**Inhibition of Lipid Signaling Enzyme Diacylglycerol Kinase ε Attenuates Mutant Huntingtin Toxicity**

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**Background:** A chemical screen using a kinase inhibitor library was carried out in a Huntington disease cellular model.

**Results:** A target kinase, diacylglycerol kinase ε, when inhibited blocked mutant huntingtin toxicity.

**Conclusion:** Inhibition of diacylglycerol kinase ε through pharmacological or siRNA knockdown prevents mutant Htt activation of caspase-3 and decreased levels of phosphoinositides.

**Significance:** Diacylglycerol kinase ε is a novel therapeutic target for Huntington disease.

Huntington disease (HD) is a dominantly inherited neurodegenerative disease caused by a polyglutamine expansion in the protein huntingtin (Htt). Striatal and cortical neuronal loss are prominent features of this disease. No disease-modifying treatments have been discovered for HD. To identify new therapeutic targets in HD, we screened a kinase inhibitor library for molecules that block mutant Htt cellular toxicity in a mouse HD striatal cell model, *Hdh*<sup>111Q/111Q</sup> cells. We found that diacylglycerol kinase (DGK) inhibitor II (R59949) decreased caspase-3/7 activity after serum withdrawal in striatal *Hdh*<sup>111Q/111Q</sup> cells. In addition, R59949 decreased the accumulation of a 513-amino acid N-terminal Htt fragment processed by caspase-3 and blocked alterations in lipid metabolism during serum withdrawal. To identify the diacylglycerol kinase mediating this effect, we knocked down all four DGK isoforms expressed in the brain (*β*, *γ*, *ε*, and *ζ*) using siRNA. Only the knockdown of the family member, DGKe, blocked striatal *Hdh*<sup>111Q/111Q</sup>-mediated toxicity. We also investigated the significance of these findings in vivo. First, we found that reduced function of the *Drosophila* DGKe homolog significantly improves Htt-induced motor dysfunction in a fly model of HD. In addition, we find that the levels of DGKe are increased in the striatum of R6/2 HD transgenic mice when compared with littermate controls. Together, these findings indicate that increased levels of kinase DGKe contribute to HD pathogenesis and suggest that reducing its levels or activity is a potential therapy for HD.

A pathological change in HD brain is the massive loss of medium spiny neurons in the striatum and loss of neurons in the cortex as the disease progresses. HD results in chorea, dementia, and eventually death. As there is no cure for HD, new therapeutic treatments are clearly needed. Protein kinases are one of the two most important groups of drug targets, after G protein-coupled receptors. The kinase superfamily, whose members are related in the sequence and structure of their catalytic domain, is the second most important group of drug targets, after G protein-coupled receptors. DGKs consist of a family of kinases that catalyze the phosphorylation of diacylglycerol (DAG) to produce phosphatidic acid (PA) (2). Both DAG and PA are important intracellular signaling molecules (2). DAG can activate protein kinase C (PKC), Ras guanyl nucleotide-releasing protein (RasGRP), and transient receptor potential channels (3, 4). Transient receptor potential channels mediate store-operated calcium entry into HD medium spiny neurons, and recently, compounds that target these channels have been shown to block HD neurotoxicity (5). There are 10 human DGK isoforms, and each has unique biochemical properties, expression patterns, and subcellular localization. These enzymes have a DAG binding C1 and catalytic domain. DGKe is unique in that it has a preference for substrate selection. DGKe favors DAG with 1-stearoyl-2-araachidonoyl (18:0, 20:4) acyl chains, which represent the prevalent acyl chain composition in phosphatidylinositol (PI) and phosphatidylcholine (PC).
its derivatives (6). The DGKε knock-out mice showed reduced seizure in response to electroconvulsive shock (7). Also, DGKε knock-out mice had lower content of PI with 1-stearoyl-2-arachidonoyl acyl chains (8).

Phosphatidylinositol and phosphatidylinositol phosphates (PIPs) have been implicated in the pathological mechanisms of HD. A vesicle-binding assay has shown that Htt binds different forms of PIPs, whereas the mutant Htt bound to a subset of PIPs more strongly than wild type Htt (9). Mutant Htt has also been shown to sensitize the type I inositol (1,4,5)-triphosphate receptor to inositol (1,4,5)-triphosphate, which is a second messenger derived from phosphatidylinositol (4,5)-bis-phosphate (PIP2) (10). When the C-terminal fragment of the type 1 inositol (1,4,5)-triphosphate receptor has been introduced to the striatum of a HD model mouse, neuroprotective effects have been observed (11). However, whether the level of 1-stearoyl-2-arachidonoyl PI and PIPs is related directly with mutant Htt-associated neurotoxicity has not been determined.

