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CEP-1347 reduces mutant huntingtin-associated neurotoxicity and restores BDNF levels in R6/2 mice

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ABSTRACT

Huntington's disease (HD) is a devastating neurodegenerative disorder caused by an expanded polyglutamine repeat within the protein Huntingtin (Htt). We previously reported that mutant Htt expression activates the ERK1/2 and JNK pathways [Apostol, B.L., Illes, K., Pallos, J., Bodai, L., Wu, J., Strand, A., Schweitzer, E.S., Olson, J.M., Kazantsev, A., Marsh, J.L., Thompson, L.M., 2006. Mutant huntingtin alters MAPK signaling pathways in PC12 and striatal cells: ERK1/2 protects against mutant huntingtin-associated toxicity. Hum. Mol. Genet. 15, 273–285]. Chemical and genetic modulation of these pathways promotes cell survival and death, respectively. Here we test the ability of two closely related compounds, CEP-11004 and CEP-1347, which inhibit Mixed Lineage Kinases (MLKs) and are neuroprotective, to suppress mutant Htt-mediated pathogenesis in multiple model systems. CEP-11004/CEP-1347 treatment significantly decreased toxicity in mutant Htt-expressing cells that evoke a strong JNK response. However, suppression of cellular dysfunction in cell lines that exhibit only mild Htt-associated toxicity and little JNK activation was associated with activation of ERK1/2. These compounds also reduced neurotoxicity in immortalized striatal neurons from mutant knock-in mice and *Drosophila* expressing a mutant Htt fragment. Finally, CEP-1347 improved motor performance in R6/2 mice and restored expression of BDNF, a critical neurotrophic factor that is reduced in HD. These studies suggest a novel therapeutic approach for a currently untreatable neurodegenerative disease, HD, via CEP-1347 up-regulation of BDNF.

Introduction

Huntington's disease (HD) is caused by expansions of CAG repeats leading to extended polyglutamine tracts within the corresponding Huntingtin (Htt) protein (Bates et al., 2002; Group, 1993; Ross, 2002; Walker, 2007). Brain pathology in HD is widespread, particularly in cortex and striatum, however the medium spiny striatal neurons appear to be most vulnerable to mutant Htt expression and show the greatest degree of cell loss (Bates et al., 2002). Neurons and other cell types respond to a wide range of external or internal stimuli by activating cellular signal transduction networks to elicit pro-survival and pro-apoptotic responses (Raman et al., 2007). One such network is the mitogen-activated protein kinase (MAPK) superfamily which is comprised of more than a dozen MAPK genes, the major pathways being the extracellular signal-regulated protein kinases (ERK1/2, ERK), the c-Jun N-terminal kinases or stress-activated protein kinases (JNK/SAPKs), and the p38 family of kinases. Following a cascade of phosphorylation events that initially activate upstream effectors, the individual MAPKs are in turn phosphorylated with subsequent changes in subcellular localization and regulation of downstream targets (Kaneko et al., 1997; McKay and Morrison, 2007; Raman et al., 2007; Turjanski et al., 2007).

MAPK signaling has been implicated in processes contributing to cell death associated with HD and other neurodegenerative diseases. The JNK pathway is activated in several HD systems (Apostol et al., 2006; Garcia et al., 2004; Liu, 1998) and in other neurodegenerative disorders such as Parkinson's and Alzheimer's (Borsello and Forloni, 2007). In mutant Htt-expressing cell systems, genetic or pharmacologic inhibition of JNK signaling blocks Htt-mediated cell death

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(Apostol et al., 2006; Garcia et al., 2004; Liu, 1998). While the role of JNK in mutant Htt-associated toxicity appears relatively straightforward, that of ERK activation in neurodegenerative diseases is more complex. Both protective (Hetman and Gozdz, 2004) and deleterious (Cheung and Slack, 2004; Chu et al., 2004; Subramaniam and Unsicker, 2006) roles have been ascribed to ERK signaling. These differing functions do not simply rely on ERK's activation state, but also on its relative kinetics, duration of activation, cellular localization and associated scaffold proteins (McKay and Morrison, 2007). ERK activation progressively increases in striatum of R6/2 transgenic mice but decreases in cerebral cortex (Lievens et al., 2002). This latter effect in cortex is consistent with the progressive decrease in brain derived neurotrophic factor (BDNF), a potential downstream target of multiple pathways, including REST/NRSF (Zuccato et al., 2003) and ERK, in HD (Zuccato and Cattaneo, 2007).

A number of proposed mechanisms of HD neurotoxicity involve the progressive depletion of BDNF and the decreased supply of BDNF to striatum through anterograde transport down corticostriatal axons (Gauthier et al., 2004). Remarkably, of all HD mouse models, transcriptional profiles of BDNF knock-out mice are the most similar to gene expression changes identified in HD human brain (Strand et al., 2007), suggesting that decreased BDNF is a key pathogenic feature in HD. Indeed, BDNF treatment of excitotoxic injury mouse models of HD and other means of up-regulating BDNF in HD transgenic mice, including exercise, enriched environments, or treatment with compounds such as cysteamine, have shown promise in ameliorating HD-related

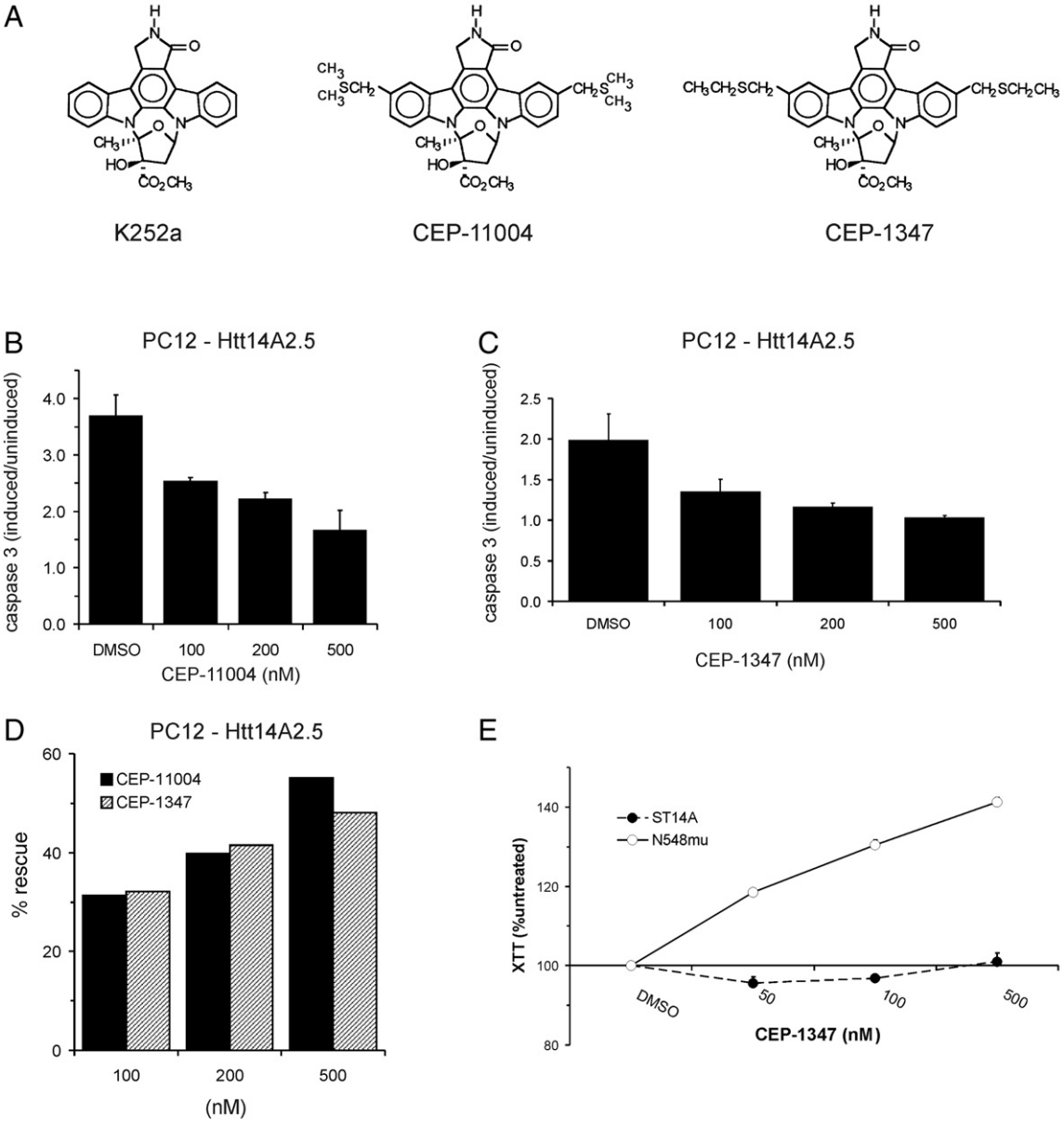

Fig. 1. CEP-11004/CEP-1347 protects against mutant Htt-associated toxicity in Htt14A2.5 and N548mu lines. (A) Structure of parent compound K252a and derivatives CEP-11004 and CEP-137. (B) Caspase 3 activity of Htt14A2.5 cells induced with 5 μM PA and treated with the indicated concentrations of CEP-11004 for 3 days. Results are expressed as the ratio between induced and uninduced cells. (C) Similar experiment to that shown in (A) except using the CEP-11004 analog CEP-1347. (D) Results from (B) and (C) expressed as the percentage of caspase 3 activity of untreated cells that is rescued by CEP-11004 or CEP-1347; both compounds exhibit similar profiles. (E) ST14A and N548mu cells were plated and the following day shifted to the nonpermissive conditions (39 °C and low serum media) with increasing concentrations of CEP-1347 as indicated. Two days later XTT assays were performed and results are presented as the percent of untreated cells. In (B), (C) and (E) error bars are the standard deviation of triplicate reactions within a representative experiment and experiments were performed at least 3 times. In (B) ANOVA test $p<0.0001$ and (C) ANOVA test $p<0.0001$. In (E), the variability in the increase in cell viability (i.e. XTT values) between replicates was low resulting in small standard deviations (i.e. small error bars) ANOVA test $p<0.0001$.

symptoms (Borrell-Pages et al., 2006; Levy et al., 2005; Pang et al., 2006; Rigamonti et al., 2007; Saydoff et al., 2006).

