Enhanced Akt Signaling Is an Early Pro-survival Response That Reflects N-Methyl-D-aspartate Receptor Activation in Huntington’s Disease Knock-in Striatal Cells*

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Huntington’s disease features the loss of striatal neurons that stems from a disease process that is initiated by mutant huntingtin. Early events in the disease cascade, which predate overt pathology in Hdh CAG knock-in mouse striatum, implicate enhanced N-methyl-D-aspartate (NMDA) receptor activation, with excitotoxicity caused by aberrant Ca2+ influx. Here we demonstrate in precise genetic Huntington’s disease mouse and striatal cell models that these early phenotypes are associated with activation of the Akt pro-survival signaling pathway. Elevated levels of activated Ser(P)473-Akt are detected in extracts of Hdh<sup>Q111/Q111</sup> striatum and cultured mutant STHdh<sup>Q111/Q111</sup> striatal cells, compared with their wild type counterparts. Akt activation in mutant striatal cells is associated with increased Akt signaling via phosphorylation of GSK3β at Ser9. Consequent decreased turnover of transcription co-factor β-catenin leads to increased levels of β-catenin target gene cyclin D1. Akt activation is phosphatidylinositol 3-kinase dependent, as demonstrated by increased levels of Ser(P)<sup>473</sup>-Akt. In contrast to wild type cells, Akt activation in mutant striatal cells can be blocked by treatment with insulin growth factor-1 and blocked by treatment with the phosphatidylinositol 3-kinase inhibitor LY294002. However, in vivo, Akt activation in mutant striatal cells can be blocked by treatment with insulin growth factor-1 and blocked by treatment with the phosphatidylinositol 3-kinase inhibitor LY294002. Akt activation in mutant striatal cells is Ca2+-dependent, because treatment with EGTA reduces levels of Ser(P)<sup>473</sup>-Akt. Thus, consistent with excitotoxicity early in the disease process, activation of the Akt pro-survival pathway in mutant knock-in striatal cells predate overt pathology and reflects mitochondrial dysfunction and enhanced NMDA receptor signaling.

Huntington’s disease (HD)<sup>1</sup> is a dominantly inherited disorder that is characterized by choreiform movements, psychiatric and cognitive decline, and the graded loss of medium spiny projection neurons in the striatum (1). HD is initiated by an unstable CAG repeat that lengthens a polyglutamine tract in huntingtin above ~37 residues, such that disease severity is increased with increased polyglutamine length (2). Disease symptoms typically manifest in mid-life caused by mutant huntingtin with about 50 glutamines or in juvenile years caused by mutant huntingtin with more than ~55 glutamines. However, although clinical symptoms manifest after decades, genotype-phenotype studies with neuropathologically graded HD post-mortem brain samples strongly suggest that the disease process is likely to begin at birth (3).

Evidence from precise genetic HD mouse and striatal cell models and HD patient cells implicates a chronic disease cascade, in which mutant cells may be progressively sensitized to further insults. Dominantly inherited abnormalities manifest in the striatum of Hdh<sup>CAG100</sup>, HdhCAG94, and Hdh<sup>Q111</sup> knock-in mice, at only a few months of age, predating signs of neuropathology, such as intranuclear inclusions, by nearly a year (4–11). These early molecular phenotypes support excitotoxicity, via Ca2+ influx, stemming from decreased mitochondrial ATP synthesis (8) and enhanced NMDA receptor activation (7). They also suggest a role for loss of neuroprotective factors, such as brain-derived neurotrophic factor (12) and proenkephalin (5), caused by reduced levels of cAMP and diminished cAMP signaling via cAMP-dependent protein kinase and CREB-binding protein/cAMP-responsive element-binding protein (CBP/CREB) (8). The dominant phenotypes detected in vivo are also manifest in a genetically precise HD cell model: immortalized STHdh<sup>Q111/Q111</sup> striatal neuronal cells, derived from Hdh<sup>Q111/Q111</sup> embryos (13). STHdh<sup>Q111/Q111</sup> striatal cells do not exhibit markers of pathology but instead display enhanced sensitivity to metabolic stressors, including 3-nitroproionic acid (8). Furthermore, lymphoblastoid cells from HD patients display polyglutamine length-dependent deficits in mitochondrial calcium handling (14, 15) and ATP synthesis (8), although these peripheral cells are not overtly affected in HD patients.