To identify potential HD therapeutic targets, we screened a kinase inhibitor library in striatal Hdh111Q/111Q mouse HD cell model. The Hdh111Q/111Q cells are immortalized striatal cells derived from a knock-in mouse model that expresses a mutant form of Htt with an expanded polyglutamine tract of 111 repeats (12). This cell line, together with the normal control line Hdh7Q/7Q cells, represents a commonly used mouse HD cell model. Using multiple assays, we identified DGK inhibitor II, also known as R59949, as an effective compound decreasing toxicity associated with mutant Htt. We evaluated whether DGK inhibition affected lipid metabolism in our Hdh111Q/111Q cellular assay. Indeed, DGK inhibition normalizes altered lipid metabolism in this HD cell model. Analysis of the 10 mouse DGK isoforms in striatal cells and tissue suggests that four family members are expressed highly in the striatum. One family member, DGKε, prevents cell death when knocked down in Hdh111Q/111Q cells. Correspondingly, partial loss of function of the Drosophila homolog of DGKε significantly improves Htt-induced motor dysfunction in a fly model of HD in vivo. Our work provides novel mechanisms of neurotoxicity from mutant Htt in HD and identifies DGKε as a therapeutic target for HD.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—All cell culture reagents were from Invitrogen unless otherwise stated. Striatal HdhQ7/Q7 and HdhQ111/Q111 cells were maintained in DMEM supplemented with 10% FBS at 33 °C. Culture plates were coated with 50 μg/ml rat tail collagen type-I (BD Biosciences) for 1 h at room temperature before immortalized striatal cells were seeded for experiments. For caspase-3/7 activity assay, Biocoat collagen I-coated 96-well plates from BD Biosciences were used. Knocking down DGK genes was achieved by transfecting corresponding Dharmacon siGENOME SMARTpool siRNAs (Thermo Scientific) via Amaxa Nucleofector kit L (Lonza).

**Caspase-3/7 Activity Assay**—The caspase activity assay was performed with Apo3 HTS kit (Cell Technology). Hdh111Q/111Q cells cultured in 96-well plates were challenged with serum deprivation along with drug treatments. We used the EMD Calbiochem InhibitorSelect™ 96-well protein kinase inhibitor library I (catalog no. 539744). Inhibitors were dissolved in dimethyl sulfoxide, and the final concentration in the treatment well was 20 μM for all kinase inhibitors except phosphatidylinositol 3-kinase γ inhibitor (10 μM for this kinase inhibitor). DGK inhibitor I (R50922) was purchased from EMD Calbiochem. Control cells were treated with 0.2% dimethyl sulfoxide, the same as the kinase inhibitor-treated cells for 24 h. Medium was removed, and 50 μl of 1× lysis buffer was added to each well. After shaking on an orbital shaker at 700 rpm for 5 min, two 10-μl aliquots of lysate were transferred to new 96-well plates for BCA protein concentration measurement, and 70 μl of substrate mix (1× lysis buffer with 1× Apo3 HTS caspase-3/7 detection reagent and 20 mM DTT) was added into the remaining 30 μl of lysate in each well. The plate was briefly shaken at 700 rpm for 30 s before reading. The Fusion-Alpha Universal Microplate Analyzer (PerkinElmer Life Sciences) was used for this fluorescence-based assay (excitation, 485 nm; emission, 530 nm) at 37 °C. For each sample, the protein concentration was measured in duplicate with Pierce BCA protein assay kit (Thermo Scientific). The caspase activity was normalized against protein concentration for each sample.

**WST Assay**—Hdh111Q/111Q cells were cultured in 96-well plates as described above. Then the medium was removed, and 100 μl 1× WST-8 reagent (Alexis) was added into each well. The cells were incubated for 2 h at 33 °C before absorbance at 450 nm was measured on a SpectraMax 190 plate reader (Molecular Devices).

