Palmitoylation and trafficking of GAD65 are impaired in a cellular model of Huntington’s disease

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INTRODUCTION

HD (Huntington’s disease) is caused by an expanded polyQ (polyglutamine) repeat in the htt (huntingtin protein). GABAergic medium spiny neurons in the striatum are mostly affected in HD. However, mhtt (mutant huntingtin)-induced molecular changes in these neurons remain largely unknown. The present study focuses on the effect of mhtt on the subcellular localization of GAD (glutamic acid decarboxylase), the enzyme responsible for synthesizing GABA (γ-aminobutyric acid). We report that the subcellular distribution of GAD is significantly altered in two neuronal cell lines that express either the N-terminus of mhtt or full-length mhtt. GAD65 is predominantly associated with the Golgi membrane in cells expressing normal htt; however, it diffuses in the cytosol of cells expressing mhtt. As a result, vesicle-associated GAD65 trafficking is impaired. Since palmitoylation of GAD65 is required for GAD65 trafficking, we then demonstrate that palmitoylation of GAD65 is reduced in the HD model. Furthermore, overexpression of HIP14 (huntingtin-interacting protein 14), the enzyme responsible for palmitoylating GAD65 in vivo, could rescue GAD65 palmitoylation and vesicle-associated GAD65 trafficking. Taken together, our data support the idea that GAD65 palmitoylation is important for the delivery of GAD65 to inhibitory synapses and suggest that impairment of GAD65 palmitoylation by mhtt may lead to altered inhibitory neurotransmission in HD.

Key words: axonal transport, glutamic acid decarboxylase 65 (GAD65), huntingtin-interacting protein 14 (HIP14), polyglutamine repeat, protein aggregate, protein palmitoylation.

Abbreviations used: biotin-BMCC, 1-biotinamido-4-[4-(maleimidomethyl)cyclohexane-carboxamido]butane; CSP, cysteine string protein; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase; GFP, green fluorescent protein; GLUT-1, glial glutamate transporter-1; GFP, green fluorescent protein; HA, hydroxylamine; HAP1, huntingtin-associated protein 1; HD, Huntington’s disease; HIP14, huntingtin-interacting protein 14; HRP, horseradish peroxidase; htt, huntingtin protein; polyQ, polyglutamine; httQ5; htt with 25 polyQ repeats; httQ10, htt with 103 polyQ repeats; mhtt, mutant huntingtin; mRFP, monomeric red fluorescent protein; MSN, medium spiny neuron; N2a, neuro-2a cell line; NEM, N-ethylmaleimide; NG2, normal goat serum; PAT, palmitoyl acyltransferase; PDI, protein disulfide-isomerase; PPT, palmitoyl protein thioesterase; PSD-95, postsynaptic density 95; SNAP-25, 25 kDa synaptosome-associated protein; iGAD, truncated GAD

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To gain insight into the mechanism underlying GABAergic neuronal dysfunction in HD, in the present study we examined the effect of mhtt on the subcellular localization of GAD65. We first demonstrate that the subcellular localization and trafficking of GAD65 are impaired in a neuronal cell line expressing the N-terminal fragment of mhtt. In addition, we also show that subcellular localization of GAD65 is altered in STHdhQ111 cells that express full-length mhtt. This is possibly due to a reduced palmitoylation of GAD65 in the presence of mhtt. Furthermore, we demonstrate that overexpression of HIP14 could restore the palmitoylation of GAD65 and improve GAD65 trafficking. Thus GAD65 palmitoylation is important for delivery of GAD65 to inhibitory synapses and disruption of this process by mhtt may lead to altered inhibitory neurotransmission in HD.

**EXPERIMENTAL**

**Cell culture and differentiation**

N2a (neuro-2a cell line; A.T.C.C.), STHdhQ7 and STHdhQ111 cells (obtained from HD Community BioRepository) were cultured in Eagle’s minimum essential medium (A.T.C.C.) supplemented with 10% (w/v) fetal bovine serum (PAA Laboratories). For N2a cell differentiation, cells were grown in the absence of serum for 48 h.

**Animals**

Breeding pairs of R6/2 transgenic mice were purchased from Jackson Laboratories, and the line was maintained by backcrossing to CBA3 C57BL/6 F1 mice in the animal facilities of Florida Atlantic University. The R6/2 mouse line, which expresses exon 1 of the human HD gene, contains 150 CAG repeats [16]. R6/2 mice display an aggressive phenotype, including deficits in motor co-ordination, altered locomotor activity, impaired cognitive performance and seizures, and therefore provide clear experimental endpoints [17]. The neuropathology of R6/2 mice is similar to human HD at the cellular level with the development of nuclear htt deposits before the onset of symptoms [18].

All animals were maintained under temperature- and light-controlled conditions (20–23°C, 12 h light/12 h dark cycle) with continuous access to food and water. Animal experiments were performed in accordance with the NIH (National Institutes of Health) Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Florida Atlantic University.

