Pathogenic huntingtin inhibits fast axonal transport by activating JNK3 and phosphorylating kinesin

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Selected vulnerability of neurons in Huntington’s disease suggests that alterations occur in a cellular process that is particularly critical for neuronal function. Supporting this idea, pathogenic Htt (polyQ-Htt) inhibits fast axonal transport (FAT) in various cellular and animal models of Huntington’s disease (mouse and squid), but the molecular basis of this effect remains unknown. We found that polyQ-Htt inhibited FAT through a mechanism involving activation of axonal cJun N-terminal kinase (JNK). Accordingly, we observed increased activation of JNK in vitro and in vivo in cellular and mouse models of Huntington’s disease. Additional experiments indicated that the effects of polyQ-Htt on FAT were mediated by neuron-specific JNK3 and not by ubiquitously expressed JNK1, providing a molecular basis for neuron-specific pathology in Huntington’s disease. Mass spectrometry identified a residue in the kinesin-1 motor domain that was phosphorylated by JNK3 and this modification reduced kinesin-1 binding to microtubules. These data identify JNK3 as a critical mediator of polyQ-Htt toxicity and provide a molecular basis for polyQ-Htt–induced inhibition of FAT.

Huntington’s disease is an autosomal-dominant, adult-onset neurodegenerative disease. Individuals with a single mutant huntingtin (HTT) gene develop symptoms in midlife with 100% penetrance. The mutation is a toxic gain of function, as the loss of a single gene has no phenotype and the Htt-null mouse is embryonic lethal. The disease-causing mutation is an expansion of a CAG trinucleotide repeat encoding a polyglutamine (polyQ) tract and polyQ expansions in other genes may also lead to adult-onset neurodegeneration, but the pathogenic mechanisms remain uncertain. Several lines of evidence indicate that pathogenesis in Huntington’s disease includes inhibition of FAT. Although the consequences of FAT inhibition in neurons are well established, the mechanisms underlying inhibition of FAT by polyQ-Htt are unknown. Proposed mechanisms include wild-type Htt loss of function, physical blockade by polyQ-Htt aggregates, sequestration of motor molecules and misregulation of FAT.

We found no evidence for direct interactions of conventional kinesin and cytoplasmic dynein (CDyn) with wild-type Htt or polyQ-Htt in vivo. However, analysis of mouse and squid models of Huntington’s disease showed that polyQ-Htt, but not wild-type Htt, increased JNK activity. Pharmacological and peptide inhibitors of JNK prevented inhibition of FAT by polyQ-Htt. To our surprise, only a subset of JNK isoforms inhibited FAT, with neuron-specific JNK3 selectively mimicking the effects of polyQ-Htt. In vitro phosphorylation and mass spectrometry studies indicated that JNK3, but not ubiquitously expressed JNK1, phosphorylated Ser176 in the kinesin heavy chain (kinesin-1, KHC) motor domain. Consistent with this location, phosphorylation of kinesin-1 by JNK3 inhibited kinesin binding to microtubules and translocation along axons. Our data indicate that polyQ-Htt inhibits FAT by a mechanism involving axonal JNK3 activation and phosphorylation of kinesin-1.

RESULTS
Htt does not interact with microtubule-based motors
PolyQ-Htt inhibits FAT in various experimental systems, including Drosophila4,5, neuroblastoma cells5,10 and isolated squid axoplasm8, but the molecular basis of this inhibition is unknown. Interactions have been reported between exogenously overexpressed Htt and conventional kinesin4 or various subunits of CDyn9–11. Alternatively, polyQ-expansion has been proposed to affect Htt function as a scaffolding protein for molecular motors9,12. However, interactions between endogenous wild-type Htt and molecular motors have not been evaluated. We tested interactions by immunoprecipitation and subcellular fractionation (Fig. 1), as described previously13,14. To avoid overexpression-related artifacts, we used brain tissue from 14-month-old homozygous HttQ109 knock-in mice and age-matched controls, which express polyQ-Htt or wild-type Htt at endogenous levels. At this age, both polyQ-Htt–derived nuclear inclusions and insoluble aggregates are found in the brains of HttQ109 knock-in mice.

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Conventional kinesin is a heterotetramer composed of two heavy chains and two light chains (KLC)\textsuperscript{13}. Antibodies recognizing kinesin-1 effectively immunoprecipitated kinesin-1 from detergent-soluble brain lysates independent of genotype (Fig. 1a). Kinesin-1 antibodies co-immunoprecipitated KLC\textsuperscript{13,14} but failed to co-immunoprecipitate either wild-type Htt or polyQ-Htt. Similarly, antibodies to dynein intermediate chains (DICs) co-immunoprecipitated dynein heavy chain (DHC)\textsuperscript{16}, but did not immunoprecipitate wild-type Htt or polyQ-Htt (Fig. 1a). Conversely, antibodies to Htt immunoprecipitated Htt from both wild-type and homozygous \textit{Htt}\textsubscript{Q109} knock-in mouse brain lysates, but kinesin-1, KLC, DIC and DHC could not be detected in Htt immunoprecipitates.

To detect substoichiometric amounts of Htt associated with conventional kinesin or CDyn, we performed three rounds of immunoprecipitation, which was sufficient to nearly deplete mouse brain lysates of kinesin-1 (Fig. 1b) or DIC (Supplementary Fig. 1 online). As in previous studies, marked reductions in kinesin-1 and KLC levels occurred with each immunoprecipitation cycle\textsuperscript{13,14}. However, Htt levels remained unchanged after three immunoprecipitation cycles, regardless of genotype.

PolyQ-Htt has been reported to sequester molecular motors in detergent-insoluble aggregates when overexpressed\textsuperscript{4,6}. To evaluate this at endogenous levels, we fractionated brain lysates from wild-type and homozygous \textit{Htt}\textsubscript{Q109} knock-in mice into detergent-soluble and detergent-insoluble fractions and analyzed partitioning of Htt and molecular motors by immunoblot (Supplementary Fig. 1). Wild-type Htt and polyQ-Htt levels were comparable in detergent-soluble and detergent-insoluble fractions, but the bulk of kinesin-1, DHC and DIC were detergent soluble. Molecular motor levels were similar for wild-type and homozygous \textit{Htt}\textsubscript{Q109} knock-in mice. Thus, wild-type Htt and polyQ-Htt expressed at endogenous levels do not interact with molecular motors; thus, FAT inhibition associated with polyQ-Htt must result from a different mechanism.

