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Huntingtin phosphorylation acts as a molecular switch for anterograde/retrograde transport in neurons

Emilie Colin[1,2,4], Diana Zala[1,2,4], Géraldine Liot[1,2], Hélène Rangone[1,2,5], Maria Borrell-Pagès[1,2,6], Xiao-Jiang Li[3], Frédéric Saudou[1,2,*] and Sandrine Humbert[1,2,*]

[1]Institut Curie, Orsay, France, [2]CNRS UMR146, Orsay, France and [3]Department of Human Genetics, Emory University, Atlanta, GA, USA

The transport of vesicles in neurons is a highly regulated process, with vesicles moving either anterogradely or retrogradely depending on the nature of the molecular motors, kinesins and dynein, respectively, which propel vesicles along microtubules (MTs). However, the mechanisms that determine the directionality of transport remain unclear. Huntingtin, the protein mutated in Huntington's disease, is a positive regulatory factor for vesicular transport. Huntingtin is phosphorylated at serine 421 by the kinase Akt but the role of this modification is unknown. Here, we demonstrate that phosphorylation of wild-type huntingtin at S421 is crucial to control the direction of vesicles in neurons. When phosphorylated, huntingtin recruits kinesin-1 to the dynactin complex on vesicles and MTs. Using brain-derived neurotrophic factor as a marker of vesicular transport, we demonstrate that huntingtin phosphorylation promotes anterograde transport. Conversely, when huntingtin is not phosphorylated, kinesin-1 detaches and vesicles are more likely to undergo retrograde transport. This also applies to other vesicles suggesting an essential role for huntingtin in the control of vesicular directionality in neurons.

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Introduction

The proper intracellular transport of membranous organelles and of other cargoes is determined by the nature of the molecular motors that propel vesicles along microtubules (MTs). Kinesins generally move vesicles anterogradely from the cell body to the tip of the neurites, whereas cytoplasmic dynein leads to the retrograde transport of vesicles back towards the cell body. *In vitro* studies have demonstrated that dynein and kinesins can function independently and that the direction of transport of a given cargo along MTs depends on the nature of the molecular motor that is present (Vale, 2003). However, the situation is more complex *in vivo*. A high level of coordination is necessary to ensure the proper transport of a given cargo to the right place. One key multiprotein complex that could have an important function in the coordination of bidirectional transport of vesicles is dynactin, which is required for attaching motors to cargoes (Holleran *et al*, 1998; Martin *et al*, 1999; Deacon *et al*, 2003; Schroer, 2004; Welte, 2004). In particular, the $p150^{Glued}$ subunit of dynactin physically interacts with MTs, the intermediate chains of dynein (DIC) and kinesin. However, the mechanisms by which the net directionality of organelle movement is achieved remain unclear and the proteins or factors that control directly or indirectly directionality remain to be identified.

The huntingtin (htt) protein is a positive regulatory factor for vesicular transport. htt associates with vesicles and MTs (Borrell-Pages *et al*, 2006) and facilitates vesicular transport in both anterograde and retrograde directions *in vitro*, in mammalian neuronal cells and in *Drosophila* (Gunawardena *et al*, 2003; Gauthier *et al*, 2004; Caviston *et al*, 2007). This mechanism involves the direct interaction of htt with dynein intermediate chain (Caviston *et al*, 2007) and with huntingtin-associated protein-1 (HAP1), which in turn interacts with the $p150^{Glued}$ subunit of dynactin and kinesin (Engelender *et al*, 1997; Li *et al*, 1998; Gauthier *et al*, 2004; McGuire *et al*, 2006). We have previously described an assay to study intracellular transport by analysing the dynamics of brain-derived neurotrophic factor (BDNF)–eGFP-containing vesicles using fast 3D videomicroscopy followed by deconvolution (Gauthier *et al*, 2004; Dompierre *et al*, 2007). BDNF is synthesized from a large precursor protein, pre-pro-BDNF, that is proteolytically processed and trafficked through the Golgi apparatus to the trans-Golgi network where it is packaged into secretory vesicles (Thomas and Davies, 2005). BDNF is important to study htt's function, and its dysfunction in disease as the striatal source of BDNF that is crucial for the survival of striatal neurons is dependent on the proper synthesis and transport of BDNF from the cortico-striatal-projecting neurons (Altar *et al*, 1997; Saudou *et al*, 1998; Zuccato *et al*, 2001; Baquet *et al*, 2004; Gauthier *et al*, 2004).

Most of the aforementioned studies have been conducted in cells rather than in primary cultures of neurons and the role of htt in the control of anterograde and retrograde transport has not been investigated. Therefore, to further characterize the function of htt in axonal transport, we studied the regulatory role of htt in transport in primary

*Corresponding authors. F Saudou or S Humbert, CNRS UMR146, Institut Curie, bat.110, centre universitaire, Orsay F-91400, France. Tel.: +33 169 863 024; Fax: +33 169 863 051;
E-mails: Frederic.Saudou@curie.fr or sandrine.humbert@curie.fr
[4]These authors contributed equally to this work
[5]Present address: Department of Genetics, University of Cambridge, Cambridge CB23EH, UK
[6]Present address: Cardiovascular Research Center, CSIC-ICCC, Hospital de la Santa Creu I Sant Pau, Barcelona, Spain

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cultures of cortical neurons, the neurons that transport BDNF *in vivo* (Altar *et al*, 1997). We show here that htt is a facilitator of anterograde and retrograde transport in neurons and that this function is regulated by the phosphorylation of htt at serine 421. When htt is phosphorylated, kinesin-1 is recruited to vesicles and MTs, facilitating anterograde transport. By contrast, retrograde transport is favoured in the absence of htt phosphorylation at S421. This study reveals that vesicular directionality can be regulated by phosphorylation of htt and identifies htt as the first regulator of the direction of transport in neurons.

Results

Huntingtin regulates anterograde and retrograde transport in neurons

To analyse transport in neurons and avoid overexpression artefacts or toxicity due to the long-term expression or down-regulation of the genes studied, experimental conditions require the electroporation technique and the analysis of the neurons about 3 days after electroporation and plating. However, at this stage, neurons are not fully mature and a cautious interpretation of the transport system needs to be done. Previous studies established that the non-uniform organization of MTs in dendrites is progressively acquired during maturation and is achieved when the neurons are fully polarized (Baas *et al*, 1989). In contrast, in 3 days *in vitro* (DIV) neurons, most if not all distinct neurites show an MT organization with the plus ends distal to the cell body. We analysed in our cultured cortical neurons the directionality of MT growth in the neurites using end-binding protein 3 (EB3) coupled to GFP. EB3 is a neuron-specific plus end-tracking protein whose dynamics reflect the 'plus' end MT polymerization state (Stepanova *et al*, 2003). We found that most of the movements of the EB3–GFP comets (90–95%) were directed distal to the cell body, indicating that outward movements correspond to 'plus' end-directed anterograde movements in three DIV cortical neurons (data not shown) (Stepanova *et al*, 2003; Gauthier *et al*, 2004). Having determined the MT orientation in our experimental system, we specifically tracked the BDNF vesicles in the neurites of three DIV cortical neurons and classified the intracellular movements as anterograde and retrograde transport according to the direction moved by the vesicles relative to the cell body (see Materials and methods in Supplementary data). We used a 480 amino-acid N-terminal fragment of wild-type htt that has the same subcellular distribution as the full-length htt in neurons (Humbert *et al*, 2002) and increases the mean velocity of BDNF vesicles as efficiently as the full-length protein in neuronal cells (Supplementary Figure S1). htt increased velocity in both directions using the 480 amino-acid N-terminal fragment of wild-type htt (Figure 1A; Supplementary Movie 1). Conversely, the reduction of htt protein levels by RNA interference (RNAi) decreased both anterograde and retrograde velocity of moving vesicles. A scramble RNA in the same conditions had no effect on BDNF anterograde and retrograde velocities (data not shown). We further validated the specificity of this assay by analysing the consequences of decreasing the levels of certain components of the molecular motors, such as $p150^{Glued}$, KHC (kinesin-1 heavy chain) and DHC (dynein heavy chain) by RNAi (Figure 1A). As we were unable to detect DHC using anti-DHC antibodies, we analysed the effect of DHC targeting on the protein levels of DIC. As previously shown, the targeting of DHC through RNAi induces the specific downregulation of DIC (Caviston *et al*, 2007). Similar to the results obtained by antibody-blocking strategies (Waterman-Storer *et al*, 1997), reducing $p150^{Glued}$ and dynein levels each induced a considerable decrease in the movement of vesicles in both directions. Also, decreasing the levels of KIF5B and KIF5C, the predominant isoforms of kinesin-1 in cortical neurons (Kanai *et al*, 2000), reduced anterograde and retrograde transport. These results are in agreement with a previous study showing that silencing kinesin-1 but not kinesin-2 affects the transport of BDNF-containing vesicles (Dompierre *et al*, 2007).

