Human Molecular Genetics, 2006, Vol. 15, No. 2 ***273–285***
doi:10.1093/hmg/ddi443
Advance Access published on December 5, 2005

Mutant huntingtin alters MAPK signaling pathways in PC12 and striatal cells: ERK1/2 protects against mutant huntingtin-associated toxicity

Barbara L. Apostol[1], Katalin Illes[1], Judit Pallos[2], Laszlo Bodai[2], Jun Wu[3], Andrew Strand[4], Erik S. Schweitzer[5], James M. Olson[4], Aleksey Kazantsev[6], J. Lawrence Marsh[2,†] and Leslie Michels Thompson[1,*,†]

[1]Department of Psychiatry and Human Behavior and [2]Developmental Biology Center and Department of Developmental and Cell Biology, University of California, Irvine, CA 92697, USA, [3]Department of Neurology, UCLA School of Medicine, Los Angeles, CA 90095, USA, [4]Division of Hematology/Oncology, Fred Hutchinson Cancer Research Center, University of Washington, Seattle, WA 98195, USA, [5]Department of Physiological Chemistry and Brain Research Institute, UCLA School of Medicine, Los Angeles, CA90095, USA and [6]Center for Aging and Neurodegeneration, Massachusetts General Hospital, Building 114-3300, 16th Street, Charlestown, MA 02129, USA

Received September 23, 2005; Revised and Accepted November 27, 2005

Huntington's disease (HD) is a devastating neurodegenerative disorder caused by an expanded polyglutamine (polyQ) tract within the huntingtin protein (Htt). Identifying the pathways that are altered in response to the mutant protein is crucial for understanding the cellular processes impacted by the disease as well as for the rational development of effective pharmacological interventions. Here, expression profiling of a cellular HD model identifies genes that implicate altered mitogen-activated protein kinase (MAPK) signaling. Targeted biochemical studies and pharmacological modulation of these MAPK pathways suggest that mutant Htt affects signaling at upstream points such that both ERK and JNK are activated. Modulation of the ERK pathway suggests that this pathway is associated with cell survival, whereas inhibition of JNK was found to effectively suppress pathogenesis. These studies suggest that pharmacological intervention in MAPK pathways, particularly at the level of ERK activation, may be an appropriate approach to HD therapy.

INTRODUCTION

Several late-onset neurodegenerative diseases, including Huntington's disease (HD), are caused by expansions of CAG repeats encoding polyglutamine (polyQ) tracts within the corresponding disease protein (1,2). The polyQ repeats elicit a complex pathogenic response in neuronal cells, the mechanisms of which are not fully understood (3–5). One common feature is the appearance of polyQ-containing visible aggregates in the affected regions of the brain, likely a result of abnormal protein folding and/or proteolytic cleavage of the Htt protein (6,7). Neurons and other cell types respond to external or internal stimuli, such as oxidative stress or abnormal protein folding and aggregation by activating cellular signal transduction networks, including the mitogen-activated protein kinases (MAPKs), to elicit pro-survival and pro-apoptotic responses (8). The MAPK superfamily comprises three major signaling pathways: the extracellular signal-regulated protein kinases (ERKs), the c-Jun N-terminal kinases or stress-activated protein kinases (JNKs/SAPKs) and the p38 family of kinases. Following a cascade of phosphorylation events that initially activate upstream effectors (e.g. MEKKs), the individual MAPKs are in turn phosphorylated and regulate transcriptional events either directly or by catalyzing phosphorylation of nuclear regulatory proteins (8–10).

MAPK signaling has been implicated in a number of neurodegenerative disorders, including HD. Early work using cell culture models showed that mutant Htt might cause activation of growth

*To whom correspondence should be addressed at: Gillespie 2121, University of California, Irvine, CA 92697-4260. Tel: +1 949 824 6756. Email: lmthompson@uci.edu
†These authors contributed equally to this work.

factor receptor signaling through direct interactions with signaling entities such as Grb2 and Ras-GAP (11). In contrast, other cell-based studies showed that expression of mutant Htt can lead to attenuated growth factor signaling (12,13), and a recent study in *Drosophila* showed that expression of mutant Htt can disrupt EGF receptor signaling (14).

Here we describe gene expression profiling of an inducible PC12 cell line expressing mutant Htt. Prominent among the genes whose expression is altered in response to mutant Htt are groups of genes that are known targets of the MAPKs: ERK1/2 (hereafter ERK), JNK and p38 that suggest induction of both neurotrophic and cell death pathways in mutant Htt-expressing cells. Consistent with the predicted MAPK activation, phosphorylation patterns of ERK and JNK are altered in cell models of HD. Inhibition of ERK activation confirms a role for this pathway in the observed gene expression changes, and the functional relevance of ERK and JNK activation is confirmed in targeted biochemical studies and through chemical modulation of these pathways. The functional relevance of these pathways has implications for novel therapeutic applications for HD.

RESULTS

Microarray analysis of PC12 cells expressing truncated mutant Htt suggests activation of MAPK pathways

To investigate processes that are relatively early pathogenic events as opposed to later death-related events, gene expression profiling was performed on inducible mutant Htt-expressing cells that become dysfunctional but continue to proliferate. A clonal rat pheochromocytoma (PC12) cell line, (Htt14A2.5), which expresses a truncated form of expanded repeat Htt exon 1 protein, fused at the C-terminus to enhanced green fluorescent protein (EGFP) (Httex1*tr*-103Q-EGFP) (Fig. 1A), was selected for robust inducible Htt expression and the production of visible aggregates (cytosolic, perinuclear and nuclear inclusions) (15). No obvious cell death or growth arrest phenotypes were observed using a variety of cell toxicity and proliferation assays in the presence and absence of exogenously applied stresses (15) (data not shown). However, when tested for caspase 3 activity, an indicator of cellular dysfunction (16), Htt14A2.5 cells induced with ponasterone (PA) show a progressive increase in activity up to 4 days following induction relative to control cells. In a representative experiment, caspase 3 activity increased 2.3-fold between induced and uninduced cells 4 days after induction (discussed subsequently, Fig. 3B). Similar assays were performed following nerve growth factor (NGF) treatment to induce PC12 cell differentiation, and no significant differences were noted in caspase 3 activity between differentiated and proliferating cells (data not shown). No increased caspase 3 activity was observed in the parental PC12ec receptor line in the presence of PA (data not shown), suggesting that this increase is specific to Htt expression as opposed to other non-Htt-related cellular events. Other studies have also reported that expression of mutant Htt causes increased caspase 3 activity (17,18). Thus, increased caspase 3 levels appear to be an early response to mutant Htt expression prior to overt cell death.

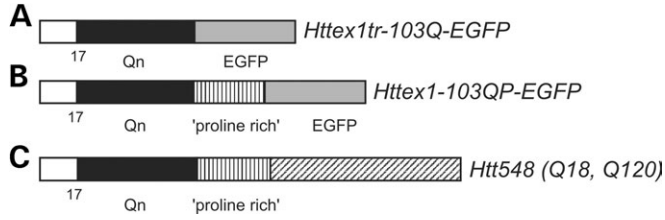

Figure 1. Htt constructs used in this study. (**A**) Httex1*tr*-103Q-EGFP encodes the first 17 amino acids of Htt with 103Qs fused to EGFP and was used to generate inducible Htt14A2.5 line (truncated exon 1). (**B**) Httex1-103QP-EGFP encodes the same sequence as in Httex1*tr*-103Q-EGFP, except that it includes the 'proline-rich' region of Htt (complete exon1) and was used to generate the Htt^{103} line. (**C**) Htt548 (Q18 and Q120) contains the first 548 amino acids of the Htt gene with either 18Qs (wild-type) or 120Qs (mutant) lengths of the polyglutamine stretch and these were used to generate the N548 wt and N548mu lines, respectively.

Gene expression analysis was performed on induced Htt14A2.5 cells relative to uninduced controls 48 h post-induction, a time point in which caspase 3 activity is increased, but prior to when cell density and other factors might complicate interpretation of results. As a control, expression profiling was performed on the parental receptor line, PC12ec, which expresses the ecdysone receptor alone. This control line showed differential expression of only one gene [increased: VGF (vacinia virus inducible growth factor), GenBank accession no. M74223], confirming that hormone induction alone does not significantly contribute to gene expression alterations observed upon mutant Htt expression. The complete data set is available through NCBI GEO Database accession no. GSE2602.