Targeted biochemical studies and pharmacologic modulation of MAPK pathways suggest that mutant Htt can alter the metabolic state of cells leading to activation of both ERK and JNK pathways. Activating ERK and JNK could potentially exert competing signals since ERK signaling is associated with cell survival while JNK inhibition effectively suppresses pathogenesis (Apostol et al., 2006). This study tested CEP-11004 and CEP-1347, first characterized as inhibitors of *M*ixed *L*ineage *K*inases (MLKs) that are upstream activators (MAPKKK) of JNK (Handley et al., 2007; Wang et al., 2004),for their effects on Htt-mediated neuropathogenesis. These inhibitors block JNK activation, but can also activate ERK signaling in certain cell settings (Chadee and Kyriakis, 2004; Roux et al., 2002), and can increase levels of the NGF and BDNF receptors TrkA and TrkB, respectively, in neuronal cells (Wang et al., 2005.). We find that these inhibitors significantly reduced neurotoxicity in cells expressing mutant Htt polypeptides through mechanisms consistent with modulation of JNK and ERK signaling. CEP-1347 also reduces neurotoxicity in striatal cell lines derived from knock-in mice, suppresses neurodegeneration in *Drosophila* and delays behavioral phenotypes in R6/2 mice. Notably, CEP-1347 increased cortical BDNF expression in R6/2 mice, restoring BDNF levels, suggesting that BDNF expression may be impacted through modulation of MAPK signaling.

Although modulation of MAPK signaling has been implicated previously in HD using proliferating and primary cell culture models (Apostol et al., 2006; Garcia et al., 2004; Roze et al., 2007), modulation of MAPK signaling has not yet been tested in mouse models of HD. These studies take a potential therapeutic compound not previously tested for its effects in any HD model from cells to a pilot preclinical study in mice together with mechanistic insights for its mode of action. We find that selective pharmacologic intervention in MAPK signaling may be an appropriate approach to HD therapy and that CEP-1347, which is safe and well tolerated in humans, represents a promising compound for treatment of HD.

Results

CEP-11004/CEP-1347 protects against mutant Htt-associated phenotypes in PC12 and ST14A cells

We previously reported that the MAP kinases ERK and JNK are activated by expression of mutant Htt in multiple cells lines (Apostol et al., 2006). Activation of JNK paralleled mutant Htt-associated cellular toxicity and a JNK inhibitor (SP600125) partially attenuated increases in caspase 3 activation, suggesting that cell dysfunction/death may involve JNK activation. Conversely, ERK activation was protective and may represent a compensatory mechanism against the deleterious effects of mutant Htt expression. Specifically, inhibiting ERK with the MEK (the upstream kinase of ERK) inhibitor U0126 increased cell dysfunction, while genetically increasing ERK activation, by transiently expressing a plasmid encoding constitutively active MEK1, partially rescued mutant Htt-associated toxicity (Apostol et al., 2006). Since JNK promotes cellular dysfunction in multiple HD cell models, we reasoned that targeting an upstream regulator of the pathway may also affect toxicity in these cells. The compounds used in this study, CEP-11004 and CEP-1347, are two closely related indolocarbazole analogs derived from a natural product, K252a (1A). Originally identified as a TrkA inhibitor with neuroprotective properties at low concentrations (for review (Wang et al., 2004) CEP-11004 and CEP-1347 were selected based upon their ability to enhance the neuroprotective qualities of K252a, while decreasing its ability to inhibit TrkA activity (Wang et al., 2004). All three compounds were subsequently shown to inhibit JNK activation through inhibition of its upstream activator, MLK1-3 (Kaneko et al., 1997; Pan et al., 2005; Roux et al., 2002; Wang et al., 2004) with MLK3 the major isoform inhibited by these compounds (Cha et al., 2006). We first obtained CEP-11004 from Cephalon, Inc and this analog was used for the majority of experiments. CEP-1347, the analog used in human clinical trials, was used in confirmatory experiments in most models and in pilot preclinical trials in mice.

To assess whether CEP-11004 has protective properties on HD phenotypes, we tested a PC12 cell line, Htt14A2.5, which inducibly expresses truncated human mutant Htt exon 1 protein fused to enhanced green fluorescent protein (EGFP) and shows a relatively mild phenotype. Htt14A2.5 cells induced to express mutant Htt exhibit visible aggregates and a progressive increase in caspase 3 activity that is typically 2–4 fold higher in induced versus uninduced cells up to 4 days after induction (Apostol et al., 2006). Caspase 3 activity is a typical marker of neuronal dysfunction that is also observed in HD transgenic mouse models (Sanchez-Mejia and Friedlander, 2001), allowing studies of the mechanistic aspects of pathogenesis relevant to neuronal dysfunction. In these lines, massive cell death is not observed and only a slight progressive increase in JNK activation is detected, consistent with the milder phenotype. Cells were induced with 5 μM ponasterone (PA) and simultaneously treated with increasing concentrations of CEP-11004 for 3 days and induced versus uninduced control lines were compared to eliminate confounding effects from variability between cell lines. In a representative experiment, caspase 3 activity levels increased 3.7-fold over uninduced levels following mutant Htt induction (Fig. 1B). Treatment with 100 and 500 nM CEP-11004 reduced this to 2.5 and 2.0, respectively. Similar experiments were performed with CEP-1347, an analog of CEP-11004, and a similar dose-dependent decrease of caspase 3 activation was observed (Fig. 1C). When these results are normalized to correct for experimental variation in the absolute level of caspase 3 activation, the two drugs show strikingly similar profiles (Fig. 1D). Both

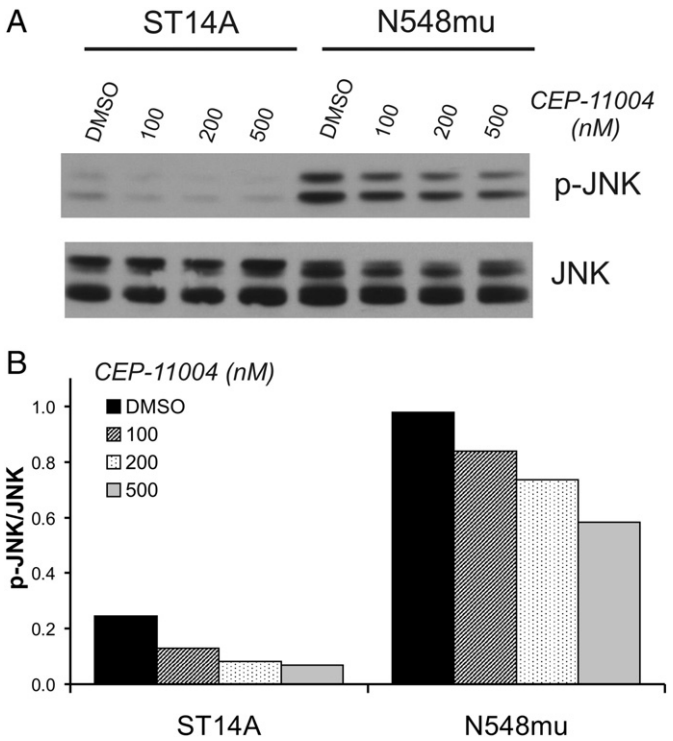

Fig. 2. CEP-11004 inhibits JNK activation in ST14A and N548mu cells. ST14A and N548mu cells were plated and the following day shifted to nonpermissive conditions (39 °C and low serum media). Approximately 30 h later, increasing concentrations of CEP-11004 were added, as indicated, for 2 h. (A) Cells were harvested and immunoblot analysis was performed using p-JNK specific antibody followed by stripping and reprobing with antibody specific for total JNK. The expected 46 and 55 kDa MW bands corresponding to JNK1 and JNK2 are detected. (B) Graphical representation of (A). Films were quantitated by densitometric analyses and the ratio of relative p-JNK/JNK intensities is shown in the graph. Experiments were performed at least 3 times. The Western in (A) and its quantitation in (B) is from a single representative experiment.

compounds also increased the number of cells containing visible Htt aggregates, a hallmark phenotype associated with HD pathology in this line (Fig. S1). These results were not associated with altered mutant Httex1p expression (data not shown).

As a means of testing MLK inhibitors in a cell model that exhibits dramatic cell death and robust JNK activation, we compared ST14A parental cells, which are conditionally immortalized striatal neuron-derived lines (Rigamonti et al., 2001), to N548mu cells, which are ST14A cells that are stably transfected with a 548 amino acid amino terminal fragment of mutant human Htt containing 120Qs. When shifted to nonpermissive conditions, these cells cease proliferating and begin to differentiate. The N548mu cells exhibit high levels of cell death as monitored by measuring a reduction in cell viability (with MTT or XTT assays, Fig. 1E) and/or measuring an increase in cell death (with LDH

