residues(Suzuki *et al.* 2007).

6.2.3 Huntingtin palmitoylation and other posttranslational modification

In many cases, co-regulation exists between palmitoylation and phosphorylation of a substrate (Soskic *et al.* 1999; Hawtin *et al.* 2001; Dorfleutner and Ruf 2003; Ponimaskin *et al.* 2005). In some other examples, palmitoylation and proteolysis also co-regulate each other. For instance, palmitoylation-deficient recombinant human A1 adenosine receptor is subject to enhanced proteolysis (Gao *et al.* 1999). Palmitoylation of

the C-terminal fragment of p75(NTR) is required for subsequent cleavage by γ-secretase(Underwood *et al.* 2008). It is also possible that phosphorylation, proteolysis and/or palmitoylation of huntingtin modulate one another and ultimately the subcellular localization of huntingtin.

6.2.4 Palmitoylation and protein nuclear localiazation

Mounting new evidence also indicates that regulated palmitoylation can control shuttling of signaling proteins between plasma membrane/and the nucleus. G protein-coupled receptor (GPCR) kinases (GRKs) specifically phosphorylate agonist-occupied GPCRs at the inner surface of the plasma membrane, leading to receptor desensitization(Jiang *et al.* 2007). Non-palmitoylated forms of GRK6A are released from the plasma membrane to the cytoplasm and then into the nucleus. A similar mechanism may also control huntingtin protein shuttling between cytosol and the nucleus. Huntingtin, the protein affected in Huntington's Disease, is N-terminally palmitoylated, and this palmitoylation is essential for its proper sorting and folding. The palmitoylation deficient form of wild type huntingtin results in its accumulation in the nucleus (Figure 1.15A) (Yanai *et al.* 2006). Likewise, the R7 family binding protein (R7BP) that binds to a subfamily of the regulator of G protein–signaling (RGS) proteins is C-terminally palmitoylated. This palmitoylation targets the R7BP/RGS7/Gβ5 complex to the plasma membrane, while nonpalmitoylated R7BP directs the complex to the nucleus(Drenan *et al.* 2005; Drenan *et al.* 2006; Song *et al.* 2006). Similarly, estrogen receptor α(Li *et al.* 2003b; Acconcia *et al.* 2005; Rai *et al.* 2005), and phospholipid scramblase 1(Wiedmer *et al.* 2003) have been demonstrated recently to localize to the plasma membrane when palmitoylated but translocate to the nucleus when depalmitoylated. Taken together, these

studies reveal a novel role for palmitoylation in controlling shuttling of several signaling proteins between the plasma membrane and the nucleus.

6.3. GLOBAL DEFFECTS IN PALMITOYLATION MAY UNDERLIE HD

The wide distribution in tissue and subcellular fractions prevent us from defining a single unique function of wildtype huntingtin. To date, huntingtin has been reported to facilitate vesicle transport in axons and regulate gene transcription. Huntingtin also functions in development and neuroprotection, probably through mechanisms such as promotion of *Bdnf* gene transcription. Although it is challenging to define wildtype huntingtin function, conclusions from studying various genetic models of HD have provided much valuble information about the wildtype protein. These studies have implied that HD is a disease of both a loss of wildtype huntingtin function and a gain of mutant huntingtin function (Cattaneo *et al.* 2005). From the work of this thesis, we identified a new role for wildtype huntingtin, which is to enhance the enzymatic activity of a PAT HIP14. *In vitro*, mutant huntingtin does not have the same capacity, indicating a loss in the wildtype function. If *in vivo* mutant huntingtin plays a dominant negative role, then palmitoylation defects of a similar pool of proteins are expected in both YAC 128 mice and huntingtin+/- mice. A comparison of the palmitoylation profile of these two mice will further provide *in vivo* evidence of this hypothesis.