htt is subjected to post-translational modifications. This is the case of htt phosphorylation by the serine/threonine kinase Akt at the S421. Although this phosphorylation is crucial to regulate the toxicity of the mutant pathogenic htt that contains the abnormal polyglutamine expansion, it was found that wild-type htt, the non-toxic form of htt, is also a physiological substrate of Akt in primary cultures of neurons and in rodent brains (Humbert *et al*, 2002; Pardo *et al*, 2006). However, the consequences of wild-type htt phosphorylation remain to be established, as phosphorylation of wild-type htt had no obvious effect on protein stability, degradation, solubility or toxicity in cells (Humbert *et al*, 2002). As wild-type htt controls both anterograde and retrograde transport, we asked whether phosphorylation at S421 could regulate htt's function in axonal transport. We analysed the consequences of htt phosphorylation on BDNF transport in neurites by expressing a form of htt that cannot be phosphorylated, htt-S421A, or a form of htt that mimicks constitutive phosphorylation, htt-S421D, in cortical neurons. Strikingly, when htt contained an unphosphorylatable S421, the vesicles moved faster in the retrograde direction than in the anterograde direction (Figure 1B and C; Supplementary Movie 2). By contrast, when the neurons were transfected with a constitutively S421-phosphorylated htt, the vesicles moved significantly faster in the anterograde direction than in the retrograde direction. These differences in velocities were not due to differences in expression levels (data not shown). We also calculated the effect of htt phosphorylation on the distance travelled (run lengths) by vesicles between two pauses and found an increase in the travelled distances by retrograde-moving vesicles when htt was not phosphorylated (Figure 1D). Conversely, htt phosphorylation at S421 increased the run lengths of anterograde but not retrograde-moving vesicles. These results show that the phosphorylation state of S421 regulates the efficiency of transport in one direction over the other.

To establish that the observed effect is physiological and specifically due to htt, we studied the dynamics of BDNF-containing vesicles in the neurites of neurons in the presence of IGF-1. This treatment leads to phosphorylation of htt at S421 (Humbert *et al*, 2002; Pardo *et al*, 2006). In the presence of IGF-1, anterograde transport was favoured (Figure 1E). We next decreased htt levels by siRNA and observed that in this condition, IGF-1 was not able to promote its effect on anterograde transport. This shows unequivocally that IGF-1 specifically increases BDNF anterograde transport along MTs through htt.