Given the evidence in support of subacute “toxicity,” we have now examined the hypothesis that mutant striatal neuronal cells may activate compensatory pro-survival pathways, countering potentially toxic metabolic changes that emanate from the expression of mutant huntingtin. Specifically, we have investigated whether serine/threonine protein kinase B, also known as Akt, which has been implicated in neuronal cell survival (16), may be activated in mutant Hdh<sup>Q111/Q111</sup> striatal cells. Akt can be activated through the interaction of a trophic factor, such as insulin growth factor-1 (IGF-1), with its cognate receptor. However, in neuronal cells...
Akt can also be activated via N-methyl-D-aspartate (NMDA) receptor signaling, because rapid glutamate-induced Ca\textsuperscript{2+}/H\textsubscript{11001}- and phosphatidylinositol (PI) 3-kinase-dependent Akt phosphorylation at Ser\textsuperscript{473} can be blocked by the NMDA receptor antagonist MK-801 (17). Furthermore, Akt signaling has been implicated in HD by findings in acute cell models that achieve pathology by overexpression of mutant huntingtin fragment. In these systems, IGF-1 treatment ameliorates mutant fragment-induced toxicity, and Akt phosphorylation of mutant fragment decreases intranuclear inclusions (18).

Now our investigations using genetically accurate HD mouse and striatal cell models reveals enhanced Ca\textsuperscript{2+} and PI 3-kinase-dependent Akt signaling that appears to be due to enhanced NMDA receptor activation. Thus, modulation of the Akt pathway may provide a means to counter the early excitotoxic effects of mutant huntingtin that predate overt neuronal cell pathology.

**EXPERIMENTAL PROCEDURES**

Chemicals and Reagents—IGF-1, LY294002, (17)-MK-801, EGTA, and cyclohexamide were obtained from Sigma. Phospho-Akt (Ser\textsuperscript{473}), phospho-PDK1 (Ser\textsuperscript{241}), phospho-PTEN (Ser\textsuperscript{380}), phospho-GSK3\textbeta (Ser\textsuperscript{9}), phospho-FKHR (Ser\textsuperscript{256}), phospho-\beta-catenin (Ser\textsuperscript{33/37/Thr\textsuperscript{41}}), total Akt, and anti-ubiquitin (PD41) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Total GSK3\textbeta antibody and NMDAR1 antibody were purchased from BD Transduction Laboratories (San Diego, CA). NMDAR2A and NMDAR2B affinity purified rabbit polyclonal antibodies were purchased from Chemicon International, Inc. (Temecula, CA). Total anti-\beta-catenin antibody was from Zymed Laboratories Inc. (San Francisco, CA). c-Myc (9E10) antibody and LEF-1 (N-17) antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Cyclin D1 (Ab-3) monoclonal antibody was from Oncogene Research Products (Boston, MA). Anti-\alpha-tubulin was from Sigma-Aldrich. pcDNA3-c-MycGSK3\textbeta mammalian cell expression vector was a kind gift of Dr. Miguel Medina (Brigham’s and Women’s Hospital).

Genetic HD Mouse and Striatal Cell Culture Models—Mice carrying the Hdh\textsuperscript{Q111/Q111} knock-in allele, expressing mutant huntingtin with 111 glutamine residues, have been described previously (10, 11). Striatum was dissected from genotyped 8-month-old homozygous mutant Hdh\textsuperscript{Q111/Q111} and wild type littermates (10, 11). Conditionally immortalized wild type STHdh\textsuperscript{Q7/Q7} striatal neuronal progenitor cells expressing endogenous normal huntingtin and homozygous mutant STHdh\textsuperscript{Q111/Q111} striatal neuronal progenitor cell lines expressing endogenous mutant huntingtin with 111-glutamines generated from Hdh\textsuperscript{Q111/Q111} and wild type Hdh\textsuperscript{Q7/Q7} littermate embryos have been described previously (13). The striatal cell lines were grown at 33° Ci n Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bo-

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**FIG. 1. Enhanced Akt activation in mutant Hdh\textsuperscript{Q111/Q111} striatum and STHdh\textsuperscript{Q111/Q111} striatal cells.** A, immunoblot of striatal extracts from three homozygous Hdh\textsuperscript{Q7/Q7} wild type (7/7) and three homozygous Hdh\textsuperscript{Q111/Q111} mutant (111/111) littermates, and in B, homozygous wild type STHdh\textsuperscript{Q111/Q111} (ST7/7) and homozygous STHdh\textsuperscript{Q111/Q111} mutant (ST111/111) striatal cells probed for Ser\textsuperscript{P473}Akt (p-Akt) or total Akt (Akt). The corresponding histograms (right panels) plot the relative Ser\textsuperscript{P473}Akt to total Akt ratio (Relative p-Akt/Akt ratio), determined by band intensities that were analyzed by Scion software, with the ratio in mutant extracts (111/111) normalized to the ratio determined in wild type extracts (7/7).