6.4. HUNTINGTIN PAMLITOYLATION AS A THERAPUETIC TARGET

The data in this thesis provide evidence that increasing huntingtin palmitoylation may be a useful strategy for therapeutical approaches, because palmitoylation deficiency of mutant huntingtin is evident and is implicated in HD. In YAC 128 mice primary cortical neurons that express mutant huntingtin, cell viability following NMDA-induced excitotoxicity is correlated with HIP14 expression. Knocking down expression of the huntingtin PAT HIP14 decreases cell resistance to excitotoxicity whereas exogenous HIP14 increases the cell viability. Therefore increasing huntingtin palmitoylation may be a viable strategy for improvement of symptoms and prognosis of HD. One possibility is to stabilize the interaction of HIP14 and mutant huntingtin, and thereby increase mutant huntingtin palmitoylation. Using the atomic insight gleaned from the 3D structure, it may be possible to employ rational drug design to increase interaction between HIP14 and huntingtin, either by increasing the binding surfaces between the two proteins, or by increasing the ionic bonding between the two, thereby stabilizing the interaction. Thioesterase inhibitors may offer another promising method of increasing the palmitoylation at huntingtin C214. For HD, the effective use of thioesterase inhibitors requires identification of the thioesterases that act on huntingtin and specifically on C214.

6.5. OTHER PALMITOYLATION SITES OF HUNTINGTIN

Cysteine mutagenesis of huntingtin was intensively investigated at the N terminus of huntingtin and only C214 was confirmed to the palmitoylation site of N terminal fragment. However, other cysteines at the C terminus may also possible to be

palmitoylated. CSS-Palm 2.0 palmitoylation site predictor (Zhou *et al.* 2006) detects several other possible palmitoylation cysteines, as are shown in the table (http://bioinformatics.lcd-ustc.org/css_palm/prediction.php).

This web-based palmitoylation site predictor uses a Clustering and Scoring Strategy. 210 experimentally verified palmitoylation sites from 83 distinct proteins were grouped into several subsets based on their sequence similarity. Each subset is characterized by the high sequence similarity between the palmitoylation sites. For each given cysteine residue, its final score as a potential palmitoylation site is defined as the highest similarity score among all similarity scores against the above partitioned subsets. Its prediction performance on the curated dataset is highly encouraging with Jack-Knife sensitivity of 82.16% and specificity of 83.17%, respectively.

Table 6.1. Predicted palmitoylation sites in full length huntingtin with high cutoff value

Cysteine site	Peptide	Score	Cutoff	Type
214	LLP**C**LTR	2.878	1.000	TypeIII: Others
1597	LQQ**C**HKE	1.235	1.000	TypeIII: Others
2284	LDC**C**CLA	1.984	1.700	TypeI: -CC-
3144	VTT**C*****	2.113	1.000	TypeIII: Others

Table 6.2. Predicted palmitoylation sites in full length huntingtin with medium cutoff value

Cysteine site	Peptide	Score	Cutoff	Type
214	LLP**C**LTR	2.878	0.800	TypeIII: Others
835	VRN**C**VMS	0.809	0.800	TypeIII: Others
1027	TFG**C**CEA	1.547	0.700	TypeI: -CC-
1028	FGC**C**EAL	1.484	0.700	TypeI: -CC-
1597	LQQ**C**HKE	1.235	0.800	TypeIII: Others
2283	GLD**C**CCL	1.359	0.700	TypeI: -CC-
2284	LDC**C**CLA	1.984	0.700	TypeI: -CC-

2285	DCC**C**LAL	1.141	0.700	TypeI: -CC-
3094	NLF**C**LVA	0.922	0.800	TypeIII: Others
3144	VTT**C*****	2.113	0.800	TypeIII: Others

Our studies have shown that C214 mutation of huntingtin loses a significant portion of the palmitoylation signal compared to wildtype, indicating C214 is a major site for palmitoylation. However, whether other putative palmitoylated cysteines participate in HD pathology still remain to be determined.

6.6.   FUTURE DIRECTIONS

The data presented in this thesis suggest numerous avenues of future investigation. This includes genetic and molecular studies ultimately aimed at identifying therapeutic targets for HD.