**Western Blotting**—Hdh111Q/111Q cells were treated with serum starvation for 24 h in the presence or absence of drugs. Then cells were scraped off, pelleted, and washed once with PBS (Cellgro). Cell pellets were lysed by sonication in mammalian protein extraction reagent (from Thermo Scientific) containing protease inhibitors (one Complete mini tablet per 10 ml lysing buffer with 10 μg/ml aprotinin, 1 mM PMSF, 1 mM EDTA). Lysates were sonicated for 10 min, and cell debris was removed by centrifugation at 13,000 × g for 10 min at 4 °C. Lysates were kept at −80 °C until electrophoresis. Lysates were diluted with 1% BSA in PBS, and 50 μl of lysate in each well. The plate was briefly shaken at 700 rpm for 30 s before reading. The Fusion-Alpha Universal Microplate Analyzer (PerkinElmer Life Sciences) was used for this fluorescence-based assay (excitation, 485 nm; emission, 530 nm) at 37 °C. For each sample, the protein concentration was measured in duplicate with Pierce BCA protein assay kit to ensure equal sample loading. Protein samples (40 μg) were run on 4−12% Bis-Tris gel (Invitrogen) at 200 V for 1 h, transferred to nitrocellulose membrane (Whatman) at constant 20 V for 14 h at 4 °C, probed with anti-Htt (1:500, MAB2166, Millipore), anti-HttNeo513 (1:750, polyclonal generated as described previously (13) Open Biosystems), and anti-GAPDH (1:10,000, Fitzgerald).

**Immunocytochemistry**—Treated cells on glass coverslips (Belco Glass) were fixed by 4% paraformaldehyde for 20 min at room temperature. Fixed cells were permeabilized with 0.1% Triton X-100 (Sigma) for 10 min and blocked with 5% donkey serum (Millipore) in PBS for 1 h. Primary antibodies were diluted with 1% BSA (Roche Applied Science) in PBS. The primary antibody (anti-PIP, 1:200 from Echelon) was incubated overnight at 4 °C. After three washes with PBS, the secondary antibody (Alexa Fluor 488-conjugated donkey anti-mouse IgG, 1:1000 from Invitrogen) was added for 1.5 h incubation at room temperature. Coverslips with stained cells were finally mounted with Prolong Gold Antifade reagent with DAPI (Invitrogen) onto glass slides (VWR) for microscopy.

**Reverse Transcription Quantitative PCR (RT-qPCR)**—Total RNA was isolated from striatal cells or mouse tissue (10.5 weeks) with the RNeasy mini kit (Qiagen) according to the
Mass Spectrometry MRM-MS Analysis—Samples were analyzed by MRM-MS on a 4000 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer (AB Sciex) equipped with a Turbo IonSpray source in negative ion mode. Lipid samples were introduced by direct infusion using a syringe pump (Mode 11 series, Harvard Apparatus) at a flow rate of 10 μl/min. A total of 18 ion transition pairs from six base phosphoinositides (34:1, 36:1, 36:4, 38:4, 38:5, and 40:6) with one, two, or three phosphate moieties (PI, PIP, and PIP₂) were quantified by MRM-MS. The negatively charged parent ion was used as the Q1 mass, and the diagnostic fragment ion of the dehydrated headgroup was chosen for Q3 (15), which differentiates phosphoinositides with different fatty acid compositions (supplemental Fig. 1). The Q1 m/z, Q3 m/z, dwell time, declustering potential, collision energy, and collision cell exit potential for each transition are listed in supplemental Table 1. Inositol 1,4,5-trisphosphate species were not included in the study due to their extremely low abundance. Data were collected with an ion spray voltage of ~4500 V, curtain gas of 10 psi, nebulizer gas of 25 psi, and an interface heater temperature of 600 °C. MRM-MS transitions were acquired and monitored at unit resolution in both Q1 and Q3. Each sample was typically infused for 3–6 min, and each sample was analyzed two times with the average signal of the runs reported. Quantification was performed using Analyst (version 1.5), and each transition was integrated individually. The quantification results were normalized against cell numbers counted by Z1 Coulter Particle Counter (Beckman Coulter).