**Plasmids and transfection**

The N-terminal coding region (amino acids 1–68) of wild-type human htt25Q (htt with 25 polyQ repeats; Addgene plasmid 1187) or htt103Q (htt with 103 polyQ repeats; Addgene plasmid 1186) was attached to an EGFP (enhanced green fluorescent protein) and subcloned into pcDNA3.1/HisB vector [19]. Human HIP14 cDNA in a pCMV6-Entry vector was purchased from Origene. Human GAD65 cDNA in pCR4-TOPO and GAD67 cDNA in PCR4-TOPO were purchased from Open Biosystems and subcloned into pcDNA3-mRFP (monomeric red fluorescent protein) which was obtained from Addgene (Addgene plasmid 13032). Truncated human GAD65 cDNA with the deletion of the amino acids 1–69 was obtained from human GAD65 cDNA by standard PCR and subcloned into pcDNA3-mRFP (forward primer, 5′-ggccgtcctagggegctggaggtg-3′; reverse primer, 5′-ggccggatccagagactgcttc-3′). Truncated human GAD67 cDNA with the deletion of amino acids 1–90 was obtained from human GAD67 cDNA by standard PCR and subcloned into pcDNA3-mRFP (forward primer, 5′-ggccggatccagagactgcttc-3′; reverse primer, 5′-ggccgtcctagggegctggaggtg-3′). Transfection was performed using the standard Lipofectamine™ 2000 method according to the manufacturer’s instructions (Invitrogen). Unless stated elsewhere, cells were analysed 48 h after transfection.

**Antibodies**

The following antibodies were used: affinity-purified rabbit polyclonal antibody against recombinant mRFP (anti-mRFP) was purchased from BioVision; affinity-purified rabbit polyclonal antibody against the sequence surrounding Ala-150 of human GAD65 isoform (anti-GAD65), monoclonal rabbit antibody against human PDI (protein disulfide-isomerase) (anti-PDI) and monoclonal rabbit antibody against a synthetic peptide corresponding to residues surrounding Gly-190 of human Rab5 protein (anti-Rab5) were purchased from Cell Signaling Technology; rabbit polyclonal antibody against amino acids 1–238 of full-length GFP (green fluorescent protein) (anti-GFP) was purchased from Santa Cruz Biotechnology; mouse monoclonal (ascites) antibody against GAD65 isoform (GAD6) was purchased from Developmental Studies Hybridoma Bank, University of Iowa; purified mouse monoclonal antibody against recombinant GAD67 isoform (anti-GAD67) was obtained from Chemicon; rabbit polyclonal antibody against C-terminus of human HIP14 (rabbit anti-HIP14), purified goat polyclonal antibodies against C-terminus of HIP14 (goat anti-HIP14), mouse monoclonal antibody against FLAG epitope (M2) were purchased from Sigma; and mouse monoclonal antibody against the Golgi matrix protein of 130 kDa (GM130) was obtained from BD Biosciences. Alexa Fluor® 405, 488 or 594 goat antibodies against rabbit or mouse were purchased from Invitrogen.

**Sample preparations**

N2a cells were lysed in lysis buffer [50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1× complete protease inhibitor cocktail (Sigma) and 1× phosphatase inhibitor cocktail (Pierce)] containing 1% Triton X-100 for 30 min at 4°C. For striatal sample preparation, striatum was first dissected from 11-week-old R6/2 mice and their control littermates and then lysed in lysis buffer containing 1% Triton X-100 at 4°C for 30 min. After lysis, samples were centrifuged at 13000 g for 20 min at 4°C to separate soluble and insoluble fractions. To dissolve insoluble fractions, the resulting pellets were further incubated with 100% formic acid at 37°C for 40 min as described in [20]. After incubation, samples were dried by vacuum centrifugation, resuspended in 1× SDS/PAGE sample buffer and neutralized with 1 M Tris. All sample mixtures were boiled for 5 min before electrophoresis. Protein concentration was determined using the Bradford method [20a].

**Co-immunoprecipitation**

Co-immunoprecipitation was performed using PureProteome™ Protein G magnetic beads (Millipore). Cells were first lysed in lysis buffer containing 0.5% NP-40 (Nonidet P40). Approximately 500 μg of cell or striatal lysates at a concentration of 2 mg/ml was pre-cleared with 30 μl of Protein G beads and then incubated with anti-mRFP (1:50), anti-GAD65 (1:50) or goat anti-HIP14 (1:50) antibodies for 2 h at 4°C with
gentle shaking. At the end of the incubation, 30 µl of Protein G beads was added to the antigen–antibody complex and further incubated with gentle mixing overnight at 4°C. The immobilized Protein G-bound complexes were washed three times with cell lysis buffer containing 0.2% Triton X-100. For the detection of protein–protein interaction, 30 µl of 2× SDS sample buffer was added and boiled for 5 min to elute the protein complex for Western blot analysis. For palmitoylation detection, the immobilized Protein G-bound complexes were further processed as described below.