6.6.1   HIP14-/- and HIP14L-/- mice

HIP14 and HIP14L are the major PATs for huntingtin (see Figure 5.2). HIP14 and HIP14L knockout mice are essential to determine (i) if these proteins are the important PATs for huntingtin; (ii) *in vivo* consequences of loss of huntingtin palmitoylation; and (iii) the contribution of each PAT to huntingtin palmitoylation. Our discovery that huntingtin regulates HIP14 activity suggests that altered palmitoylation of neuronal substrates—other than huntingtin itself—may underlie the selective striatal loss in HD. Thus, it is a possibility that HIP14, HIP14L or HIP14/HIP14L double knockout mice will show similar phenoypes to those observed in our YAC128 HD mice. To this end, both HIP14 and HIP14L knockout mice are being generated to determine the phenotype. We expect that, because huntingtin may act as an auxiliary subunit for HIP14, proteins

showing altered palmitoylation in huntingtin+/- mice and YAC128 mice may also show altered palmitoylation in HIP14-/- and HIP14L-/- mice.

6.6.2   HIP14 and/or HIP14L transgenic mice cross YAC128 mice

Generation of HIP14 and HIP14L transgenic mice will be valuble to determine the effect of overexpression of HIP14 and HIP14L on the palmitoylation of huntingtin in vivo. Crossing either HIP14 or HIP14L to the YAC128 mice will determine if the increase in palmitoylation of huntingtin will result in an abrogation or delay of any aspect of the HD phenotype.

6.7. CONCLUSIONS

The discovery of mammalian palmitoyl acyl transferases has significantly promoted the progress of the palmitoylation field. Recent studies reveal a role of palmitoylation in many aspects of health and disease including Huntington Disease. Palmitoylation is a dynamic means of regulating huntingtin and is implicated in HD because it is reduced by the polyglutamine-expansion and modifies the cell viability. The findings from the characterization of C214 palmitoylation of huntingtin suggest that posttranslational modifications of huntingtin can modify crucial pathogenic processes and open new therapeutic possibilities for HD.

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APPENDIX

A.1 REAGENTS AND SAMPLES

A.1.1   Mouse Lines

All in vivo work, including YAC and wildtype mice, was done with mice from the FVB strain. Mice were housed either at the CMMT or the Wesbrook Building at UBC. The YAC18Q (line 212) and YAC128Q (line 53) mouse models contain the full-length genomic human HD gene with its endogenous promoter elements, providing appropriate developmental and tissue specific expression of the huntingtin protein (Slow *et al.* 2003). Huntingtin+/- mouse was created to have a disruption in exon 5 of the Hdh gene. The targeting construct pHdhNeo6 contained approximately half of exon 5 plus intron sequences and a PGKneobpA cassette. Disruption of murine exon 5 results in stop codons in all reading frames. Cognitive, behavioural and neuropathological features of the YAC128 mouse model and huntingtin+/- mice have been studied extensively (Nasir *et al.* 1995; O'Kusky *et al.* 1999; Slow *et al.* 2003; Van Raamsdonk *et al.* 2005a). The use of mice for research was approved in UBC animal care certificate A04-0073.

A.1.2   Antibodies

<u>Commercial antibodies</u>

1° ANTIBODY	SUPPLIER	CAT#	SOURCE	EPITOPE	WORKING DILUTION
Actin	Sigma	A2066	rabbit	SGPSIVHRKCF	1:200
Huntingtin	Chemicon	mAb2166	mouse	aa 443 - 457	1:2000
γ-Tubulin	Sigma	T3559	mouse mAb	aa 38-53	1:1000
HA	BABCO	AFC-101P	mouse	YPYDVPDYA	1:1000
GM130	BD	610822	mouse	aa869-982	1:200