After probe pairs encoding identical genes were combined, 192 gene expression differences were noted (107 increased and 75 decreased) (Table 1). Genes from expressed sequence tags (ESTs) without a well-established biochemical function or which show only moderate or weak similarity to a known gene were not further evaluated (Supplementary Material, Table S1A and B), leaving 85 increased and 53 decreased mRNAs for further evaluation. Of these altered genes, only 20% (17/85) of the mRNAs that increased exhibited a $\geq$3-fold change, whereas the majority of the changes were relatively small. This was also true for the mRNAs that decreased, where only $\sim$3.8% (2/53) exhibited a $\leq$3-fold change. Altered genes were sorted into broad functional groups to simplify discussion and several patterns emerged. One group of genes whose expression increased encodes proteins classically associated with stress responses (Table 1). Included in this class are several molecular chaperones: *Hsp70*, *Hsp27* and *BiP/GRP78* that are involved in facilitating proper protein-folding in the cytosol and endoplasmic reticulum. Also included are mRNAs that encode proteins involved in the reduction of reactive oxygen species: glutathione *S*-transferase (GST, multiple subunits), thiol-specific antioxidant (peroxiredoxin) and cathespin L. A second class of genes includes those associated with apoptosis, particularly p53-regulated genes, including *GADD34*, *GADD45* and $p21^{WAF1/CIP1}$. We also observed an increase in caspase 3 expression consistent with the increase in caspase 3 activity discussed earlier.

Within the groups of altered genes, many are identified as genes upregulated in response to ERK, JNK and/or p38

Table 1. Genes with altered expression in induced Htt14A2.5 cells relative to uninduced controls

GenBank accession no.	Gene product	FC
(**A**)		
	Stress response	
X62660, X62660	Glutathione transferase subunit 8	1.6
H32189, J02810, X04229	GST, mu type 2 (Yb2)	2.1
U86635, U86635	GST, mu 5	2.4
AA998683, AI176658, M86389	Heat shock 27 kDa protein	2.3
M14050	Heat shock 70 kDa protein 5	1.7
S63521	Glucose-regulated protein GRP78	2.2
AI176170, AI228738, AA875594	Peptidyl-prolyl *cis–trans* isomerase	1.6
Y17295, AF014009, AA892041	Acidic calcium-independent phospholipase A2	1.6
AI176595	Cathepsin L	2.0
S85184, S85184	Cathepsin L proenzyme	1.8
E03424, M58364	GTP cyclohydrolase I	2.6
	Cytoskeletal/adhesion	
U30938	Microtubule-associated protein 2 (MAP2)	1.8
AA859757	Collagen, type V, alpha 1	1.6
U75405, M27207	Alpha 1 type I collagen	2.7
X81448	Keratin 18	1.9
S76054	Cytokeratin-8	2.1
AI072634	Keratin complex 1, acidic, gene 18	2.1
AA800948	ESTs, Highly similar tubulin alpha	1.8
X67788	Ezrin p81	1.5
AF004811	Moesin	1.9
X60651, S61865	Syndecan	1.7
X81193	Muscle LIM protein	26.6
J02962	Lectin, galactose binding, soluble 3	1.7
AF028784	Glial fibrillary acidic protein alpha (GFAP)	9.3
X71898	uPAR-1	17.8
M23697	*Tissue-type plasminogen activator (t-PA)	3.3
M24067	Plasminogen activator inhibitor-1 (PAI-1)	2.1
X76985	Latexin	1.6
	Growth factors/signaling	
AF014827	Vascular endothelial growth factor D (VEFG-D)	3.1
L05489	Heparin-binding EGF-like growth factor	6.7
AI014163	*Interferon-related developmental regulator 1 (IFRD1)	4.8
M74223	*VGF (vacinia virus inducible growth factor)	5.8
L21192	*GAP-43	1.6
U75397, AF023087, M18416	*egr1/ Krox-24/NGFIA	3.9
AF030089	Neurotransmitter-induced early gene 4 (ania-4)	2.5
U42627	*Dual specificity phosphatase 6 (MKP-3)	5.0
S74351, U02553, S81478	*3CH1134/CL100 PTPase (MKP-1)	4.5
D38222	Tyrosine phosphatase-like protein	2.0
S61973, S61973	NMDA receptor glutamate-binding subunit	1.5
D30734	Ras GTPase-activating protein	1.7
AA900505	rhoB gene	1.7
AA891940	ESTs, highly similar to RhoC mouse	1.7
AA859878, AI639318	*Ret proto-oncogene	2.9
Y08355	PKC-zeta-interacting protein	1.8
X06916	*p9Ka homologous to calcium-binding protein	2.4
S57478	Lipocortin I/ annexin I	2.0
L13039	Annexin II	1.5
AI171962	Annexin A1	2.2
J03627	*S-100 related protein	2.6
U88958	Neuritin	1.7
	Cell cycle/apoptosis	
AF020618	Progression elevated gene 3 protein (GADD34)	1.9
U24174, L41275	p21 (WAF1)	1.8
X63594, X63594	IkappaBalpha	1.7
L32591, L32591, AI070295	GADD45	2.7
U49930, U49930, U84410	Caspase 3	1.8
AI639488	ESTs, highly similar to A42772 mdm2 protein	1.9
M60921	PC3 NGF-inducible anti-proliferative putative secreted protein	1.7
AA944156	B-cell translocation gene 2	1.6
AF087037	B-cell translocation gene 3	1.6
	Transcription	
AA945867	v-jun sarcoma virus 17 oncogene homolog (avian)	3.7
X17163, AI175959	*c-jun oncogene mRNA for transcription factor AP-1	3.3
M19651	*fos-related antigen (Fra-1)	10.7
M63282	Activating transcription factor 3	2.4
AI102031, AI102031	myc box-dependent interacting protein 1	1.6
AF009330	Enhancer-of-split and hairy-related protein 2 (SHARP-2)	1.9
AF001417	Kruppel-like transcription factor 6	6.7
AJ011969	SBF gene, exon 1 and joined CDS	5.0
AF026529	Stathmin-like 4	3.5
	Metabolism	
M36410, M36410	Sepiapterin reductase	1.6
X07467	Glucose-6-phosphate dehydrogenase	1.6
S49760, S49760	Diacylglycerol kinase	2.4
X02610, X02610	Enolase 1, alpha	1.6
J05210, J05210, L27075	ATP citrate lyase	1.6
J02791	Acyl coenzyme A dehydrogenase medium chain	1.9
U35774	Cytosolic branch chain aminotransferase	1.9
M57664, M57664	Creatine kinase-B	1.7
AF036537	Homocysteine respondent protein HCYP2	1.5
AI236145	Hydroxysteroid dehydrogenase 17 beta, type 7	1.7
AB000280	Peptide/histidine transporter	1.9
M96601	Taurine transporter	2.9
	Intracellular trafficking/secretion	
X53363	Calreticulin/Ca(2+)-binding chaperone/ ER lumen	1.5
M58758	ATPase, H+ transporting, lysosomal accessory protein 1a	1.5
AA891068, U52663, AA799575, E03428	Peptidylglycine alpha-amidating monooxygenase	1.9
U14398, U14398	Synaptotagmin 4	2.5
X96437, X06832	*PRG1/prechromogranin A	2.8
(**B**)		
	Stress response	
AB000362	Cold inducible RNA-binding protein	−1.6
	Cytoskeletal/adhesion	
D83348	Long type PB-cadherin	−3.8
M74067	Claudin 3	−1.6
AB015946	Tubulin, gamma 1	−1.6
U18314, U18314	Thymopoietin (lamina-associated polypeptide 2)	−1.7
	Growth factors/signaling	
AF084576	Delta 3/ 14-3-3 protein	−1.6
L02529	*Drosophila* polarity gene (frizzled) homolog	−2.2