The relative Ser\textsuperscript{P473}Akt/totalAkt ratio is significantly increased in mutant Hdh\textsuperscript{Q111/Q111} striatum (n = 3 experiments; *, p = 0.0045) and in mutant STHdh\textsuperscript{Q111/Q111} striatal cells (n = 8 experiments; *, p = 0.0006).

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Akt, and anti-ubiquitin (PD41) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Total GSK3\textbeta antibody and NMDAR1 antibody were purchased from BD Transduction Laboratories (San Diego, CA). NMDAR2A and NMDAR2B affinity purified rabbit polyclonal antibodies were purchased from Chemicon International, Inc. (Temecula, CA). Total anti-\beta-catenin antibody was from Zymed Laboratories Inc. (San Francisco, CA). c-Myc (9E10) antibody and LEF-1 (N-17) antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Cyclin D1 (Ab-3) monoclonal antibody was from Oncogene Research Products (Boston, MA). Anti-\alpha-tubulin was from Sigma-Aldrich. pcDNA3-c-MycGSK3\textbeta mammalian cell expression vector was a kind gift of Dr. Miguel Medina (Brigham’s and Women’s Hospital).
vine serum, 1% nonessential amino acids, 2 mM t-glutamine, and 400 μg/ml G418 (Geneticin; Invitrogen). For overexpression of Myc-GSK3β, striatal cells were transfected with pcDNA3-Myc-GSK3β or, in the control with pcDNA3 vector alone, using LipofectAMINE™ 2000, according to the protocol of the supplier (Invitrogen). The protein extracts were prepared 24 h post-transfection.

Immunoblot Analysis—In general, the protein extracts were prepared from striatal tissue and from cultured striatal cells by lysis on ice for 30 min in a buffer containing 50 mM Tris base (pH 7.4), 150 mM NaCl, 2 mM EDTA, protease inhibitor mixture, 10 μg/ml leupeptin, 10 μg/ml pepstatin and supplemented with phosphatase inhibitors (1 mM Na3VO4 and 50 μm NaF). The total cell lysates were then cleared by centrifugation at 10,000 × g, and the supernatants were collected.

To analyze Akt activation, striatal cells were placed in Dulbecco’s modified Eagle’s serum-free medium 3 h before protein extracts were prepared. For treatment with drugs, striatal cells were first placed in Dulbecco’s modified Eagle’s serum-free medium for 3 h and then exposed to IGF-1 (50 ng/ml for 30 min), LY294002 (25 μM), and the cell lysates were centrifuged at 20,000 × g for 15 min. The supernatant was further centrifuged at 100,000 × g. The membrane fraction was obtained by resuspending the high speed pellet in buffer A containing 1% Nonidet P-40.

Following determination of protein concentration, by Bio-Rad (detergent compatible) protein assay, 30 μg of total protein extract prepared as detailed above was mixed with 4× SDS sample buffer, boiled for 5 min, and resolved on 8% SDS-PAGE. For immunoblot analysis the proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) and incubated for 30 min in blocking buffer containing 10% nonfat powdered milk in TBS-T (50 mM Tris-HCl, 150 mM NaCl, pH 7.4, 0.5% Tween 20). The blots were then probed overnight at 4 °C with primary antibodies: phospho-Akt (Ser473) (1:1,000), phospho-PDK1 (Ser445) (1:1,000), phospho-PTEN (Ser380) (1:1,000), phospho-GSK3β (Ser9) (1:1,000), phospho-FOXO1a (Ser256) (1:1,000), total Akt (1:1,000), total GSK3β (1:1,000), cyclin D1 (1:100), NMDAR1 (1:1,000), NMDAR2A (1:500), or NMDAR2B (1:500). Immunoblots were rinsed three times for 10 min in TBS-T and incubated 1 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse (1:10,000) or anti-rabbit (1:10,000) antibodies. After being washed extensively for 30 min, the membranes were processed using an ECL chemiluminescence substrate kit (New England Biolabs, Beverly, MA) and exposed to autoradiographic film (Hyperfilm ECL; Amersham Biosciences). Quan-
tification of the immunoreactive bands was performed by scanning and analysis using the Scion densitometry program (Scion Images for Windows-Release Beta 4.0.2).