Ubiquitin	Upstate	07-375	rabbit	bovine ubiquitin	NT
α-adaptin	Transduction Laboratories	610501	mouse	aa 38-215	1:2000
Rab5	Transduction Laboratories	610281	mouse	aa 1-215	1:500
Rab8	Transduction Laboratories	610845	mouse	aa 84-205	1:4000
GST	Santa Cruz Biotechnology	Sc-138	mouse	Full length GST	1:1000
PSD-95	Affinity Bioreagents	MA1-045	mouse	Full length PSD-95	1:2000
SNAP25	Stressgen	VAM-SV012E	mouse	ND	1:1000
EEA1	BD Biosciences	610457	mouse	aa 3-281	1:500
Hsp70	Neomarker	RB-080-A0	rabbit	ND	1:200
Transferrin receptor	Zymed	A-11130	mouse	ND	1:1000
Calnexin	Sigma	C4731	Rabbit	aa 573-592	1:200
GFP	Synaptic system	132 002	Rabbit	full-length GFP	1:2000
GAD65	Abcam	ab26113	mouse	near the C-terminus	1:1000
Synaptotagmin I	Synaptic system	105-221	mouse	lumenal domain	1:100
SCAMP	Upstate Biotech	05-741	mouse	Purified GLUT4 vesicles	1:1000
Syntaxin 1B	Synaptic system	110402	rabbit	GKLAIFTDD IKMDSQMT	1:1000
Glutamate Transporter (EAAC1)	Chemicon	MAB1587	mouse	KDKSDTISF TQTSQF	1:500
Flag M2	Sigma	F1804	mouse	DYKDDDDK	1:1000

ND: Not Determined

Antibodies generated in El-Husseini lab or Hayden Lab

1° ANTIBODY	REFERENCE	SOURCE	EPITOPE	WORKING DILUTION
BKP1 poly	(Kalchman *et al.* 1996)	Rabbit	aa 1-17	1:500
BKP1mono	(Kalchman *et al.* 1996)	mouse	aa 1-17	1:500

HD650	(Slow *et al.* 2003)	mouse	human specific @ aa 650	1:85
HIP14	(Singaraja *et al.* 2002)	Rabbit	aa 49-60	1:200
PSD-95	(Arstikaitis *et al.* 2008)	Rabbit	GST-PSD95 (1-PDZ2)	1:1000
GFP	(Huang *et al.* 2004)	Guinea pig	Full length GST-GFP	3μg for IP
GFP	N/A	Rabbit	Full length GST-GFP	3μg for IP

N/A: Not Applicable

A.2 EXPERIMENT PROCEDURES

A.2.1   Primary Neuronal Culture and Transfection

Neuronal cultures were prepared from hippocampi of E18/E19 rats. The hippocampi were dissociated by enzyme digestion with papain followed by brief mechanical trituration. Cells are plated on poly-D-lysine– (Sigma) treated glass coverslips (12 mm in diameter, Fisher) in 24-well plates (Falcon) and maintained in Neurobasal media (Gibco) supplemented with B27, penicillin, streptomycin, and L-glutamine. Hippocampal or cortical cultures are transfected with lipofectamine 2000 (Invitrogen) based on factory mannul. Briefly, cells were transfected between DIV 5 to 7. For each well of the 24 well plate, 1μl of DNA and 0.5μl of lipofectamine 2000 reagent are mixed in 50 µl of OPTIMEN (Invitrogen) and incubated at room temperature for 15 minutes. After the mixture is added to the well, the cells are incubated for 3 hr at 37°C and 5% CO_2 and then cell media that contains the transfection reagent is replaced with fresh pre-warmed media.

A.2.2   Cell Lysis and Tissue Preparation

<u>Cultured Cells</u>

To harvest the cultured cells, 1ml of PBS was added to each well of the plates and cells were scraped into a 1.5ml eppendof tube. After spinning the tube at 14000rpm for 1min, we collected the cell pellete and added 500ul of lysis buffer. The cells were sonicated briefly (3 x 10s on ice) and centrifuged at 14,000 rpm for 15 min at 4°C.

Lysis buffer for extracting PSD-95: 50 mM Tris pH 7.5, 150 mM NaCl, 2mM EDTA, 2mM EGTA, 0.2% SDS, 1% Triton-X100, 1X protease inhibitors (Roche) and 1mM PMSF).

Lysis buffer for extracting huntingtin and HIP14: 50 mM Tris pH 8.0, 150 mM NaCl, 1% Igepal, 1 mM PMSF, 1X protease inhibitors (Roche) and 5μM zVAD.

Lysis buffer for extracting other soluble proteins: 50 mM Tris pH 7.5, 150 mM NaCl, 2mM EDTA, 2mM EGTA, 1% Triton-X100, 1X protease inhibitors (Roche) and 1mM PMSF).

