Inhibition of Metabotropic Glutamate Receptor Signaling by the Huntingtin-binding Protein Optineurin

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Huntington disease is caused by a polyglutamine expansion in the huntingtin protein (Htt) and is associated with excitotoxic death of striatal neurons. Group I metabotropic glutamate receptors (mGluRs) that are coupled to inositol 1,4,5-triphosphate formation and the release of intracellular Ca2+ stores play an important role in regulating neuronal function. We show here that mGluRs interact with the Htt-binding protein optineurin that is also linked to normal pressure open angle glaucoma and, when expressed in HEK 293 cells, optineurin functions to antagonize agonist-stimulated mGluR1a signaling. We find that Htt is co-precipitated with mGluR1a and that mutant Htt functions to facilitate optineurin-mediated attenuation of mGluR1a signaling. In striatal cell lines derived from HttQ111/Q111 mutant knock-in mice mGluR5-stimulated inositol phosphate formation is also severely impaired when compared with striatal cells derived from HttQ7/Q7 knock-in mice. mGluR desensitization and an additional biochemical link between optineurin and mGluR1a, optineurin H486R-dependent attenuation of mGluR1a, is not enhanced by the expression of mutant Htt. Because G protein-coupled receptor kinase 2 (GRK2) protein expression is relatively low in striatal tissue, we propose that optineurin may substitute for GRK2 in the striatum to mediate mGluR desensitization. Taken together, these studies identify a novel mechanism for mGluR desensitization and an additional biochemical link between altered glutamate receptor signaling and Huntington disease.

Huntington disease (HD) is an autosomal-dominant neurodegenerative disorder manifested by symptoms of involuntary body movement, loss of cognitive function, and psychiatric disturbance, which inevitably leads to death (1–4). The HD gene mutation consists of an unstable CAG repeat resulting in a polyglutamine expansion in the amino-terminal region of the huntingtin (Htt) protein, a ubiquitously expressed and evolutionary conserved protein (1). It is the polyglutamine expansion of the Htt amino terminus that is proposed to cause progressive widespread neuronal death in the neocortex and the striatum of HD patients. Although the precise function of Htt in cells is not completely understood, analysis of the proteins with which Htt interacts suggests that Htt plays a role in regulating clathrin-coated vesicle-mediated endocytosis, neuronal survival, vesicle transport, morphogenesis, calcium homeostasis, and transcriptional regulation (4).

Glutamate-mediated neurotoxicity has been postulated to play an important role in the pathogenesis and excitotoxic neuronal cell loss in HD (5–9). The receptors for glutamate are classified into two types: ionotropic and metabotropic (10). The ionotropic receptors comprise cation-specific ion channels that mediate fast excitatory glutamate responses and are subdivided into AMPA/kainate and NMDA receptors. There is a considerable body of evidence to support the idea that glutamate released from cortical afferents may regulate the excitotoxic damage observed in HD by activating both NMDA and AMPA/kainate receptors (5–7, 11, 12). In particular, there is increased sensitivity to NMDA receptor-mediated excitotoxic cell death in a YAC128 transgenic mouse model of HD (11) and NR2A and NR2B receptor gene variations modify the age of HD onset (12). More recently, Group I metabotropic glutamate receptors (mGluRs), which are G protein-coupled receptors (GPCRs) linked to the activation of phospholipase C, increase in intracellular inositol 1,4,5-triphosphate (IP3) formation, and the release of intracellular Ca2+ stores have been proposed to contribute to the underlying pathophysiology of HD. Specifically, the survival of R6/2 HD transgenic mice is significantly increased following treatment with mGluR5 antagonists (13). In addition, mutant Htt and Htt-associated protein 1 interactions with the IP3 receptor result in altered mGluR5-stimulated Ca2+ signaling (14). This increased Ca2+ release is associated with increased apoptosis of medium spiny striatal neurons derived from a YAC128 transgenic mouse model of HD (15). Thus, glutamate signaling via both ionotropic and metabotropic receptors may be linked with Htt function and HD.

Optineurin (OPTN) is one of a number of recently identified Htt-interacting proteins (4). OPTN is a coiled-coiled protein that was first identified as a positive regulator of tumor necrosis factor-α-mediated apoptosis (16). OPTN interacts with a variety of proteins that link it to the regulation of cellular morphogenesis and membrane trafficking (Rab8), vesicular trafficking (Htt), and transcription activation (TFIIA) (17–19). Mutations in OPTN are responsible for ~17% of hereditary forms of normal tension glaucoma (20). The link between OPTN, apoptosis, and the retinal degeneration observed in glaucoma has lead to the suggestion that OPTN mutants may directly induce optic neuropathy leading to visual loss (20). This has lead to the suggestion that OPTN

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4The abbreviations used are: HD, Huntington disease; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; Htt, huntingtin; IP3, inositol 1,4,5-triphosphate; mGluR, metabotropic glutamate receptor; OPTN, optineurin; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA, N-methyl-D-aspartate; HA, hemagglutinin; MITT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; GST, glutathione S-transferase; HBSS, HEPES-buffered saline solution; PBS, phosphate-buffered saline; C-tail, carboxyl-terminal tail; N-Htt, amino-terminal domain of Htt.