Detection of GAD palmitoylation

Palmitoylated GAD was detected using the ‘fatty acyl exchange labelling’ method as described by Drisdel et al. [21,22]. Free cysteines in GAD were first blocked with excessive NEM (N-ethylmaleimide). The cysteine–palmitoyl thioester linkage in palmitoylated cysteines was then cleaved by HA (hydroxyamine), which made the palmitoylated cysteine available for biotin labelling and detection by streptavidin–HRP (horseradish peroxidase). Briefly, immunopurified GAD65 attached to Protein G–agarose beads was resuspended in 150 µl of lysis buffer containing 50 mM NEM and 0.2% Triton X-100 followed by incubation at 4°C overnight. After incubation, excess NEM was removed by washing the sample extensively with the lysis buffer. The complex was further incubated with 1 M HA (pH 7.4) for 1 h to cleave the cysteine-palmitoyl thioester linkage. After removal of excess HA, the complex was further incubated with 80 µM biotin-BMCC (1-biotinamido-4-[4-(maleimidomethyl)cyclohexane-carboxamido] butane; in lysis buffer, pH 6.2) for 2 h at 4°C. At the end of incubation, the beads were washed extensively with lysis buffer and eluted with 2× SDS sample buffer. The palmitoylated GAD was then detected by Western blotting using streptavidin–HRP antibodies (Cell Signaling Technology). The coverslips were then mounted in Prolong Gold.

Statistical analysis

Data are expressed as means ± S.E.M. To establish significance, data were subjected to an unpaired Student’s t test using StatView 5.0 (SAS Institute) or GraphPad Prism 5.0. The criterion for significance was set at P ≤ 0.05.

RESULTS

The subcellular distribution of GAD65 and GAD67 is altered in the presence of mhtt

We initially examined whether mhtt expression would affect the subcellular distribution of GAD in a neuronal N2a cell line. For easier visualization of GAD expression, we attached mRFP to the C-terminus of GAD. In all cells that were co-transfected with GAD65–mRFP and htt25Q–EGFP, confocal analysis revealed that GAD65–mRFP accumulated in vesicular structures in the perinuclear region of N2a cells, which is largely co-localized with the Golgi marker GM130 (Figures 1A and 1E). This is consistent with the report that GAD65–GFPLocalizes predominantly to the Golgi membrane in the cell body of neurons and COS-7 cells [9]. In contrast, GAD65–mRFP was detected in a diffuse pattern in the cytosol of N2a cells expressing htt103Q and correlated poorly with GM130 staining (Figures 1B and 1E). We also noticed that, in approximately 50% of the cells that had htt103Q aggregate formation, part of GAD65–mRFP accumulated around htt103Q aggregates (Supplementary Figure S1 available at http://www.BiochemJ.org/bj/442/bj4420039add.htm).

Since GAD exists in two isoforms, we also examined the subcellular distribution of GAD67 in N2a cells expressing htt25Q or htt103Q. Similar to GAD65–mRFP, GAD67–mRFP was also largely associated with Golgi membrane (Figure 1C compared with Figure 1A). However, more GAD67–mRFP was also found outside of the Golgi compartment (Manders’ coefficient with GM130 is 0.386 ± 0.036, n = 10). Unexpectedly, we found that in all cells expressing htt103Q aggregates, GAD67–mRFP formed tight aggregates around htt103Q aggregates and no diffuse expression pattern of GAD67 in the cytosol was observed compared with that of GAD65–mRFP (Figure 1D compared with Figure 1B). Furthermore, the aggregates did not co-localize with...
Golgi marker (Figure 1D). It is necessary to mention that mhtt had no effect on the expression pattern of RFP alone (results not shown). To rule out the possibility that the attachment of mRFP may cause GAD to form aggregates or change its subcellular localization, we compared the expression pattern of GAD–mRFP to that of GAD without the attachment of mRFP in N2a cells. We did not find any significant difference in their expression patterns. Both GAD65 and GAD67 were predominantly distributed in the Golgi compartment regardless of the presence of mRFP (Supplementary Figures S2A and S2B available at http://www.BiochemJ.org/bj/442/bj4420039add.htm). This was also confirmed with endogenous GAD staining in primary cortical neurons (Supplementary Figures S2C and S2D).

It was reported that mhtt forms detergent-insoluble aggregates, which can be further dissolved using formic acid [20]. To investigate whether GAD forms insoluble aggregates with mhtt, we carried out immunoblotting studies on the detergent-soluble and -insoluble fractions in N2a cells expressing mhtt. As shown in the lower panels of Figures 1(F) and 1(G), htt25Q was present in the soluble fraction, whereas htt103Q was mainly present in the detergent-insoluble fraction due to the aggregate formation. We also detected the presence of htt25Q in the insoluble fraction. This was probably due to the insufficient wash of the insoluble pellet before dissolving with formic acid. GAD65–mRFP was present in the soluble fractions in both htt25Q- and htt103Q-expressing cells (upper panels in Figure 1F). In contrast, GAD67–mRFP was found to be present in both soluble and insoluble fractions (upper panels in Figure 1G).