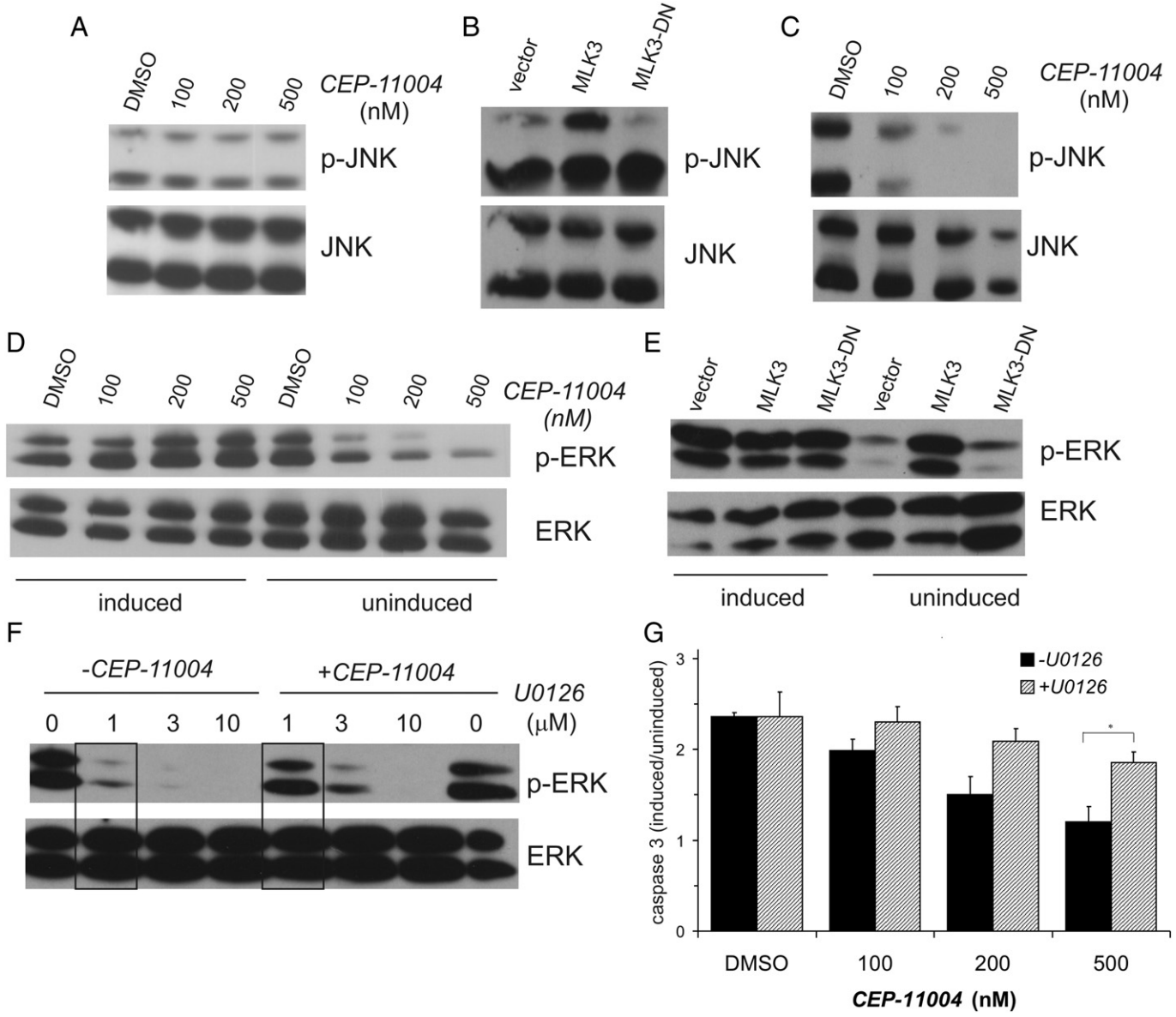

Fig. 3. CEP-11004 does not alter JNK activation in the Htt14A2.5 line but promotes sustained ERK activation. (A) Htt14A2.5 cells were induced with 5 μM PA and increasing concentrations of CEP-11004 were added for 3 days prior to harvesting. Immunoblot analysis was performed using a p-JNK specific antibody followed by stripping and reprobing with a total JNK antibody. Increasing concentrations of CEP-11004 do not decrease the levels of activated JNK. (B) Htt14A2.5 cells were induced with 5 μM PA for 1 day and the following day transiently transfected with wild-type MLK3 (e.g. overexpression of MLK3 results in a constitutively active phenotype), dominant negative (DN) MLK3, or vector only. Two days later cells were harvested and immunoblot analysis was performed as described in (A). (C) Immunoblot of induced Htt14A2.5 cells transiently transfected with MLK3 in the presence of increasing concentrations of CEP-11004, as indicated. Cells were harvested 2 days post-transfection and immunoblots were performed with p-JNK followed by total JNK antibodies as described in (A). (D) Htt14A2.5 cells were either uninduced or induced with 5 μM PA and treated with increasing concentrations of CEP-11004 for 3 days prior to harvesting. Immunoblot analysis was performed using a p-ERK antibody followed by stripping and reprobing with a total ERK antibody. In induced cells CEP-11004 appeared to activate ERK. (E) Htt14A2.5 cells were induced with 5 μM PA or uninduced for 1 day and the following day transiently transfected with MLK3, MLK3-DN, or vector only. Two days later cells were harvested and immunoblot analysis was performed as indicated. MLK3 overexpression activated ERK in the Htt14A2.5 line in the absence of PA induction and has no effect in the presence of PA. (F) Htt14A2.5 cells were induced for 3 days and treated with the increasing concentrations of the MEK1/2 inhibitor U0126 and 300 nM CEP-11004 for 2 h prior to harvesting. In the presence of CEP-11004, increased amounts of U0126 are required to inhibit ERK activation, suggesting that ERK activation is maintained in the presence of CEP-11004 (see boxed lanes). (G) Htt14A2.5 cells were induced with 5 μM PA or uninduced and simultaneously treated with increasing concentrations of CEP-11004 and 3 μM U0126 for 3 days prior to harvesting and caspase 3 assays were performed. Data are presented as the ratio of caspase 3 activity in induced versus uninduced cells under the indicated conditions and shown is the mean ± SD from triplicate samples of a representative experiment. The protective effect of CEP-11004 on mutant Htt-associated caspase 3 activation is partially diminished when ERK activation is inhibited. Experiments shown in (A–F) were performed at least 2 times and (G) was performed at least 3 times. ANOVA test for + U0126 $p = .0003$, and −U0126 $p = 0.08$. * $p < 0.05$.

assays, data not shown) (Apostol et al., 2006). Expression of the 548 amino acid segment with a normal repeat length human Htt (N548wt) in ST14A cells has previously been reported to exhibit protective effects and thus parental ST14A lines are used as controls. N548mu cells typically exhibit a >50% Htt-mediated decrease in cell viability after ~48 h following shift as measured by XTT, compared to a <30% decrease in the parental ST14A line. ST14A and N548mu lines cells were shifted to the nonpermissive conditions, simultaneously treated with increasing concentrations of CEP-1347 or CEP-11004, and subjected to XTT assays 48 h later. An Htt-specific, dose dependent increase in cell viability relative to the untreated control was observed when treated with CEP-1347 (Fig. 1E) or CEP-11004 (data not shown). For example, CEP-1347 at 500 nM increased cell viability of the N548mu line by ~40% whereas no significant change was observed in the parental ST14A line.

CEP-11004/CEP-1347 differentially regulates JNK activation in ST14A and PC12 HD models

To determine whether the rescue from neurotoxicity observed in the cell lines is due to reduced JNK activation, we examined p-JNK levels in lysates from treated and untreated ST14A and N548mu cells (Figs. 2A–B). As previously reported, when N548mu cells are shifted to nonpermissive conditions a dramatic increase in activated JNK is observed (Apostol et al., 2006). As anticipated for inhibition of an upstream activator of JNK, when cells were shifted to the nonpermissive conditions for ~24 h followed by treatment with increasing concentrations of CEP-11004 for 2 h, the levels of activated JNK decreased (a single representative experiment is shown in Figs. 2A–B). Longer treatments with the inhibitor resulted in more dramatic decreases of p-JNK, however total JNK levels were also reduced (data not shown).

Despite the ability of CEP-11004 to inhibit JNK in the N548mu line and to reduce toxicity in the inducible Htt14A2.5 cells, p-JNK levels were not affected by increasing concentrations of up to 500 nM in Htt14A2.5 cells (Fig. 3A) nor were components downstream of MLK altered (p-MKK4/SEK1 and p-c-*jun*; data not shown). This was surprising given our previous results showing that SP600125, a inhibitor of JNK activity, partially decreased Htt-associated toxicity in the Htt14A2.5 line, indicating that inhibiting JNK activation can be protective in these cells (Apostol et al., 2006).

Since JNK can be activated by other MAPKKKs in addition to MLKs, an MLK-independent mechanism may activate JNK in the Htt-expressing PC12 cell line Htt14A2.5. To determine if the MLK pathway is active in these cells, we tested whether exogenous MLKs can activate JNK and whether CEP-11004 treatment inhibits this JNK activation. Induced Htt14A2.5 cells were transiently transfected with MLK3 or a dominant negative MLK3 (MLK3-DN) and levels of p-JNK were examined by immunoblot analysis (Teramoto et al., 1996). As shown in Fig. 3B, overexpression of wild-type MLK3 can activate JNK in induced cells whereas overexpression of MLK3-DN prevents JNK activation, suggesting that the inherent ability of the cells to mount this response is intact. Furthermore, activation of JNK induced by exogenous MLK3 is suppressed in the presence of increasing concentrations of CEP-11004 (Fig. 3C). Similar results were observed in uninduced cells (data not shown). These results suggest that the effects of CEP-11004 on caspase 3 activation in mutant Htt-expressing cells that do not produce a robust JNK response, may be due to other survival-promoting activities of the compound rather than its inhibition of JNK, or that inhibition of MLK3 in this context elicits other protective effects.

Sustained ERK activation is stimulated by CEP-11004 treatment of Htt14A2.5 PC12 cells

CEP-11004 (and CEP-1347) can exert neuroprotective effects through pathways independent of JNK inhibition (Lund et al., 2005; Roux et al., 2002; Shen et al., 2003; Wang et al., 2005). MLKs have an established role in activating ERK (Chadee and Kyriakis, 2004; Handley et al., 2007) and inhibition of MLK activity by CEP-11004/CEP-1347 treatment in these systems would be expected to prevent ERK activation. In some cellular environments, however, such as mouse primary cortical neurons and PC12 cells, ERK activation can be induced by CEP-11004/CEP-1347 treatment (Roux et al., 2002; Shen et al., 2003). To determine if rescue of Htt14A2.5 toxicity by CEP-11004 may be associated with ERK activation versus inhibition, cells were induced and simultaneously treated with increasing concentrations of CEP-11004. Following treatment, ERK phosphorylation was not reduced and indeed appeared to increase slightly (Fig. 3D). However this effect was only observed in cells expressing mutant Htt. In the absence of mutant Htt (uninduced Htt14A2.5 cells), CEP-11004 inhibited ERK activation in a dose dependent manner (Fig. 3D), as would be predicted if ERK activation is related to MLK activity (Kim et al., 2004). When MLK3 is overexpressed in uninduced cells, p-ERK levels, which are normally low in these lines compared to induced lines (Apostol et al., 2006), are elevated, whereas this increase is not observed following overexpression of a dominant negative form of MLK3 (MLK3-DN) (Fig. 3E, uninduced, see model). In contrast, phosphorylation of ERK, which is already elevated in mutant Htt-expressing cells as a possible compensatory response to mutant Htt insult (Apostol et al., 2006), was not altered by overexpression of MLK3 or MLK3-DN (Fig. 3E, induced). These data suggest that, when challenged with mutant Htt, ERK activation occurs that is not potentiated by exogenous MLK3 and that CEP-11004 does not have an inhibitory effect on p-ERK activity in these cells as compared to uninduced cells.