**Figure 1** Endogenous Htt does not interact with molecular motors. (a) Detergent-soluble brain lysates obtained from wild type (WT) and 14-month-old \textit{Htt}\textsubscript{Q109} knock-in (polyQ) mice were immunoprecipitated with antibodies to Htt, KHC or DIC. Immunoblots of resulting immunoprecipitates (IPP) showed that antibody to kinesin-1 (ref. 13) effectively precipitated both KHC and KLC, but failed to precipitate Htt, DHC or DIC. Similarly, antibody to DIC\textsuperscript{16} immunoprecipitated DIC and DHC, but not Htt. Conversely, antibodies to Htt effectively immunoprecipitated Htt, but not KHC, KLC, DHC or DIC. Immunoprecipitation with non-immune mouse IgG (NMIgG) were used as a control for nonspecific immunoprecipitation. An aliquot of each brain lysate before immunoprecipitation (input) was used as a positive control. (b) Detergent-soluble brain lysates from wild-type and \textit{Htt}\textsubscript{Q109} knock-in (polyQ-Htt) mice were subjected to three cycles of immunoprecipitation with antibodies to kinesin-1. Aliquots of each supernatant (SN1–3) were analyzed by immunoblot. Both KHC and KLC immunoreactivity were reduced with each cycle. In contrast, we detected no change in Htt levels, regardless of mouse genotype. Immunoprecipitations with a non-immune mouse IgG served as a control for nonspecific precipitation of proteins.

JNK activity mediates FAT defects induced by polyQ-Htt

We assayed the effects of wild-type Htt and polyQ-Htt on FAT in isolated squid axoplasm\textsuperscript{8}. Perfusion of wild-type Htt showed no effect (Fig. 2a), but perfusion of polyQ-Htt at a concentration 100–1,000-fold lower than that of kinesin-1 (refs. 3,8) markedly inhibited both anterograde (kinesin dependent) and retrograde (CDyn dependent) FAT (Fig. 2b). In axoplasm, anterograde FAT of membrane-bounded organelles depends primarily on conventional kinesin\textsuperscript{17}, which is regulated by phosphorylation\textsuperscript{11,18,19}. Multiple kinase activities may be deregulated in Huntington’s disease\textsuperscript{20–22} and inhibition of FAT by pathogenic androgen receptor (polyQ-AR) depends on JNK activation in axoplasm\textsuperscript{3}. This led us to evaluate the role of JNK in polyQ-Htt-induced inhibition of FAT. We assessed axoplasmic JNK activity \textit{in vitro} using recombinant GST-cJun, a well-characterized JNK substrate\textsuperscript{23} (Fig. 2c). Incubation with axoplasm lysates produced a time-dependent increase in cJun phosphorylation at JNK sites (serines 63 and 73), reflecting basal JNK activity in axoplasm. We validated the pharmacological properties of endogenous axoplasmic JNK with two JNK inhibitors (SP600125 and JNK inhibitor 1 peptide, JIP)\textsuperscript{23}. SP600125 is a pharmacological inhibitor of JNK, and shows a >20-fold selectivity for JNK over a range of kinases\textsuperscript{24}. JIP is a 20–amino acid peptide derived from a JNK-binding protein that selectively inhibits JNK, but not p38, activity\textsuperscript{25}. Both SP600125 and JIP markedly inhibited cJun phosphorylation, suggesting that squid and mammalian JNKs have common pharmacological properties (Fig. 2c).

As with polyQ-AR\textsuperscript{7}, perfusion of axoplasm with polyQ-Htt and either SP600125 (500 nM; Fig. 2d) or JIP (100 nM; Fig. 2e) blocked the effects of polyQ-Htt on FAT, suggesting that polyQ-Htt inhibition of FAT requires JNK activity. In cultured cells, pharmacological inhibitors of histone deacetylase (HDAC) have been reported to rescue defects in FAT induced by polyQ-Htt by increasing tubulin acetylation\textsuperscript{26}. However, perfusion of polyQ-Htt with the HDAC6 inhibitor BC-6-25 (10 mM, Compound 2)\textsuperscript{27} did not prevent inhibition of FAT by polyQ-Htt in axoplasm (Fig. 2f). Thus, inhibition of FAT by polyQ-Htt involves the activation of endogenous axonal JNK.

**PolyQ-Htt expression increases JNK activity**

Our vesicle motility and biochemical assays indicated that JNK activity mediates polyQ-Htt inhibition of FAT. JNK activation involves phosphorylation by upstream mitogen-activated protein kinase kinases (MKKs, typically MKK4/MKK7)\textsuperscript{28}. We evaluated the effects of polyQ-Htt on JNK phosphorylation in cellular and animal models of Huntington’s disease, using antibodies to total JNK (pan-JNK, phosphorylation independent) or to phosphorylated, catalytically active.
Htt (Q18) or polyQ-Htt (Q56)29. The expression of exogenous Htt in NSC34 mouse neuroblastoma cells were transfected with wild-type constructs were similar to that of endogenous Htt, as shown by immunoblotting. Pan-JNK and pJNK antibodies recognized two increased in genotype, whereas the amount of active JNK (pJNK) that was present was increased in polyQ-Htt mice. Akt activation (pAkt) was similar for all mice. Immunoblot using NSC34 (top) and mouse striatum (bottom) showed JNK1 comigrating with p46, whereas JNK2 and JNK3 comigrated with p54. Quantitative analysis of immunoblots in (c) showed activation of both p54 and p46 Htt in HttQ109 mice, pJNK/JNK ratios for JNK1 (p46) or JNK2/3 (p54) indicated that both JNK2/3 and JNK1 activity was higher in homozygous than in heterozygous HttQ109 mice. JNK2/3 was activated to a greater extent than JNK1, suggesting differential activation of JNKs by polyQ-Htt. Error bars represent s.e.m. The differences between JNK2/3 activity in wild-type and HttQ109 mice were significant (* indicates P < 0.01 in a pooled t-test).