Continued

Continued

Table 1. *Continued*

GenBank accession no.	Gene product	FC
D16840, D43778, U01908	Angiotensin II type 2 receptor	−3.0
M91590	Arrestin, beta 2	−1.6
AA892470	Calmodulin 1 (phosphorylase kinase, delta)	−2.0
AI145490	Lamin B receptor	−1.5
AF042714	Neurexophilin 4 (Nph4)	−1.8
U73030	Pituitary tumor-transforming 1	−1.6
D84336	ZOG	−1.9
S54008	Fibroblast growth factor receptor 1	−1.6
	Cell cycle/apoptosis	
U77933	Caspase 2	−1.5
X60767	Cell division cycle 2 homolog A (*Schizosaccharomyces pombe*)	−1.7
AA893235	ESTs, Highly similar to G0S2_mouse G0/G1 switch protein 2	−2.3
	DNA replication/repair/chromatin structure	
X93591, AA874919	Mismatch repair protein, MSH2	−1.7
M24604, M24604	Proliferating cell nuclear antigen	−1.7
AJ011605	DNA polymerase alpha subunit I	−2.2
AJ222691	DNA polymerase delta, catalytic subunit	−2.2
D14045, AA899854, Z19552	DNA topoisomerase IIA	−1.8
U17565, U17565, AI639082	Mini chromosome maintenance deficient 6 (*Saccharomyces cerevisiae*)	−1.6
AA858636	ESTs, highly similar to mini chromosome maintenance deficient 7	−1.7
U95920	Pericentriolar material 1	−1.6
X98490	p32-subunit of replication protein A	−1.6
AI232374, AA874802	Histone H1-0	−1.5
AI008836, AA996401, D84418	High mobility group box 2	−1.7
AB012214	DNA cytosine 5 methyltransferase	−1.6
	Transcription	
AB012230	NF1-B1	−1.6
AB012231	NF1-B2	−1.6
AB012234	NF1-X1	−1.6
AF009329	Enhancer-of-split and hairy-related protein 1 (SHARP-1)	−1.7
U91679	ETS domain transcription factor Pet-1	−1.6
U25967	Arix1 homeodomain protein	−1.5
AI231821	Stathmin 1 (SCG10)	−1.5
AB019576	Timeless (Drosophila) homolog	−1.9
AI102868	Inhibitor of DNA binding 3, dominant-negative helix–loop–helix	−1.6
	Metabolism	
AA686870, AA686870, AA685376, AA684960, AA684929	NADH-ubiquinone oxidoreductase, MLRQ subunit	−1.9
M11794, AI102562, AI176456	Metallothionein	−1.7
AF015304	Equilbrative nitrobenzylthioinosine-sensitive nucleoside transporter	−1.9
U64030	Deoxyuridinetriphosphatase (dUTPase)	−1.8
AA848421	ESTs, highly similar to uracil-DNA glycosylase mouse	−1.8
U32314	Pyruvate carboxylase	−1.5
H31313	Selenoprotein P, plasma, 1	−1.7
AA799729	Phosphodiesterase 4B, cAMP-specific	−2.5
L03294, L03294	Lipoprotein lipase	−1.5
	Intracellular trafficking/secretion	
AF093569	XLas protein (XLas)	−1.8
Y12635	ATPase, H+ transporting, lysosomal (vacuolar proton pump)	−1.7
AF035951	Kinesin-related protein KRP1	−1.7
AF035955	Kinesin-related protein KRP6	−1.7
AF063102, AF063102	Calcium-independent alpha-latrotoxin receptor homolog 2	−1.6

Affymetrix U34A GeneChips were hybridized to target cRNA from Htt14A2.5 cells induced for 48 h with PA or uninduced and genes listed were called changed in MAS v5.0 in at least 75% of the pairwise comparisons. Multiple probe pair sets were identified for several genes and the corresponding GenBank accession nos are given. FC refers to the average fold change values determined in MAS v5.0 for all possible pairwise comparisons and for all probe pair sets identified. Genes with average fold changes less than −1.5 or greater than +1.5 were not considered. (A) and (B) include genes with increased and decreased expression, respectively. The asterisk indicates genes altered by NGF treatment (20).

signaling, such as *ATF3, c-jun, egr-1, Fra-1, Hsp27*, *MKP-3, PAI-1*, *VEGF-D*, *GAP-43* and *u-PAR* (19–21). Among the increased genes, there is extensive overlap with upregulated genes specific to NGF treatment of PC12 cells, including several tyrosine or dual specificity phosphatases (e.g. 3CH134/CL100 PTPase/*MKP-1*, dual specificity phosphatase 6/*MKP-3*, *uPAR-1, egr-1, c-jun* and *Fra-1*) (20). Genes that overlap with NGF treatment are annotated in Table 1. These results suggest that the expression of Htt mimics the intracellular transcriptional responses induced by cellular stress (e.g. oxidative stress) and growth factor (e.g. NGF) stimulation through MAPK signaling.

Genes whose expression was decreased can also be sorted into broad functional classes (Table 1). A notable group of mRNAs that were decreased includes genes encoding proteins involved in DNA replication and repair and chromosome structure. Interestingly, decreases in the expression of this class of genes are typically associated with either heat shock responses or cell cycle arrest and apoptosis similar to that observed with activation of MAPK and/or p53 pathways (22). We also detected the decreased expression of several isoforms of nuclear factor 1 (*NF1*), a group of transcription factors that have previously been shown to be repressed by stress in HepG2 cells (23). Similar classes of genes have been identified in other HD model systems (24,25).

Gene expression changes were verified by testing 13 altered mRNAs and one unchanged mRNA (EF1-α), using northern blot analysis (Supplementary Material, Fig. S1). A high confirmation rate between northern blot analysis and microarray results was found (13/13), although the absolute fold changes varied (Fig. 2). Specifically, *GST-YB-2*, *Creatine kinase* (brain), *Diacylglycerol kinase*, *GTP cyclohydrolase*, *p21$^{WAF1/CIP1}$*, *Taurine transporter*, *Egr-1*, *GAP-43*, *Fra-1* and *MKP-1* mRNAs were confirmed as increased, and *angiotensin II type 2 receptor, DNA topoisomerase IIa* and *PCNA* mRNAs were confirmed as decreased.

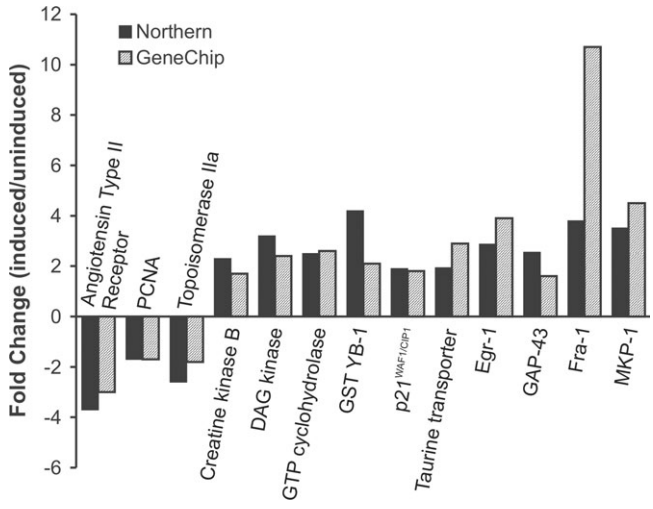

Figure 2. Comparison of fold change values of selected genes determined from the GeneChip and northern blot analysis. Fold changes were determined by quantitation of blots using phosphoimager scanning and normalization for loading differences by stripping and reprobing with EF1-α (unchanged transcript). For northern blots, probe sequences are provided in Materials and Methods. For the GeneChip data, fold change values are the average fold change values of all possible pairwise comparisons (four replicates, 16 pairwise comparisons) determined using Affymetrix MAS v5.0 software as described in Materials and Methods.

Expression of mutant Htt causes increased activation of JNK in PC12 and ST14A cells which parallels toxicity

As described earlier, a notable group of genes whose transcription is altered is that which can be upregulated in response to stress- or growth factor-induced activation of signaling pathways including the MAPKs JNK, ERK and p38. These pathways tend to regulate pro-apoptotic and pro-survival responses of the cell. To determine which signaling pathways might be modulated by mutant Htt expression, we first examined the phosphorylation status of two MAPKs typically associated with cell death in mammalian cells JNK and p38. Htt14A2.5 cells were induced for 48 h, when transgene expression and inclusion formation are maximal. Levels of activated (i.e. phosphorylated) forms of these proteins were evaluated by immunoblotting, using antibodies that detect the phospho- and total-protein forms. Phospho-JNK (p-JNK) levels increased slightly over a 48 h time course, showing an approximate 2-fold increase (Fig. 3A). A progressive increase in caspase 3 activation was observed over time, corresponding to the observed JNK activation (Fig. 3B). Phospho-p38 levels were unchanged (data not shown). Similar increases in the parental PC12ec line were not observed at the corresponding time points (data not shown). For an independent measure of JNK kinase activity, the ability of p-JNK in cell lysates to phosphorylate an exogenously added substrate, GST-c-jun(1–79), was measured. In a representative experiment (Fig. 3C), at 2 days post-induction the level of JNK kinase activity was 1.6-fold higher in induced cells, and at 3 days this ratio was 1.7-fold. These results are consistent with immunoblot analysis showing elevated p-JNK. To confirm that JNK has a role in the observed Htt-mediated cell dysfunction, the contribution of activated JNK to caspase 3 activation was investigated by treating Htt14A2.5 cells with a competitive inhibitor of JNK, SP600125 (26). Cells were simultaneously induced and treated with SP600125 for 48 h. In the presence of the inhibitor, caspase 3 activity was reduced by 21% (Fig. 3D). Levels of the downstream target of JNK, c-jun, were evaluated by immunoblot analysis to verify inhibition of JNK activity (Fig. 3E). In both uninduced and induced Htt14A2.5 cells, p-c-jun levels are decreased in the presence of SP600125. Taken together, these results suggest that JNK activation may contribute to the cellular dysfunction observed in the presence of mutant Htt.