Drosophila Motor Performance Analysis—Tests were carried out using 15 age-matched virgin females. Animals were placed in an empty vial and tapped down. The number of animals able to climb 9 cm after 15 s was recorded as a percentage of the total. This was repeated 10 consecutive times, and the average of the ten observations was plotted for each day shown in the chart. Two replicates were tested in parallel for each genotype. Animals were raised at 26.5 °C. The nervous system driver line elav-GAL4 (155) was obtained from the Bloomington Drosophila Stock Center at University of Indiana. The inducible shRNA line targeting the Drosophila homolog of DGKε (4659GD) was obtained from the Vienna Drosophila RNAi Center. NT-Htt112Q animals express an N-terminal Htt fragment comprising exons 1–4 (first 336 amino acids with 128Q polyglutamine expansion) have been described previously (16).

RESULTS

Kinase Inhibitor Library Screening Identifies DGK Inhibitor II as Therapeutic Target for HD—We screened a kinase inhibitor library that contained 80 different inhibitors (see supplemental Table 2) in the mouse HdhQ111Q/Q111Q cell model utilizing caspase activity and WST-8 as endpoints during serum withdrawal (Fig. 1). As reported previously, serum withdrawal results in a dramatic increase in caspase activity in HdhQ111Q/Q111Q when compared with HdhQ7Q/Q7Q cells (13, 17). We found several inhibitors that reduce caspase-3/7 activity when HdhQ111Q/Q111Q cells undergo serum withdrawal. These include Akt inhibitor X, PDK1/Akt/Fkt dual pathway inhibitor, chelerythrine chloride, DGK inhibitor II, IGF-1R inhibitor II, Lck inhibitor, PDGF RTK inhibitor III, PKCβ II/EGFR inhibitor, rapamycin, SU11652,
VEGF receptor 3 kinase inhibitor, VEGF receptor 2 kinase inhibitor III, and VEGF receptor 2 kinase inhibitor IV (Fig. 1A).

However, when coupled with WST-8 assay, which measures bioreduction of WST-8 mostly dependent on the glycolytic NAD(P)H production of viable cells, DGK inhibitor II (R59949) and rapamycin were the only kinase inhibitors that protected

FIGURE 1. Screening of the kinase inhibitor library identified DGK inhibitor as a potential drug candidate. A, the screening of the kinase inhibitor library in Hdh\textsuperscript{111Q/111Q} cells during serum withdrawal using caspase activity assay as an end point. Several kinase inhibitor hits, including DGK inhibitor II, decreased caspase-3/7 activity. B, screening the same library using viability WST-8 assay library in Hdh\textsuperscript{111Q/111Q} cells during serum withdrawal. The WST-8 assay and caspase activity assay identified DGK inhibitor II along with rapamycin, a known protective molecule, as a hit. See supplemental Table 2 for further details of kinase inhibitors used in this screen. neg contr, negative control; pos contr, positive control; DMSO, dimethyl sulfoxide. The compounds are defined in supplemental Table 2.
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Mutant Huntingtin Proteolysis Is Reduced in Cells Treated with DGK Inhibitor II—The proteolysis of mutant Htt by caspases to generate the toxic N-terminal fragments is linked directly to the cell death mechanisms in HD. In Hdh^{111Q/111Q} cells, proteolysis of Htt was detected upon serum withdrawal (Fig. 2B, lane 2) using Western blot analysis. Addition of either DGK inhibitor I or II reduced the level of Htt proteolysis (Fig. 2B, lanes 3, 4, and 6). Using an antibody specific to the caspase-3 cleavage site at the 513 amino acid of Htt (NeoHtt513), we found that Htt proteolysis at this site was induced by serum starvation (Fig. 2B, lane 2) and reduced by treatment with DGK inhibitors (Fig. 2B, lanes 3, 4, and 6). We did not detect cleavage of Htt at amino acids 552 or 586 using neoHtt552 or neoHtt586 under these conditions (data not shown). Immunocytochemistry revealed that cleaved active caspase-3 was induced in Hdh^{111Q/111Q} cells upon serum withdrawal (data not shown). Treatment with DGK inhibitor II prevented the generation of cleaved caspase-3 (data not shown).