**Turnover of Cytosolic β-Catenin—**Wild type STHdh<sup>Q7/Q7</sup> and mutant STHdh<sup>Q111/Q111</sup> cells plated on 10-mm tissue culture dishes were incubated in growth medium containing cyclohexamide at a final concentration of 30 μg/ml and chased at 0, 0.5, 1, and 2 h after the addition of cyclohexamide. At each time point the cells were washed once with ice-cold phosphate-buffered saline and mechanically lysed by incubation for 10 min in ice-cold buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, protease inhibitor mixture, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin) followed by 40 strokes in a Dounce homogenizer. The cell lysates were then centrifuged at 2,000 × g for 15 min, and the supernatant was further centrifuged at 100,000 × g for 30 min to provide the cytoplasmic fraction. The protein concentration of the cytoplasmic extracts was quantified by Bio-Rad (detergent compatible) protein assay, and equal amounts of protein from each lysate were resolved by 8% SDS-PAGE. The proteins were then transferred to nitrocellulose membranes, blocked in 10% nonfat milk TBS-T, and incubated overnight at 4 °C with a monoclonal anti-β-catenin antibody. The immunoblot was then probed with horseradish peroxidase-conjugated secondary antibody and visualized by ECL reagents (New England Biolabs).

**Immunoprecipitation—**To isolate cytosolic and nuclear fractions, wild type STHdh<sup>Q7/Q7</sup> and mutant STHdh<sup>Q111/Q111</sup> cells were incubated in ice-cold hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, protease inhibitor mixture, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin) for 10 min and mechanically lysed by 40 strokes in a Dounce homogenizer. The homogenate was centrifuged at 2,000 × g for 15 min, and the resulting supernatant was further centrifuged at 100,000 × g for 30 min to isolate the cytoplasmic fraction. The washed pellet from the 2,000 × g centrifugation was resuspended in buffer C (20 mM HEPES, pH 7.9, containing 25% glycerol, 0.42 M KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA protease inhibitor mixture, and 1 mM phenylmethylsulfonyl fluoride) with gentle rotation for up to 1 h at 4 °C and centrifuged at 12,000 × g for 10 min, and the supernatant was collected to provide the nuclear fraction.

**Immunoprecipitation was performed by incubation of cytosolic or nuclear fractions (500 μg of protein) with 3 μg of anti-β-catenin antibody overnight at 4 °C followed by a 2-h incubation with 50 μl of protein A-Sepharose Cl-4B (Sigma). The beads were washed by centrifugation three times, then resuspended in ice-cold phosphate-buffered saline, and then boiled for 5 min in reducing SDS loading buffer. The immunocomplexes were resolved by SDS-PAGE on 12% polyacrylamide gel and transferred to nitrocellulose membranes. Immunoblot analysis was carried out as described above. Briefly, the blots were incubated with anti-β-catenin, anti-phospho-β-catenin, anti-ubiquitin, or anti-LEF-1 antibody and detected using ECL chemiluminescent reagents.

**Statistical Analysis—**The statistical significance of observations from independent experiments was determined by one-way analysis of variance and Scheffe’s S post-test for comparisons between multiple groups.

**RESULTS**

**STHdh<sup>Q111/Q111</sup> Striatal Cells Exhibit Enhanced Akt Activation—**Akt can be activated as a direct downstream target of PI 3-kinase. Upon stimulation of PI 3-kinase, PDK1 can activate Akt by phosphorylation of Thr<sup>308</sup> and Ser<sup>473</sup>. Phosphorylation of Akt kinase at Ser<sup>473</sup> is required for its full activation (19). Therefore, we evaluated Akt activation in Hdh CAG knock-in mice by using an antibody that specifically recognizes Akt phosphorylated at Ser<sup>473</sup>. Extracts of striatum dissected from three homozygous mutant Hdh<sup>Q111/Q111</sup> and three wild type Hdh<sup>Q7/Q7</sup> littermate mice were individually analyzed by immunoblotting with antibodies that detect Ser<sup>473</sup>-Akt or total Akt, as shown in Fig. 1A. Quantification of band intensity revealed that the average levels of Ser<sup>473</sup>-Akt in the striata of mutant mice was significantly increased by 1.8-fold (p = 0.0045), compared with levels in wild type striata. Similar results were obtained at 2 and 18 months of age (data not shown). These data indicate that enhanced Akt activation is a consequence of the disease process in vivo, in Hdh knock-in striatum. Next, Akt kinase activation was measured in extracts prepared from immortalized wild type ST T Hdh<sup>Q7/Q7</sup> and mutant STHdh<sup>Q111/Q111</sup> striatal cells, a manipulable genetic HD neuronal striatal cell model. As shown in Fig. 1B, a significant ~4-fold increase in the ratio of Ser<sup>473</sup>-Akt signal versus the total Akt signal (p = 0.0006) was apparent in mutant cell extracts compared with wild type cell extracts. This finding, which is consistent with the in vivo results, demonstrates an increase in Akt activation in mutant striatal neuronal cells.