**Figure 3** PolyQ-Htt increases JNK activity in models of Huntington’s disease. JNK activation was evaluated by immunoblots with antibodies to active JNK (pJNK) and total JNK (pan-JNK), which mainly recognized bands at p54 and p46. (a) NSC34 cells transfected with wild-type Htt or polyQ-Htt constructs were analyzed after 24 h. Both endogenous (end) and exogenous (exo) Htt were detectable. Total JNK (pan-JNK) was similar for untransfected (control) cells, and cells transfected with either wild-type Htt or polyQ-Htt (polyQ), but the amount of phosphorylated p54 JNK (pJNK) that was present increased with polyQ-Htt expression. (b) JNK1, JNK2 and JNK3 antibody specificity was validated with GST-JNKs (left) and striatal lysates from mice with individual JNK gene ablations (right). JNK1, JNK2 and JNK3 antibodies showed no immunoreactivity in brains from corresponding knockout samples. (c) Striatum from wild-type, heterozygous (hetero) and homozygous (homo) HttQ109 mice showed similar total JNK (pan-JNK) levels regardless of genotype, whereas the amount of active JNK (pJNK) that was present was increased in HttQ109 mice. Akt activation (pAkt) was similar for all mice. Immunoblot using NSC34 (top) and mouse striatum (bottom) showed JNK1 comigrating with p46, whereas JNK2 and JNK3 comigrated with p54. Quantitative analysis of immunoblots in (c) showed activation of both p54 and p46 JNK in HttQ109 mice, pJNK/JNK ratios for JNK1 (p46) or JNK2/3 (p54) indicated that both JNK2/3 and JNK1 activity was higher in homozygous than in heterozygous HttQ109 mice. JNK2/3 was activated to a greater extent than JNK1, suggesting differential activation of JNKs by polyQ-Htt. Error bars represent s.e.m. The differences between JNK2/3 activity in wild-type and HttQ109 mice were significant (* indicates P < 0.01 in a pooled t-test).

**Figure 2** JNK inhibitors prevent polyQ-Htt-induced FAT inhibition. In vesicle motility assays in isolated axoplasm, we measured individual velocity (arrowheads) as a function of time. The dark arrowheads and black lines represent the anterograde kinesin-dependent FAT rates and the reversed gray arrowheads and gray lines indicate retrograde, cytoplasmic dynein-dependent FAT rates. In vitro translated Htt constructs were perfused as described previously8. (a) Perfusion of wild-type Htt did not affect FAT8. (b) Perfusion of polyQ-Htt inhibited both anterograde and retrograde FAT8. (c) Endogenous JNK activity in squid axoplasm was examined by *in vitro* phosphorylation assays. We observed a time-dependent increase in cJun phosphorylation at JNK sites (cJun S63/73) when GST-cJun was incubated with squid axoplasm lysates (axo). Phosphorylation of GST-cJun was completely inhibited by either SP600125 or JIP, two well-characterized JNK inhibitors. Antibody to GST revealed similar GST-cJun levels for each reaction (cJun total). (d,e) Perfusion of polyQ-Htt with either (d) SP600125 (500 nM) or (e) JIP (100 µM) completely prevented the inhibition of FAT elicited by polyQ-Htt (compare d and e with b). (f) In contrast, perfusion of polyQ-Htt with the HDAC inhibitor BC-6-25 (10 µM)17 failed to prevent the effects of polyQ-Htt on FAT. These results indicate that polyQ-Htt-mediated inhibition of FAT involves activation of endogenous axoplasmic JNK.

mammalian JNKs in NSC34 cell lysates and striatal mouse tissue (Fig. 3a,c). The expression levels for both pan-JNK immunoreactive bands were comparable in cells expressing wild-type Htt and polyQ-Htt. However, antibodies to pJNK showed a clear increase in p54 immunoreactivity in cells expressing polyQ-Htt (Fig. 3a), which is consistent with previous reports20,22,31.

Differential activation of p54 and p46 JNKs led us to evaluate their identity using antibodies selective for JNK1, JNK2 and JNK3. Specificity was validated by immunoblotting with recombinant GST-tagged JNKs and brain lysates from mice lacking individual JNK genes30 (Fig. 3b). Antibodies to JNK1, 2 and 3 selectively recognized their target JNKs (Fig. 3b) and showed no immunoreactivity to brain lysates from mice lacking Mapk8 (JNK1), Mapk9 (JNK2) or Mapk10 (JNK3) (Fig. 3b).

We examined JNK expression in striatal tissue from 8-month-old wild-type mice and heterozygous and homozygous HttQ109 mice (Fig. 3c). HttQ109 knock-in mice are asymptomatic at this age15. Phosphorylation of Akt at Ser473 (Fig. 3c) and pan-JNK

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immunoreactivity did not change, regardless of genotype. However, pJNK immunoreactivity was substantially greater in lysates from heterozygous and homozygous Htt\(^{Q109}\) knock-in mice relative to wild-type mice (Fig. 3c). Cortical lysates gave similar results (data not shown). The p54 and p46 forms of JNK were differentially activated, as observed in transfected cells. P54 showed greater increases in pJNK immunoreactivity than p46 in Htt\(^{Q109}\) mice relative to wild type in both striatum (Fig. 3e) and cortex (data not shown). Consistent with the autosomal dominance of Huntington’s disease, JNK activation increased in both heterozygous and homozygous Htt\(^{Q109}\) mice, but activation was greater in homozygotes than in heterozygotes.

JNK activation ratio in wild-type mice was 1.097 ± 0.146 (mean ± s.e.m.) and 1.527 ± 0.061 in homozygote and 1.342 ± 0.133 in heterozygote Htt\(^{Q109}\) mice (\(n = 4\) in all cases). The difference in JNK1 activity between wild-type and Htt\(^{Q109}\) homozygote mice was significant (\(P < 0.05\) in a pooled \(t\) test), but the difference between wild-type and Htt\(^{Q109}\) heterozygote mice was not (\(P > 0.05\)). The JNK2/3 activity ratio for wild-type mice was 0.242 ± 0.012 (mean ± s.e.m.) and 0.590 ± 0.055 for homozygote and 0.440 ± 0.035 for heterozygote mice (\(n = 4\) in all cases). The JNK2/3 activity ratio significantly increased in both homozygote and heterozygote Htt\(^{Q109}\) mice relative to wild-type mice (\(P < 0.01\) in a pooled \(t\) test). Both JNK1 and JNK2/3 activity ratios were increased by polyQ-Htt, but polyQ-Htt had a greater effect on JNK2/3 than JNK1.