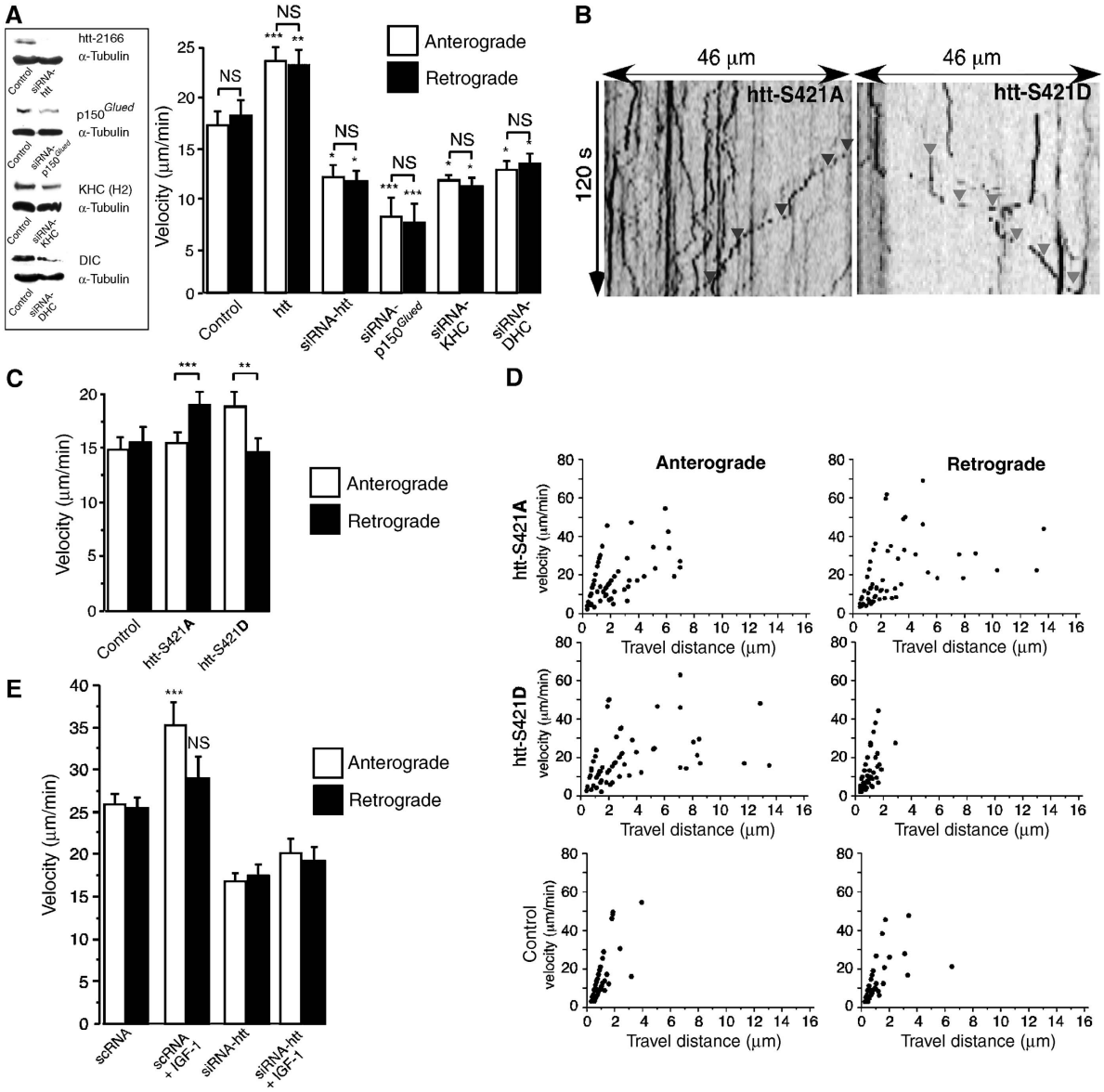

Figure 1 Phosphorylation of htt regulates BDNF transport directionality. (**A**) Anterograde and retrograde BDNF vesicular velocities are stimulated by htt in cortical neurites and are inhibited by siRNA targeting htt, $p150^{Glued}$, KHC or DHC. The reduction of protein levels by siRNA is shown by immunoblotting. (**B**) Expression of htt-S421A stimulates retrograde transport, whereas htt-S421D stimulates anterograde transport as shown by kymographs. (**C**) Mean velocity, (**D**) scatter plots of translocation velocities as a function of the distance travelled between two pauses are shown. (**E**) IGF-1 promotes anterograde transport of BDNF vesicles in the scramble RNA condition. This effect is lost in the absence of htt (siRNA-htt). Control: corresponding empty vector ($*P<0.05$, $**P<0.01$, $***P<0.001$; see Supplementary data for detailed statistical analyses and number of measures).

S421 stimulation of transport is an MT-based mechanism but does not affect the MT binding of huntingtin and BDNF or MT dynamics

BDNF vesicular transport is inhibited by the treatment of cells with nocodazole, an MT-depolymerizing drug, indicating that this transport requires an intact MT network (Gauthier *et al*, 2004). However, a report revealed that htt regulates the motility of endosomes by redistributing them from an MT localization to a preferential association with actin filaments (Pal *et al*, 2006). To test whether the modification of BDNF transport directionality observed in our assay could result from a switch to actin filaments, we treated cells with latrunculin B, a known actin-depolymerizing agent, and analysed BDNF transport (Figure 2A). Whereas nocodazole disrupted BDNF movement in neurons, latrunculin B treatment had no obvious effect on htt localization with respect to actin filaments and the movement of BDNF vesicles in neurites could still be detected (Figure 2A; Supplementary Figure S2A; Supplementary Movies 3 and 4). We also investigated whether the change in BDNF vesicular dynamics upon phosphorylation might be due to a change in the distribution of BDNF with markers of the endocytic pathway such as LAMP1, which labels late endosomes as well as lysosomes, and EEA1, which is specific to the early endosomal compartment. We observed a very low colocalization of BDNF-containing vesicles with such markers, and the relative distribution of BDNF vesicles to these markers was not affected by the phosphorylation state of htt at S421

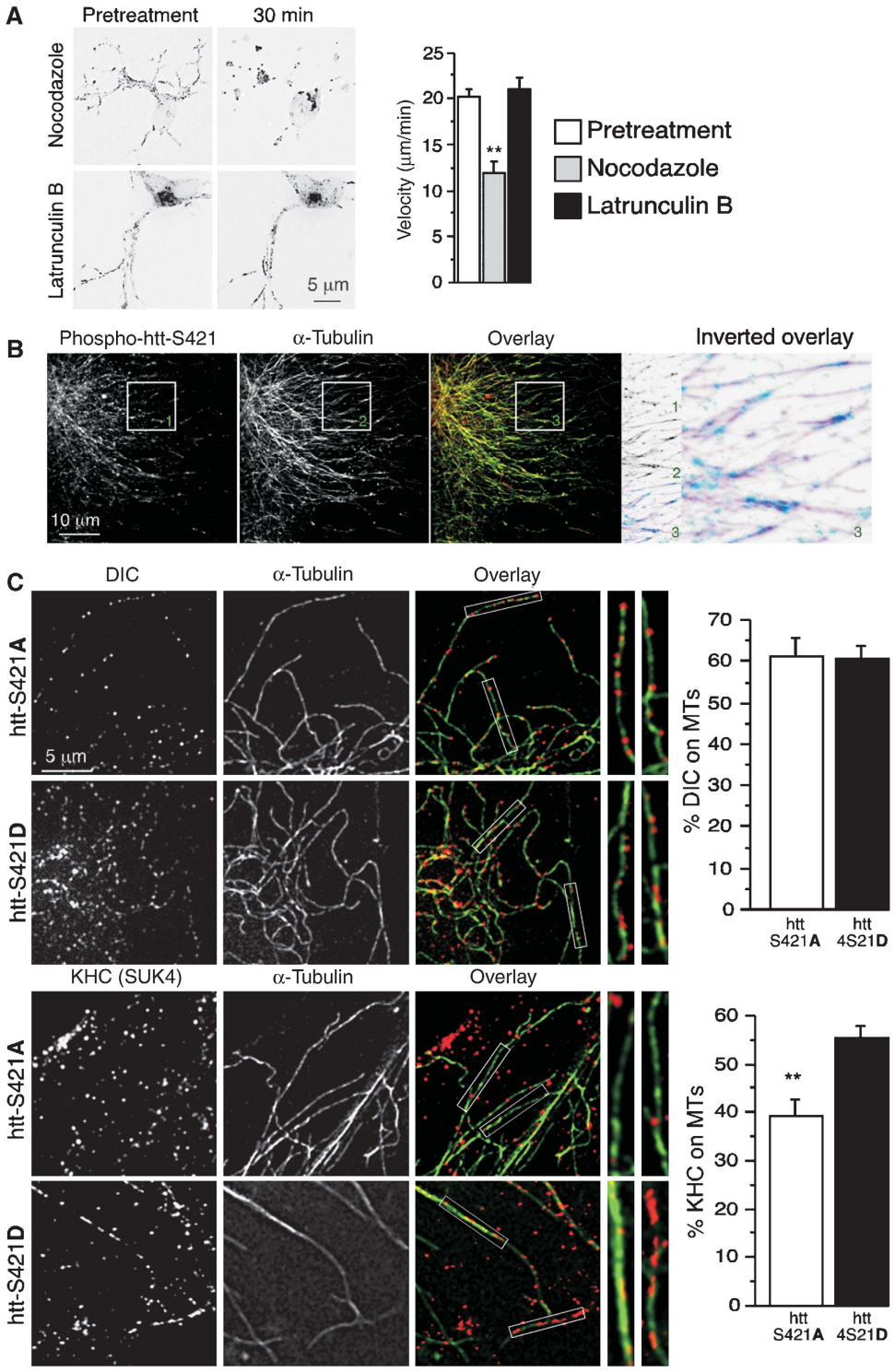

Figure 2 Phosphorylation of htt increases localization of kinesin-1 to microtubules. (**A**) Huntingtin-mediated control of BDNF trafficking is MT dependent. Time projection and velocity show that nocodazole treatment of cortical neurons significantly reduces motility of BDNF vesicles, whereas latrunculin B has no marked effect. (**B**) S421-phosphorylated htt is present on MTs in COS7 cells. Cells are immunostained using anti-phospho-htt-S421–714 and anti-α-tubulin antibodies. (**C**) Compared with unphosphorylatable htt (htt-S421A), expression of S421 constitutively phosphorylated htt (htt-S421D) in neuronal cells significantly increases the percentage of KHC-positive dots decorating microtubules. The localization of DIC is unchanged in both conditions (**$P<0.01$; see Supplementary data for detailed statistical analyses and number of measures).

(Supplementary Figure S2B). We next investigated whether htt phosphorylation regulates the association of htt to MTs or modifies the attachment of BDNF-containing vesicles to MTs and found no significant effect of htt phosphorylation on the association of htt and BDNF to MTs (Supplementary Figure S3A).