Our data suggest that while CEP-11004 can inhibit MLKs when this pathway is dominant, it can also protect under certain cellular conditions either via a mechanism that is independent of both ERK and MLK or via a mechanism that leads to additional ERK activation. To test whether ERK signaling is necessary for these neuroprotective effects, we asked whether the MEK1/2 inhibitor, U0126, can block the protective action of CEP-11004. Cells were treated with CEP-11004 (300 nM) and limiting (less than that typically used for maximal inhibition) U0126 (1, 3 or 10 μM) for 2 h following 3 days of induction. Treatment of cells with U0126 in the absence of CEP-11004 reduced p-ERK in a dose-dependent manner, however higher concentrations of U0126 were required in the presence of CEP-11004 for the same magnitude of reduction (Fig. 3F; compare p-ERK levels at 1 μM U0126 in the presence and absence of CEP-11004, boxed), suggesting that CEP-11004 is maintaining or increasing ERK phosphorylation. To determine if ERK inhibition also decreased the protective effect of CEP-11004, cells were simultaneously induced and treated for 3 days with increasing concentrations of CEP-11004 with or without addition of U0126 (5 μM). In the presence of U0126, caspase 3 activity remained higher than with CEP-11004 alone, although it continued to show a dose-dependent decrease in activity as concentrations of CEP-11004 increased (Fig. 3G). These results indicate that the protective effect of CEP-11004 may involve sustained ERK activation.

Since ERK signaling can suppress toxicity in the Htt14A2.5 line and we previously showed it is protective in the N548mu line (Apostol et al., 2006), we tested the ST14A and N548mu lines to determine if CEP-11004 could also maintain ERK activation in these neuronal-like cells. CEP-11004 did not have a differential effect on p-ERK levels, but did suppress cell death when cells were grown under nonpermissive conditions; U0126 did not suppress the CEP-11004 mediated rescue (data not shown). These results indicate that the metabolic pathways that respond to stress can be regulated uniquely in different cell types and that they can change in response to specific cellular insults. In the case of the striatal cells, the shift to the nonpermissive conditions is an additional stress that results in massive cell death which is rescued in an ERK-independent manner by CEP-11004. In the case of the inducible PC12 cells, mutant Htt challenge alone without additional stressors changes the cellular response to CEP-11004 such that challenged cells maintain activated ERK while unchallenged cells do not.

CEP-1100/CEP-1347 decreases mutant Htt toxicity in full length Htt expressing cells and in vivo in Drosophila

To gain further insight into the potential therapeutic application of these compounds, we tested the ability of both CEP-11004 and CEP-1347 to rescue mutant Htt-related phenotypes in two additional HD models that recapitulate unique properties relevant to human disease. Shown in the following figures is treatment with CEP-1347, which is the analog used for *in vivo* experiments.

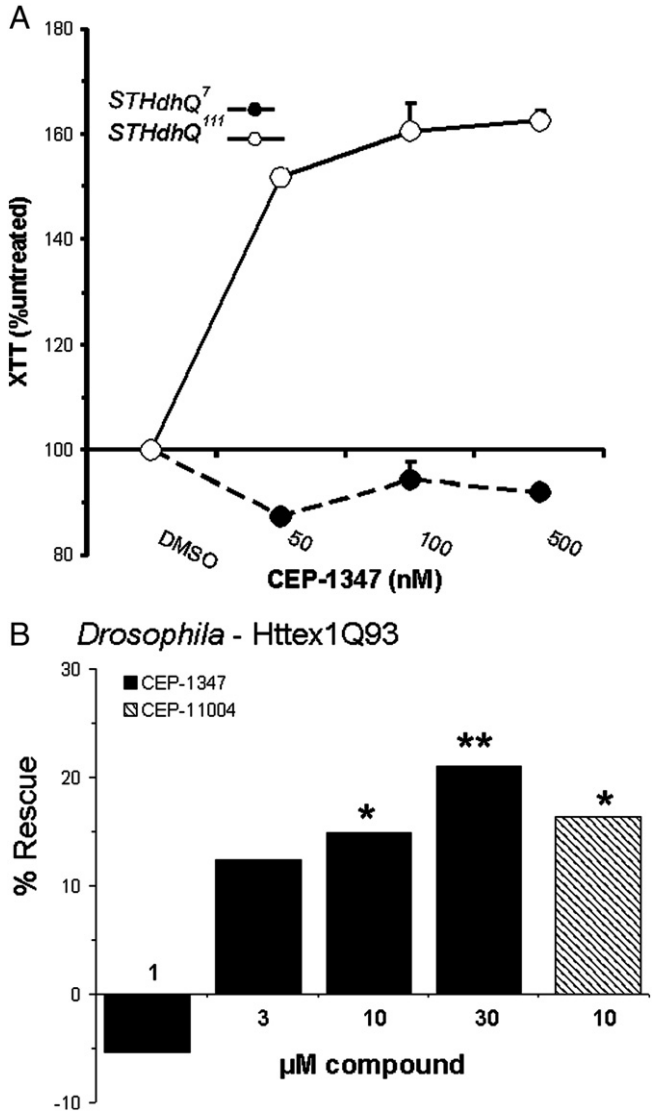

Fig. 4. CEP-11004/CEP-1347 rescue toxicity in $STHdh^{Q7}$ and $STHdh^{Q111}$ cell lines and photoreceptor neurodegeneration in *Drosophila*. (A) The day after $STHdh^{Q7}$ and $STHdh^{Q111}$ cells were plated, increasing concentrations of CEP-1347 were added to the plates and cells were simultaneously shifted to nonpermissive conditions as described in the Materials and Methods section. Two days later XTT assays were performed. Results are presented as the percent relative to untreated cells and each experiment was performed 3 times. ANOVA test $p<0.0001$. (B) Httexon1Q93 transgenic flies were fed the indicated concentrations of CEP-1347. As a readout for neuronal toxicity, the percent rescue of the average number of photoreceptors per ommatidia is shown at 7 days post-eclosion. Due to the inability to feed flies during development in the pupal case, the eyes have already begun to degenerate when flies emerge (day 0 values are <7), however the compounds appear to significantly reduce further progression. Day 0 refers to the average number of photoreceptor neurons per ommatidia on the first day after eclosion. (0) indicates flies fed DMSO without compound. Experiments were also performed with CEP-11004 with similar results (data not shown). Data is plotted as percent rescue and values were calculated by dividing two averages from unpaired data sets. * p value = 0.01 for the 10 µM of CEP-1347 and CEP-11004 and ** p value = 0.002 for 30 uM of CEP-1347.

Striatal $STHdh^{Q7}$ and $STHdh^{Q111}$ cell lines, which are derived from either wild-type ($HdhQ^7$) or homozygous mutant knock-in mice ($HdhQ^{111}$), express full length Htt from its normal chromosomal location (Trettel et al., 2000). Similar to the ST14A cells, these cell lines are striatal neuronal progenitor cells which contain a temperature sensitive Large T Antigen to immortalize cells and exhibit dramatic cell death when shifted to nonpermissive conditions. In a typical experiment, we observe a >50% decrease in cell viability as measured by XTT for the $STHdh^{Q111}$ line compared to a <10% decrease for the $STHdh^{Q7}$ line after shifting to the nonpermissive conditions for 24 h. However, when cells are simultaneously shifted and treated with increasing concentrations of CEP-1347, a robust increase in cell viability is observed (i.e. ~60% increase at 100 nM, Fig. 4A), whereas $STHdh^{Q7}$ lines are unaffected.

We next tested whether CEP-11004 and CEP-1347 are neuroprotective *in vivo* by feeding the compounds in a *Drosophila* model expressing a truncated mutant Htt protein using a photoreceptor neuron degeneration assay as a readout for neurodegeneration. The pathways altered by these compounds are conserved in *Drosophila*; single JNK (i.e. dJNK/*basket-1*) and MLK orthologs (i.e. dMLK/*slipper*) have been reported (Sathyanarayana et al., 2003; Sluss et al., 1996), and a single ERK (i.e. dERK/*rolled*) gene is conserved (Biggs et al., 1994; Kussick and Cooper, 1992; Takahashi et al., 1996). Transgenic flies expressing Httexon1Q93 in all neurons from embryogenesis on, using a neuron-specific *elav* promoter to drive expression, show a progressive decrease in the number of visible photoreceptor neurons beginning at day 1 post-eclosion (emergence from pupal case as adult flies) (Marsh and Thompson, 2006); a robust degenerative phenotype is observed at day 7. Flies were fed CEP-11004 or CEP-1347 and the number of visible photoreceptor neurons was counted at day 7 post-eclosion and compared to vehicle treated control flies at the same time point. Both compounds show a dose-dependent suppression of photoreceptor neurodegeneration up to a maximal rescue at 30 µM (Fig. 4B, CEP-1347 results shown). Data is plotted as percent rescue as absolute numbers of photoreceptor neurons vary between experiments. It is unclear if the mechanism of rescue by these compounds involves inhibition of JNK via MLKs and/or activation of ERK, however, consistent with a protective role for modulation of MAPK signaling in cells, the JNK inhibitor SP600125 (J. Pallos and M. Roarke, unpublished results) and constitutively active MEK are both protective (L. Bodai, unpublished results).