To determine whether Htt-dependent increases in p-JNK are unique to this cell line or common to other mutant Htt-expressing cells, the Htt14A2.5 line was compared with Htt^{103} (Fig. 1B), an independent, inducible, mutant Htt exon1 (Httex1-103QP-EGFP)-expressing PC12 line that exhibits extensive and rapid cell death (27). These PC12 cells exhibit ∼55% cell death as measured by LDH activity after 48 h (data not shown), an ∼2.5-fold increase in caspase 3 activity after 48 h of induction with PA (Fig. 4B) and ∼6.8-fold increase after 72 h of induction (Fig. 4B). Consistent with a greater increase in caspase 3 activity, the Htt^{103} line exhibits a more rapid and higher level of p-JNK (Fig. 4A), beginning at ∼4 h, peaking by ∼8 h and remaining at an ∼15-fold increased level up to 48 h. Later measurements were not possible due to cell death.

Finally, we examined p-JNK levels in ST14A cells: a conditionally immortalized striatal-derived line that expresses a 548-amino acid N-terminal segment of either wild-type (N548wt) or mutant (N548mu) human Htt (Fig. 1C) (28). These cells constitutively express Htt; however, they contain a temperature-sensitive large T-antigen, which, upon shifting cells to the non-permissive temperature (39°C), is inactivated and cells stop dividing and partially differentiate. Upon expression of mutant Htt (N548mu), cells show rapid cell death (28). Consistent with significant levels of cell death, p-JNK levels were dramatically increased in the N548mu line relative to N548 wt or the parental ST14A cells (Fig. 4C). To confirm that increased p-JNK correlates with cell dysfunction and death, both caspase 3 activation and MTT were assessed. Upon simultaneous shift to 39°C and to serum deprived media (SDM) for 12 h, control, N548 wt and N548mu cells all demonstrate increased caspase 3 activation; however, N548mu cells show a significantly higher activation than controls (>2-fold, Fig. 4D). As cell death in this line is so prominent, we were unable to use caspase 3 activity as a measure of dysfunction for periods longer than ∼16 h following temperature and media shift. Using MTT assays, N548mu cells undergo dramatic cell death when simultaneously shifted to 39°C and to SDM over a 3-day time course (Fig. 4E).

In summary, three separate cell lines expressing various regions of Htt support the hypothesis that levels of activated JNK directly correlate with the extent of Htt-associated cell death.

Expression of mutant Htt causes sustained activation of ERK in PC12 and ST14A cells

In the PC12 Htt14A2.5 line, gene expression profiling suggests that pro-survival signaling responses are induced in

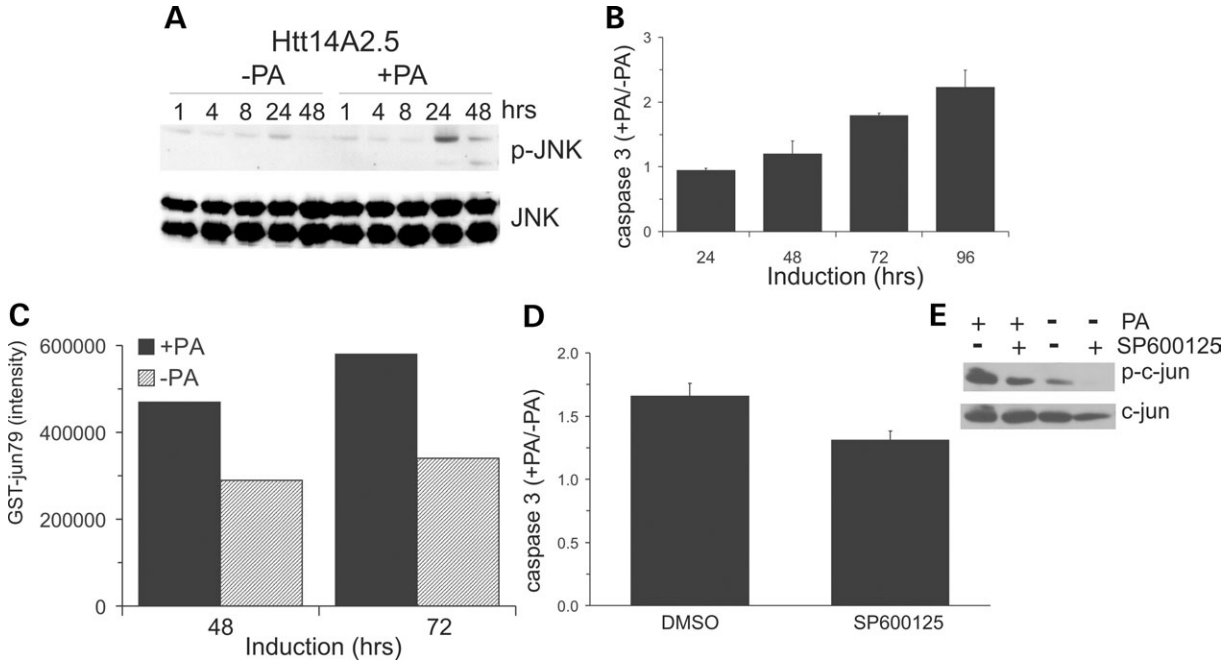

Figure 3. Expression of mutant Htt in the Htt14A2.5 line causes activation of JNK signaling and an increase in caspase 3 activity. (**A**) Cells were induced with 5 μM PA for the indicated times, and 75 μg of total protein was electrophoresed on 10% SDS–polyacrylamide gels and probed with phospho-JNK specific antibody. Blots were stripped and reprobed with antibody that detects total JNK protein. The doublets represent different gene products (e.g. JNK 1 and 2) that are activated in a similar manner and both are recognized by the indicated antibodies. The two proteins, represented by the two bands, are independently phosphorylated. Although activation of both proteins is typically observed simultaneously and shows the same trends, absolute levels of phosphorylation may not always increase to the same degree and display identical ratios. (**B**) Cells were grown as described in (A) and caspase 3 assays were performed as described in Materials and Methods. Caspase 3 activity is expressed as the ratio of induced to uninduced cells. (**C**) JNK kinase activity. Htt14A2.5 cells were induced for 48 or 72 h, cells were lysed and crude lysates were incubated with the substrate GST-c-jun(1-79) and [γ-^{32}P]ATP. Samples were resolved on 12% SDS–polyacrylamide gels and the relative intensity of bands corresponding to the phosphorylation of GST-c-jun quantiated by phosphoimager scanning is shown in the figure. A representative experiment is shown. (**D**) Htt14A2.5 cells were induced for ~8 h and 10 μM SP600126 added. Cells were harvested ~40 h later and caspase 3 activity was measured as described in Materials and Methods. (**E**) Samples from experiments as described in (D) were electrophoresed on 12% polyacrylamide gels and probed with a phospho-specific c-jun antibody. Blots were stripped and reprobed with antibody detecting total c-jun protein. Caspase 3 assay was performed in triplicate and experiments were repeated at least three times. Graphs are the mean and SE from a representative experiment.

addition to pro-cell death responses upon expression of mutant Htt and that these pathways may act together to produce the relatively mild cell dysfunction observed. Therefore, we examined the activation of two kinases associated with signaling pathways typically involved in cell survival: the MAPKs ERK and AKT (29,30). In the Htt14A2.5 cells, in response to Htt challenge, phospho-ERK (p-ERK) levels increased over time to a maximum at ~24 h and were elevated ~5-fold relative to uninduced cells (Fig. 5A). AKT levels were unchanged (data not shown). To confirm that the phosphorylation results from the immunoblot analysis correspond to increases in ERK kinase activity, we performed direct measurements of the levels of activated ERK in induced versus uninduced cells using the Stratagene PathDetect Trans-Reporting System, which measures the ability of ERK to activate an Elk1 fusion protein resulting in luciferase production. After 3 days induction, ERK kinase activity was ~6-fold higher in induced cells than in uninduced cells (Fig. 5D).

We also measured the levels of p-ERK in the Htt^{103} line over a 48 h time course. Similar to the Htt14A2.5 line, p-ERK showed an increase in induced cells over the 48 h period, but to a lesser extent (Fig. 5B). In these cells, a higher basal level of p-ERK with a moderate increase in p-ERK was observed beginning at 4 h and reaching 2–4-fold maximal levels by 8–12 h. Finally, p-ERK levels were also elevated at the permissive temperature (33°C) in the N548mu cells relative to the parental ST14A or to the N548 wt lines (~3-fold relative to ST14A, Fig. 5C). We did not observe a change in the level of p-ERK when cells were shifted to the non-permissive temperature and SDM in any of the lines (data not shown), likely because of the rapid cell death that occurs. These results are consistent with a role for ERK in cell survival and suggest a possible compensatory mechanism by the cell to counteract the deleterious effects of mutant Htt expression.