To test the possibility that htt phosphorylation alters MT dynamics, we analysed the effect of htt phosphorylation on

MT growth in mouse neuronal cells using EB3–eGFP. Transfecting cells with htt, htt-S421A and htt-S421D constructs had no significant effect on the patterning of MT growth in cells (Supplementary Figure S3B). We next measured the MT polymerization rate and found that htt phosphorylation state had no detectable effect on MT growth and on the nucleation of MTs (growth of new MTs) in cells (Supplementary Figure S3C and D). Taken together, these results indicate that, though the change in BDNF dynamics upon htt phosphorylation depends on MTs, the phosphorylation status of htt has no effect on the binding of htt and BDNF to MTs or on MT dynamics.

Kinesin-1 is recruited on MTs upon S421 huntingtin phosphorylation

Previous studies have reported a localization of htt on MTs (Gutekunst *et al*, 1995). However, these studies used antibodies that recognized the protein whatever its phosphorylation status. To make sure that htt phosphorylation at S421 is relevant to the mechanism studied, we examined by immunofluorescence the localization of phosphorylated htt with respect to the MT cytoskeleton using a previously described anti-phospho-htt-S421–714 antibody raised against the phospho-S421 epitope (Pardo *et al*, 2006). We observed phospho-specific immunostaining along MTs, as shown by the partial colocalization with α-tubulin (Figure 2B). We next analysed the localization of DIC and kinesin-1 to MTs in htt-S421A- or htt-S421D-transfected cells. Whereas htt phosphorylation had no profound effect on the colocalization of DIC with α-tubulin, we found a marked increase in the localization of kinesin-1 to MTs in cells expressing phosphorylated htt (Figure 2C). We quantified in a random and blinded manner the recruitment of the motor proteins to MTs and found a statistically significant increase in the association of KHC but not DIC to the MTs (Figure 2C). In agreement, we also observed a preferential colocalization of endogenous KHC with S421-phosphorylated htt on structures that could correspond to MT stretches (data not shown) using conditions in which S421 of htt is phosphorylated (Humbert *et al*, 2002). By contrast, in serum-starved cells treated with wortmannin, a condition that leads to loss of phosphorylation at S421, we did not observe such colocalization (data not shown).

Huntingtin phosphorylation at S421 leads to kinesin-1 recruitment to vesicles

Having shown that htt phosphorylation leads to an increased recruitment of KHC to MTs, we analysed the association of molecular motors with BDNF-containing vesicles. We transfected mouse neuronal cells with htt-S421A or htt-S421D constructs and performed subcellular fractionation obtaining a cytosolic (S3) and a pellet (P3) fraction that is enriched in small vesicles and vesicle-associated proteins. This fraction contains htt and BDNF-containing vesicles (Figure 3A) (Gauthier *et al*, 2004). The P3 fraction was also enriched in kinesin-1 and in the dynein–dynactin complex. Interestingly, compared with cells transfected with the unphosphorylatable form of htt, cells transfected with htt-S421D showed a statistically significant higher level of kinesin-1 in the P3 fraction compared with the S3 fraction (P3/S3 ratio) (Figure 3A). In contrast, htt phosphorylation status had no significant effect on the P3/S3 ratio of DIC (data not shown). To confirm these findings, we performed subcellular fractionation in mouse neuronal cells that were cultured in conditions in which the PI3K/Akt pathway was either inhibited (1% serum + 100 nM wortmannin) or activated (20% serum + 50 ng/ml IGF-1). These conditions lead to the phosphorylation of endogenous htt at S421 as shown by immunoblotting experiments with the anti-phospho-htt-S421–714 antibody (Figure 3B). After IGF-1 treatment, kinesin-1 was enriched in the P3 fraction, whereas after serum starvation and wortmannin treatment, most of the kinesin-1 was found in the S3 fraction corresponding to the cytosol (Figure 3C). Thus, when htt is phosphorylated at S421, kinesin-1 is enriched in the fraction that contains synaptic vesicles. To make sure that the observed changes are due to htt, we analysed the consequences of decreasing the levels of htt by RNAi on the distribution of kinesin-1 after S3/P3 subcellular fractionation (Figure 3D). In cells with low htt levels, the kinesin-1 levels in the P3 fraction were significantly reduced compared with the scramble situation. Taken together, these results demonstrate that htt phosphorylation at S421 leads to the recruitment of kinesin-1 to vesicles.

S421-phosphorylated huntingtin co-sediments with kinesin-1

We studied the nature of the motor complexes upon htt phosphorylation through sucrose gradient fractionation. We and others have previously used this approach to show that a fraction of soluble wild-type htt co-sediments with the $p150^{Glued}$–dynein complex (Li *et al*, 1998; Gauthier *et al*, 2004). We examined whether an increased phosphorylation of htt modifies the sedimentation pattern of htt, $p150^{Glued}$, dynein or kinesin-1 in linear sucrose gradients. Mouse neuronal cells were cultured as in Figure 3B and C. Cell extracts were fractionated on a linear sucrose gradient (7.5–25%) revealing that the htt and kinesin-1 sedimentation pattern was different between low and high htt phosphorylation conditions (Figure 4). S421-phosphorylated htt co-migrated with kinesin-1 in IGF-1/high serum condition, whereas htt and kinesin-1 were not present in the same fractions in wortmannin/low serum condition. We obtained similar results with the human neuroblastoma SH-SY5Y cell line (data not shown). This indicates that the nature of the cytoplasmic complex of htt and molecular motors is modified upon serum treatment. The preferential co-sedimentation of S421-phosphorylated htt with kinesin-1 suggests that htt and kinesin-1 may be present in the same native cytoplasmic complex upon htt phosphorylation. Taken together, the results of our biochemical and immunofluorescence experiments demonstrate that when htt is phosphorylated at S421, kinesin-1 colocalizes with htt and MTs and is associated with BDNF-containing vesicles at MTs. Conversely, in conditions that decrease the fraction of phosphorylated htt, KHC is weakly associated with htt, MTs and vesicles.

Phosphorylation of huntingtin increases the level of kinesin-1 in motor complex

How does htt phosphorylation lead to the recruitment of kinesin-1 to MTs and vesicles? One mechanism could involve the association of htt with HAP1. Indeed, htt associates with HAP1, and HAP1 associates with the $p150^{Glued}$ subunit of dynactin and the light chain of kinesin-1 (Engelender *et al*, 1997; Li *et al*, 1998; Gauthier *et al*, 2004; McGuire *et al*, 2006). We have shown that this complex is essential for stimulating