**JNK3 mediates the effects of polyQ-Htt on FAT**

JNK2/3 is selectively activated in neurons by stress in the presence of constitutive JNK1 activity. Because we observed differential activation of JNKs (Fig. 3a,b), we decided to evaluate the effects of specific JNK isoforms on FAT. SB203580 differentially affects JNK1 and JNK2/3 in vitro. At a concentration of 10 µM, SB203580 preferentially inhibits JNK2/3 and inhibits JNK1 at a tenfold higher concentration. Axoplasmic JNK activity was almost eliminated by 100 µM SB203580, but substantial JNK activity remained after treatment with 10 µM SB203580 (Fig. 4a). However, 10 µM SB203580 completely prevented inhibition of FAT by polyQ-Htt (Fig. 4b), suggesting that the effects of polyQ-Htt involved JNK2 or JNK3. Notably, 10 µM SB203580 also prevented inhibition of FAT by polyQ-AR. JNK isoforms were distinguished using biochemical approaches. JSAP1 is a scaffold protein regulating JNK activation. We identified four JSAP1 isoforms (a, b, c and d), all of which contained a 17–amino acid JNK-binding domain homologous to JIP (Fig. 4c). A 31–amino acid insert in JSAP1c/JSAP1d selectively reduces binding to JNK3 (ref. 34). As a result, JSAP1d binds JNK3 with a lower affinity than JSAP1a and inhibits JNK3 less efficiently. Recombinant JSAP1a (amino acids 115–233) and JSAP1d (amino acids 115–264) polypeptides were expressed and purified, and then perfused at 5 µM with polyQ-Htt (Fig. 4d). Perfusion of JSAP1a and JSAP1d alone had no effect on FAT (data not shown). As with JIP (Fig. 2e), perfusion of polyQ-Htt and JSAP1a completely prevented inhibition of FAT by polyQ-Htt (Fig. 4d). In contrast, JSAP1d reduced, but did not eliminate, the effects of polyQ-Htt on FAT (Fig. 4e), suggesting that JNK3 mediates the effects of poly-Q-Htt on FAT.

We evaluated the effects of specific JNK isoforms on FAT in isolated axoplasm (Fig. 5). The activity of recombinant JNKs was normalized in vitro using c-Jun as a substrate (data not shown). JNK1 (200 nM) did not affect FAT (Fig. 5a), which is consistent with reports of constitutive JNK1 activity in neurons. JNK2 (100 nM) decreased anterograde FAT rates slightly (1.25 ± 0.01, two-sample \(t\) test; Fig. 5b), but did not affect retrograde FAT (Fig. 5d). As with polyQ-Htt perfusion (Fig. 5d), JNK3 (100 nM) inhibited both directions of FAT (Fig. 5c). Quantitative analysis showed that JNK3 inhibited both anterograde (0.9 ± 0.01, compared with 1.6 µm s\(^{-1}\) in control buffer) and retrograde (1.25 ± 0.01, compared with 1.4 µm s\(^{-1}\) in control buffer) FAT (\(P < 0.01\), two-sample \(t\) test), suggesting that JNK3 is principally responsible for the effects of poly-Q-Htt on FAT.
LC/MS/MS analysis, we used site-directed mutagenesis to substitute peptides with the SEQUEST algorithm. KHC584 identity was confirmed not reveal phosphorylation at this site (KHC584-S176A). Bacterially expressed mutant proteins were purified Supplementary Fig. 2 and mass spectra, cross correlation and delta correlation values (phosphorylation by JNK3 using analysis of protease cleavage sites, several peptides (data not shown) and we identified a unique peptide Supplementary Fig. 2 by identification of multiple kinesin-1C peptides (coverage of 72%; Supplementary Fig. 2). We detected residual phosphorylation of several peptides (data not shown) and we identified a unique peptide corresponding to amino acids 173–188 with unequivocal evidence of phosphorylation by JNK3 using analysis of protease cleavage sites, mass spectra, cross correlation and delta correlation values (Fig. 6a and Supplementary Fig. 2). Phosphatase treatment reduced the phosphorylated peak intensity compared with that of neighboring peaks (data not shown). This JNK3-phosphorylated peptide was in the kinesin-1c motor domain and contained two series (Fig. 6a). LC/MS/MS allowed us to precisely map the specific serine residue carrying the phosphorylation. Tandem mass spectrometry analysis by collision-induced dissociation indicated that phosphorylation occurred on Ser176, but not Ser175 (Fig. 6 and Supplementary Fig. 2). The presence of a proline residue adjacent to Ser176 (a hallmark of JNK substrates) supported our identification of Ser176 as the JNK3 phosphorylation site.

Mass spectrometric analysis of JNK1-phosphorylated KHC584 did not reveal phosphorylation at this site (Supplementary Fig. 3 online), consistent with the differential effects of JNK1 and JNK3 on FAT that we observed (Fig. 5a,c). To confirm electrospray ionization-LC/MS/MS analysis, we used site-directed mutagenesis to substitute an alanine for either Ser175 or Ser176 of KHC584 (KHC584-S175A and KHC584-S176A). Bacterially expressed mutant proteins were purified trypsin for LC/MS/MS analysis or for phosphopeptide enrichment by metal ion affinity chromatography (IMAC) and LC/MS/MS analysis (Supplementary Fig. 2). We identified mass spectrometry–generated peptides with the SEQUEST algorithm. KHC584 identity was confirmed by identification of multiple kinesin-1C peptides (coverage of 72% Supplementary Fig. 2). We detected residual phosphorylation of several peptides (data not shown) and we identified a unique peptide corresponding to amino acids 173–188 with unequivocal evidence of phosphorylation by JNK3 using analysis of protease cleavage sites, mass spectra, cross correlation and delta correlation values (Fig. 6a and Supplementary Fig. 2). Phosphatase treatment reduced the phosphorylated peak intensity compared with that of neighboring peaks (data not shown). This JNK3-phosphorylated peptide was in the kinesin-1c motor domain and contained two series (Fig. 6a). LC/MS/MS allowed us to precisely map the specific serine residue carrying the phosphorylation. Tandem mass spectrometry analysis by collision-induced dissociation indicated that phosphorylation occurred on Ser176, but not Ser175 (Fig. 6 and Supplementary Fig. 2). The presence of a proline residue adjacent to Ser176 (a hallmark of JNK substrates) supported our identification of Ser176 as the JNK3 phosphorylation site.