As described earlier, gene expression changes identified in the microarray analysis indicated alterations in the expression of a number of genes that can be regulated by MAPKs, including several associated with ERK (*Fra-1*, *Egf-1* and *GAP-43*) (20). To determine whether the increased expression of a subset of these genes could be directly associated with the increase in ERK activation, we performed northern blot analysis to examine the levels of *Fra-1*, *Egf-1* and *GAP-43* mRNAs in induced cells in the presence of U0126, a specific inhibitor of MEK1/2 (MEK), which is the upstream kinase of ERK. Cells were induced for 24 h followed by an additional 24 h of treatment with 0, 1 or

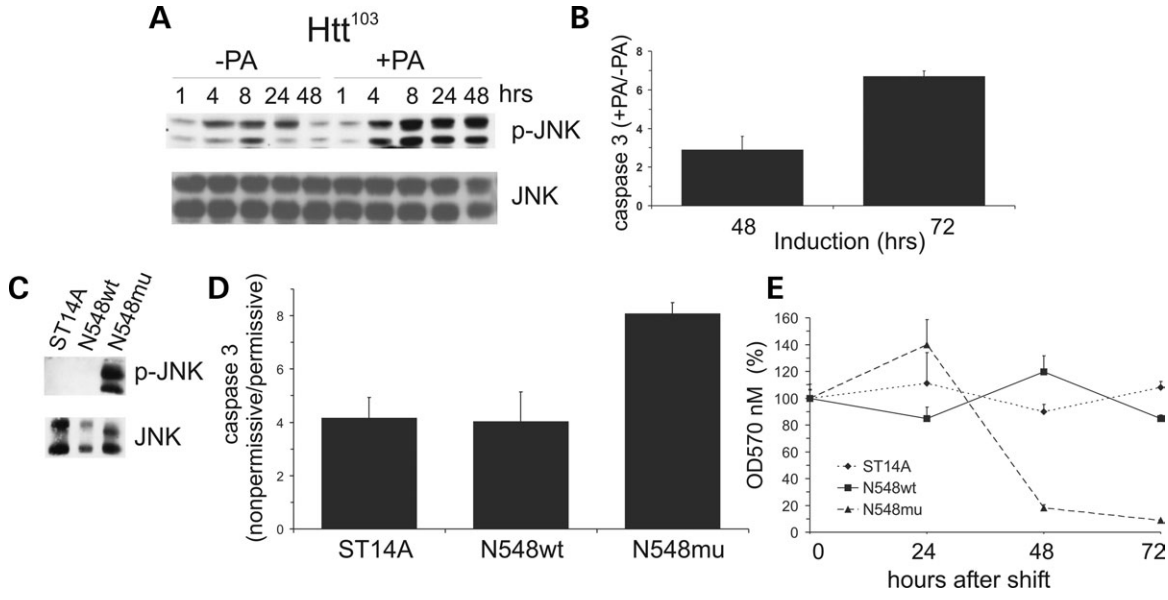

Figure 4. Expression of mutant Htt causes activation of JNK signaling in the Htt^{103} and N548mu lines and an increase in Htt-associated cellular dysfuntion. (**A**) Htt^{103} (Httex1-103QP-EFGP expressing) cells were induced with 2 μM PA for the indicated times and 75 μg of total protein was electrophoresed on 10% SDS–polyacrylamide gels and probed with phospho-JNK specific antibody followed by reprobing with total JNK antibody. (**B**) Caspase 3 activity was determined at the indicated times and expressed as the ratio of induced to uninduced cells. (**C**) ST14A, N548wt (Htt548-Q18) and N548mu (Htt548-Q120) cells were grown under the permissive condition (33°C and CM) overnight and then shifted to the non-permissive condition (39°C and SDM) for 1 day. Immunoblots were performed as described in (A). (**D**) Caspase 3 assays were performed 16 h after shifting cells to the non-permissive conditions and expressed as a ratio of cells grown at 33°C in CM to those grown at 39°C in SDM. (**E**) MTT assays were performed on the ST14A, N548wt and N548mu lines at the indicated times after shifting to non-permissive conditions. Cellular MTT was solubilized and absorbance was determined at 570 nm. Caspase 3 and MTT assays were performed in triplicate and experiments were repeated at least three times. Graphs are the mean and SE from a representative experiment.

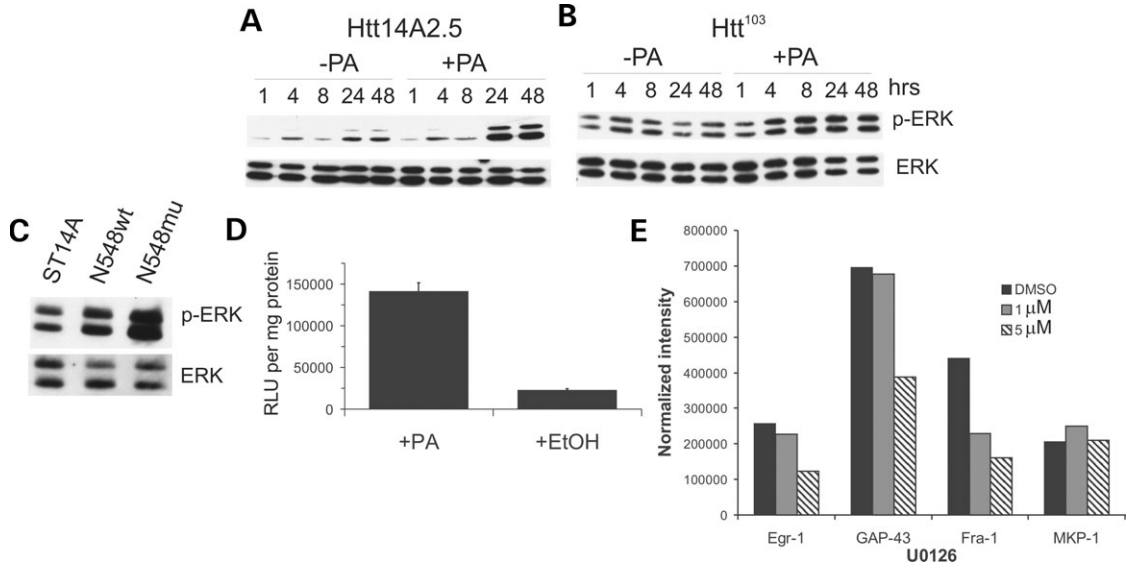

Figure 5. Expression of mutant Htt causes sustained activation of ERK in the Htt14A2.5 line. (**A** and **B**) Htt14A2.5 and Htt^{103} cells were induced with 5 or 2 μM PA, respectively, and harvested at the indicated times. Twenty-five micrograms of total protein was electrophoresed on 12% SDS–polyacrylamide gels and probed with phospho-ERK specific antibody followed by stripping and reprobing with antibody detecting total ERK. The doublets represent different gene products (e.g. ERK 1 and 2) that are activated in a similar manner and both are recognized by the indicated antibodies. The two proteins, represented by the two bands, are independently phosphorylated. Although activation of both proteins is typically observed simultaneously and shows the same trends, absolute levels of phosphorylation may not always increase to the same degree. (**C**) ST14A, N548 wt and N548mu cells were grown at 33°C in CM overnight, harvested and immunoblots performed as described earlier. (**D**) Uninduced Htt14A2.5 cells or cells induced with 5 μM PA were transiently transfected with pFA2-Elk1 and pFR-Luc plasmids. Two days later (3 days of post-induction), cells were lysed and luciferase assays were performed. RLUs are expressed per milligram of total protein. (**E**) Htt14A2.5 cells were induced with 5 μM PA for 24 h followed by addition of the indicated concentrations of U0126 for an additional 24 h. Northern blots were performed using probes specific for the indicated genes and blots were quantitated using phosphoimager scanning and normalization for loading differences by stripping and reprobing with EF1-α (unchanged transcript). Probe sequences are provided in Materials and Methods.

5 μM U0126. Immunoblot analysis confirmed a progressive inhibition of p-ERK under these conditions with no detectable p-ERK at 5 μM. As would be predicted, if gene expression changes were dependent upon ERK activation, the levels of *Fra-1*, *Egf-1* and *GAP-43* mRNAs decreased as concentrations of U0126 were increased (Fig. 5E) (Supplementary Material, Fig. S2). As a control, *MKP-1*, whose expression does not appear to be regulated by ERK, was tested. Consistent with a specific role for ERK, increasing concentrations of U0126 had no effect on the level of *MKP-1* expression (31). These results suggest that at least a subset of the gene expression changes identified in the microarray analysis and predicted to be due to ERK activation is indeed sensitive to changes in ERK activity levels in the Htt14A2.5 line.