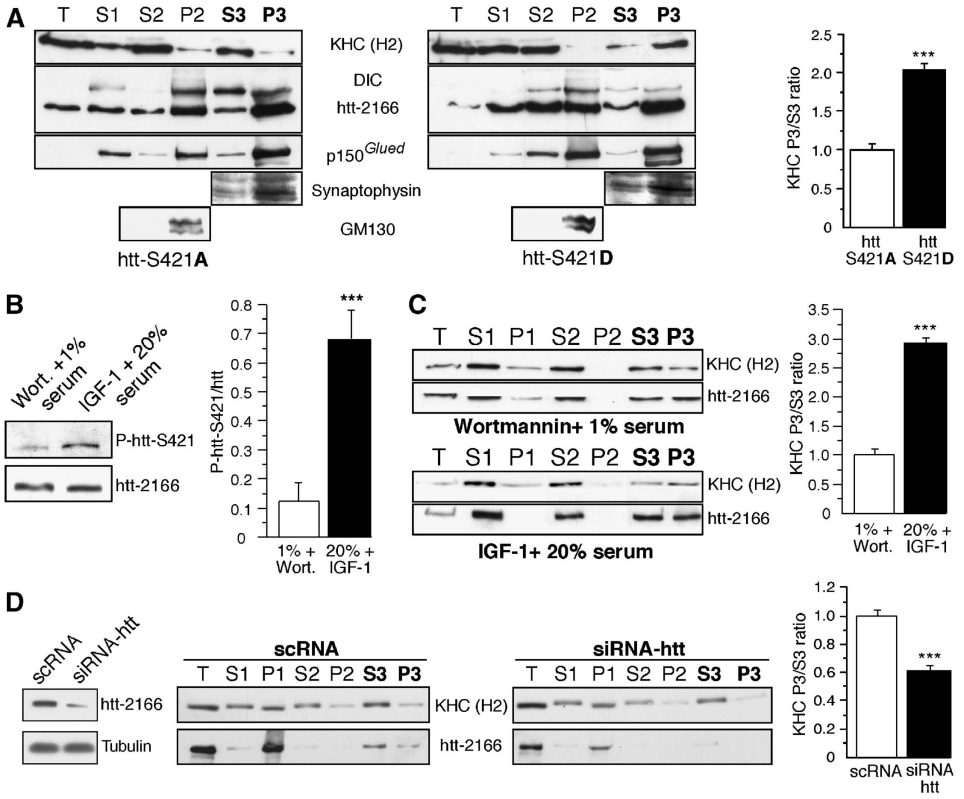

Figure 3 Phosphorylation of htt leads to a recruitment of kinesin-1 to vesicles. (**A**) Subcellular fractionation of mouse neuronal cell extracts by successive centrifugation steps is analysed by immunoblotting for the presence of kinesin-1 (KHC), dynein (DIC), htt, p150^{Glued}, GM130 (Golgi marker) and synaptophysin (small vesicles marker). Quantitative assessment of the optical density of KHC is expressed as P3/S3 ratio and shows a recruitment of KHC from cytosol to vesicles in cells expressing htt-S421D. (**B**) Phosphorylation of endogenous htt at S421 is increased by IGF-1 treatment. Total extracts from mouse neuronal cells treated with wortmannin (wort)/1% serum or IGF-1/20% serum are immunoprobed with the anti-phospho-htt-S421–714 and anti-htt antibodies. Quantification of phosphorylated htt (P-htt) is expressed as a ratio of P-htt/total htt optical densities. (**C**) Phosphorylation of endogenous htt leads to kinesin-1 recruitment to vesicles. Extracts treated as in (B) are subjected to subcellular fractionation, analysed and quantified as in (A). (**D**) Loss of htt reduces kinesin-1 recruitment to vesicles. Mouse neuronal cells treated with siRNA targeting htt or control scramble RNA (scRNA) were subjected to subcellular fractionation, analysed and quantified as in (A). (***$P<0.001$; see Supplementary data for detailed statistical analyses and number of measures).

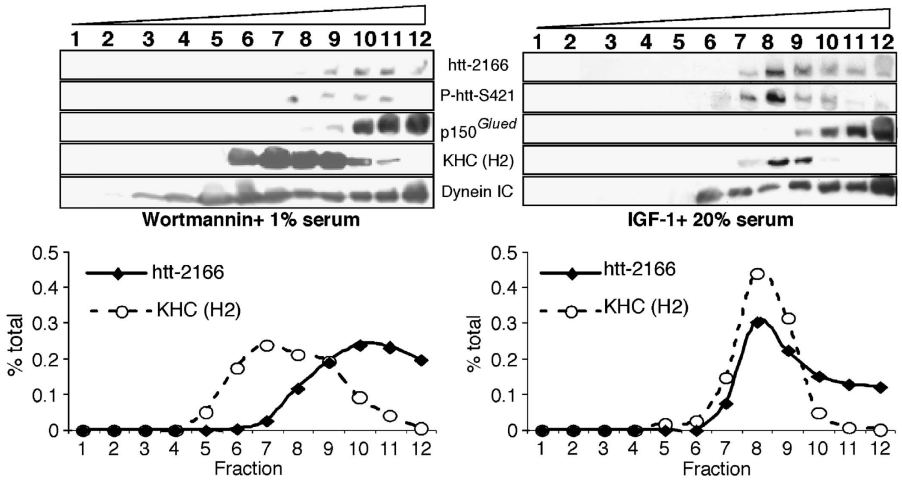

Figure 4 Phosphorylation of htt modifies soluble molecular motor complexes. The co-sedimentation of htt with kinesin in sucrose gradients is modified by phosphorylation at S421. Total extracts from mouse neuronal cells treated with wortmannin/1% serum or IGF-1/20% serum (as in Figure 3B) are fractionated by sucrose gradient and analysed by immunoblotting. The distribution of the optical density of endogenous KHC and htt in the different fractions is shown in the graphs.

MT-based transport and requires the HAP1 protein. In agreement, the first exon of htt, which does not contain the HAP1-binding domain cannot stimulate transport, whereas a longer fragment does (Figure 1A) (Gauthier *et al*, 2004). Furthermore, HAP1-deficient cells have an impaired capacity to transport BDNF- and amyloid precursor protein (APP)-containing vesicles efficiently (Gauthier *et al*, 2004; McGuire *et al*, 2006). We first investigated whether the