<u>Fresh Brain Lysates</u>

To generate fresh brain lysates, FVB wildtype or huntingtin+/- mice or YAC transgenic mice were sacrificed with Halothane and the brain was isolated and homogenized in lysis buffer without the detergent supplement. Samples were homogenized for 20 times on ice and then sonicated briefly (3 x 10s on ice). Next, detergent was supplemented and the samples were rotated at 4°C for an hour to for lysis. Samples were then centrifuged at 14,000 rpm for 30 min at 4°C. Protein concentration was assessed with the BCA assay (Pierce).

A.2.3   Immunoprecipitations

<u>Huntingtin immunoprecipitation</u>

Immunopurification of huntingtin was performed to purify huntingtin to quantify its bound protein interactors or to be used for *ex vitro* pamitoylation assay. Sepharose G beads (25uL) were washed and pre-bound with anti huntingtin mAb2166 for roughly 1-2 hours. Lysates (250ug to 2mg) were incubated with the beads for two hours, rotating at 4 °C and then washed three times with lysis buffer. When huntingtin is purified for *ex vitro* palmitoylation assay, lysis buffer that is used for washing the beads does not contain Igepal or Triton because the palmiotylation assay is very sensitive to the amount of detergent.

<u>HIP14 immunoprecipitation</u>

Immunopurification of HIP14 was performed to purify HIP14 to quantify its bound protein interactors or to be used for *ex vitro* pamitoylation assay. For HIP14 mAb (HD26), Sepharose G beads (25μL) were washed and pre-bound with 4 μg of HIP14 mAb for an hour. For HIP14 pAb (HD27), Sepharose A beads (25μL) were washed and pre-bound with 4 μg of HIP14 pAb for an hour. Lysates (2-4 mg) were incubated with the beads for two hours, rotating at 4 °C and then washed three times with lysis buffer. When HIP14 is purified for *ex vitro* palmitoylation assay, lysis buffer that is used for washing the beads does not contain Igepal or Triton because the palmiotylation assay is very sensitive to the amount of detergent.

A.2.4   Immunoblotting

Procedure

Standard procedures were used for most immunoblotting. Briefly, sample preparation involved denaturing the lysates or immunoprecipitates in SDS gel loading buffer and heating for 10mins at 90°C. For proteins of molecular weight between 40kDa to 200kDa, 10% polyacrylamide gels were used. For proteins of smaller molecular weight (20 – 30kDa), 4-12% bis-tris (Invitrogen) gels were used. For proteins of big molecular weight such as huntingtin 350kDa, 3-8% Tris-acetate gels (Invitrogen) were used. The gels were run for 1-2 hours at 150-200V. Rainbow marker (GE health, RPN800) was used for indicating the molecular weight. Transfers were performed onto Nitrocellular membranes (Amersham) at 0.5A for 60-90 minutes using transfer buffer (11.6g Tris, 58g glycine, 400ml MeOH and 2g SDS in 2L H_2O). Blocking was performed with 5% milk or 5% BSA depending on the primary antibody.

Quantitative Immunoblotting

All immunoblots were used with fluorescently labeled secondaries and the Licor Odyssey immunoblotting system. Reliable quantitation relies on clean, crisp separation of bands that are of moderate exposure (not over/underexposed). For statistical analysis of immunoblotting, groups were compared based on the numerical densitometry results from Image J software. Student T-test was performed with 2 groups. P values <0.05 were considered to be statistically significant.