Protective Effect of DGK Inhibitors in HD Does Not Require PKC—The reaction catalyzed by DGKs is the phosphorylation of DAG into PA. DAG is a well known PKC activator, and the inhibition of DGKs would likely lead to decreased DAG consumption and increased PKC signaling. To test whether this could be the mechanism underlying the protective effects of DGK inhibitors, we treated cells with two PKC inhibitors, bisindolylmaleimide I and G66983. Both bisindolylmaleimide I and G66983 inhibit PKC isozymes belonging to conventional and novel PKC subfamilies, the two subgroups of PKCs responsive to DAG. Caspase activity, WST-8 and mutant Htt proteolysis were evaluated during serum withdrawal conditions with these two inhibitors. The protective effect achieved by DGK inhibitor II treatment in Hdh^{111Q/111Q} cells was not altered by co-treatment of bisindolylmaleimide I or G66983 using caspase activity (Fig. 3A), WST-8 (Fig. 3B) or the proteolysis of Htt (Fig. 3C) as end points. DGK inhibitor I did not affect Htt proteolysis in Hdh^{7Q/7Q} cells in the presence or absence of PKC inhibitors as expected (supplemental Fig. 3). Both inhibitors showed strong inhibition of PKCs as measured by their effects on phorbol ester-induced PKC activation (data not shown). Therefore, the protective effect of DGK inhibitors on Hdh^{111Q/111Q} cells does not require PKC signaling as a downstream mediator.

Altered Phosphoinositides in Hdh^{111Q/111Q} Cells and DGK Inhibition Normalizes Lipid Metabolism During Serum Withdrawal—DGKs are critical enzymes involved in lipid metabolism. When certain DGK family members are knocked out in mice, a decrease in polyphosphoinositides is observed (8). Therefore, a critical mechanism for rescue of HD cytotoxicity may be through altered lipid metabolism. Lipid profiling of cellular phosphatidylinositol monophosphate/phosphatidylinositol biphosphate (PI/PIP/PIP2) was carried out using mass spectrometry in total extracts from Hdh^{7Q/7Q} and Hdh^{111Q/111Q}. Representative MALDI-MS spectra are shown in supplemental Fig. 4–8. The predominant species is PI, but we were also able to detect PIP and PIP2. We also noted that the distribution and pattern between the Hdh^{7Q/7Q} and Hdh^{111Q/111Q} extracts is distinct for these lipids (data not shown).

Having established that the PI, PIP, and PIP2 could be detected by MALDI-MS, we quantified the levels in Hdh^{7Q/7Q} and Hdh^{111Q/111Q} cells against serum withdrawal (Fig. 1B), thus indicating that these compounds act specifically on Hdh^{111Q/111Q}-induced toxicity. Rapamycin has been reported to reduce HD-associated toxicity through induction of autophagy (18). The identification of DGK inhibitor II as blocking mutant Htt toxicity is novel, and the mechanism of action for this effect is not known. To confirm that other DGK inhibitors were effective in this assay, we tested another DGK inhibitor, DGK inhibitor I (also known as R59022). As shown in Fig. 2A, both DGK inhibitors showed reduced caspase activity in a dose-dependent manner, suggesting a role for DGKs in toxicity caused by the polyglutamine mutation in Htt. Hdh^{7Q/7Q} cells generally show very modest caspase activation after serum starvation in 24 h, and DGK inhibitors do not alter the caspase-3/7 activity in serum starved Hdh^{7Q/7Q} cells (supplemental Fig. 2). Given this result, we utilized Hdh^{111Q/111Q} cells for most of our studies.

**Figure 2.** Both DGK inhibitor I (R59022) and II (R59949) reduced caspase activity and mutant Htt proteolysis in Hdh^{111Q/111Q} cells undergoing serum withdrawal. A, retesting DGK inhibitor II and the evaluation of related compound DGK inhibitor I confirmed the protective effect of these kinase inhibitors in Hdh^{111Q/111Q} cells. We found a dose-dependent reduction of caspase-3/7 activity in Hdh^{111Q/111Q} cells after serum withdrawal with DGK inhibitor I and II. DGK inhibitor treatment conditions were compared with FBS withdrawal only condition by one-way analysis of variance (*, p < 0.05; **, p < 0.01; ***, p < 0.005 for all figures). B, Hdh^{111Q/111Q} cells were treated with different doses of DGK inhibitor I or II during serum starvation. Western analysis demonstrates that serum withdrawal produces huntingtin product detected by Htt 2166 and neoHtt513 antibody, and this fragment was reduced by DGK inhibitors at 10 µM. Control levels of GAPDH suggest equal protein loading. Quantification of the Htt fragment bands was also shown.