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Ser176 phosphorylation affects kinesin-1 function

The effects of polyQ-AR on FAT involve JNK activation, increase kinesin-1 phosphorylation and reduce microtubule binding of kinesin-1 (ref. 7). Ser176 localized to loop 8, a sequence in the motor domain of kinesin-1 that has been implicated in kinesin-1 interaction with microtubules (Supplementary Fig. 4) and polyQ-AR (Fig. 6b). Similar effects were observed when using immortalized striatal cell lines derived from wild-type and HttQ109 mice (data not shown). This was consistent with previous reports of reduced kinesin-1 binding to microtubules in cells expressing polyQ-AR.\(^7\)}
Recombinant KHC\(^{584}\) was incubated with radiolabeled \(^{32}\)P-\(\gamma\)ATP (100 \(\mu\)M) in the presence or absence of active JNK3 (Fig. 7b) and then incubated with purified microtubules in the presence of 2 mM AMP-PNP or 2 mM ATP (to control for nonspecific pelleting, data not shown). We analyzed microtubule pellets and corresponding supernatants by immunoblot to visualize nonphosphorylated KHC\(^{584}\) and by autoradiography to visualize JNK3-phosphorylated KHC\(^{584}\). In the presence of AMP-PNP, kinesin-1 forms a rigid structure with microtubules\(^1\). Virtually all nonphosphorylated KHC\(^{584}\) partitioned with microtubules by immunoblot (Fig. 7b), but a large fraction of JNK3-phosphorylated KHC\(^{584}\) remained in the supernatant (Fig. 7b), suggesting that phosphorylation of kinesin-1 by JNK3 inhibits kinesin-1 binding to microtubules. The presence of JNK3-phosphorylated KHC\(^{584}\) in pellet fractions was consistent with residual phosphorylation occurring at residues outside of the motor domain. JNK-3 phosphorylation of kinesin-1 reduced binding to microtubules with AMP-PNP by ~50\% (Supplementary Fig. 5 online).

We assessed the effects of S176 phosphorylation on kinesin-1 motility in vivo by expressing GFP-tagged, truncated kinesin-1 constructs in cultured hippocampal neurons (Fig. 8). When expressed in hippocampal cells, a GFP-tagged kinesin-1 construct (KHC\(^{560}\)-GFP), which consists of the first 560 amino acids of wild-type KHC, selectively translocates to axon distal ends and accumulates there, but not in dendrites, indicating that this motor translocates preferentially along axonal microtubules\(^17\). KHC\(^{560}\)-GFP wild-type translocation is so efficient that little or no fluorescence can be detected in cell bodies or axons\(^17\). To evaluate whether S176 phosphorylation affects kinesin translocation efficiency, we compared the localization of the phosphorylation-mimicking construct KHC\(^{560}\)-GFP-S176E and its nonphosphorylatable counterpart KHC\(^{560}\)-GFP-S176A with that of KHC\(^{560}\)-GFP by quantitative fluorescence microscopy (Fig. 8a-f).

Figure 7 PolyQ-Htt expression inhibits kinesin-1 binding to microtubules. (a) Lysates of NSC34 cells transfected with wild-type Htt or polyQ-Htt (as in Fig. 3a) were analyzed by immunoblot. The total levels (input) of KHC, DHC and tubulin (Tub) were comparable for untransfected (ctrl) cells and cells expressing wild-type Htt or polyQ-Htt. However, the fraction of kinesin-1 recovered in association with microtubules was reduced in lysates from polyQ-Htt-expressing cells when compared with untransfected and wild-type Htt-expressing cells. (b) Microtubule-binding assays using KHC\(^{584}\). Immunoblotting (WB) showed that nonphosphorylated KHC\(^{584}\) was mainly recovered in association with microtubules (pellet). An autoradiogram (32P) revealed that a substantial fraction of JNK3-phosphorylated KHC\(^{584}\) remained in the supernatant.

**DISCUSSION**

Despite ubiquitous expression of Htt, neuronal cells are uniquely vulnerable to a toxic gain-of-function polyQ expansion, suggesting that processes that are critical for proper neuronal function and survival are selectively altered in Huntington’s disease\(^1\). Axonal transport is essential for neurons because of their large size and complex functional architecture\(^1\). Notably, alterations in kinesin-1 function and regulation are associated with various human neuropathologies\(^3,7,8,10,19\).

Several reports link FAT inhibition to pathogenesis in Huntington’s disease\(^3,5,10,19\) and other polyQ-expansion diseases\(^3,7\). For example, overexpression of polyQ-Htt in cultured cells\(^10\) and *Drosophila*\(^3,5\) leads to reduced transport and accumulation of vesicular cargos in axons. PolyQ-Htt and polyQ-AR, two otherwise unrelated proteins, similarly inhibit FAT vesicle motility in isolated axoplasm independently of changes in gene transcription or aggregate formation\(^7,8\). Such observations implicate FAT defects in Huntington’s disease pathogenesis, which would partially explain the selective vulnerability of neurons in Huntington’s disease and other polyQ-expanded diseases\(^1\).

Several models have been proposed to explain the inhibition of FAT that is induced by polyQ-Htt. Some require physical interactions...
between Htt and conventional kinesin or CDyn subunits. PolyQ-Htt aggregates have been proposed to sequester molecular motors or to physically block FAT. Alternatively, it has been suggested that Htt mutations interfere with the normal functions of Htt as a scaffold linking molecular motors to transported cargoes or that Htt mutations affect the subunit composition and function of molecular motors. However, axoplasm experiments indicate that polyQ-Htt inhibits FAT at concentrations 100–1,000-fold lower than molecular motors, suggesting activation of enzymatic activities.