A.2.5   Expression and Purification of GST-Fusion Proteins

GST fusion proteins were produced in E. coli using the pGEX 6p3 expression system (Amersham). For GST-SNAP25, PSD95 (1-PDZ2), GAD65 (1-80aa), paralemmin (C-terminal 12aa) and synaptotagmin VII (C-terminal 12aa), the induction was performed with 300μM IPTG at 30°C for 5 hours. For GST-HIP14, the induction was performed at RT with 100uM IPTG for overnight. For 1L of induced bacteria culture, cells were resuspended in 10ml of ice cold lysis buffer containing 50mM Tris, 150mM NaCl, 1% Triton X-100, 0.1mg/ml DNAase, 1mg/ml Lysosome, 1mM PMSF and 1X protease inhibitor (Roche). The lysis solution was then sonicated on ice for 4X 15 seconds, followed by incubation at 4°C for an hour. GST-HIP14 was purified with no detergent to maintain proper protein folding. Next, the lysis solution was then centrifuged at 45000rpm, 4°C for 60 minutes to remove the bacteria debris. Supernatant was then subject to Glutathione Sepharose^{TM} 4B (Amersham) to extract the GST fusion proteins. After the sepharose was washed 6 times with wash buffer containing 25mM Tris, 150mM NaCl, 1mM PMSF, the GST fusion proteins were then eluted with 20mM Glutathione and dialyzed with 25Mm Tris pH7.0. To generate a free N-terminus of PSD-95, GST-PSD-95 protein was cleaved using thrombin (50mM Tris-HCl pH 8.0, 150mM NaCl, 2.5mM CaCl2, 0.1% 2-mercapto-ethanol).

A.2.6   Synthesis of Palmitoyl CoA

[^{3}H]palmitoyl-CoA, [^{3}H]myristoyl-CoA and palmitoyl-CoA were synthesized enzymatically from [9,10-^{3}H(N)]palmitic acid (5 mCi/ml; Perkin Elmer Life Sciences), [9,10-^{3}H(N)]myristic acid (1mCi/ml; Perkin Elmer Life Sciences) and palmitic acid (Sigma-Aldrich), respectively. Synthesis components also included co-enzyme A (CoA), ATP and acyl-CoA synthase (Sigma-Aldrich). Synthesized products were purified as

previously described (Dunphy *et al.* 1996). Synthesized [³H]palmitoyl-CoA and [³H]myristoyl-CoA were subjected to TLC (TLC aluminium sheet, silica gel, EM Science, Germany) to determine the efficiency. Ninhydrin Staining was used for the detection of synthesized non-radiolabeled palmitoyl-CoA. The synthesis was highly efficient, with >95% conversion of acid to fatty acid-CoA. Specific activity for [³H]palmitoyl-CoA was 60 Ci/mmol and for [³H]myristoyl-CoA was 30Ci/mmol.

A.2.7   Palmitoylation Assay

Palmitoylation reaction (60 µl) contained 5 µCi of [³H]palmitoyl-CoA, 0.33 µg/µl substrate protein, 1 mM ATP, 50 mM MES, pH 6.4, 0.2 mg/ml bovine liver lipids, and either 10µl of COS cell lysates expressing full length or N terminus huntingtin, or immunoprecipitated huntingtin. After 15min incubation at 37°C, sample buffer was added with final concentration of 5mM DTT was added and samples were subjected to SDS-PAGE analysis (Roth *et al.* 2002; Huang *et al.* 2004).

A.2.8   Btn-BMCC assay

After the immunoprecipitation was performed with lysis buffer containing 50mM HEPES pH7.0, 150mM NaCl, 1.0%Triton and 1X Protease inhibitors (Roche), Protein sepharose that contained the IPed protein was washed 3X with lysis buffer and incubated with 50mM NEM (Sigma) in lysis buffer at 4°C for an hour. The beads were washed 4X with lysis buffer very well and split evenly into two tubes: one tube was treated with 1M Hydroxylamine (HAM) (Sigma) supplemented with 1X protease inhibitor in 50mM HEPES pH7.0 at RT for an hour; the other tube was incubated with 50mM HEPES pH7.0 containing no HAM as a control. After an hour, each sample was washed 3X with lysis buffer and incubated with sulfhydryl-specific biotinylating reagent Btn-BMCC at 20 µM

in lysis buffer for 2 hours at 4°C. Samples were then washed 3X with lysis buffer and boiled in 5mM DTT SDS loading buffer for 10min before SDS electrophoresis and transferring to nitrocellular membranes. After blocking with BSA, blots are probed with streptavidin-conjugated horseradish peroxidase (Pierce) at a dilution of 1:10000 followed by ECL detection(Drisdel and Green 2004).