More importantly, we also investigated the subcellular localization of GAD in striatal cell lines that express full-length murine htt with either seven (STHdhQ7) or 111 (STHdhQ111) glutamine residues [23]. In STHdhQ7 cells, most GAD65–mRFP accumulated in the Golgi compartment, as suggested by GM130 staining (Figures 1H and 1J). However, this pattern changed in STHdhQ111 cells. GAD65–mRFP was found to be more diffusely expressed in STHdhQ111 cells (Figures 1I and 1J), which is similar to what we observed in N2a cells expressing the N-terminal fragment of mhtt (Figure 1B). Since the N-terminal fragment of mhtt is more toxic, it is reasonable to expect that htt103Q has a more severe effect on GAD65 subcellular expression than full-length mhtt (Figure 1E compared with Figure 1J). Interestingly, GAD67–mRFP also showed aggregate formation or diffuse...
GAD is largely re-located to ER membrane in the presence of mhtt in N2a cells

Since GAD65 appeared in a diffuse pattern in cells expressing mhtt, we next investigated whether GAD65 in htt103Q-expressing cells is associated with the ER membrane with a specific ER marker, PDI. Due to the heavy bleed-through of EGFP aggregate signal into the blue channel, we transfected N2a cells with htt25Q or htt103Q that contains no EGFP. In N2a cells expressing htt25Q, GAD65 was found to have little overlap with PDI staining (Figure 2A), indicating that the majority of GAD65–mRFP is not present in ER. However, in the presence of mhtt, GAD65 was re-directed to ER membrane as shown in Figure 2(B). Co-localization coefficient analysis revealed that there is a significant degree of co-localization between GAD65 and PDI in cells expressing htt103Q (Figure 2C).

N-terminus of GAD is responsible for the different subcellular distribution of GAD in the presence of mhtt

GAD65 and GAD67 showed different subcellular expression patterns in N2a cells expressing htt103Q (Figure 1), suggesting that GAD65 and GAD67 respond differently to the presence of mhtt. Since GAD65 and GAD67 differ substantially at the N-terminus only [24,25], we reasoned that the N-terminus of GAD may be responsible for the different responses to mhtt expression. To test this hypothesis, we generated a truncated GAD65 with the deletion of the first 69 amino acids, tGAD65–mRFP [26], and a truncated GAD67 with the deletion of the first 90 amino acids, tGAD67–mRFP [27]. Both truncated GADs were linked to mRFP at the C-terminus. In N2a cells that were co-transfected with htt25Q–EGFP and tGAD65–mRFP, tGAD65 was detected in a diffuse appearance (Figure 3A). A similar pattern was found for tGAD67–mRFP in N2a cells expressing htt25Q (Figures 3C and 3E). Interestingly, both tGAD65 and tGAD67 were present as aggregates in cells expressing htt103Q (Figures 3B and 3D), indicating that the N-terminus of GAD65 may prevent the full-length GAD65 from forming aggregates in cells expressing htt103Q (Figure 1B). We further determined the subcellular localization of tGAD65 in N2a cells transfected with htt25Q. Compared with full-length GAD65, removal of the N-terminal fragment of GAD65 appeared to abolish the association of GAD65 with the Golgi membrane as indicated by a lack of co-staining with GM130 (Figure 3E and Supplementary Figure S4 available at http://www.BiochemJ.org/bj/442/bj4420039add.htm). Moreover, tGAD65 had a different staining pattern from PDI staining, indicating that tGAD65 mainly diffuses in the cytosol (Supplementary Figure S4). This is consistent with the report that the N-terminus of GAD65 is important for membrane association [28]. Similar findings were obtained for tGAD67 when co-stained with GM130 or PDI (results not shown).

Palmitoylation of GAD65 is reduced in the presence of mhtt

Palmitoylation is an important post-translational modification of GAD65 that regulates its subcellular localization [9]. This prompted us to investigate whether GAD65 palmitoylation was affected in N2a cells expressing mhtt. The extent of palmitoylation was determined using the ‘fatty acyl exchange labelling’ method [21]. We first determined whether GAD was palmitoylated in N2a cells that were transfected with GAD65–mRFP or GAD67–mRFP alone. We demonstrated that GAD65, but not GAD67, was palmitoylated in N2a cells (Figure 4A). As expected, in the absence of HA treatment, no signal was detected since no free cysteine was available for biotin-BMCC labelling (Figure 4A). We then investigated whether GAD65 palmitoylation was affected by m htt expression. Palmitoylation of GAD65 in cells expressing htt103Q was greatly reduced compared with cells expressing htt25Q, whereas the total GAD65 input remained the same between groups (Figure 4B). We estimated the percentage of GAD65 palmitoylation by densitometry analysis and found that GAD65 palmitoylation in cells expressing htt103Q was reduced to ~20% of the control group (Figure 4B). Additionally, we also analysed the palmitoylation of endogenous GAD65 in R6/2 mice. As shown in Figure 4(C), palmitoylation of GAD65 in the striatum of R6/2 mice was also significantly decreased. Palmitoylation of endogenous GAD65 was mediated by HIP14 [13]. Recently, it was reported that the enzymatic activity of HIP14 is regulated by palmitoylation and HIP14 palmitoylation is decreased in the YAC128 HD mouse model [29,30]. We therefore also checked the palmitoylation of HIP14 in R6/2 mice. Compared with the control animals, HIP14 palmitoylation was decreased in R6/2 mice (Figure 4D).