We used cell fractionation and immunoprecipitation to evaluate the interactions between Htt and molecular motors in brain tissue of wild-type and homozygous Htt mice. Regardless of genotype, we found similar levels of full-length Htt in detergent-soluble and detergent-insoluble fractions. In contrast, KHCs, KLCs, DIC and DHC levels were much higher in soluble fractions, suggesting that most molecular motors are not sequestered by polyQ-Htt. Unlike experiments involving Htt overexpression, immunoprecipitation failed to detect interactions between endogenous Htt and kinesin or CDyn. (Fig. 1.) Detection of KLCs in KHC immunoprecipitates and DHC in DIC immunoprecipitates confirmed that physiologically relevant protein complexes were preserved. Such observations are inconsistent with models in which polyQ-Htt inhibits FAT through direct interactions with molecular motors.

Another model proposes that pathogenic polyQ-Htt activates the neuronal regulatory pathways that affect FAT. Kinesin-1 and CDyn are regulated by phosphorylation of specific motor subunits. Kinase activities that regulate FAT, including GSK-3, Akt, and JNK, are altered in Huntington’s disease, and polyQ-AR activates JNK. JNK inhibitors blocked the effects of polyQ-Htt on FAT and we found JNK activation in polyQ-Htt-expressing cells and Htt mice. (Fig. 3.) JNK activation was more pronounced in homozygous Htt mice than in heterozygous littermates, but was increased in both, which is consistent with the autosomal dominant inheritance of Huntington’s disease. JNK activation in presymptomatic Htt mice indicates that this change represents an early event in Huntington’s disease.

Mammals have three JNK genes. We observed that polyQ-Htt increased JNK2/3 activity more than JNK1 in Htt mice and polyQ-Htt–transfected cells. (Fig. 3,) which is consistent with reports showing constitutive JNK1 activity and selective JNK2/3 activation in neurons with stress. This prompted us to examine the roles of specific JNK isoforms in mediating the effects of polyQ-Htt on FAT. SB203580 inhibited JNK2/3 at 10 μM, but JNK1 inhibition required >100 μM SB203580 (ref. 23). Substantial JNK activity (presumably JNK1) remained in axoplasms treated with 10 μM SB203580 (Fig. 4a), but 10 μM SB203580 prevented the effects of polyQ-Htt on FAT (Fig. 4b), suggesting that the effects of polyQ-Htt on FAT involve JNK2/3. JSA1 polypeptides further supported a role for JNK3, as JSA1a, which inhibits all JNKs, preserved FAT effectively, but JSA1d, with a 31–amino acid insert that selectively interferes with JNK3 binding, did not. (Fig. 4c,d.)

To evaluate the role of individual JNKs on FAT, we examined the effects of active recombinant JNK1, JNK2 and JNK3 on FAT in axoplasms. Active JNK1 had no effect on FAT (Fig. 5a), consistent with constitutive JNK1 activity in axons. JNK2 produced a selective, moderate effect on anterograde FAT, but had no effect on retrograde FAT. In contrast, the effects of JNK3 on FAT paralleled those of polyQ-Htt by inhibiting both anterograde and retrograde FAT. The effects of polyQ-Htt on FAT appear to be primarily mediated by JNK3, although JNK2 may contribute. Notably, JNK3 is preferentially expressed in neuronal tissues and polyQ-Htt pathology is restricted to neurons.

To provide a molecular basis for FAT inhibition by JNK3, we identified relevant phosphorylation sites. Mass spectrometry mapped JNK3 phosphorylation of kinesin-1 to S176, a conserved residue in the motor domain. (Fig. 6a and Supplementary Figs. 2 and 3.) S176 was also modified in kinesin-1 that was immunoprecipitated from mouse brain (data not shown). Mutagenesis studies confirmed that JNK3 phosphorylated Ser176 (Fig. 6b). JNK1 did not phosphorylate kinesin-1 at Ser176 (Supplementary Fig. 3), which is consistent with unique substrate preferences for JNK3 (ref. 48). Ser176 was conserved among squid, mouse and human kinesin-1 (Supplementary Fig. 4), validating the squid axoplasm model. Collectively, these data demonstrate that JNK3 activity is elevated in the brains of Htt mice and axoplasm perfused with polyQ-Htt. (Fig. 3 and 4), that JNK3 phosphorylates kinesin-1 at Ser176 (Fig. 6 and Supplementary Figs. 2 and 3), and that Ser176 modification is sufficient to inhibit trafficking of kinesin-1. (Fig. 8). Antibodies selective for this epitope are under development and should allow us to quantify the fraction of neuronal kinesin-1 modified by JNK3 in Huntington’s disease brains.

On the basis of Ser176’s position in the motor domain, we evaluated the effects of JNK3 phosphorylation on kinesin-1 binding to microtubules. JNK3-treated recombinant kinesin-1 and kinesin-1 in polyQ-Htt–transfected cells showed reduced binding to microtubules (Fig. 7). Furthermore, pseudo-phosphorylation at Ser176 markedly decreased kinesin-1 translocation in vivo (Fig. 8), indicating that Ser176 phosphorylation interferes with kinesin-1 function.

Our results suggest that polyQ-Htt inhibits FAT by activation of axonal JNKs. In addition, JNK activation by polyQ-Htt is also consistent with changes in transcription and JNKs can be pro-apoptotic (Supplementary Fig. 6 online). Notably, JNK3, but not JNK1, activity mediated the effects of polyQ-Htt on FAT, providing a partial explanation for selective neuronal vulnerability in Huntington’s disease. JNK1 and JNK2 are ubiquitously expressed, whereas JNK3 is selectively expressed in brain and testes. The identification of JNK3 as a JNK3 substrate provides a molecular basis for changes in FAT that is consistent with the neuron-selective pathology of polyQ-Htt.