ERK activity is protective against Htt-mediated cell dysfunction in PC12 cells

As the earlier studies suggest a potential protective effect of ERK activation against deleterious cellular effects associated with mutant Htt expression, we investigated whether ERK activation influenced the degree of caspase 3 activation in the Htt14A2.5 line. When ERK activation is prevented by treating cells with 10 μM U0126, a corresponding increase in caspase 3 activity is observed (Fig. 6A), consistent with a pro-survival function for activated ERK.

To further test the hypothesis that activation of ERK may be protective, the role of ERK was evaluated in the Htt^{103} cell line. Owing to the profound cell death phenotype of this line, effects of chemical compounds upon toxicity are difficult to evaluate. Therefore, the effect of overexpressing a constitutively active form of MEK1 (MEK1-CA) in both the Htt14A2.5 and the Htt^{103} lines was assessed. The Htt14A2.5 line was induced for 24 h followed by transient transfection with MEK1-CA or control plasmids and cells were harvested 48 h after transfection. As the Htt^{103} cells show relatively rapid cell death following induction, these cells were transfected with MEK1-CA-expressing plasmids first, induced with PA 24 h later and then harvested an additional 24 h following induction. Caspase 3 activity was determined, and p-ERK levels were evaluated in both lines (Fig. 6B). Consistent with the inhibitor results in Htt14A2.5 lines, increasing ERK activity in both PC12 cell lines with MEK1-CA resulted in decreased caspase 3 activity (Fig. 6B–D).

ERK activity is protective against Htt-mediated cell dysfunction in ST14A cells

As ERK activation is protective in two separate mutant Htt-containing PC12 lines, we next examined whether the increased levels of p-ERK seen in the N548mu line might also served a protective role. At 33°C, these cells do not exhibit appreciable cell death; however, p-ERK levels are higher in the N548mu line than in either the ST14A or the N548 wt line, suggesting that either cells are attempting to mount a protective response or ERK is deleterious in these lines. ST14A and N548mu cells were grown under permissive conditions in the presence of increasing concentrations of U0126, and caspase 3 assays were performed ~20 h later. Consistent with a protective role for ERK activation, a dose-dependent increase in caspase 3 activity and decreased p-ERK were observed in the N548mu line relative to ST14A parental cells with increasing concentrations of U0126 (Fig. 6E and F). These results are consistent with a compensatory upregulation of p-ERK in the proliferating N548mu cell line that constitutively expresses mutant Htt, but is not sufficient to prevent rapid cell death upon transition to non-permissive conditions.

To determine whether additional ERK activation could reduce Htt-associated toxicity when N548mu cells were shifted to 39°C and SDM, transient transfections with MEK1-CA were formed as described in Materials and Methods. As shown using MTT assays (Fig. 6G), increasing the amount of activated ERK genetically decreases toxicity associated with mutant Htt by ~2-fold, again consistent with a protective role for ERK.

DISCUSSION

Gene expression profiling and targeted biochemical studies presented here implicate pathways modulated by ERK and JNK signaling in HD pathogenesis. These studies suggest that among potential pathways altered, ERK promotes survival, whereas JNK promotes cell death. Therefore, expression of mutant Htt upregulates both protective and deleterious pathways, and the balance between these opposing pathways determines the ultimate fate of the cell: life or death. The ability to mount a protective response (p-ERK) as opposed to robust activation of cell death-promoting pathways (p-JNK) is reflected in the degree and timing of toxicity and cell death in the cell lines used. The cell line used for the gene expression profiling (Htt14A2.5) seems to reveal early pathogenic and compensatory cellular events as opposed to massive cell death that may reflect later or secondary events. The number of genes altered is moderate with the majority of genes increased in their expression, and changes are largely reflective of simultaneous apopotic and neuroprotective signals elicited by a cell to cope with molecular insults, such as a misfolded protein (1). Our results are distinct from gene expression profiling results from several other HD model systems (both whole animals and cell culture models), in which the number of decreased genes is typically greater than those with increased expression (24,25,32,33); however, the results presented here may reflect early events as the cell begins to cope with the mutant Htt insult. In support of the hypothesis that at least some of the gene expression changes reflect compensatory events, the induction of molecular chaperones such as *Hsp27*, *Hsp70* and *GRP78* is a classic indicator of stress responses and is generally protective in HD and other polyQ disorders (reviewed in 34–42).

JNK

We observed a positive correlation between the cell dysfunction (i.e. increased caspase 3 activity and/or decreased MTT) and the levels of activated JNK. Treatment with the JNK inhibitor, SP600125, decreased caspase 3 activation and reduced phospho-c-jun levels in the Htt14A2.5 line. In separate studies, SP600125 was also found to rescue photoreceptor