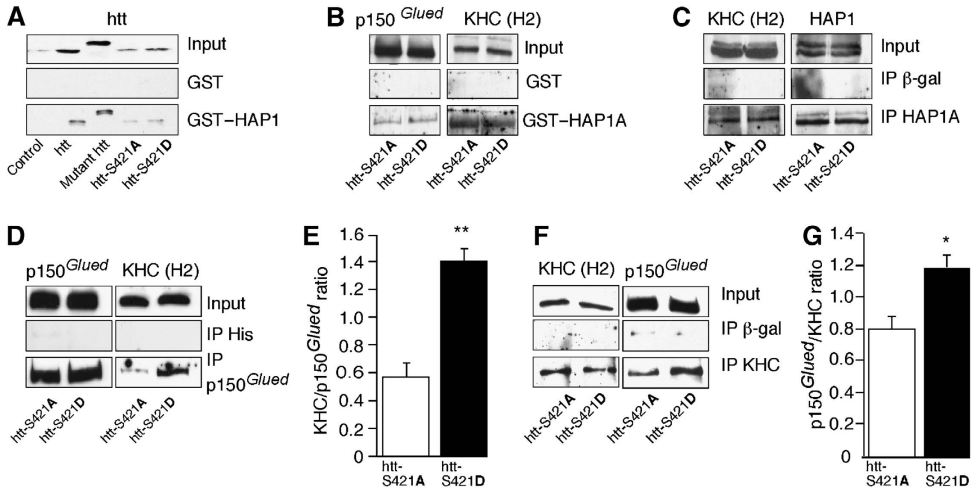

Figure 5 Phosphorylation of huntingtin at S421 modifies the nature of the molecular motor complexes. (**A**) GST–HAP1 pull-down experiments of protein extracts from HEK cells transfected with htt, mutant htt-polyQ, htt-S421A or htt-S421D reveal that the binding of HAP1 with the htt 480 amino-acid fragment is not modulated by phosphorylation. (**B**) GST–HAP1A pull-down experiments from HEK cells transfected with htt-S421A or htt-S421D show that the interactions between HAP1A and $p150^{Glued}$ and between HAP1A and KHC are not modified by phosphorylation. (**C**) The HAP1A–KHC interaction is unchanged whether htt S421 is phosphorylated or not. Extracts of htt-S421A- and htt-S421D-transfected mouse neuronal cells are immunoprecipitated using anti-β-galactosidase (control) or anti-HAP1A antibodies and immunoprobed with anti-KHC (H2) and anti-HAP1 (EM78) antibodies. (**D–G**) The $p150^{Glued}$–KHC interaction is significantly increased when S421 is phosphorylated. (D) Mouse neuronal cell extracts are immunoprecipitated using anti-His (control) or anti-$p150^{Glued}$ antibodies and immunoprobed with anti-$p150^{Glued}$ and anti-KHC antibodies. (F) Mouse neuronal cell extracts are immunoprecipitated using anti-β-galactosidase (control) or anti-KHC antibodies and immunoprobed as in (D). Quantitative assessments of the optical densities were performed and expressed as KHC/$p150^{Glued}$ (E) or $p150^{Glued}$/KHC ratios (G) ($^{*}P<0.05$, $^{**}P<0.01$; see Supplementary data for detailed statistical analyses and number of measures).

phosphorylation of htt at S421 regulates the interaction between HAP1 and htt. GST–HAP1 pull-down assays revealed no differences in the association of HAP1 to htt using extracts from cells transfected with either the htt-S421A or the htt-S421D construct (Figure 5A). As previously reported, in these conditions, polyQ-expanded htt has a higher affinity to HAP1 (Figure 5A) (Li *et al*, 1995). We next determined the association between HAP1 and $p150^{Glued}$ or kinesin-1. We used a full-length HAP1A construct as the HAP1A isoform interacts with the kinesin light chain-2 (KLC2) C-terminal domain (McGuire *et al*, 2006). However, htt phosphorylation at S421 had no effect on the association of $p150^{Glued}$ or kinesin-1 to HAP1A (Figure 5B). Immunoprecipitation experiments using anti-HAP1A antibodies further confirmed that htt phosphorylation does not modify the ability of HAP1 to bind to kinesin-1 (Figure 5C). We next tested whether htt phosphorylation could affect the association of kinesin-1 to the other components of the motor complex (Ligon *et al*, 2004). In cells expressing htt-S421D, the ability of $p150^{Glued}$ subunit of dynactin to co-immunoprecipitate KHC was significantly greater than in cells expressing htt-S421A (Figure 5D and E). This difference was not due to differences in the expression levels of htt as these were similar (data not shown). We confirmed this increased co-immunoprecipitation by the complementary experiment. When htt contained the S421D mutation, KHC antibodies co-immunoprecipitated $p150^{Glued}$ more efficiently (Figure 5F and G). Although the exact nature of the interaction between kinesin-1 and $p150^{Glued}$ that is modified upon htt phosphorylation remains to be determined (direct versus indirect), our results suggest a mechanism by which kinesin-1 is recruited to the BDNF-containing vesicles on MTs.

To unequivocally establish that the htt phosphorylation effect on anterograde transport occurs through a kinesin-1-dependent mechanism, we decreased the levels of kinesin-1 in cortical neurons and observed the effect of htt-S421A or htt-S421D constructs on the direction of BDNF transport. As previously described (Figure 1A) (Gauthier *et al*, 2004; Dompierre *et al*, 2007), decreasing kinesin-1 levels resulted in a decrease in the velocity of BDNF vesicles in the anterograde and retrograde directions (Figure 6A). In this situation, htt-S421A or htt-S421D had no stimulatory effects on retrograde or anterograde transport, respectively. This confirms our biochemical data and indicates that the S421 regulatory effect on transport depends on the presence of kinesin-1.

Huntingtin's function is not restricted to BDNF vesicles

We have found that the direction of BDNF vesicles, which are classical post-Golgi secretory vesicles, is controlled by htt phosphorylation in primary cultures of cortical neurons. We next investigated to what extent htt controls axonal transport in neurons. We choose to analyse the dynamics of vesicles involved in neuritogenesis that bear the vesicular SNARE tetanus neurotoxin-insensitive vesicle-associated membrane protein (TI-VAMP/VAMP7) fused to GFP and that can be used to follow synaptic vesicles (Martinez-Arca *et al*, 2000). Similar to the results obtained for BDNF vesicles, we found that expressing htt increased both anterograde and retrograde transport of TI-VAMP in cortical neurons (Figure 6B, left graph). When htt contained an unphosphorylatable S421, the TI-VAMP vesicles moved faster in the retrograde direction than in the anterograde direction (Figure 6B, right graph). Conversely, when the neurons were transfected with the htt-S421D construct, the vesicles moved significantly faster in the anterograde direction than in the retrograde direction. We conclude that htt's function in the control of bidirectional axonal transport is a

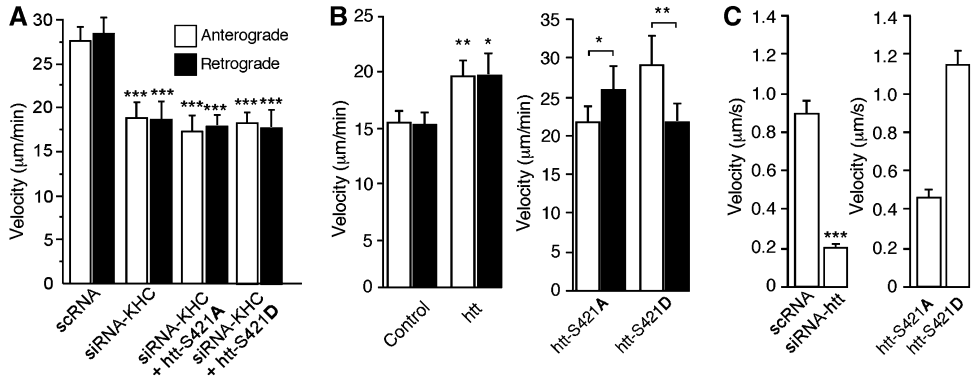

Figure 6 Huntingtin is an essential regulator of axonal transport. (**A**) Anterograde and retrograde transports are inhibited by siRNA targeting kinesin-1 (KHC) compared with the scramble situation. Expressing htt-S421A and htt-S421D in the presence of siRNA-KHC has no stimulatory effects on retrograde or anterograde transport. (**B**) htt stimulates anterograde and retrograde transport of TI-VAMP-GFP vesicles in cortical neurons (left graph). Absence of phosphorylation at S421 (htt-S421A) favours retrograde transport, and constitutive phosphorylation (htt-S421D) specifically increases anterograde transport (right graph). (**C**) APP anterograde movement depends on htt and its phosphorylation at S421. The velocity of APP-containing vesicles is reduced in cells with low htt levels (siRNA-htt) compared with a scramble situation. Expressing htt-S421D significantly increases the anterograde velocity of APP-containing organelles compared with htt-S421A ($*P<0.05$, $**P<0.01$, $***P<0.001$; see Supplementary data for detailed statistical analyses and number of measures).

general mechanism that is not restricted to BDNF-containing vesicles.