**Increased Akt Activation Is Associated with GSK3β Inactivation—**To test whether increased Akt activation might be associated with altered Akt signaling in STHdh<sup>Q111/Q111</sup> striatal cells, we assessed Ser<sup>9</sup>-GSK3β, which is a well known downstream target of the Akt pathway that is implicated in neuronal cell survival (16). Phosphorylation of GSK3β at Ser<sup>9</sup> by Akt inhibits GSK3β kinase activity. Immunoblot analyses of Ser<sup>9</sup>-GSK3β and total GSK3β in extracts prepared from wild type ST Hdh<sup>Q7/Q7</sup> and mutant STHdh<sup>Q111/Q111</sup> cells are compared from immortalized wild type ST Hdh<sup>Q7/Q7</sup> and mutant STHdh<sup>Q111/Q111</sup> striatal cells, a manipulable genetic HD neuronal striatal cell model. As shown in Fig. 1B, a significant ~4-fold increase in the ratio of Ser<sup>9</sup>-Akt signal versus the total Akt signal (p = 0.0006) was apparent in mutant cell extracts compared with wild type cell extracts. This finding, which is consistent with the in vivo results, demonstrates an increase in Akt activation in mutant striatal neuronal cells.

**Supplemental Figure 1: Enhanced Akt Activation in Mutant Striatal Cells**

**A** Immunoblot of proteins immunoprecipitated (IP) with a monoclonal β-catenin antibody, or control IgG, from nuclear ST Hdh<sup>Q7/Q7</sup> (ST7/7) and STHdh<sup>Q111/Q111</sup> (ST111/111) striatal cell extracts, probed for β-catenin and transcription factor LEF-1. The IgG band is indicated. **B** Immunoblot of proteins extracted from striata of three wild type (7/7) and three homozygous mutant Hdh<sup>Q111/Q111</sup> mice (111/111) at 8 months of age, probed for cyclin D1. Analysis of the ratio of cyclin D1/α-tubulin band intensities revealed an average ~1.8-fold increase (ratios of 0.14, 0.19, and 0.18 for wild type mice and 0.3, 0.38, and 0.27 for mutant mice). **C** Immunoblot of total protein extracts prepared from STHdh<sup>Q7/Q7</sup> (ST7/7) and STHdh<sup>Q111/Q111</sup> (ST111/111) striatal cells, probed with specific antibodies to detect cyclin D1 or α-tubulin, as a loading control. Analysis of the ratio of cyclin D1/α-tubulin band intensities indicated an ~2-fold increase (n = 2 experiments; p = 0.03) in cyclin D1 levels in extracts from mutant striatal cells, compared with their wild type counterparts.
shown in Fig. 2A. Consistent with increased Akt activation, the ratio of Ser(P)9-GSK3β versus total GSK3β was increased 3-fold (p = 0.0028) in mutant cell extracts compared with wild type cell extracts.

To determine whether increased levels of Ser9-GSK3β phosphorylation might be a specific consequence of enhanced Akt activation in STHdhQ111/Q111 cells, we examined the Akt-regulated phosphorylation of pro-apoptotic transcription factor FKHR at Ser256 by immunoblot analysis. In contrast to GSK3β, levels of Ser(P)256-FKHR, normalized to α-tubulin levels, were similar in mutant compared with wild type striatal extracts (Fig. 2B). Thus, increased Akt activation in STHdhQ111/Q111 cells does not appear to have consequences for a downstream target that is involved in apoptosis but instead is associated with neuronal cell pro-survival signaling via GSK3β inactivation.