mHtt affects the transport of GAD65 in differentiated N2a cells

Since palmitoylation is required for post-Golgi trafficking of GAD65 to synaptic nerve terminals [10], it is reasonable to...
Figure 3  N-terminus deletion affects the subcellular localization of GAD in N2a cells expressing htt25Q or htt103Q

(A–D) N2a cells were co-transfected with (A) tGAD65–mRFP and htt25Q–EGFP, (B) tGAD65–mRFP and htt103Q–GFP, (C) tGAD67–mRFP and htt25Q–EGFP or (D) tGAD67–mRFP and htt103Q–GFP. Co-transfected N2a cells were fixed 48 h after transfection and the nuclei were stained with Hoechst 33342 (blue in merged images). Scale bar, 5 μm. (E) Correlation coefficient analysis for co-localization of tGAD65–mRFP with GM130 in N2a cells expressing htt25Q. n = 10 cells per group. ***P < 0.0001.

Figure 4  GAD65 palmitoylation is reduced in the presence of mhtt

(A) GAD65 but not GAD67 was palmitoylated in N2a cells with HA treatment (upper panel). Immunoprecipitated (ip) GAD65 and GAD67 were detected using GAD6 and anti-GAD67 antibodies respectively (lower panel). (B) Palmitoylation of GAD65 was reduced in N2a cells expressing htt103Q. N2a cells were co-transfected with GAD65 and htt plasmids as indicated. Immunoprecipitated GAD65–mRFP was detected using GAD6. Inset, upper panel: representative image from three independent experiments. Inset, lower panel: densitometry analysis of the percentage of palmitoylated GAD65 in cells expressing htt103Q compared with cells expressing htt25Q. ** P < 0.01, n = 3, unpaired Student’s t test. (C) Palmitoylation of GAD65 was reduced in R6/2 mice. Upper panel: palmitoylated GAD65. Lower panel: total input of immunoprecipitated GAD65. (D) Palmitoylation of HIP14 was reduced in R6/2 mice. Upper panel: palmitoylated HIP14. Lower panel: total input of immunoprecipitated HIP14.
Reduced GAD65 palmitoylation and trafficking in Huntington's disease

Overexpression of HIP14 improves GAD65 trafficking in cells expressing htt103Q

We next investigated whether increasing GAD65 palmitoylation would rescue the defective trafficking of GAD65 in N2a cells expressing mhtt. Accordingly, we performed triple transfection in N2a cells using HIP14–FLAG, GAD65–mRFP and htt plasmids. Overexpression of all three proteins was first confirmed by Western blotting (Figure 6A). We noticed that GAD65 and htt103Q expression in the triple transfection was lower than those in the double transfection. This is probably due to lesser amounts of plasmids used in the triple transfection. We then investigated the effect of HIP14 overexpression on GAD65 subcellular localization by immunofluorescence. Consistently, when HIP14 was overexpressed, GAD65–mRFP was re-associated with vesicular structures in N2a cells expressing htt103Q (Figure 6C). Lastly, we tested whether overexpression of HIP14 increased GAD65 palmitoylation, which causes the redistribution of GAD65. Indeed, we found that GAD65 palmitoylation was increased when HIP14 was overexpressed (Figure 6D).

HIP14 does not interact with GAD65

Finally, we wanted to address how mhtt expression would impair GAD65 palmitoylation at the molecular level. Could mhtt down-regulate the expression of HIP14, which directly reduces its activity? We tested this hypothesis in R6/2 mice. As shown in Figure 7(A), HIP14 expression level was not significantly affected by mhtt expression in the striatum of R6/2 mice at 11 weeks of age. In addition, the predominant localization of HIP14 in the Golgi compartment was not affected in R6/2 mice (Supplementary Figure S5 available at http://www.BiochemJ.org/bj/442/bj4420039add.htm). Increasing evidence suggests that HIP14, as a PAT, interacts with its substrates such as htt [12] and PSD-95 [31]. This may also be true for other DHHC palmitoylating enzymes [31]. We therefore tested whether HIP14 also interacts with GAD65 and, if so, whether mhtt expression disrupts the interaction. We first performed a co-immunoprecipitation assay using lysates of N2a cells that...