CEP-1347 reduces the decline in motor performance in R6/2 mice

Previous studies indicate that CEP-11004/CEP-1347 exert CNS effects, including neuroprotection, in rats and/or mice *in vivo* (Ciallella et al., 2005; Muller et al., 2006; Saporito et al., 1999, 1998). We therefore examined the ability of CEP-1347 to reduce mutant Htt-associated phenotypes in a mouse model of the disease. The R6/2 transgenic mouse model expresses human mutant exon 1 of huntingtin with a highly expanded repeat from the mouse Htt promoter and is often used as a first test of a therapeutic compound *in vivo* due to the high reproducibility of phenotypes and relatively rapid progression (Hockly et al., 2003). In addition, gene expression profiling suggests that this model shares similar transcriptional signatures as human HD brain (Hodges et al., 2006). In the mice tested for these studies, neurological defects are observed by 10 weeks of age with death at about 15 weeks. To perform a pilot preclinical trial over a defined time period beginning when symptoms are present and progressive, CEP-1347 (0.5 mg/kg, s.c.) or vehicle was administered to genotyped R6/2 mice and wild-type littermate controls once daily for 4 weeks starting at 6 weeks of age. This specific dose of CEP-1347 was chosen because it is in the range of doses that were previously shown to attenuate neurodegeneration in rodents (Muller et al., 2006; Saporito et al., 1999, 1998). The ability of the drug to reduce the decline in motor performance reported for R6/2 mice verses wild-type littermates was measured by performing weekly rotarod and clasping behavior tests during treatment as described (Hockly et al., 2003).

As expected, wild type and R6/2 groups differed in their motor ability over the four weeks of testing (ANOVA $p=0.002$, $F=5.7$, for set speed tests; $p<0.001$, $F=15.9$, for variable speed). Regardless of treatment, motor performance was impaired in R6/2 mice compared to wild-type at each testing week on both set and variable rotarod speeds. Latency to fall from the rotarod at the set speed was 110±15.8 s (mean±SEM of all 4 weeks) for wild-type and 50±8.7 for R6/2 mice ($p=0.0003$, Bonferroni/Dunn test); for the variable speed it was 264±17.6 s and 123±17.6, respectively ($p<0.0001$, Bonferroni/Dunn test) (Fig. 5A).

CEP-1347 delayed the decline in motor performance seen in R6/2 mice. As shown in Fig. 5A, by the third and fouth week of treatment, CEP-1347-treated R6/2 mice stayed on the rotarod at the variable speed 1.5 and 1.8 times longer, respectively, than those given vehicle

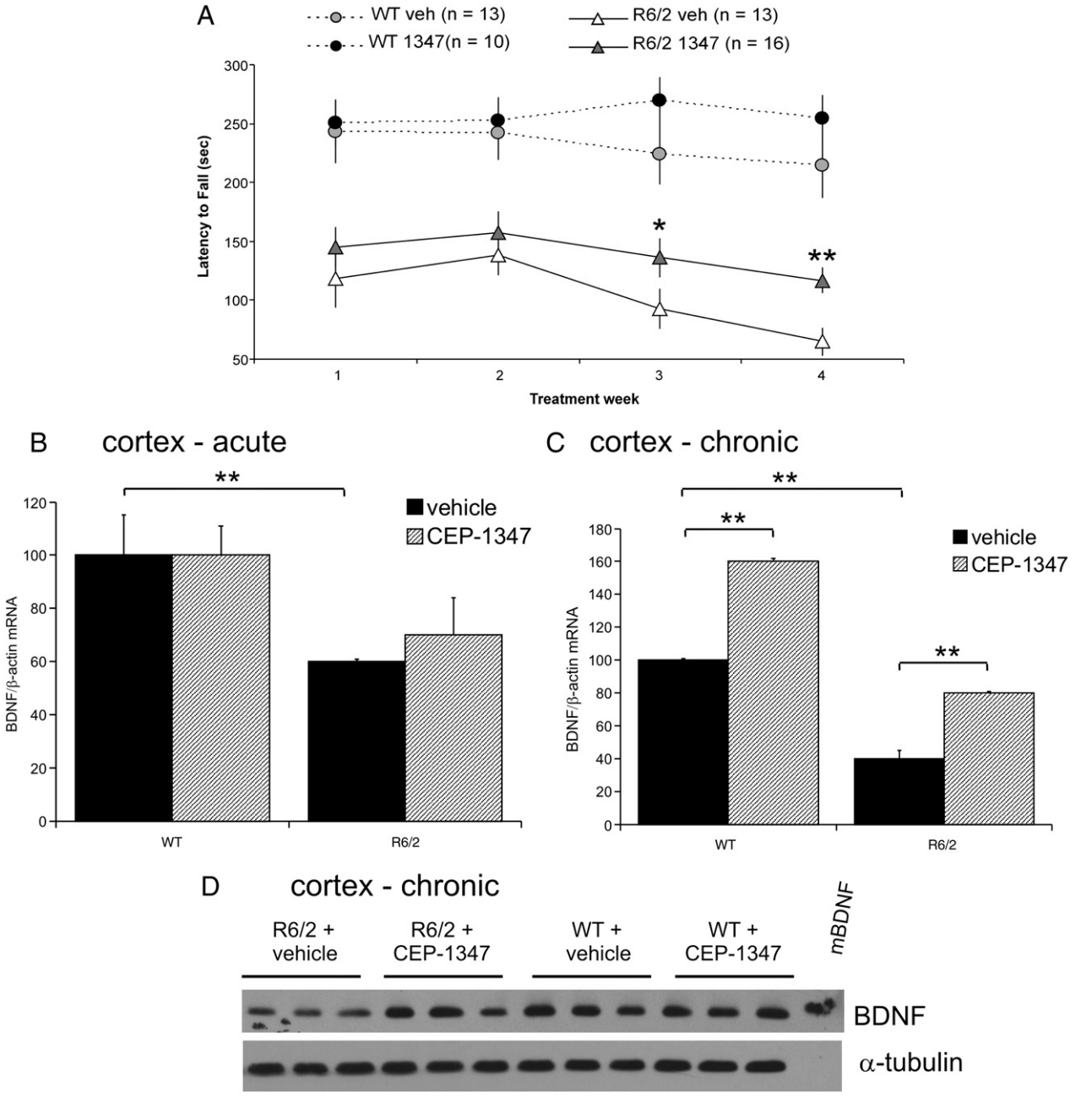

Fig. 5. CEP-1347 prevents the decline of motor performance in R6/2 mice in a pilot preclinical test and increases cortical BDNF levels. (A) R6/2 and wild-type (WT) littermates were given CEP-1347 (0.5 mg/kg in vehicle; 10 ml/kg s.c.) once daily beginning at 6 weeks of age and continued daily for 4 weeks. At each testing week, the latency (s) to fall from the rotarod at a variable speed (4–40 rpm) is significantly shorter for R6/2 mice than WT administered CEP-1347 or vehicle (repeated measures ANOVA: $p<0.001$, $F=15.9$). R6/2 mice treated with CEP-1347 remained on the rotarod longer than those given vehicle on the third and fourth weeks of testing. Data are expressed means±SEM. * $p<0.05$, ** $p=0.001$ compared to vehicle group. (B) Six week old R6/2 and WT mice were given one injection of CEP-1347 (1.0 mg/kg in vehicle; 10 ml/kg s.c.) 4 h prior to sacrificing (acute treatment) and cortical regions were isolated. Cortical BDNF mRNA levels were determined by quantitative Real Time PCR in R6/2 and age-matched littermates (WT control). (B) For chronic treatment mice were given CEP-1347 (0.5 mg/kg in vehicle; 10 ml/kg s.c.) once daily beginning at 6 weeks of age and continued daily for 4 weeks followed by determination of cortical BDNF mRNA levels. Data in (B) and (C) are presented as the average of 6 independent Real Time-PCR analyses and are expressed as percentage of control (100% was assigned to vehicle-treated control mice). (A) ** $p<0.01$ versus WT mice treated with vehicle, ANOVA test. (B) ** $p<0.01$ versus WT and R6/2 mice treated with vehicle, ANOVA test. (D) R6/2 and WT mice were given CEP-1347 as described in (A). Protein lysates (50 μg of total protein) from the cortex were resolved on 15% SDS-PAGE and immunoblot analysis was performed using BDNF antibody followed by stripping and reprobing with α-tubulin antibodies to confirm equal loading. mBDNF (~14-kDa) in the last lane is purified mature BDNF (3 ng) used as a marker.

($p<0.05$ and $p=0.001$ for weeks 3 and 4, respectively). This improvement is significant in that this model progresses rapidly and it is relatively difficult to overcome deficits. The latency to fall from the rotarod at the set speed was longer for R6/2 mice given CEP-1347 than those given vehicle during the fourth treatment week, but this difference was not significant (53.9±14.5 and 34.6±11.8 s, respectively, $p=0.16$). CEP-1347 did not alter motor performance of wild-type mice (means for all 4 weeks: for set speed, vehicle: 98±0.6 vs. CEP-1347: 127±1.3 s, $p=0.2$; for variable speed, vehicle: 231±1.0 vs. CEP-1347: 257±0.1 s, $p=0.4$), although by the last two weeks of administration CEP-1347-treated wild-types tended to remain on the rotarod longer than those given vehicle (not significant).

R6/2 mice exhibit a characteristic limb clasping phenotype that is exacerbated with age (Mangiarini et al., 1996). On week 4 of testing, the latency to clasp was prolonged by about 60% in R6/2 mice treated with CEP-1347 compared to those left untreated (20.3±4.6 and 32.0±4.8 s, respectively, $p=0.04$, Student's t-test, data not shown). R6/2 mice weighed less than wild-type controls by the third and fourth week of treatment, however this difference was not significant and CEP-1347 did not affect the body weights of mice of either genotype. In all, CEP-1347 prevented the decline in performance on two motor tasks in R6/2 mice in this pilot preclinical trial.