Increased β-Catenin Stabilization in STHdhQ111/Q111 Striatal Cells—To further study the functional significance of Akt phosphorylation in mutant striatal cells, we determined whether elevated Ser(P)9 GSK3β in STHdhQ111/Q111 might alter the levels of β-catenin, an essential transcriptional co-activator downstream of GSK3β whose turnover and translocation to the nucleus is regulated by GSK3β phosphorylation (20). We first tested whether the turnover of cytosolic β-catenin differs in STHdhQ111/Q111 and wild type STHdhQ7/Q7 striatal cells. Cyclohexamide, an inhibitor of protein synthesis, was added to the growth medium, and cells were then harvested after 0, 0.5, 1, and 2 h and processed by differential centrifugation to isolate cytosolic and nuclear protein fractions. β-Catenin levels in equal amounts of cytosolic protein fractions were analyzed by immunoblot analysis. Fig. 3A shows that although the half-life of the 92-kDa β-catenin-reactive band in wild type cytosolic extracts was about 30 min, the β-catenin band in STHdhQ111/Q111 cytosolic extracts remained stable over the 2-h chase period, implying decreased turnover.

Because β-catenin turnover is regulated by GSK3β-mediated...
phosphorylation, which targets β-catenin for degradation by the ubiquitin/proteosome pathway, we next assessed the levels of ubiquitinated and phosphorylated β-catenin, immunoprecipitated from wild type and mutant cytosolic extracts. Detection of immunoblots with anti-ubiquitin antibody (Fig. 3B) revealed that multiple bands of ubiquitinated β-catenin are immunoprecipitated from wild type striatal cell cytosolic extracts, whereas the β-catenin band precipitated from mutant striatal cell extracts is not detected by the anti-ubiquitin reagent. Reprobing immunoblots for phosphorylated β-catenin using a phospho-specific anti-β-catenin antibody that detects Ser33-, Ser37-, or Thr41-β-catenin revealed phosphorylated β-catenin bands in wild type immunoprecipitates. By contrast, phosphorylated β-catenin was almost undetectable in anti-β-catenin immunocomplexes from mutant cytosolic extracts.

To determine whether the increased β-catenin levels that are
PI 3-kinase Mediates Akt Activation in STHdh<sup>Q111/Q111</sup> Striatal Cells—To explore the basis of enhanced Akt signaling in STHdh<sup>Q111/Q111</sup> striatal cells, we analyzed the upstream signaling molecules that regulate Akt activation. PDK1, the Ser/Thr kinase that transduces the PI 3-kinase signal and regulates Akt phosphorylation, is activated by phosphorylation at Ser<sup>473</sup>. Phosphatase-PTEN, which blocks PI 3-kinase signaling, thereby reducing Akt activation, is activated by phosphorylation at Ser<sup>400</sup> and Thr<sup>370</sup>. Ser<sup>400</sup>/Thr<sup>370</sup>-dual-specific ATP phosphatase activity was detected in STHdh<sup>Q111/Q111</sup> cell extracts. Thr<sup>370</sup>-specific immunoblot analysis of cytosolic protein extracts from wild type and mutant striatal cells suggested a decreased level of Thr<sup>370</sup>-Ser<sup>400</sup> activity. Immunoblot analysis of total cell extracts from wild type and mutant striatal cells demonstrated a decrease in PI 3-kinase-dependent Akt phosphorylation at Thr<sup>370</sup> in mutant compared with wild type cells. 

To confirm PI 3-kinase regulation of Akt activation, wild type and mutant striatal cells were treated with LY294002, a selective inhibitor of PI 3-kinases. The immunoblot results presented in Fig. 5B demonstrate that pretreatment of cells with LY294002 completely prevented Akt phosphorylation at Ser<sup>473</sup> in wild type and mutant cell extracts, whereas the total levels of Akt were not altered. Thus, as it is in wild type striatal cells, Akt activation in mutant striatal cells is appropriately regulated by PI 3-kinase. This finding implies that increased Akt activation in mutant cells may reflect enhanced upstream receptor signaling.