Figure 5  GAD65 trafficking is impaired in differentiated N2a cells expressing mhtt

Cells were then allowed to differentiate after transfection in the absence of serum for 48 h. (A) N2a cells were transfected with GAD65–mRFP alone. Arrowheads indicate GAD65 present in vesicle-like structures. (B) N2a cells were co-transfected with GAD65–mRFP and htt25Q–EGFP. (C) N2a cells were co-transfected with GAD65–mRFP and htt25Q. Cells were stained with anti-Rab5 (green in the merged image). (D) N2a cells were co-transfected with GAD65–mRFP and htt103Q. GAD65 is present as diffuse pattern in the process of differentiated N2a cells. (E) N2a cells were co-transfected with GAD65–mRFP and htt103Q. Cells were stained with anti-Rab5 (green in the merged image). (A–E) Scale bar, 5 μm.
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Figure 6 Overexpression of HIP14 improves GAD65 trafficking and palmitoylation

(A) Western blot (WB) analysis of HIP14 overexpression in cells triple-transfected with GAD65–mRFP, htt103Q–EGFP and HIP14–FLAG. HIP14–FLAG was detected using M2 antibodies. GAD65–mRFP was detected using GAD6 antibodies and htt103Q–EGFP was detected using anti-GFP antibodies. (B and C) Effect of HIP14 overexpression on the subcellular localization of GAD65. N2a cells were co-transfected with (B) htt103Q–EGFP and GAD65–mRFP or (C) triple-transfected with HIP14–FLAG, htt103Q–EGFP and GAD65–mRFP. After 48 h, cells were fixed and stained with M2 antibody. Scale bar, 2 μm. (D) Overexpression of HIP14 increased GAD65 palmitoylation in N2a cells expressing htt103Q.

had overexpressed HIP14–FLAG and GAD65–mRFP. As shown in Figure 7(B), we detected the immunoprecipitated GAD65 (lower panel); however, we did not detect the presence of HIP14 in the immunoprecipitated complexes (lower panel). To rule out the possibility that the tags we used in the present study may interfere with the interaction between HIP14 and GAD65, we immunoprecipitated endogenous GAD65 from striatal lysates of 11-week-old wild-type and R6/2 mice. As seen in Figure 7(C), HIP14 was not present in the immunoprecipitated complex from the striatal lysates of either wild-type or R6/2 mice. Taken together, our data suggest that GAD65 does not interact with HIP14.

DISCUSSION

Palmitoylation plays an important role in regulating the trafficking and function of neuronal proteins. The reversible nature of S-palmitoylation provides a potential mechanism for protein shuttling between different intracellular compartments [32]. GAD65 is predominantly associated with synaptic vesicles in nerve terminals [7] and regulates GABAergic neurotransmission [5,6]. Palmitoylation is crucial for efficient targeting of GAD65 to presynaptic clusters. It has been shown that palmitoylation-deficient GAD65 mutants have a diffuse appearance in dendrites and proximal axons of primary hippocampal neurons [10]. However, whether GAD65 palmitoylation is affected under neuropathological conditions has not been reported. In the present study, we demonstrate that palmitoylation of GAD65 is decreased in HD, which further impairs the axonal transport of GAD65 and its function.

GAD65 and GAD67 are the two isoforms of GAD in the brain with different subcellular localizations and functions. The results of the present study strongly support the notion that GAD65 and GAD67 are subject to different regulatory mechanisms in neurons. GAD65 is palmitoylated at Cys-30 and Cys-45 [11]. Although GAD67 has similar cysteine-containing motifs in the N-terminus (Cys-33 and Cys-48), there have been no reports of GAD67 palmitoylation. Instead, evidence from the present study and studies by others [33,34] indicates that unlike GAD65, GAD67 is not palmitoylated in neurons. Therefore the transport of GAD67 to synaptic nerve terminals may be mediated through distinct mechanisms that involve GAD65-dependent and -independent pathways [35]. Interestingly, we found that GAD65 and GAD67 show different subcellular expression patterns in neuronal cell lines expressing mhtt. GAD65 remains soluble and diffuse in the presence of mhtt, whereas GAD67 becomes partially insoluble and forms aggregates with mhtt. What causes this difference remains to be investigated. The results of the present study suggest that the N-terminus of GAD65 may play an essential role since the removal of the N-terminus in GAD65 led to the formation of GAD65 aggregates with mhtt.