CEP-1347 restores cortical and serum BDNF levels in R6/2 mice

Since CEP-1347 treatment was protective in mice and in the above cell studies appeared to impact MAPK signaling, we investigated JNK and ERK activation in mouse tissues isolated from CEP-1347-treated and vehicle-treated mice, focusing on areas most strongly dysfunctional in HD (Bates et al., 2002). p-JNK and total JNK levels were assessed in tissue lysates from striatum and cortex of wild-type and R6/2 mice tested for motor performance with or without chronic CEP-1347 treatment. Neither brain area showed significant decreases in p-JNK relative to total JNK following CEP-1347 treatment (data not shown). To capture possible transient signaling changes, we acutely treated wild-type and R6/2 mice with CEP-1347 and harvested brains 2 or 4 h later. Similar results were obtained and no alterations in JNK activation in the presence of CEP-1347 were observed. Likewise, using immunoblot analysis and quantitation of the ratio of p-ERK relative to total ERK in individual mice, it was difficult to detect a consistent change in ERK phosphorylation in the presence of CEP-1347, although a trend toward increased phosphorylation was noted (data not shown). Since activation of signaling pathways are often transient at the level of phosphorylation cascades, and since variability was observed between samples, we next monitored the levels of a potential downstream target of ERK, namely BDNF, that is strongly implicated in HD pathogenesis.

BDNF is a critical neurotrophic factor expressed in the cortex and its expression is reduced in the cortex of HD rodent models, including R6/2 mice, and in human HD patients (Ferrer et al., 2000; Zuccato and Cattaneo, 2007; Zuccato et al., 2001). Forebrain-specific BDNF knockout mice exhibit a striatal gene expression profile that is more similar to human HD than any other HD mouse model examined, validating the importance of the corticostriatal pathway in the disease (Kuhn et al., 2007; Strand et al., 2007). In addition, increasing BDNF levels, by injecting adenoviral BDNF and Noggin into striatum of R6/2 mice, significantly decreased HD phenotypes (Cho et al., 2007). Finally, of direct relevance to the present study, activation of the ERK/MAPK pathway in primary rat cortical neurons orchestrates the expression of endogenous BDNF (Yasuda et al., 2007). Therefore, BDNF expression at both the protein and mRNA level was examined in cortical tissues from CEP-1347- and vehicle-treated mice.

BDNF mRNA levels were first examined using quantitative RT-PCR of RNA from cortex of 6 week old wild-type and R6/2 mice administered a single injection of 1.0 mg/kg CEP-1347 or vehicle and sacrificed 4 h later (acute treatment). As previously reported (Zuccato and Cattaneo, 2007; Zuccato et al., 2005), total BDNF mRNA levels were significantly reduced in cortex of early symptomatic R6/2 mice given vehicle, with respect to age-matched wild-types ($p<0.01$, ANOVA test) (Figs. 5B and C). However, R6/2 mice that received CEP-1347 acute treatment did not show a significant increase in cortical BDNF mRNA levels compared to those given vehicle; similar negative results were seen in wild-types (Fig. 5B).

To test the potential effects of longer term treatment with CEP-1347, the R6/2 and wild-type mice tested for motor performance (as described above) that received daily treatments of vehicle or CEP-1347 for 4 weeks beginning at 6 weeks old were evaluated for cortical BDNF mRNA levels. Quantitative RT-PCR experiments indicated that BDNF mRNA was reduced in 10 week old vehicle-treated R6/2 mice compared to wild-types ($p<0.01$, ANOVA test), further confirming the presence of reduced BDNF mRNA level in this mouse model (Fig. 5C). CEP-1347 treatment significantly increased BDNF mRNA levels in wild-type mice compared to those treated with vehicle ($p<0.01$, ANOVA test) (Fig. 5C), suggesting that treatment with this compound can enhance BDNF gene transcription in a wild-type background. More importantly, chronic treatment with CEP-1347 increased BDNF mRNA in R6/2 mice to levels observed in vehicle-treated wild-types ($p<0.01$, ANOVA test) (Fig. 5C).

mRNA results were confirmed at the protein level in R6/2 mice given chronic CEP-1347 treatment. A representative Western blot (Fig. 5D) shows decreased levels of the mature form of BDNF (~14-kDa) in cortex of vehicle-treated R6/2 compared to wild-type mice [ratio of BDNF/α-tubulin: 1.02±0.05 and 2.27±0.31 for vehicle-treated R6/2 and wild-type, respectively; $n=3$/group]. CEP-1347 increased mature BDNF in R6/2 but not wild-type mice [BDNF/α-tubulin ratio: 1.95±0.28 and 2.21±0.22 for CEP-1347 treated R6/2 and wild-type mice, respectively; $n=3$/group]. Remarkably, blood levels of BDNF mirrored the above results (Conforti et al., accompanying manuscript). In vehicle-treated R6/2 mice, BDNF mRNA levels were decreased compared to age-matched wild-type littermates. Both acute and chronic CEP-1347 treatment increased blood levels of BDNF in R6/2 mice compared to those given vehicle. These data indicate that long-term treatment with CEP-1347 restores deficits in cortical BDNF mRNA and protein levels associated with mutant Htt.

Discussion

The results presented here suggest a novel therapeutic approach for a currently untreatable neurodegenerative disease, HD, via the previously unreported mechanism of increasing *in vivo* BDNF levels using pharmacologic MAPK modulation. CEP-1347 and CEP-11004, MLK inhibitors with neurotrophic properties, inhibit neurotoxicity upon expression of mutant Htt in each model system tested including cells, *Drosophila* and R6/2 mice. The mechanism of rescue in cell lines is accompanied by modulation of MAPK signaling. In general, we find that if cell death is rapid and associated with JNK activation, then CEP-1347 inhibition of MLK and downstream JNK signaling predominates and appears to be associated with rescue from neurotoxicity. Keeping with other known functions of these compounds in cell models and in the presence of mutant Htt, CEP-1347 is associated with sustained ERK activation. Finally, increased levels of the key neurotrophin, BDNF, which is known to progressively decrease in HD mouse and human brain, and whose restored expression is associated with improved symptoms in mouse models of HD, correlates with the protective effect of CEP-1347 on motor function in a limited treatment regimen of symptomatic mice with CEP-1347. Since feasibility of direct delivery of BDNF is problematic (Levy et al., 2005), and other methods of increasing BDNF levels may be toxic or less bioavailable in humans, a compound shown previously to be safe and well tolerated in humans that can restore BNDF expression to cortical neurons is highly significant. This restorative effect on BDNF is also observed using a non-invasive approach of measuring BDNF levels in blood (Conforti et al.,

accompanying manuscript) from CEP-1347 treated mice. We conclude the CEP-1347 may be a useful option for further testing as a therapeutic intervention in HD.

CEP-11004/CEP-1347 can inhibit JNK activation

There is extensive evidence that CEP-1347 and CEP-11004 can prevent JNK activation through inhibition of the upstream activators of JNK, namely the MLKs ((Morrison and Davis, 2003), Fig. 6). MLKs are therapeutically attractive targets as a greater degree of selectivity may be allowed by targeting upstream kinases that often have tissue-restricted expression in these signaling cascades as opposed to targeting more general pathways through inhibition of terminal kinases. Several indolocarbazole analogs have been developed that inhibit JNK signaling via MLK inhibition and thereby prevent cell death (Morrison and Davis, 2003; Murakata et al., 2002; Wang et al., 2004), including CEP-11004 and CEP-1347.

JNK is activated in HD and other neurodegenerative disorders (Garcia et al., 2004; Harris et al., 2002) and inhibition of this pathway has protective effects (Garcia et al., 2004). Immortalized hippocampal cell lines expressing full length mutant Htt protein show JNK activation while inhibition with a dominant negative MKK4/SEK1 mutant blocks Htt-mediated apoptosis (Liu, 1998; Liu et al., 2000). Similar results were found using primary striatal neurons; overexpression of a dominant negative form of c-*jun* or pharmacological inhibition with SP600125 abolished cell death (Garcia et al., 2004), suggesting a role for JNK activation in HD pathogenesis. The upstream kinase, MLK2, can also be activated in response to mutant Htt expression (Liu et al., 2000). Further, Htt-mediated neurotoxicity is reduced with SP600125 in both PC12 cells (Apostol et al., 2006) and in *Drosophila* (J. Pallos and M. Roarke, unpublished results). This activity, which is associated with overt cell death, may reflect later stages of HD, where neuronal cell loss is observed.

CEP-11004/CEP-1347 can upregulate neurotrophic pathways

While MLKs have long been known to function upstream of JNKs (Gallo and Johnson, 2002; Xu et al., 2001), more recent studies indicate that MLKs may in fact have a more general upstream role (Handley et al., 2007). For instance, MLKs can activate Raf, and thus MEK and ERK, in some settings (Chadee and Kyriakis, 2004) (Fig. 6). Under this scenario, inhibition of MLK should suppress both JNK and ERK activation, as is observed in PC12 cells that do not express mutant Htt or in cells overexpressing MLK3. While CEP-11004/CEP-1347 suppressed mutant Htt-mediated phenotypes in every HD model tested, in cell models where cell death is less overt, CEP-11004/CEP-1347 appear to be protective by a JNK-independent mechanism. In these settings, the compound appears to rescue via a mechanism that involves sustained ERK phosphorylation (Fig. 6). This effect was reduced by inhibition of MEK with U0126, suggesting a requirement for ERK in protection by CEP-11004, similar that that observed for CEP-1347 in primary cortical neurons and PC12 cells in other studies (Roux et al., 2002).

The role of ERK in neurodegenerative diseases is complex as both protective and deleterious roles have been described in neurons (Cheung and Slack, 2004; Chu et al., 2004; Colucci-D'Amato et al., 2003). While ERK is classically associated with mediating the neuroprotective activities of neurotrophins such as NGF (Marek et al., 2004) and has an important role in integrated CNS functions that require neuronal plasticity (Colucci-D'Amato et al., 2003; Hetman and Gozdz, 2004), other studies find that MEK inhibition can exert a protective effect against chronic ERK activation in cells treated with glutamate or the neurotoxin 6-OHDA (Chu et al., 2004; Colucci-D'Amato et al., 2003). Additional evidence for a protective role for specific functions of ERK in neurodegeneration include the reduction of striatal cell death upon overexpression of mitogen- and stress-activated protein kinase-1, a downstream target of ERK that is depleted in R6/2 mouse brain and is required for proper histone H3 phosphorylation and transcriptional control (Roze et al., 2007), and the requirement for MEK activity in the neurotrophic effect of progesterone upon glutamate-induced toxicity in cerebral cortex slice cultures (Kaur et al., 2007). This effect on histone H3 phosphorylation was shown to involve MLK3 in certain cell settings (Cha et al., 2006), again highlighting the interrelationship of ERK and MLKs. The results presented here are consistent with a protective effect for ERK, possibly reflecting neurotrophic effects upon early stages of HD neuronal dysfunction.