Finally, we analysed the transport of APP-containing organelles in cortical neurons. APP is a characteristic marker of fast axonal transport in neurons whose transport is kinesin-1 dependent and mainly occurs in the anterograde direction (Kaether *et al*, 2000; Araki *et al*, 2007). We thus asked what would be the effect of htt and of S421 phosphorylation on APP anterograde transport. Compared with the velocity of BDNF or TI-VAMP vesicles, APP-containing vesicles moved at a much higher speed (Figure 6C). We analysed whether APP anterograde movements depends on htt by silencing htt and found that the velocity of APP-containing vesicles was reduced in cells with low htt levels compared with a scramble situation (Figure 6C). Furthermore, expressing the htt-S421D significantly increased the anterograde velocity of APP-containing vesicles compared with the htt-S421A. This further shows that the S421 effect on transport is kinesin-1 dependent and extends to the APP organelles.

BDNF flow and release is increased through huntingtin phosphorylation

Finally, we specifically assessed the physiological consequence of the stimulatory effect of S421 phosphorylation on anterograde transport by analysing whether phosphorylation affects the flow of vesicles in a given direction. Neurons were transfected with htt-S421A or htt-S421D and BDNF. We measured the total distances travelled by the vesicles in both directions and expressed this as a ratio of anterograde flux over retrograde flux (Figure 7A). We observed a marked increase in the anterograde flux when htt was phosphorylated at S421, indicating that htt phosphorylation increases the outward flux of BDNF-containing vesicles in neurons.

Thus, we addressed the effect of an increased BDNF flux on the ability of neurons to release BDNF. As phosphorylation of htt at S421 is induced by the IGF-1/Akt pathway, we first treated cortical neurons with IGF-1 every 2 days for 1 week and measured the BDNF content in the supernatant. We observed a significant increase in BDNF content in the supernatant of IGF-1-treated cortical neurons (Figure 7B). We assessed whether this effect was linked to phosphorylation at S421 by electroporating primary cultures of cortical neurons with htt-S421A and htt-S421D and BDNF constructs, and measuring the ability of these neurons to release BDNF in the supernatant (Figure 7C). We detected no obvious increase in BDNF release in the supernatant when neurons expressed the htt-S421A construct, whereas the htt-S421D induced a significant BDNF release. Taken together, these results indicate that phosphorylating htt at S421 preferentially promotes anterograde transport and an outward flow of BDNF-containing vesicles, leading to a significant release of BDNF into supernatant.

Discussion

Since the discovery of the abnormal polyglutamine expansion in htt as the mutation responsible for HD, most of the studies in the field have focused on understanding the gain of the toxic function elicited by this mutation. Although little is known about the function of wild-type htt, growing evidences reveal that loss of function also contributes to pathogenesis supporting the importance of understanding htt normal function (Zuccato *et al*, 2001; Gauthier *et al*, 2004). We demonstrate here that htt is a key protein that regulates bidirectional axonal transport and we reveal a new cellular mechanism by which the direction of vesicular transport is controlled in neurons.

It is well accepted that anterograde transport of vesicles is mainly mediated by kinesins, whereas DIC moves organelles in the retrograde direction. *In vitro*, these motors can function independently. *In vivo*, the cell needs to regulate and coordinate this transport efficiently to allow cargoes to move rapidly and reverse their direction. The dynactin complex may have a function in coordinating organelle transport directionality, and several studies have suggested that dynactin may accomplish this by serving as a platform protein (Burkhardt *et al*, 1997; Waterman-Storer *et al*, 1997; Deacon *et al*, 2003). Kinesins and dyneins are interdependent. For example, in *Drosophila*, depletion of DHC by siRNA or by genetic approaches results in a decrease in both retrograde and anterograde transport (Martin *et al*, 1999). Similarly, immunodepletion of conventional kinesin inhibits both plus

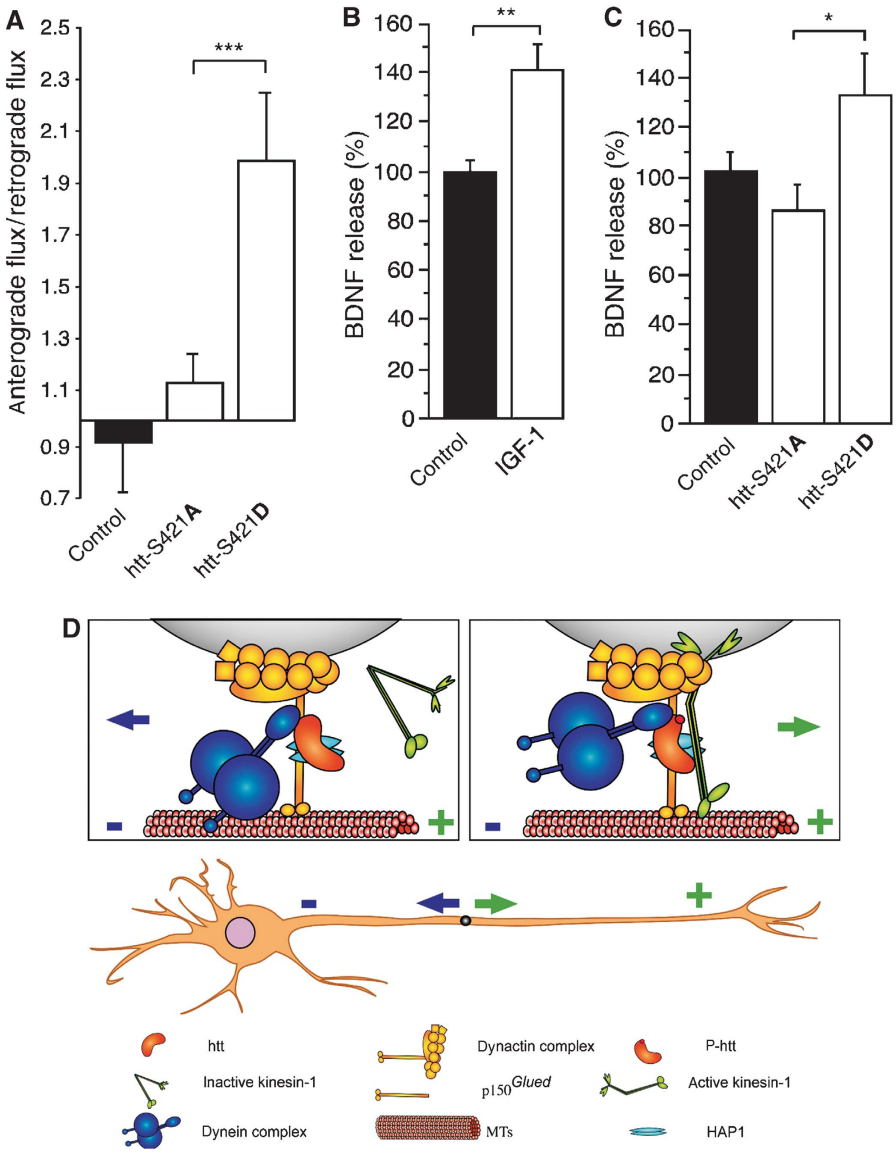

Figure 7 Phosphorylation of htt at S421 increases anterograde flux and BDNF release. (**A**) Phosphorylation of htt at S421 increases anterograde flux as quantified by the ratio of the total anterograde travelled distance over the total retrograde travelled distance by BDNF-containing vesicles. (**B**, **C**) BDNF release is increased in cortical neurons after IGF-1 treatment or after transfection with the htt-S421D construct. (**D**) Proposed model of the directionality switch induced by phosphorylation of htt. When htt is not phosphorylated, the kinesin-1 interaction with motor complex is weak, kinesin-1 is detached from MTs and vesicles leading to retrograde transport. When htt is phosphorylated, the kinesin-1 association with motor complex is increased and kinesin-1 is recruited to vesicles, therefore inducing a switch to anterograde transport (*$P<0.05$, **$P<0.01$, ***$P<0.001$; see Supplementary data for detailed statistical analyses and number of measures).