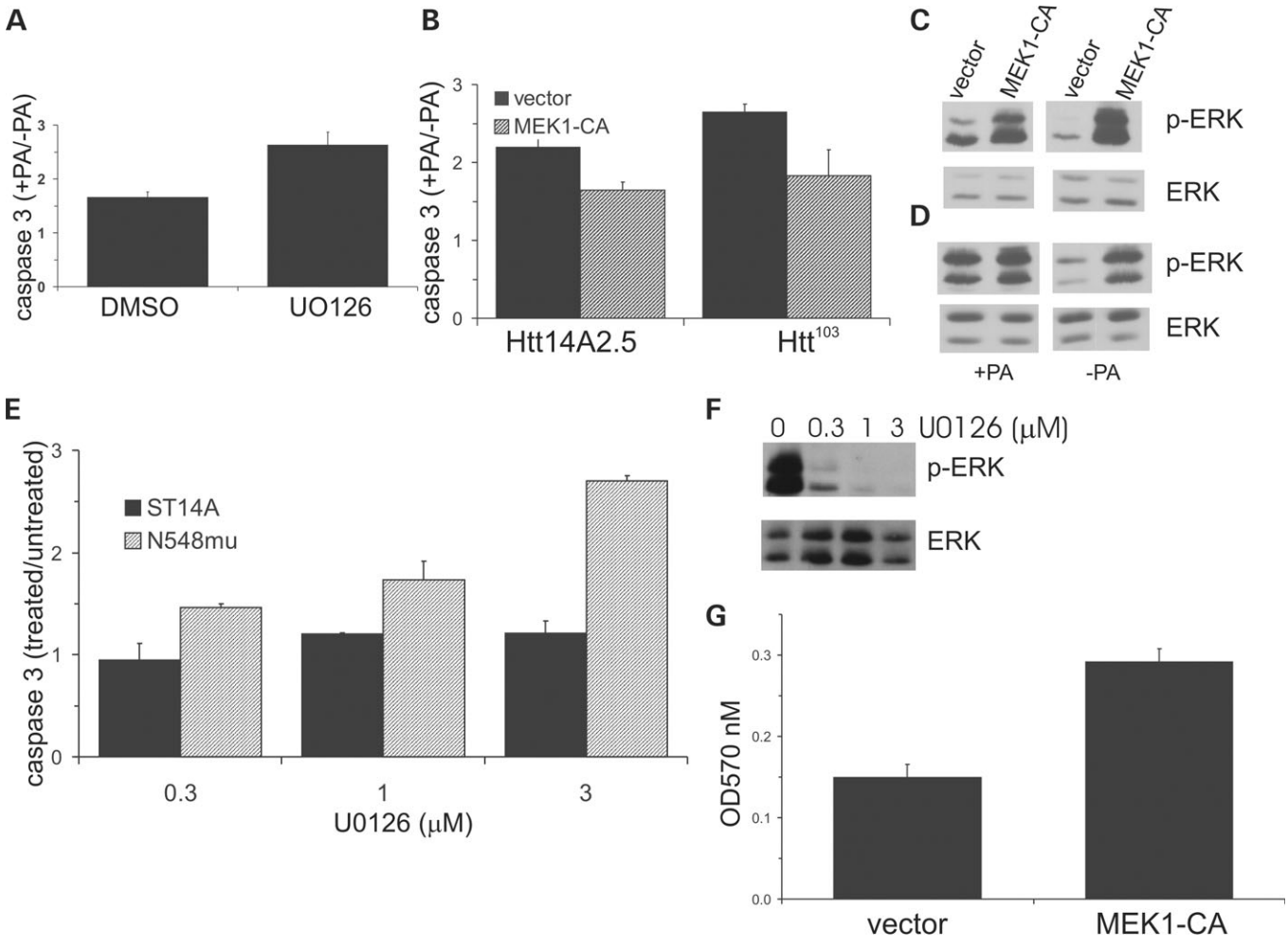

Figure 6. Alterations in ERK MAPK signaling pathway impact caspase 3 activation in the Htt-expressing PC12 lines. (**A**) Htt14A2.5 cells were induced for ~8 h, 10 μM U0126 was added and cells were harvested ~40 h later. Caspase 3 activity was measured as described in Materials and Methods and expressed as the ratio between induced and uninduced cells. (**B**) Transient transfections were performed in the Htt14A2.5 and Htt^{103} lines either with vector alone or with a constitutive active MEK1 (MEK1-CA), as described in Materials and Methods and Results. Caspase 3 activity was determined 48 h after transfection. Immunoblots of p-ERK and total ERK levels in the presence and absence of MEK1-CA as in (B) for Htt14A2.5 (**C**) or Htt^{103} line (**D**). Twenty-five micrograms of total protein was electrophoresed on 12% SDS–polyacrylamide gels and probed with phospho-ERK specific antibody followed by stripping and reprobing with antibody detecting total ERK. (**E**) ST14A and N548mu cells were treated with the indicated concentrations of U0126 and the following day caspase 3 assays were performed. Results are presented as the ratio of treated to untreated cells. (**F**) Immunoblot showing decrease in p-ERK levels relative to total ERK levels for N548mu cells treated with increasing concentrations of U0126 as indicated in (E). (**G**) N548mu cells were transfected with MEK1-CA or vector only and 2 days later shifted to 39°C and SDM. MTT assays were performed the following day, and results are presented as arbitrary units of OD570 nm. Caspase 3 and MTT assays were performed in triplicate, and experiments were repeated at least three times. Graphs are the mean and SE from a representative experiment.

neurodegeneration *in vivo* in a *Drosophila* model of HD (unpublished data). Furthermore, treatment with a mixed lineage kinase (MLK) inhibitor (an upstream kinase in the JNK pathway), CEP-11004 (43–45), strongly inhibits JNK activation and cell death in the striatal N548mu cell line (unpublished data). Other studies have implicated JNK activation in mutant Htt-associated toxicity as well. For example, JNK has been shown to be activated in primary striatal neurons expressing mutant Htt, and inhibition of this pathway with SP600125 as well as overexpression of a dominant-negative form of c-jun protects against cell death (46). In immortalized hippocampal cell lines expressing a full-length mutant Htt protein, JNK activation promotes cell death, whereas inhibition of this pathway with a dominant-negative MKK4/SEK1 mutant blocks Htt-mediated apoptosis (47,48). JNK activation has also been found in a number of other neurodegenerative disease models including SBMA (49), Alzheimer's (50) and Parkinson's diseases (51). Here we confirm the relationship of JNK activation to mutant Htt-associated toxicity in three different stably transfected cell lines that exhibit varying degrees of cell dysfunction/death. Interference with this activation is protective, suggesting that inhibition of JNK activation might represent a potential therapeutic target.

ERK

Although the role of JNK in mutant Htt-associated toxicity seems relatively straightforward, the role of ERK activation

in HD and other neurodegenerative diseases is likely to be more complex, as both protective and deleterious roles for ERK activation in neuronal cells have been described, and the reader is referred to several excellent reviews (19,52,53).

In this study, we present novel findings that strongly suggest that activation of ERK elicits pro-survival and protective responses in mutant Htt-expressing cells. In two different PC12 cell lines and in an immortalized striatal neuronal line, we found higher levels of activated ERK in cells expressing mutant Htt when compared with controls. Inhibition of this activated ERK with U0126 preferentially increases cell dysfunction/death in mutant Htt-expressing cells when compared with controls, suggesting that the higher levels of activated ERK in mutant Htt-expressing cells are a protective cellular response. Increasing the levels of activated ERK in these cells by co-expression of a constitutive active MEK1 protects against cellular dysfunction/death, further reinforcing a protective role for ERK. Additional support comes from two separate studies. First, we find that treatment with a compound that increases ERK activation in the Htt14A2.5 line (CEP-11004) (43–45) is also protective and this protection is at least partially due to ERK activation (unpublished data). Secondly, studies using orthovanadate, a phosphatase inhibitor, which reduces the dephosphorylation/inactivation of ERK and other kinases, were found to result in decreased caspase 3 activation in Htt14A2.5 cells (data not shown) and reduced cell death in other HD PC12 cell models (54).

A pro-survival role for ERK activation is consistent with a number of studies which suggest that ERK can mediate neuroprotective activities, following a number of neuronal insults including DNA damage, excitotoxicity, calcium overload, oxidative injury or hypoxia (reviewed in 55,56). Notably, a number of the above cellular processes are impacted upon mutant Htt expression, (reviewed in 3). In contrast to a potential protective role for ERK in neurons, several studies suggest that ERK activation can be detrimental to neurons and in particular contribute to neuronal degeneration (50). For example, in a 6-hydroxydopamine model of Parkinson's disease, chronic ERK activation is associated with cell death and MEK inhibition exerts a protective effect (53). Additionally, several Alzheimer's disease studies have shown a positive correlation between the amount of Aβ and the level of ERK activation (reviewed in 53). In addition to eliciting pro-survival and pro-death responses in the nervous system, ERK has an important role in integrating CNS functions that require neuronal plasticity, such as long-term potentiation and depression, and in memory (19,55). Relevant to this proposed function, a recent study shows that transgenic mice susceptible to HD have very early problems with learning and memory prior to any movement phenotypes (57).

Although studies investigating a role for ERK in HD are limited, there is support for an involvement of ERK activity in pathogenesis. In a *Drosophila* study, mutant Htt disrupted EGFR/ras/ERK signaling in glial cells, and reduced signaling through this pathway was associated with inclusion formation and reduced life span (14). The complexity of the role of ERK signaling in HD is exemplified by the R6/2 transgenic mouse model (expressing a mutant Htt exon 1 protein) (58). In this model, a progressive increase in ERK activation was found in the striatum (8–12 weeks), whereas in the cerebral cortex, the opposite was found. These results suggest distinct roles for activated ERK in these neuronal subtypes or that these cells respond uniquely to mutant Htt expression. One possibility is that the decreased ERK activation observed in cortical neurons is accompanied by decreased levels of BDNF, a downstream target of ERK. This in turn could result in reduced trophic support to striatal neurons, which may then mount a compensatory stress response and increase activated ERK in an attempt to prevent cell death. Consistent with the earlier observations and with this hypothesis, cortical neurons in HD patients exhibit reduced levels of BDNF, and consequently, its trophic effects upon striatal neurons appear to be reduced (59).

The diversity of consequences of ERK activation is not surprising, as the ERK signaling pathway plays a pivotal role in numerous cellular functions, including proliferation, differentiation, migration and survival. It is probable that this diversity is likely to be mediated by a number of factors which are relevant to ERK activity within a given cell or tissue type, including the intensity and duration (e.g. transient, sustained or chronic) of activation, as well as the subcellular localization and presence of other signaling mediators (60). These parameters are the basis for continued investigation of ERK in HD pathogenesis. However, the results presented here suggest that a possible therapeutic approach to prevent early neuronal dysfunction may involve upregulation of ERK activity.

MATERIALS AND METHODS

Propagation of cell lines and use of chemical inhibitors

PC12 cells. Generation and propagation of the Htt14A2.5 line were performed as previously described (15) and cells were grown on collagen-coated plates (BD Biosciences). These lines express a truncated Htt peptide containing the first 17 amino acids and 103Qs fused in frame to EGFP (15). A separate, independently derived, ecdysone-inducible PC12 cell line containing complete Htt exon 1 (Htt^{Q103} = Httex1-103QP-EGFP) was also analyzed and has been previously described (27). These cells were maintained in complete media (CM) with continued selection: Dulbecco's modified Eagle's medium (DMEM) (5% glucose) with 10% HS, 5% FBS, 1% penicillin/streptomycin and 200 μg/ml G418. Htt expression was induced with PA (5 μM for Htt14A2.5 cells and 2 μM for Htt^{103} cells) for the indicated times. For chemical inhibitor experiments, cells were plated to ∼30–40% confluency in six-well plates.

ST14A cells. The parental (ST14A) and derived lines (N548wt and N548mu) were propagated as previously described (61). Briefly, cells were plated at high density in six-well plates (1×10^5 cells/well) in CM (DMEM/5% glucose, 10% FBS and 1% penicillin/streptomycin) at 33°C. To inactivate the temperature-sensitive large T-antigen, cells were rinsed in phosphate-buffered saline (PBS) followed by shifting to SDM (SDM: DMEM-F12 containing 15 mM HEPES, L-glutamine, pyridoxine hydrochloride and N2 supplement) at 39°C.