Further evidence for neurotrophic effects of CEP-11004/CEP-1347 that may specifically modulate HD phentoypes is the increased

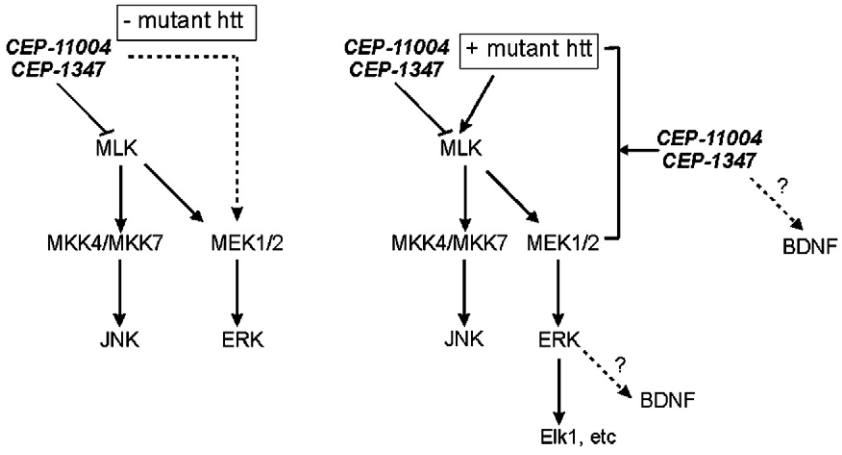

Fig. 6. Model for the protective effects of CEP-1347 and CEP-11004 on mutant Htt-mediated neurotoxicity. In cells not expressing mutant Htt, ERK activation is mediated through upstream activation of signaling pathways leading to activation of MEK1/2 (MAPKK) which in turn activates ERK1/2 (MAPK) (left panel). Phosphorylated ERKs either translocate to the nucleus directly or activate cytosolic proteins which then translocate to the nucleus where they can activate transcription factors such as Elk and CREB, and regulate expression of groups of genes having multiple outcomes in terms of cell survival. For the JNK pathway, MAPKKK (such as MLKs), activate MKK4/SEK1 and/or MKK7 (MAPKK), which in turn activate JNKs (MAPK). MLKs can also activate ERKs via MEK1/2 activation. CEP-11004 and CEP-1347 can decrease p-JNK levels by inhibition of MLK activation, as well as decrease p-ERK levels via inhibition of MEK1/2 activation. However, CEP-1347 can also activate ERK through an as yet undefined mechanism in some cell settings (28). Phosphorylated JNKs are translocated to the nucleus and activate transcription factors such as c-*jun* to transcribe genes which tend to be associated with cell death. In our PC12 cells not expressing exogenous mutant Httex1p, CEP-1347 or CEP-11004 treatment represses ERK activation. In the presence of mutant Htt (right panel), ERK is activated, likely as a compensatory response, which activates as yet uncharacterized pathways. In this scenario CEP-1347 and CEP-11004 can also inhibit JNK via inhibition of MLKs and can also promote a sustained ERK activation that appears to be protective in nature. Increased BDNF observed in CEP-1347 and CEP-11004 is proposed to be one consequence of this ERK activation and from modulation of MAPK signaling in general, however it may also be derived from some other yet as unidentified mechanism.

expression of total cortical BDNF mRNA compared to vehicle treated littermate controls, correlating with reduction of disease progression in a pilot preclinical trial in R6/2 mice. BDNF gene transcription is induced by wild-type, but not mutant, Htt, (Zuccato et al., 2007; Zuccato et al., 2001), and BDNF mRNA and protein levels are severely decreased in the cortices of numerous HD murine models ((Zuccato and Cattaneo, 2007) review). The significance of the latter finding is substantiated by the marked reduction in BDNF mRNA and protein in postmortem human HD cortex (Ferrer et al., 2000; Zuccato and Cattaneo, 2007) and reduced BDNF transport from cortex to striatum has been proposed (Gauthier et al., 2004). In a parallel study, Conforti et al. (accompanying manuscript), observed this same up-regulation of BDNF mRNA in blood samples from the same CEP-1347 treated mice, highlighting a novel outcome measure for BDNF levels in peripheral tissues. The effect upon BDNF is critical, given the recent finding that gene expression profiling of multiple HD mouse models and comparison to human HD brain, suggests that BDNF depletion plays a major role in striatal degeneration (Strand et al., 2007).

A possible explanation of how these compounds might modulate ERK activation and increase the expression and activity of neurotrophic factors comes from studies showing that the TrkA (NGF) and TrkB (BDNF) receptors are upregulated in the presence of CEP-1347 in nerve growth factor (NGF)-deprived sympathetic neurons and rat cerebellar granule cells, respectively (Wang et al., 2005). In PC12 cells, which express the TrkA receptor, preliminary data suggests that CEP-11004 treatment causes an increase in receptor levels and that some ligand independent activation is observed as well, which could contribute to an increase in activated ERK (unpublished results). These results are consistent with both the trend for increased ERK and increased BDNF in modulating neurotoxicity in R6/2 mice with CEP-1347 treatment. These underlying mechanisms, including whether reduced TrkB receptor levels observed in HD mouse and human brain (Gines et al., 2006) are restored, will be investigated in future studies. Studies are also in progress to determine the mechanism(s) through which mutant Htt can increase ERK activity as a putative compensatory response, how CEP-1347 treatment may increase activation of protective signaling pathways in HD neurons and the relationship of MLKs to HD pathology.

Therapeutic potential of CEP-1347

JNK and MLK inhibitors are in active development for treatment of neurodegeneration (see (Bogoyevitch et al., 2004; Wang et al., 2004) for reviews) as are compounds that increase neurotrophic responses. Our studies suggest that compounds such as CEP-1347 can productively affect these pathways. Intriguingly, in PD cytoplasmic hybrid cells (cybrids) made from mitochondrial DNA from idiopathic PD subjects, H_2O_2-induced cell death is protected by BDNF. When ERK pathways are blocked, the protection by BDNF is blocked; whereas if JNK pathways are inhibited, this protection by BDNF is enhanced (Onyango et al., 2005). These findings, together with the results presented here, ultimately suggest that CEP-1347 may act synergistically to inhibit JNK and activate protective MAPK signaling pathways to increase BDNF levels, a therapeutically sound strategy for HD.

CEP-1347 was found to be safe and well tolerated in a randomized, blinded, placebo-controlled study in PD patients (Group, 2004), although ultimately was not found to be effective as a disease modifying agent for early PD (Investigators, 2007). In this case the compound was tested originally for efficacy in toxin (MPTP) treatment models, which have limitations as a model for PD (Waldmeier et al., 2006) as opposed to genetic models of PD (Silva et al., 2005). In contrast, human PD patients in the clinical trials will elicit disease caused by a range of mutations or environmental effects that may or may not each be modulated in the same way. Therefore, it remains an open question whether CEP-1347 will modulate effects from expression of mutant α-synuclein or other genetic causes of PD in cell based or rodent models of disease. In contrast, an advantage in testing HD is that all patients enrolled in clinical trials exhibit disease based upon the same mutation, a dominantly acting expanded polyglutamine repeat. When a variety of HD mouse models, including genetic models expressing the repeat expansion and 3NP mitochondrial toxin treated mice, were tested by gene expression profiling and compared to human HD brain, the findings suggested that striatal lesions caused by mitochondrial toxins may arise via pathways that are different from those that drive neurodegeneration in HD (Strand et al., 2007). Taken together, these findings suggest that while CEP-1347 did not perform well in mixed populations of early PD patients, testing of this compound in genetically inherited HD could prove efficacious. In the pilot mouse preclinical trial, the efficacy of the CEP-1347 compound in restoring motor performance in the R6/2 mouse is modest. However this study was performed over a relatively short period of time and at a single dose and it is anticipated that the effect will be improved as these variables are rigorously tested in subsequent full-scale studies and other mouse models with milder phenotypes are tested.

There is precedence for modulation of BDNF as a desirable outcome for compounds that may be effective in HD. Several compounds that reduce HD phenotypes have been shown to increase BDNF levels including cystamine and cysteamine (Borrell-Pages et al., 2006) and PN401 (Saydoff et al., 2006). More recently, compounds increasing BDNF level have been isolated via a cell based drug-screening assay that uses an element of the BDNF promoter (Rigamonti et al., 2007). Finally, direct administration of BDNF restores deficits caused by mutant Htt including rescue of long-term potentiation in HD knock-in mice (Lynch et al., 2007) and reduced frequency of spontaneous GABAergic synaptic currents in MSNs from R6/2 mice (Cepeda et al., 2004). While neurotrophic factors are rational candidates to treat neurodegenerative diseases such as HD, difficulties in protein delivery and pharmacokinetics in the CNS limit their application (Levy et al., 2005). Therefore, a safe and efficacious compound that increases BDNF levels and shows protection in a transgenic mouse model of HD would be highly desirable as a therapeutic agent. Further studies have been initiated to determine whether, in addition to BDNF deficits, CEP-1347 alleviates other HD-related pathologies (e.g. IBs, inflammation) in HD murine models, since CEP-1347 can inhibit microglial activation (Lund et al., 2005), also a hallmark of HD (Pavese et al., 2006).

In summary, our results provide evidence for modulation of MAPK signaling as a novel therapeutic option for HD treatment and point to the compound, CEP-1347, an inhibitor of MLKs, as a potentially effective therapeutic agent that rescues neurotoxicity in every HD system tested to date including cell lines, *Drosophila* and mouse HD models. This compound appears to rescue through dual mechanisms of inhibition of a pro-apoptotic JNK pathway and activation of neurotrophic pathways including up-regulation of the critical neurotrophin BDNF in mice. The precise underlying mechanisms of compound action await further study as do confirmation studies in other HD animal models.