In mammals, up to 23 different kinds of PATs have been reported using a systemic screening method [36]. They all share the conserved DHHC domain and exhibit distinct, but overlapping, substrate specificity [31]. For instance, DHHC-3 specifically enhances palmitoylation of GABAA receptor γ2 subunit in vivo [37]. It is reported that four PATs (DHHC-3,
DHHC-8, DHHC-13 and HIP14) can enhance the palmitoylation of GAD65 in co-transfected COS cells [31]. This raises the possibility that other PATs may also palmitoylate GAD65. However, palmitoylation of endogenous GAD65 in neuronal culture is decreased by knocking down HIP14, but is not affected by DHHC-3 knockdown [31]. Consistently, it has been shown that overexpression of HIP14 enhances perinuclear accumulation of GAD65 in cultured neurons, whereas HIP14 small interfering RNA significantly reduces axonal clustering of GFP-tagged GAD65 [13]. Consequently, it is suggested that HIP14 is the major endogenous enzyme for GAD65 palmitoylation.

We have demonstrated that GAD65 palmitoylation is decreased in HD. In addition to GAD65, reduced palmitoylation of other neuronal proteins in HD has also been reported in several studies. Palmitoylation of htt by HIP14 is essential for its trafficking and function. In HD, expansion of polyQ in htt results in a significant reduction in htt palmitoylation, leading to the accelerated formation of inclusion bodies [14]. Another study has shown that the palmitoylation and function of GLT-1 (glial glutamate transporter-1) are reduced in the YAC128 mouse model of HD [38]. The enzyme that palmitoylates GLT-1 has not been identified. On the other hand, not all palmitoylated proteins are affected in HD. For instance, it has been reported that palmitoylation of SNAP-25 is not altered in HD. It is possible that other DHHC enzymes such as DHHC-3 can palmitoylate SNAP-25 in vivo [31]. Another possibility is that different palmitate turnover rates exist among proteins. It has been shown that palmitate on PSD-95, CDC42 and two G-proteins (H-RAS and N-RAS) rapidly and continually turns over. In contrast, palmitate on SNAP-25 and synaptotagmin I is relatively stable since 2-bromopalmitate treatment, an inhibitor of protein palmitoylation, has little or no effect [39]. Conceivably, GAD65 may have a higher palmitoylation turnover rate; hence changes in GAD65 palmitoylation can be readily detected under pathological conditions.

The molecular basis for reduced palmitoylation of neuronal proteins in HD, except for htt, remains elusive. The decreased palmitoylation of htt in HD is believed to be due to a decreased direct interaction between mhtt and HIP14, which is mediated through the ankryin repeat domain in HIP14 [12,14]. Furthermore, it has been reported that other DHHC palmitoylating enzymes can interact with their respective substrates in overexpressed cell lysates, but not in brain extracts. For example, DHHC-8 interacts with paralemmin and DHHC-3 interacts with SNAP-25 [31]. Therefore it is reasonable to hypothesize that HIP14 interacts with GAD65. Unexpectedly, we could not detect any interaction between GAD65 and HIP14 by co-immunoprecipitation in either overexpressed cell extracts or brain samples. Although the results of the present study suggest that there is no interaction between HIP14 and GAD65, there is still a possibility that GAD65 and HIP14 do not form a long-lived enzymatic reaction intermediate as others [31], which prevents the detection of the complex formation between GAD65 and HIP14 in the overexpressed cell lysates.

In the present study, we showed that HIP14 protein levels are not affected in HD. Therefore if there is any change of HIP14 activity in HD, it is more likely related to some post-translational modifications of HIP14, such as palmitoylation. Recently, it was reported that palmitoylation of HIP14 is essential for the enzymatic activity of HIP14, which is modulated by wild-type htt [29]. HIP14 palmitoylation is decreased in the brains of mice lacking one htt allele and further reduced in neuronal culture with htt knockdown [29]. It has also been shown that exon 1 of htt, which does not interact with HIP14, can further compromise the ability of mhtt to modulate HIP14 activity [29]. Soon afterwards, the same group showed that HIP14 activity is indeed reduced in a full-length HD mouse model, YAC128 [30]. Consistently, we also showed that HIP14 palmitoylation was reduced in R6/2 mice. Therefore it is likely that HIP14 activity is decreased in HD, which accounts for the reduced palmitoylation of GAD65.

Since protein palmitoylation is a reversible process, another possibility that cannot be excluded is that GAD65 de-palmitoylation is increased, which results in the net decrease in palmitoylated GAD65. Protein de-palmitoylation is mediated by PPTs (palmitoyl protein thioesterases). The identified PPTs are very limited and include APT1 (acyl-protein thioesterase 1), PPT1 and PPT2. No specific PPT has been reported to de-palmitoylate GAD65. PPT1 is a promising candidate for neuronal proteins as it has been reported that PPT1 deficiency impairs synaptic vesicle recycling at nerve terminals [40]. It would be interesting to investigate whether PPT1 de-palmitoylates GAD65 and whether its activity is up-regulated in HD.