and minus end-directed vesicle transport (Schroer *et al*, 1988; Brady *et al*, 1990). The notion that the various motors are interdependent is also supported by experiments on peroxisome transport (Kural *et al*, 2005). Finally, the direct interaction between kinesin-1 and dynein is in agreement with a coordinated movement between anterograde and retrograde motors (Ligon *et al*, 2004). Using fast 3D videomicroscopy to record intracellular transport of BDNF-containing vesicles in primary neurons and RNAi experiments, we also found that kinesin-1, DIC and dynactin are essential for the bidirectional transport of vesicles. Our results together with work from previous studies demonstrate that htt also has a function in the bidirectional transport of organelles by associating with motor complexes (Engelender *et al*, 1997; Li *et al*, 1998; Gauthier *et al*, 2004; McGuire *et al*, 2006). Consistently, htt is found at the distal and proximal sites after sciatic nerve ligation, indicating that htt is actively transported in both directions in axons *in vivo* (Block-Galarza *et al*, 1997).

Although it is clear that motors and certain accessory proteins such as htt are involved in bidirectional transport, the mechanisms that control the directionality of transport are poorly understood. Studies strongly suggest these events are somehow coordinated. For example, in *Drosophila*, the Klar and Halo proteins may coordinate antagonistic motors and regulate the net directionality of lipid droplets and vesicles (Welte *et al*, 1998; Gross *et al*, 2003). Also, substitutions of single amino acids by mutagenesis in the *Drosophila* retrograde motor *non-claret disjunctional* (ncd) have been shown to lead to a reversal of motor direction (Endow and Higuchi, 2000). This indicates that components of motor

complexes or trans-acting factors such as htt could regulate direction of transport through modifications such as phosphorylation. Our study has established for the first time that a single phosphorylation in htt dictates the direction of the movements of vesicles along MTs in neurons. htt phosphorylation does not affect basic MT properties such as nucleation, dynamics or stability. Phosphorylation also fails to modify the localization of BDNF to other motile organelles or of BDNF vesicles to MTs. In contrast, we show by various complementary approaches that htt phosphorylation leads to the recruitment of the anterograde motor kinesin-1 to vesicles and MTs and preferentially increases the overall anterograde transport of organelles. When htt is dephosphorylated,
kinesin-1 is released from MTs and vesicles, and these vesicles that still contain the htt–dynein–dynactin complex move back towards the nucleus (Figure 7D).

How does htt phosphorylation lead to kinesin-1 recruitment to vesicles and MTs? htt binds to HAP1 and to the dynein complex through the dynein intermediate chain (Li *et al*, 1995; Caviston *et al*, 2007). However, no direct interaction with kinesin-1 has thus far been reported. HAP1 interacts with $p150^{Glued}$ and the heavy chain of kinesin-1 through its coiled-coil domain (Engelender *et al*, 1997; Li *et al*, 1998). More recently, HAP1 has been shown to interact with the C-terminal part of the KLC (McGuire *et al*, 2006). We found that htt S421 phosphorylation leads to no binding differences between htt and HAP1 or between HAP1 and $p150^{Glued}$ or kinesin-1. However, the marked increase in the interaction between $p150^{Glued}$ and kinesin-1 suggests that htt phosphorylation stabilizes the interaction of kinesin-1 to dynactin. We propose that recruitment of kinesin-1 upon htt phosphorylation to vesicles and MTs is a regulatory process that changes transport direction in neurons.

htt's function in the control of bidirectional transport is not only restricted to the transport of BDNF vesicles in cortical neurons but also applies to TI-VAMP vesicles and APP-labelled organelles, suggesting this previously unknown phenomenon to be of broad significance. The specificity of the htt-stimulated transport may depend on the nature of the organelles and of its associated motors. Indeed, our results show that the htt-dependent anterograde transport requires kinesin-1. The observation that APP-containing vesicles, whose transport depends on kinesin-1 (Kaether *et al*, 2000; Araki *et al*, 2007), are also regulated by htt support our findings. However, not all kinesin-1-dependent organelles are regulated by htt. For example, mitochondrial transport, although kinesin-1 dependent, is not altered in early stage of disease (Gauthier *et al*, 2004; Hollenbeck and Saxton, 2005). This specificity could be determined by proteins such as the htt-interacting protein HAP1 and its *Drosophila* orthologue Milton that exert an effect as adaptors for cargoes. Indeed, whereas Milton is essential for mitochondrial axonal transport by recruiting kinesin-1 (Glater *et al*, 2006) it has no effect on BDNF vesicles (data not shown). Conversely, HAP1 is required for the transport of BDNF- and APP-containing organelles but does not alter the trafficking of mitochondria (Gauthier *et al*, 2004; McGuire *et al*, 2006).

htt is phosphorylated by Akt upon IGF-1 stimulation. Phosphorylation of htt at S421 leads to an increased anterograde transport and flux that lead to an increased BDNF release from cortical neurons. We suggest a new mechanism by which IGF-1/Akt regulates survival, through the activation of intracellular trafficking. BDNF is produced by cortical neurons and is transported from the cortex to the striatum where it exerts an effect as a pro-survival factor for striatal neurons. The direct phosphorylation of htt by Akt promotes the anterograde transport of the BDNF factor and therefore provides an essential trophic support to striatal neurons.

Materials and methods

Cell cultures

COS7, HEK, mouse neuronal cells, rat primary cortical neurons were prepared and cultured as previously described (Saudou *et al*, 1998; Gauthier *et al*, 2004). Cell lines were transfected with Lipofectamine (Invitrogen) and neurons were electroporated with rat neuron Nucleofector (Amaxa).

Videomicroscopy experiments

Videomicroscopy experiments were done 2–3 days after transfection and the methods and materials used have been previously described in detail (Gauthier *et al*, 2004; Dompierre *et al*, 2007). Further information is described in Supplementary data.

Analysis of 3D images

Quantification of DIC and KHC signal associated with MTs was achieved by a binary mask from the MT image. For further details, see Dompierre *et al* (2007) and Supplementary data.

Cell fractionation, GST pull-down assays, immunoprecipitation and sucrose gradients

Preparation, experimental treatment and analysis of cell lysates for fractionation, immunoprecipitation, GST pull-down assays and sucrose gradients are described in detail in Supplementary data.

Cytoskeleton depolymerization and MT preparation

For MTs and actin depolymerization (Figure 2; Supplementary Figure S2), cortical neurons expressing BDNF-eGFP were imaged for 2.5 min, followed by 30 min of 10 μM nocodazole treatment to depolymerize MTs or 10 μM latrunculin B treatment to depolymerize actin and imaged again for 2.5 min or subjected to immunofluorescence.

BDNF immunoenzyme assays

The amount of BDNF was measured in supernatants and cell lysates using the BDNF Emax Immunoassay system (Promega) and expressed relative to the content of proteins in cells. For IGF-1 experiment, cortical neurons were treated with 50 ng/μl IGF-1 every 2 days for 1 week after plating and BDNF ELISA assays were performed. In Figure 7C, constructs encoding htt-S421A or htt-S421D and BDNF were electroporated into cortical neurons. At 36 h after electroporation, supernatants and lysates were collected and the amount of BDNF was measured.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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