Hdh^{111Q/111Q} cells
and Hdh$^{111Q/111Q}$ cells with or without DGK inhibitor II using MRM-MS (see supplemental Table 1 for MRM transitions). As shown in Fig. 4A, the levels of PI (38:4) are distinct in the Hdh$^{7Q/7Q}$ and Hdh$^{111Q/111Q}$ cells. The level of PI (38:4), PIP (38:4), and PIP$_2$ (38:4) in Hdh$^{7Q/7Q}$ cells is 1.8- to 2.4-fold higher, respectively, when compared with Hdh$^{111Q/111Q}$ cells in resting cells. Thus, mutant Htt expression results in lower levels of polyphosphoinositides. Strikingly, cells undergoing serum withdrawal have increased levels of PIP (38:4) and PIP$_2$ (38:4) (Fig. 4B). Treatment with 20 $\mu$M DGK inhibitor II in Hdh$^{111Q/111Q}$ cells reduces the levels of PI(38:4) and PIP$_2$(38:4) to 44 and 54%, respectively (Fig. 4B).

To further analyze alterations in lipid metabolism, we also performed immunocytochemistry on Hdh$^{7Q/7Q}$ and Hdh$^{111Q/111Q}$ cells using a pan-lipid antibody that recognizes PIPs. As shown in Fig. 4C, we detected less PIP staining in Hdh$^{111Q/111Q}$ cells than in Hdh$^{7Q/7Q}$ cells with or without DGK inhibitor II using Western blotting. Serum-starved Hdh$^{111Q/111Q}$ cells were co-treated with 10 $\mu$M DGK inhibitor II (DGKi) with or without 1 $\mu$M bisindolylmaleimide I or 1 $\mu$M G66983. Western blot using the antibody Htt2166 against Htt was performed.

Identification of DGK e as Therapeutic Target for HD—There are 10 DGK mammalian isoforms, and therefore it is possible that one or several of these family members are possible therapeutic targets of the DGK inhibitors in the context of HD toxicity. Five DGK isoforms, $\alpha$, $\beta$, $\gamma$, $\epsilon$, and $\xi$ have been shown to be expressed in the brain (19). To identify the isoforms most abundant in the striatum, we used RT-qPCR to quantify the isoforms in this region of the brain. $\beta$, $\gamma$, $\epsilon$, and $\xi$ DGK isoforms were expressed in the striatum (Fig. 5). We then knocked down these four DGKs in striatal Hdh$^{111Q/111Q}$ cells individually with siRNA and performed the caspase activity assay after serum starvation. Only the siRNA specific to DGKe resulted in decreased caspase-3/7 activity, similar to treatment of DGK inhibitors (Fig. 6A, data not shown for other DGKs). We confirmed the knockdown of DGKe using RT-qPCR (Fig. 6B), and the level was similar in magnitude to that found for the reduction in caspase-3/7 activity (Fig. 6A).

We also investigated whether the levels of DGKe are altered in response to mutant Htt. RT-qPCR for DGKe showed that Hdh$^{111Q/111Q}$ cells have higher levels of DGKe than Hdh$^{7Q/7Q}$ cells (Fig. 6C), indicating that DGKe levels are increased in response to mutant Htt.

In Vivo Evidence for DGKe Reduction in Levels of DGK $\epsilon$ Homolog Improves Neuronal Function in Drosophila Model of HD—We next sought to validate this observation in vivo. We found that in the striatum of R6/2 HD transgenic mice, a HD mouse model with overexpression of N-terminal fragment of Htt containing $\sim$120 polyglutamines, the levels of DGKe are higher than in the striatum of littermate control mice (Fig. 7A). These results suggest a specific role of DGKe in HD toxicity.
The above data suggest that DGKε is involved in HD and may function as a suppressor of Htt-induced toxicity in vivo. To investigate this possibility, we studied the effect of decreasing DGKε levels in a well established Drosophila HD model (16). Expression of N-terminal Htt with a 128Q (NT-Htt128Q) expansion in the Drosophila nervous system (using the neuronal elav-GAL4 driver) leads to progressive motor performance deficits when compared with normal animals (Fig. 7B, compare blue discontinuous line with black dotted lines). Consistent with Hdh111Q/111Q cell data, decreasing the levels of the Drosophila DGKε in the nervous system by means of an inducible shRNA line (NT-Htt128Q/DGKe<sub>shRNA</sub> 4659GD), significantly ameliorates the toxic effects of expanded Htt (Fig. 7B, compare solid red lines with black dotted lines). NT-Htt128Q/DGKe<sub>shRNA</sub> 4659GD show a delay in disease onset, and they perform better than animals expressing NT-Htt128Q with normal levels of DGKε. These data, together with the observation in the striatum of R6/2 mice, confirm that the Htt-DGKε interaction is relevant in vivo.