Experimental and methods

Propagation of cell lines

Generation and propagation of the Htt14A2.5 line was performed as previously described (Apostol et al., 2003). Htt expression was induced with PA (2.5 or 5 μM) for the indicated times. The ST14A and N548mu and the wild-type STHdh^{Q7}/Hdh^{Q7} and homozygous mutant STHdh^{Q111}/Hdh^{Q111} cell lines were propagated essentially as previously described (Apostol et al., 2006; Cattaneo and Conti, 1998). Briefly, cells were plated in 6-well plates (1×10^5 cells/well) in complete media (DMEM/5% glucose, 10% FBS, 1% penicillin/streptomycin) at 33 °C. To inactivate the temperature sensitive Large T-Antigen, cells were rinsed

in PBS followed by shifting to low serum media [DMEM (phenol red free for XTT assays)/5% glucose, 0.5% FBS heat inactivated, 1% penicillin/streptomycin] at 39 °C. U0126 (Calbiochem) and CEP-11004 and CEP-1347 (supplied by Cephalon Inc, West Chester, Pennsylvania) were resuspended at 10 mM in DMSO. For longer time courses, media and other components were changed every 2 days.

Cell toxicity assays

For caspase 3 assays, cells were harvested from 6-well plates, washed with cold PBS and the pellets were frozen. Caspase 3 assays were performed in triplicate using EnZCheck Caspase 3 Assay Kit #1 (Molecular Probes) as described by the manufacturer and activities were corrected for variations in protein concentration. For XTT assays, ST14A and N548mu cell lines and ST*Hdh*^{Q7} and ST*Hdh*^{Q111} and cell lines were plated in 24 well plates (0.75×10^5 cells per well) in complete media. The following day cells were shifted to nonpermissive conditions (i.e. 39 °C and low serum media) for ~48 h for the ST14A and N548mu lines and 24 h for ST*Hdh*^{Q111} and ST*Hdh*^{Q7} lines followed by incubation for 4 h with XTT and phenazine methosulfate (0.2 mg/ml and 0.2 μg/ml respectively; Sigma) for 4 h and plates were read at 450 nM.

Antibodies and immunoblots

Cell lysates were made and immunoblots were performed as previously described (Apostol et al., 2003). Detection of hybridized HRP was performed using ECL (Pierce) and when indicated, quantitation of photographic films was performed using ImageJ 1.32j software from NIH. p-ERK (Thr^{202}/Tyr^{204}), p-JNK (Thr^{183}/Tyr^{185}), ERK, JNK antibodies (Cell Signaling Technology) were used at a 1:1000 dilution. BDNF antibody (Santa Cruz Biotechnology) was used at 1:2000. Anti-α-tubulin monoclonal antibody B-5-1-2 (Sigma) was used at 1:5000 dilution. Purified recombinant human brain derived BDNF (Chemicon) was used as a positive control for immunoblots.

Transient transfections and constructs

Htt14A2.5 cells were transfected in 6-well plates using Lipofectamine 2000 according to manufacturer's instructions (Invitrogen). Htt14A2.5 cells were induced with 5 μM PA two days after plating and transfected the following day using 1 μg DNA/well or 1.25 μl Lipofectamine 2000/well and cells were harvested 48 h after transfection. MLK3 and MLK3-DN constructs were kind gifts from Dr. Lloyd Greene at Columbia University (Xu et al., 2001).

Drosophila feeding and ommatidia analysis

UAS-Httexon1Q93 (line P463) flies were mated to elav>Gal4 at 25 °C and the freshly eclosed polyQ-expressing adults transferred to vials containing standard *Drosophila* medium supplemented with either CEP-11004, CEP-1347 or vehicle (DMSO). Flies were transferred to fresh food every day, and assayed for neurodegeneration immediately after eclosion (day0) and at day 7 post-eclosion, using the pseudopupil technique (Marsh et al., 2000; Steffan et al., 2001). At least 30 ommatidia in each of the 9–11 flies were scored and the average number of rhabdomeres per ommatidium calculated for each fly. The plotted value (relative rescue) was calculated as: 100*(exp−ctrl)/(day0−ctrl), where "exp", "ctrl" and "day0" are the average number of photoreceptor neurons per ommatidium at day 7 post-eclosion of drug-fed flies, vehicle (DMSO) fed flies and the freshly eclosed flies, respectively. Significance of the difference of each value from the no compound (DMSO) control was evaluated using a Wilcoxon rank-sum test.

Mice and CEP-1347 treatment

All animal procedures were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and with protocols approved by the Institutional Animal Care and Use Committee of the University of California at Irvine. This includes efforts to minimize animal suffering and numbers of mice used.

Breeding pairs of R6/2 mice, composed of an ovarian transplant hemizygote female and a B6CBAF1/J male both with background strain C57BL/6J, were purchased from Jackson Laboratories [Bar Harbor, ME; code name B6CBA-TgN (HDexon1)62]. They were bred and reared in the Department of Psychiatry and Human Behavior colony at the University of California, Irvine. Male R6/2 mice, which are transgenic for the 5′ end of the human HD gene carrying about 100–150 glutamine (CAG) repeats (for actual number see below), and their gender-matched WT littermates were used. All mice received rodent chow *ad libitum* and were maintained with a 12 h light/dark cycle (on 2 am, off 2 pm).

R6/2 and WT mice were divided into two groups/genotype ($n=10$–16 per group): (1) Controls that were administered vehicle [0.15% Cremophor E.L. (Sigma, St. Louis, MO) in 0.9% saline, 10 ml/kg s.c.] and (2) Experimentals given CEP-1347 (0.5 mg/kg in vehicle). Injections were given once daily (10 ml/kg, s.c.) starting when mice were 6 weeks of age and continuing for 4 weeks.

Mouse genotyping

Before weaning, genotypes were identified by PCR of tail-tip DNA, using standard procedures [as described previously (Simmons et al., 2007)]. Tail-tip DNA procured at the time of sacrifice was used to confirm genotypes via RT-PCR and CAG repeat numbers were measured with ABIGeneMapper 4.0 by Laragen, Inc. (Los Angeles, CA). R6/2 mice used in this study had an average of 104±5.4 (mean±SEM) CAG repeats which is reduced in number compared to the original line instituted at Jackson Laboratories since these mice were purchased before the line was restored during the winter of 2006/7.

Motor behavior and statistical analyses

Starting 2 days after the first injection, mice were tested for motor impairment twice a week using a Rotarod (Med Associates Inc., St. Albans, VT) during the dark phase of the light cycle. The first rotarod test session consisted of three 120 s trials at a set speed of 16 rpm; trials were averaged. The second test consisted of one trial performed at a variable speed of 4–40 rpm for 300 s. In both tests, latency to fall from the apparatus was recorded. Clasping behavior was also tested once a week; mice were suspended by the tail for 60 s and the latency for the hind paws to clasp was recorded. One R6/2 mouse that received vehicle died during the first week of testing and was not included in the analysis. Data is presented as mean±SEM. Statview software (SAS Institute, Inc., Cary, NC) was used for group comparisons using a repeated measures ANOVA and, when appropriate, a Bonferroni/Dunn post-hoc test or Student's *t*-test (one-tailed with unequal variance, unless noted); statistical significance was set at $p\leq0.05$.

Mouse brain dissection and tissue lysates

Mice were sacrificed via asphyxiation; brains were microdissected into striatum and parietal cortex and and blood was collected from the heart for BDNF analysis. All samples were quickly frozen in liquid nitrogen and stored at −80 °C. For protein lysates brain sections were placed in 300 μl of 50 mM Tris–HCl pH 8.0, 10% glycerol, 5 mM EDTA and 150 mM KCl supplemented with protease inhibitors (complete protease inhibitor, Roche) and phosphatase inhibitor cocktail I and II (Sigma) and disrupted using a dounce homogenizer followed by

sonicating for 2×30 s on ice (Zourlidou et al., 2007). Immunoblots were performed as described above for cell lysates.

RNA isolation and reverse transcription

Total RNA was isolated from mouse cortices with TRIZOL Reagent (Invitrogen). Genomic DNA was digested with DNA-free™ (Ambion-Applied Biosystems, Foster City, CA) at 37 °C for 15 min. Total RNA (1 μg) was reverse-transcribed to single-stranded cDNA using Superscript III RNaseH reverse transcriptase (Invitrogen, Carlsbad, CA) and random primers in a volume of 20 μl, according to manufacturer's instructions.

RT-PCR for BDNF mRNA level

Two independent reverse transcriptase reactions were set up for every RNA stock. RT-PCR analyses were done in triplicate to obtain replicates for statistical analyses. iCycler Thermal Cycler with Multicolor RT-PCR Detection System (Biorad, Hercules, CA) was used. All reactions were performed in a total volume of 25 μl containing 50 ng of cDNA, 50 mM KCl, 20 mM Tris–HCl, pH 8.4, 0.2 mM dNTPs, iTaq DNA polymerase, 25 U/ml, 3 mM $MgCl_2$, SYBR Green I, 10 nM fluorescein, and stabilizers (iQ™ SYBR Green Supermix-Biorad) and 0.2 μM of forward and reverse primers. Amplification cycles consisted of an initial denaturing cycle at 95 °C 3 min, followed by 45 cycles of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. Fluorescence was quantified during the 60 °C annealing step and product formation confirmed by a melting curve analysis (55 °C–94 °C). Amounts of target gene mRNA were normalized to a reference gene (β-actin) according to (Pfaffl, 2001). Primer sequences are the following:

- mBDNF S: 5′-TCGTTCCTTTCGAGTTAGCC-3′;
- mBDNF AS: 5′-TTGGTAAACGGCACAAAAC-3′;
- mβ-actin S: 5′-AGTGTGACGTTGACATCCGTA-3′;
- mβ-actin AS: 5′-GCCAGAGCAGTAATCTCCTTCT-3′.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mcn.2008.04.007.

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