In sum, neuronal JNK3 is activated by polyQ-Htt. JNK3 in turn phosphorylates kinesin-1, decreasing its ability to bind microtubules and move cargoes in FAT. (Fig. 8 and Supplementary Fig. 6.) Identification of Ser176-phosphorylated kinesin-1 in normal mouse brain suggests that JNK3 is involved in a normal pathway for regulating kinesin and delivering specific membrane-bounded organelle cargoes to particular neuronal domains. Increased activation of this pathway by polyQ-Htt would lead to reductions in kinesin-based transport, deficits in synaptic and axonal function, and eventual dying-back neuronal degeneration. Inhibition of JNK3 sufficed to prevent inhibition of FAT resulting from polyQ-Htt. Our results suggest that elevation of JNK activity and consequent inhibition of FAT may represent primary pathogenic events in Huntington’s disease. Further studies should illuminate the molecular components mediating JNK activation by polyQ-Htt. Regardless, inhibition of JNK activity to protect FAT represents a promising therapeutic target for the treatment of Huntington’s disease.

METHODS
Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Note: Supplementary information is available on the Nature Neuroscience website.

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19. E.T.C., C.B. and D.H. carried out the mass spectrometry studies. C.-F.H. and G.B. biochemistry experiments. K.Y. provided and characterized recombinant JIP. B.B.,


ONLINE METHODS

Antibodies and reagents. In our experiments, we used antibodies to KH C (H2 clone, Chemicon)\(^3\), pan-KLC (63-90 clone, Chemicon)\(^5\), DIC (Santa Cruz \#15324), DHC (Santa Cruz \#9115), phospho-Akt (44-622G, Biosource), Htt (2166, Chemicon), pan-JNK (Upstate \#06-748), JNK1 (Pharmingen \#53268), JNK2 (Cell Signaling \#6762), JNK3 (Cell Signaling \#2305), phospho-JNK (Cell Signaling \#9251), GST (Sigma \#G7781), phospho–c-Jun (Santa \#16312) and tubulin (DM1A, Sigma). All antibodies were characterized for specificity against the tissues that we used (see Supplementary Fig. 7 online). For secondary antibodies, we used horseradish peroxidase (HRP)-conjugated goat antibody to rabbit IgG (Jackson 111-035-045), HRP-conjugated goat antibody to mouse IgG (Jackson 115-035-146) and HRP-conjugated bovine antibody to goat IgG (Jackson 805-035-180).

SB203580, SP600125 (JNK inhibitor II), okadaic acid, staurosporine, K252a, 50 nM okadaic acid, microcystin and c-Jun were obtained from Calbiochem. The HDAC inhibitor BC-6-25 (ref. 27) was a generous gift from A. Kozikowski.

Immunoblots. Proteins were separated by SDS-PAGE on 4–12% Bis-Tris gels (NuPage minigels, Invitrogen), using MOPS running buffer (Invitrogen) and transferred to PVDF using Towbin buffer supplemented with 10% (vol/vol) methanol (90 min at 400 mA using Hoeffer TE22 apparatus). Immunoblots were blocked with 1% (wt/vol) non-fat dried milk in tris-buffered saline (25 mM Tris (pH 7.2), 2.68 mM KCl and 136.8 mM NaCl). Membranes were incubated with primary antibodies overnight at 4 °C in 1% IgG-free BSA (wt/vol, Jackson ImmunoResearch) and washed four times with 0.1% Tween-20 (w/vol) in tris-buffered saline. Primary antibody binding was detected with HRP-conjugated antibodies to mouse, rabbit or goat (Jackson Immunoresearch) and visualized by chemiluminescence (ECL, Amersham). Quantitative immunoblotting was performed as described previously\(^39\).

Immunoprecipitation. Mouse brains from wild-type and Htg(\(\text{H2}\))\(^{109}\) knock-in mice were homogenized in lysis buffer (25 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100 (wt/vol), and mammalian protease inhibitor cocktail (Sigma, 1:100 dilution)). Lysates were centrifuged twice for 5 min at 55,000 rpm (163,640 g(\text{max})) using a TLA100.3 rotor (Beckman Instruments). The resulting supernatant fractions were pre-cleared using a mixture of Protein G agarose beads (Pierce) and non-immune mouse IgG–conjugated Sepharose beads (Jackson ImmunoResearch) for 1 h at 18–22 °C. We brought 400 μg of each pre-cleared brain lysate to 1 ml with lysis buffer and incubated with them with 5 μg of the appropriate antibody plus 10 μl of Protein G agarose beads at 4 °C for 3 h. Immunocomplexes were recovered by centrifugation (3,000 g\text{(max)} for 30 s), washed four times with 1 ml of lysis buffer, once with 50 mM HEPES (pH 7.4), and resuspended in Laemmli buffer. Primary antibody binding was analyzed on a Zeiss Axiomat with a 100\,\times\,1.3 n.a. objective, and DIC optics.

Recombinant constructs. Expression vectors encoding amino acids 1–548 of human Htt with an N-terminal FLAG epitope tag were translated \textit{in vitro} as described previously\(^8\) and used for vesicle motility assays. Expression vectors encoding amino acids 1–969 of human Htt (wild type (Q16) and pathogenic (Q46)) with an N-terminal FLAG tag were used for transfection studies (Figs. 3a and 7a)\(^2\). His-tagged KHC\(^{385}\) and KHC\(^{434}\) and GFP-tagged KHC\(^{296}\) mutant constructs were generated using the Quick-site mutagenesis kit (Stratagene).

Axoplasmic JNK activity assays. For experiments on kinase activity in axoplasm (Figs. 2c and 4a), we triturated 3–4 axoplasms in 280 μl of buffer containing 10 mM HEPES (pH 7.4), 10 mM MgCl\(_2\) and 1 mM DTT, without ATP. Reactions (50 μl) were incubated at 18–22 °C and were started by adding ATP (50 μM). Aliquots were collected at various time points up to 20 min and analyzed by immunoblotting.

Brain tissue/cell lysate preparation. Mouse brain tissue and cultured cells were homogenized in ROLB buffer (10 mM HEPES (pH 7.4), 0.5% Triton X-100, 80 mM β-glycerophosphate, 50 mM sodium fluoride, 2 mM sodium orthovanadate, 100 mM staurosporine, 100 nM K252a, 50 nM okadaic acid, 50 nM microcystin, 100 mM potassium phosphate and mammalian protease inhibitor cocktail (Sigma)). Lysates were clarified by centrifugation and protein concentration was determined using BCA kit (Pierce). All of our experiments involving mice were approved by the Institutional Animal Care and Use Committee at the University of Illinois at Chicago and followed US National Institutes of Health guidelines for vertebrate animal use.