MK-801 Blocks Akt Phosphorylation in STHdh<sup>Q111/Q111</sup> Striatal Cells—Consequently, when evidence of excitotoxicity in HD patient cells (14, 15) and genetic knock-in mouse models (5–8), we tested whether increased activation of Akt in STHdh<sup>Q111/Q111</sup> striatal cells might be mediated through NMDA receptor signaling. Wild type STHdh<sup>Q111/Q111</sup> and mutant STHdh<sup>Q111/Q111</sup> striatal cells express NMDA receptor subunits, NMDAR1, NMDAR2A, and NMDAR2B, as demonstrated by immunoblot analyses of proteins extracted from cell membrane fractions (Fig. 6A). To test whether Akt activation is associated with NMDA receptor activity, wild type and mutant striatal cells were treated with MK-801, a specific NMDA receptor antagonist that blocks Ca<sup>2+</sup> influx associated with NMDA receptor signaling. Akt activation was judged by monitoring the levels of Ser<sup>473</sup>-Akt in total cell extracts as assayed by immunoblot.
munoblot analyses. The results presented in Fig. 6B reveal that phosphorylation of Akt at Ser173 in STHdhQ111/Q111 cell extracts was dramatically decreased (−10-fold, p = 0.0002) by treatment with MK-801. By contrast, the same MK-801 pretreatment had no significant effect (−1.2-fold, p = 0.1) on the levels of Ser(P)473 Akt in wild type cell extracts. These data indicate that Akt activation in mutant but not wild type cells is directly associated with stimulation of NMDA receptors.

Therefore, to address whether Ca2+ influx through the NMDA receptor pore might be involved in Akt phosphorylation in mutant cells, EGTA (400 μM) was added to the growth medium to chelate extracellular Ca2+. Immunoblot analyses of protein extracts prepared from untreated and treated mutant cells (Fig. 6C) revealed that EGTA treatment reduced the levels of phospho-Akt by −3-fold (p = 0.001). These data strongly implicate Ca2+ influx via NMDA receptors in the PI 3-kinase-dependent enhanced activation of Akt signaling in STHdhQ111/Q111 cells.

**DISCUSSION**

HD is a chronic neurodegenerative disorder that is initiated by a novel property that is conferred on mutant huntingtin by a lengthened amino-terminal polyglutamine segment. Evidence from HD patient samples and precise genetic Hdh CAG knock-in mouse and STHdhQ111/Q111 striatal neuronal cell models has revealed abnormal biochemical phenotypes that suggest excitotoxicity and loss of neuroprotective factors that must precede later pathologic markers and even later neuronal cell death.

Now our data demonstrate that mutant huntingtin leads to enhanced activation of protein kinase B/Akt signaling via GSK3β inhibition and β-catenin stabilization. These data are consistent with a pro-survival response. For example, constitutive β-catenin signaling via LEF-1 has been shown to protect against neuronal cell death induced by the toxic amyloid protein (21). Therefore, enhanced β-catenin signaling via LEF-1 may be expected to protect mutant STHdhQ111/Q111 striatal cells from the effects of mutant huntingtin. Notably, the precise downstream β-catenin target genes that mediate neuronal cell survival remain to be elucidated.

In STHdhQ111/Q111 mutant striatal cells, the activation of Akt is appropriately dependent on PI 3-kinase and Ca2+. However, in contrast to Akt activation in wild type striatal cells, the enhanced Akt activation that is observed in mutant striatal cells can be abrogated by MK-801 and therefore is largely determined by Ca2+ influx via the NMDA receptor. Thus, as depicted in Fig. 7, our results link the PI 3-kinase-dependent activation of the Akt pathway with the enhanced NMDA receptor activation and mitochondrial deficits that support excitotoxicity in mutant striatal cells (7, 8, 14, 22). In the latter long standing hypothesis, decrements in mitochondrial calcium handling and ATP synthesis lead to lowered resting membrane potential alleviating the voltage-dependent Mg2+ blockade on the NMDA receptor, permitting Ca2+ influx via the NMDA receptor at physiologic glutamate concentrations (22–25). Therefore, the early disease cascade that is initiated by mutant huntingtin comprises a mixture of events. Some of these can lead to detrimental consequences, whereas other events represent the recruitment of signaling pathways that are activated in response to these deficits. Thus, the pro-survival Akt pathway is activated as an early disease event in mutant striatal neuronal cells in conjunction with NMDA receptor-mediated excitotoxicity.

Importantly, these consequences of mutant huntingtin are manifest before mutant striatal neuronal cells become more severely compromised and start to exhibit markers of overt pathology (6–8). Therefore, in genetically accurate HD models, in vivo and in cell culture, the end products of cellular dysfunc-

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