**DISCUSSION**

To identify new therapeutic targets in HD, we screened a kinase inhibitor library for molecules that block mutant Htt cellular toxicity. We found that DGK inhibitor II decreased caspase-3/7...
activity after serum withdrawal in striatal \( \text{Hdh}^{111Q/111Q} \) cells. Our screen also identified rapamycin as a kinase inhibitor that blocked mutant Htt-mediated cell death. The DGK inhibitor II decreased the level of cleaved caspase-3, the accumulation of the 513-amino acid N-terminal Htt fragment processed by caspase-3, and blocked alterations in lipid metabolism during serum withdrawal. We also tested the DGK inhibitor I, which was not in the initial library of 80 compounds, and this compound was effective as well.

There are 10 distinct mammalian isoforms of DGKs. To identify the possible diacylglycerol kinase family member that was responsible for rescuing striatal \( \text{Hdh}^{111Q/111Q} \)-mediated toxicity, we knocked down all four DGK isoforms expressed in the brain (\( \text{H9252}, \text{H9253}, \text{H9280}, \text{H9256} \)) using siRNA. Only the knockdown of the family member, DGK \( \text{H9280} \), blocked striatal \( \text{Hdh}^{111Q/111Q} \)-mediated toxicity.

FIGURE 5. Expression of DGK isoforms in mouse striatum. Total RNA sample from mouse striatum tissue was subjected to RT-qPCR for all 10 mammalian DGK isoforms. Actb stands for \( \beta \)-actin, a housekeeping gene that we used for normalization. \( \beta, \gamma, \epsilon, \) and \( \xi \) DGKs showed relatively higher expression levels. rel., relative.

FIGURE 6. DGKe in immortalized \( \text{Hdh}^{111Q/111Q} \) cells. A, DGKe was knocked down by siRNA in \( \text{Hdh}^{111Q/111Q} \) cells before serum withdrawal. Caspase activity showed decreased levels of caspase-3/7 activity with siRNA specific to DGKe compared with non-targeting (NT) siRNA in serum-starved \( \text{Hdh}^{111Q/111Q} \) cells. B, RT-qPCR confirmed knockdown of DGKe in \( \text{Hdh}^{111Q/111Q} \) cells. C, RT-qPCR showed higher expression level of DGKe in \( \text{Hdh}^{111Q/111Q} \) cells than in normal \( \text{Hdh}^{7Q/7Q} \) cells. Serum starvation further increased DGKe expression. One-way analysis of variance was used for statistical analysis. rel., relative.

FIGURE 7. DGKe in mouse and \( \text{Drosophila} \) HD models. A, RT-qPCR showed higher DGKe level in transgenic HD R6/2 striatum than in wild type (WT) mouse striatum tissue at the age of 10.5 weeks (Student’s \( t \) test, \( p < 0.001 \)). B, reduction in DGKe homolog improves neuronal function in a \( \text{Drosophila} \) model of HD. Chart represents motor performance as a function of age in control flies and flies expressing NT-Htt128Q either alone or together with an inducible siRNA targeting the \( \text{Drosophila} \) DGKe homolog (CG8657), in the nervous system. Test measures the ability of the animals to climb 9.5 cm in 15 s (see “Experimental Procedures” for details). Control animals (blue dashed line) perform well in the motor performance test for the complete duration of the experiment. Animals expressing NT-Htt128Q in the nervous system show progressive impairment of motor performance starting at day 12 (black dotted line). Animals expressing NT-Htt128Q in the nervous system together with an shRNA targeting the \( \text{Drosophila} \) DGKe homolog show a suppression of the Htt-induced motor performance impairment (solid red lines). Notice that the Htt128Q/DGKe\textsuperscript{shiRNA} 4659GD animals show delayed disease onset (impairments begin at day 14) and continue to perform better than the NT-Htt128Q for the remainder of the experiment. Genotypes are as follows: normal control, \( \text{Elav-GAL4/w1118; NT-Htt128Q} \); \( \text{Elav-GAL4/w1118; NT-Htt128Q/+}\),\textsuperscript{+}; \( \text{UAS-NT-Htt128Q(f33A)/+}; \text{DGKe\textsuperscript{shiRNA}4659GD; Elav-GAL4/w1118; DGKe\textsuperscript{shiRNA}4659GD/+}; \text{UAS-NT-Htt128Q(f33A)/+}. \) Error bars represent S.E. Two experimental replicates are shown for the NT-Htt128Q and the Htt128Q/DGKe\textsuperscript{shiRNA} animals.
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diated toxicity. This isoform DGKe is unique in that it has a hydrophobic domain that facilitates attachment to membranes (20), and furthermore, it has specificity for diacylglycerol containing arachidonyl chains (21, 22). Loss of function of the Dro- sophila DGKe homolog was evaluated in an HD Drosofihia model, and we found significant improvement of Htt-induced motor dysfunction. In addition, the levels of DGKe were higher in the striatum of R6/2 HD transgenic mice when compared with controls, confirming the relevance of the interaction in vivo.