As to the functional consequences of reduced GAD65 palmitoylation in HD, we have shown that GAD65 trafficking is affected. It is well established that normal htt is involved in neuronal protein trafficking, such as BDNF (brain-derived neurotrophic factor) [41] and GABAA receptor [42], by forming a complex with HAP1 (huntingtin-associated protein-1), which is disrupted by mhtt [43,44]. It is not clear whether huntingtin is directly involved in GAD65 trafficking. Our data indicate that it is less likely since the shorter fragment of htt (amino acids 1–68) used in the present study may not interact with HAP1 and possesses the function of full-length huntingtin in vesicular trafficking. Moreover, the N-terminal fragment of mhtt used in the present study is missing the palmitoylation site Cys-214, which is essential for htt trafficking and function on palmitoylation [14]. Therefore we believe that disrupted GAD65 trafficking is more likely due to the decreased GAD65 palmitoylation rather than a direct involvement of htt. In summary, our results indicate that GAD65 palmitoylation is decreased in HD, which leads to disrupted GAD65 trafficking and reduced synaptic clustering of GAD65 in nerve terminals. This contributes to the understanding of the altered synaptic inhibition and increased neuronal excitability observed in HD.

**AUTHOR CONTRIBUTION**

Daniel Rush conducted the experiments, contributed to data analysis and helped with manuscript preparation. Rebecca Leon helped with HIP14 experiments and contributed to data analysis. Mark McCollum contributed to experiments and data discussion. Ryan Treu performed molecular cloning of truncated GADs. Jianing Wei designed the experiments, analysed the data and wrote the paper.

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SUPPLEMENTARY ONLINE DATA
Palmitoylation and trafficking of GAD65 are impaired in a cellular model of Huntington’s disease

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Figure S1 Confocal analysis of the subcellular expression of GAD65 in N2a cells expressing htt103Q
GAD65–mRFP appeared in diffused pattern in N2a cells expressing htt103Q (A). In approximately 50% of the co-transfected cells, GAD65–mRFP started to accumulate around htt103Q–EGFP aggregates in addition to the diffuse pattern (B). Co-transfected N2a cells were fixed 48 h later and the nuclei were stained with Hoechst 33342 (blue in merged images). Scale bars, 5 μm.

Figure S2 The attachment of mRFP to GAD has no effect on GAD expression pattern
(A and B) GAD65 (A) and GAD67 (B) have a similar expression pattern in N2a cells as GAD65–mRFP and GAD67–mRFP. N2a cells were transfected with GAD65 or GAD67 without mRFP and fixed 48 h later. Both GAD65 (red in merged images) and GAD67 (red in merged images) are co-localized with the Golgi marker GM130 (green in merged images). Scale bars, 5 μm. (C) Endogenous GAD65 in primary cortical neurons was stained with a monoclonal antibody, GAD6. Scale bar, 10 μm. (D) Endogenous GAD67 in primary cortical neurons was stained with a monoclonal antibody, anti-GAD67. Scale bar, 10 μm. Both GAD65 and GAD67 in the cell bodies of primary cortical neurons are accumulated in vesicular structures in the perinuclear region.

Figure S3 Confocal analysis of the subcellular expression of GAD67–mRFP in STHdhQ7 (A) and STHdhQ111 cells (B–D)
Cells were fixed 48 h after transfection with GAD67–mRFP and co-stained with GM130 (green in merged images) and Hoechst 33342 (blue in merged images). Scale bars, 5 μm. GAD67–mRFP is accumulated in Golgi structure in STHdhQ7 cells (A) but appears in aggregate formation (B) or diffuse pattern (D) in STHdhQ111 cells.

Figure S4 Representative images showing the subcellular localization of tGAD65–mRFP in cells expressing htt25Q
N2a cells were fixed 48 h after transfection with tGAD65–mRFP and htt25Q. Cells were then stained with (A) the Golgi marker GM130 (green in the merged image) or (B) the ER marker PDI (green in the merged image). Scale bars, 5 μm.

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Figure S5  HIP14 is localized in Golgi and its expression pattern is not changed in (A and B) wild-type or (C and D) R6/2 mice

Scale bar in (A) and (C), 10 μm. Scale bar in (B) and (D), 5 μm. Mice at 8 weeks of age were transcardially perfused with 50 ml of 0.9% saline followed by 50 ml of 4% (w/v) PFA (paraformaldehyde) in PBS. Brains were removed, post-fixed overnight in 4% PFA at 4°C and incubated in 30% sucrose in PBS at 4°C until equilibrated. Sagittal sections of 30 μm were cut using a microtome and processed as free-floating sections. Sections were first blocked in blocking buffer (10% normal goat serum, 1% BSA and 0.3% Triton X-100 in PBS) for 1 h at room temperature before incubating with antibodies against HIP14 (1:500) and GM130 (1:500) for 18 h at 4°C. For fluorescent visualization, sections were incubated with the respective secondary antibody conjugated to Alexa Fluor® 488 or 594 (1:2000). Between steps, sections were washed three times for 5 min in PBS. After immunostaining, the sections were mounted with Prolong® Gold antifade reagent containing DAPI (4',6-diamidino-2-phenylindole).

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