Inhibitors. U0126 (Calbiochem) was resuspended at 10 mM and SP600125 (Calbiochem) at 20 mM in dimethyl sulfoxide. For longer time courses, media and other components were changed every 2 days.

Htt14A2.5 sample preparation and microarrays

For total RNA preparation, $\sim 3 \times 10^5$ cells were plated on 150 mm plates and the following day either induced with 5 μM PA or treated with ethanol alone (uninduced) for 48 h, followed by direct lysis using the RNeasy total RNA prep as described by the manufacturer (Qiagen, Valencia, CA, USA). Cells were harvested at ~40–60% confluency. RNA was quantitated spectrophotometrically and integrity tested by capillary electrophoresis (Agilent 2100 Bioanalyzer). Total RNA of 25 μg was used to generate target cRNAs for hybridization to Affymetrix Rat Genome U34A oligonucleotide arrays (UCI DNA Array Core Facility). Four separate cell growths were performed for Htt14A2.5 and PC12ec lines for the PA induced versus uninduced comparisons. Target cRNA was synthesized separately for each of these cell growths and hybridized individually to the oligonucleotide microarrays.

Microarray data analysis

For all the microarrays, the average fluorescence intensity was scaled to 500 arbitrary units so that chips could be directly compared and all 16 possible pairwise comparisons were made between the PA induced and the uninduced lines using the Affymetrix Microarray Suite v5.0 (Santa Clara, CA, USA). Genes were considered significant if 75% of the pairwise comparisons were called changed (i.e. difference call was increased, moderately increased or decreased and moderately decreased). MAS v5.0 signal log ratio values were converted to fold change values and averaged across all pairwise comparisons. Probe pairs were selected that had an average fold change of at least ± 1.5.

Probes for northern hybridizations and northern blots

The following probes were generated by reverse transcription–polymerase chain reaction of total RNA using the indicated primers: diacylglycerol kinase (CTCGATCGATGGCT CCTGGAAGTG) (forward) and (CTCAAGGCAGATGGCA TCCATCCTC) (reverse) (GenBank accession no. S49760), GST Yb-2 subunit (CCGATCCGCCTGCTCCTGGAATAC) (forward) and (GGACTGTGAGGTTGTGTTAAGGTGTC) (reverse) (GenBank accession no. X04229), angiotensin II type 2 receptor (CTCTATGAAGCAGTGGGCAGCAG) (forward) and (CATCACAGGTCCAAAGAGCCAGTC) (reverse) (GenBank accession no. D43778), GTP cyclohydrolase I (CTCATTCGGTGCAGAACTCCTGTC) (forward) (GCTCCTGATGAGTGTGAGGAACTC) (reverse) (GenBank accession no. E03424), DNA topoisomerase IIa (GACT CCGGCAGAGACTAGAGAAGAG) (forward) and (ACTT AATCGGCTTCCTTGCTCGTCCG) (reverse) (GenBank accession no. Z19552), EF1-α (GACTTCATCAAGAACAT GAT) (forward) and (GTGCCAATGCCGCCAAT) (reverse) (GenBank accession no. X13661), taurine transporter (CCT GTTTGGGAGCGGCCTGCCTGTG) (forward) and (GCAT CCCAGCAGCATACAGTCCCTAC) (reverse) (GenBank accession no. M96601), EGR-1 (CCTCTTAGGTCAGAT GGAAGATCTC) (forward) and (GGAACAACACTCTGAC ACATGCTCC) (reverse) (GenBank accession no. U75397), Fra-1 (CGAAAGAGTAGCAGCAGCAGTGGTC) (forward) and (GCTTCTCAGAGCTAGGGAGTACAGC) (reverse) (GenBank accession no. M19654), MKP-1 (CACCTCTAC TACAACGGTCTTCAAC) (forward) and (CACTGGCTTC GTCCATCAGTGCTG) (reverse) (GenBank accession no. S74351) and GAP-43 (GTTGCTGATGGTGTGGAGAAG AAGG) (forward) and (CACCTCAGAACGGAACATTG CACAC) (reverse) (GenBank accession no. L21192). $p21^{WAF1/CIP1}$ protein (WAF1) gene (GenBank accession no. AA956793), early growth response factor 1 (GenBank accession no. BF521744), PCNA (GenBank accession no. AA924358) and creatine phosphokinase (brain) (GenBank accession no. AA818791) were ESTs obtained from Research Genetics. EST cDNAs were amplified using T7 and T3 primers complementary to plasmid sequences. All probes were sequence verified and labeled by random priming using the method of Feinberg and Vogelstein (62).

Northern blot analysis was performed using 20–30 μg of total RNA isolated from uninduced Htt14A2.5 cells or cells induced with 5 μM PA for 48 h. Northern blots were performed as previously described (63). Signals were normalized to that of EF1-α, which was called unchanged on the microarrays. The ratio of induced to uninduced mRNA levels (e.g. fold change) was calculated from the normalized intensities.

Cell toxicity assays

For caspase 3 assays, cells were harvested from six-well plates, washed with cold PBS and frozen. Caspase 3 assays were performed in triplicate using EnZCheck Caspase 3 Assay Kit no. 1 (Molecular Probes) as described by the manufacturer. PC12 cells were not used if above ~70% confluency, and caspase 3 activities were corrected for variations in protein concentration. For MTT assays, ST14A and derivative cell lines were plated in six-well plates and incubated with MTT (1.25 mg/well) for 4 h at the indicated temperature. Cellular MTT was solubilized with acidic isopropanol and absorbance was measured at 570 nm.

Transient transfections and constructs

Htt14A2.5 and Htt^{103}cells were transfected in six-well plates using Lipofectamine 2000, according to manufacturer's instructions (Invitrogen). Htt14A2.5 cells were induced with 5 μM PA 2 days after plating and transfected the following day. Htt^{103} cells were transfected 2 days after plating and induced with 2 μM PA the following day. Both cell lines were transfected with 1 μg DNA/well and 1.25 μl Lipofectamine 2000/well, and cells were harvested 48 h after transfection. ST14A and derivative lines were plated in six-well plates and the following day transfected as described above except using 1.5 μl Lipofectamine 2000. Two days after transfection, cells were shifted to SDM and 39°C. MEK1-CA and MEK1-DN were kind gifts from Dr Guang Bai, University of Maryland Dental School (64).

ERK and JNK activity assays

p-ERK activity was measured using the PathDetect Elk1 *trans*-Reporting System (Stratagene, La Jolla, CA). Briefly, cells were plated in six-well plates and induced the following day with 5 μM PA. The next day cells were transfected as described earlier, using 50 ng of pFA2-Elk1 (fusion *trans*-activator plasmid) and 1 μg of pFR-Luc (reporter plasmid). Two days later (3 days of total induction), luciferase activity was measured using an Enhanced Luciferase Assay Kit as described by the manufacturer (BD Pharmingen) and readings performed using an Eritas microplate luminometer (Turner Biosystems, Sunnyvale, CA). Relative light unit (RLU) activity was corrected for variations in protein lysate concentrations.

p-JNK activity was measured from cell lysates using a c-Jun Terminal Kinase Assay Kit (Stratagene). Twenty micrograms of crude lysate was incubated with 4 μg GST-c-jun(1–79), reaction buffer and 0.2 μCi [α-^{32}P]ATP (6000 Ci/mmol) and incubated at 30°C for 30 min. Reactions were stopped by adding sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) sample loading buffer, proteins were resolved on 12% SDS–polyacrylamide gels and the amount of ^{32}P incorporated into the substrate was quantitated by phosphoimager scanning.

Antibodies and immunoblots

Cell lysates were made and immunoblots were performed as previously described (15). p-ERK (Thr^{202}/Tyr^{204}), p-JNK (Thr^{183}/Tyr^{185}), p-c-jun (Ser^{73}), total ERK, total c-jun and total JNK antibodies were from Cell Signaling Technology and all were used at a 1:1000 dilution. Quantitation of immnuoblots was performed using ImageJ 1.32j software from NIH.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

This work was supported by the Hereditary Disease Foundation (to L.M.T. and J.L.M.), a Huntington's Disease Society of America Coalition for the Cure grant (to L.M.T.), the High Q Foundation, NIH awards HD36081 (J.L.M.), NS 42157-04 (J.M.O. and A.S.) and NS045283 (J.L.M. and L.M.T.). We gratefully acknowledge Dr. Elena Cattaneo for the ST14A cells and the DNA Array Core Facility at UCI.

Conflict of Interest statement. We have no involvements that might raise the question of bias in the work reported or in the conclusions, interpretations or opinions stated.

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