We have discovered that inhibition of the enzyme DGK is protective to immortalized striatal cells expressing mutant Htt. Because the enzymatic activity of this protein is to catalyze the phosphorylation of DAG into PA, we expect that inhibition would lead to a rise in DAG production and decreased PA production. Classically, DGKs are known as regulators of PKC family members. However, we tested PKCs inhibitors and did not find the protection imparted by DGK inhibition was dependent upon PKCs for mutant Htt toxicity.

Because generation of PA through DAG phosphorylation also is the first step in PI resynthesis, we evaluated whether the inhibition of DGK could impact lipid metabolism in Hdh7Q/7Q and Hdh111Q/111Q cells. First, we found that the levels of PI, PIP, and PIP2 in Hdh7Q/7Q cells are higher when compared with Hdh111Q/111Q cells. It is possible that the lower levels of PIs in the Hdh111Q/111Q cells reflect a compensatory mechanism to block striatal cell death. Consistent with this notion, we found that both Hdh7Q/7Q and Hdh111Q/111Q cells undergoing serum withdrawal had elevated levels of PIs. When we evaluated the impact of DGK inhibition on lipid levels in Hdh111Q/111Q cells undergoing serum withdrawal, we found the levels of PIP and PIP2 were reduced significantly. In addition, immunocytochemistry demonstrated an increase in PIP signaling during serum withdrawal, which was blocked by DGK inhibition. This may be relevant to the protective effect of DGK inhibition.

A number of alterations in lipid metabolism have been noted before in HD. This includes lower cholesterol levels in affected areas of the brain (23) and reduced ganglioside (GM1) synthesis (24). Further modulation of GM1 restores normal motor behavior in HD mouse model YAC128 (25). Our findings further emphasize the role of altered lipid metabolism in HD, particularly with respect to phosphatidylinositols, which are already known to have distinct binding to the mutant Htt protein. This may be particularly important as the normal function of Htt appears to be involved in vesicular trafficking, and many protein complexes involved in this process require PIPs. Htt binds different forms of PIPs, whereas the mutant Htt bound to a subset of PIPs more strongly than wild type Htt in vesicle-binding assays (9). Therefore, inhibition of DGKs may alter the interaction of mutant Htt with PIPs.

DGKs levels as measured by RT-qPCR appear to be modulated by polyglutamine expansion in the Htt protein. We found higher levels of DGKe in immortalized striatal cells expressing mutant Htt and in the mouse model of R6/2 HD transgenic mouse model. Furthermore, cells undergoing cell death had increased levels of DGKe. This may increase the levels of PIP2 which is known to regulate cell death through P2X7 receptors (26).

In conclusion, we have identified DGKe as a therapeutic target for HD. We have shown that pharmacological inhibition of DGK activity can improve Htt-induced toxicity in cells. In addition, we show that the abnormal phospholipid levels induced by mutant Htt are restored upon decreasing DGK activity. Finally, we also have observed in vivo that DGKe levels are increased in response to Htt and that decreasing DGKs levels rescues Htt toxicity in an animal model. There are DGKs mutant mice available (7, 8), and our future work will be aimed at how modulation of this enzyme in mammalians affects disease progression and neuropathology in HD mouse models.

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