NSC34 cell culture and transfection. NSC34 cells were plated at 10,000 cells per cm\(^2\) on 10-cm tissue culture dishes for biochemical studies and grown on DMEM containing 1 mM glutamine and 10% fetal bovine serum (vol/vol, HyClone). When cells reached 60–70% confluency, media was replaced by DMEM with 1 mM glutamine and cells were transfected with wild-type Htt and polyQ-Htt constructs (1–969)\(^{70}\) using Lipofectamine 2000 (Invitrogen), as previously described\(^3\). JNK activation was evaluated 24 h after transfection.

Microtubule-binding assays. NSC34 cells were transfected with wild-type Htt and polyQ-Htt constructs as described above. After 24 h, cells were scraped in BRB80 buffer (80 mM PIPES (pH 7), 1 mM MgCl\(_2\), 1 mM EGTA, 1 μM staurosporine, 1 μM K252a, 50 nM okadaic acid, 200 mM microcystin, 1:100 dilution of phosphatase inhibitor cocktail II (Calbiochem) and 1:100 dilution of mammalian protease inhibitor cocktail (Sigma)) at 4 °C and homogenized using a 30 gauge syringe (500 μl of BRB80 per 10-cm dish). Lysates were centrifuged at 18,000 g for 15 min at 4 °C in a Beckman TLA-100.3 rotor at 18–22 °C. Microtubule-enriched pellets were resuspended in Laemmli buffer and analyzed by immunoblotting. For microtubule binding experiments (Fig. 7b), 4 μl aliquots of \textit{in vitro} phosphorylated KHC\(^{84}\) (prepared as above) were incubated with 64 μg of taxol-stabilized microtubules in BRB80 buffer and either 2 mM AMP-PNP or 2 mM ATP (100 μl total volume). After a 30-min incubation, these mixtures were centrifuged for 15 min at 106,000 g over a cushion of 20% in BRB80 and centrifuged for 15 min at 106,000 g in a Beckman TLA-100.3 rotor at 18–22 °C. Pellets and supernatants were collected and the levels of kinesin in each fraction were quantified by phosphorimager scanning of radioactivity or with fluorescently labeled secondary antibodies.

Kinesin-1 translocation assays. Accumulation of wild-type KHC\(^{560}\)-GFP and KHC\(^{560}\)-GFP-S176E constructs in neurites of cultured hippocampal neurons was evaluated as described previously\(^7\). Statistical analysis. All experiments were repeated at least three times. Unless otherwise stated, the data was analyzed by ANOVA followed by \textit{post hoc} Student-Newman-Keul’s test to make all possible comparisons. Data was expressed as mean ± s.e.m. and significance was assessed at \(P < 0.05\) or \(P < 0.01\) as noted.
**Mouse brain fractionation.** Brains from wild-type (12 months old) and HttQ109 knock-in (14 months old) mice were homogenized in 3 ml of buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl and 1% Triton X-100, centrifuged twice at 2,500 g for 10 min and centrifuged once at 7,000 g for 10 min to pellet debris and nuclei. Clarified lysates were spun at 150,000 g for 20 min in a Beckman TLA 100.3 rotor at 55,000 rpm. The pellets and supernatant represented the detergent-insoluble and detergent-soluble fractions, respectively. Detergent-insoluble pellets were resuspended in buffer. Equal amounts of protein from each fraction were analyzed by immunoblotting.

**In vitro phosphorylation.** In vitro phosphorylation experiments (40 µl volume) were performed by incubating KHC584 protein constructs (3 µM) with 0.1 µM JNK3 (Upstate) or 0.2 µM JNK1 (Upstate) in HEM buffer (50 mM HEPES, 12 mM MgSO4 and 100 µM ATP, pH 7.4), as described previously7.

**Mass spectrometry studies.** Phosphorylated KHC584 protein was subjected to trypsin digestion in solution. Briefly, dried samples were resuspended in 30 mM HEPES and 30 mM NaF in the presence of 1 µg of trypsin (Sigma, proteomics grade) and incubated at 37 °C overnight (16–18 h). The resulting tryptic peptides were later resuspended in buffer A (5% acetonitrile, 0.4% acetic acid, 0.005% heptfluorobutyric acid (vol/vol) in water) for mass spectrometry analysis or prepared for IMAC50. Samples were analyzed by liquid chromatography on line with a LTQ (a two-dimensional ion trap) instrument equipped with a commercial nanospray source (Thermo Finnigan). Samples were automatically loaded by a microautosampler (Famos, LC Packings) onto an 11-cm bore 100-Å Magic beads, Michrom Bioresources. The solvent system was delivered by an HP1100 pump (Agilent Technologies). Samples were analyzed by performing full scan followed by tandem mass spectrometry or MS/MS of the five most intense ions (top five) by collision-induced dissociation. Sample loading, solvent delivery and scan function were controlled with Xcalibur software (Thermo Finnigan). LC/MS/mS files were searched using a SEQUEST algorithm against a generated database containing KIF5C_Rat and KIF5C_Mouse, among others proteins. SEQUEST search parameters included mass tolerance of ±1.5 Da, trypsin specificity and a differential search for serine, threonine and tyrosine phosphorylation. The generated dataset was filtered using INTERACT50 based on the following criteria: delta correlation of 0.1, and X correlation values for +1 peptides ≥ 1.8, +2 ≥ 2.2 and +3 ≥ 3.2.

Erratum: Pathogenic huntingtin inhibits fast axonal transport by activating JNK3 and phosphorylating kinesin

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In the version of this article initially published, a critical word was missing. The sentence should read: “Conversely, antibodies to Htt immunoprecipitated Htt from both wild-type and homozygous HttQ109 knock-in mouse brain lysates, but kinesin-1, KLC, DIC and DHC could not be detected in Htt immunoprecipitates.” The error has been corrected in the HTML and PDF versions of the article.