A.2.9   Immunofluorescence techniques

Immunofluorescence microscopy was performed in cultured cells that were grown on coverslips. One of two fixation methods was used depending on the primary antibody: 2% (w/v) paraformaldehyde for 10min at room temperature or ice-cold MeOH for 5 mins at a -20°C freezer. Many antibodies produced similar staining with both fixation methods, however some antibodies such as synaptic proteins only stained appropriately with MeOH fixation. Following fixation, cells were washed briefly with PBS containing 0.3% Triton X-100 (PBS-T) 3 times. Cells were then incubated with the primary antibody in 2% normal goat serum in PBS for 1h at RT or overnight at 4°C. Cells were washed 3x with PBS-T, and then incubated in the secondary antibody (Goat anti Mouse Alexa 568 (red): 1/1000, Goat anti Rabbit Alexa 568 (red): 1/1000, Goat anti Mouse Alexa 488 (green): 1/1000, or Goat anti Rabbit Alexa 488 (green): 1/1000) in 2% normal goat serum in PBS for 1h at RT. Cells were washed 3x PBS-T before mounting. Fluoromount-G (Southern Biotech Cat. #0100-01) was used to mount coverlips on slides. Immunofluorescence was detected using a laser confocal microscope (Zeiss) or conventional immunofluorescence microscopy (Zeiss) with a CCD camera (Princeton Instruments Inc.). Images were captured using Zeiss software and stored as separate JPG or TIFF files for each channel.

A.2.10 Electron microscopy

Electron microscopy on HIP14 in the brain was performed as previously described (Singaraja *et al.* 2002). Ultra-small colloidal gold conjugated secondary antibody (Aurion, Wageningen, The Netherlands) was used. Following a post-fixation with 2.5% glutaraldehyde, gold particles were intensified using the R-gent SE-EM silver enhancement kit (Aurion). Sections were further fixed with 0.5% OsO_4 in 0.1 M PB, dehydrated in ethanol and propylene oxide (1:1) and flat-embedded in Eponate 12 (Ted Pella, Redding, CA). Ultrathin sections (90 nm) were cut using a Leica Ultracut S ultramicrotome, and counterstained with 5% aqueous uranyl acetate for 5 min followed by lead citrate for 5 min. Thin sections were examined using a HITACHI H-7500 electron microscope.

A.2.11 Cell Radiolabeling with 3H palmitate

COS cells were labeled with 1 mCi/ml [^{3}H]palmitate (57 Ci/mmol; Perkin Elmer Life Sciences, Inc.) for 3 h while expressing the transfected proteins. Labeled cells were washed with ice-cold PBS and re-suspended briefly in 0.1 ml of lysis buffer containing 50 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% SDS. Triton X-100 was added to 1% to neutralize the SDS in a final volume of 0.5 ml. 26-Gage syringe was applied to break the nuclear chromosome DNAs. Insoluble material was removed by centrifugation at 14,000 rpm for 15 min at 4^0C. Immunoprecipitation procedure was performed as described above. Immunoprecipitates were washed three times with buffer containing 50 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1 mM EGTA, 150

mM NaCl, and 1% Triton X-100, boiled in SDS-PAGE sample buffer with 1 mM DTT for 10 min, and separated by SDS-PAGE and dried under vacuum. Gels were exposed to Hyper Film (Amersham) with intensifying screens at -80^0C for 3 to 20 days.

A.2.12   Bioinformatics

The prediction of potential palmitoylation sites was performed by submitting huntingtin protein sequence (P42858) to CSS-Palm site (http://bioinformatics.lcd-ustc.org/css_palm/prediction.php). This prediction program is based on the hypothesis that a short peptide surrounding the Cysteine amino acid contains sufficient information for whether the residue could be palmitoylated. However, the features for palmitoylation are quite elusive and almost all the existing studies proposed that there is no consensus sequence/motif for palmitoylation (el-Husseini Ael and Bredt 2002; Smotrys and Linder 2004). Therefore, CSS-Palm predictor divided the set of known palmitoylation sites into several clusters according to the similarities between them. Then, an algorithm to score the possibility of a potential palmitoylation site peptide was applied and the result was compared to the already known palmitoylated peptides.

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