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represents a common factor involved in protection against neuronal cell death. Here we show that OPTN is also a Group I mGluR-interacting protein that functions to inhibit mGluR G protein coupling to phospholipase C and IP₃ signaling. Moreover, we show that polyglutamine-expanded mutant Htt but not wild-type Htt potentiates the inhibition of mGluR signaling. The association of OPTN with both Htt and mGluRs may provide an additional biochemical link that may help shed light upon the relationship between glutamate-induced neurotoxicity and HD.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human embryonic kidney cells (HEK 293) were provided by the American Type Culture Collection (Manassas, VA). Tissue culture medium was from Invitrogen. Bovine serum albumin was obtained from Roche Applied Science. Horseradish peroxidase-conjugated anti-mouse IgG secondary antibodies were from Amersham Biosciences. Quisqualate was purchased from Tocris Cookson Inc. (Ellisville, MO). [3H]myo-inositol was acquired from PerkinElmer Life Sciences. The Dowex 1-X8 (formic form) resin with 200–400 mesh was purchased from Bio-Rad. LysoTracker Red was purchased from Molecular Probes (Eugene, OR). Fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody, mouse anti-FLAG M2 monoclonal antibody, and all other biochemical reagents were purchased from Sigma.

**Yeast Two-hybrid Screening**—A human brain cDNA library in the pGAD10 vector, used in the two-hybrid screen, was purchased from Clontech. pGAD10 contains the ADH promoter expressing the GAL4 transactivation domain (amino acids 768 to 881). The yeast reporter strain Y190 harbors two reporter gene constructs. Two-hybrid interactions activate the transcription of the HIS3 gene, allowing screening for growth in the absence of histidine, and of the lacZ gene, allowing screening for β-galactosidase activity. Expression of the mGluR1α-Ct-GAL4-binding domain fusion protein from pAS2-mGluR1-Ct in yeast strain Y190 was verified by immunoblotting with anti-mGluR1α antibodies (Upstate Biotechnology, Inc., Lake Placid, NY). Y190 was cotransfected with pAS2-mGluR1-Ct and the pGAD10-human brain cDNA library and plated at a density of 5 × 10⁴ colonies/plate on synthetic minimal medium lacking leucine and tryptophan (for plasmid Htt constructs were then digested with BamHI and NotI. The Htt-pGEX4T1 (Amersham Biosciences) digested with XmaI. The pGEX4T-BspEII followed by cloning the Htt amino-terminal fragments into N1-Htt-Q138 1–704 or peGFP-N1-Htt-Q15 1–704, respectively, with myc-tagged amino-terminal Htt mutant (Q138) and wild-type GRK2 constructs were described previously. Myc-tagged amino-terminal Htt constructs were then digested with BamHI and NotI. The Htt-containing fragments were cloned into pcDNA3myc. Full-length human OPTN was cloned by PCR amplification using a Quick Clone kit (Clontech). Amino-terminal myc- or HA-tagged wild-type OPTN and HA-tagged OPTN-H486R were constructed by PCR amplification and mutagenesis of human OPTN cDNA and inserted into the BamHI/EcoRI sites of pcDNA3. The sequence integrity of each of the constructs was confirmed by automated DNA sequencing. The bait for the yeast two-hybrid screen was constructed as follows. The rat mGluR1α carboxyl-terminal tail (amino acids 841–1199) was PCR-amplified and cloned into pAS2–1 (Clontech) using BamHI and EcoRI restriction sites to generate pAS2-mGluR1-Ct. For expression of amino-terminal glutathione S-transferase (GST) fusion proteins in mammalian cells, the cDNA encoding the mGluR1α and angiotensin II type 1A receptor carboxyl-terminal tails (C-tails) were cloned into pEBG using BamHI and NotI restriction sites (23).

**Cell Culture and Transfection**—HEK 293 cells and COS7 cells were grown at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 100 μg/μl gentamicin. The mouse striatal cell lines expressing wild-type and mutant Htt were grown at 33 °C in Dulbecco’s modified Eagle’s medium. Cells were transfected with plasmid cDNAs using a modified calcium phosphate method (24). After transfection (18 h), the cells were incubated with fresh medium, allowed to recover for 6–8 h, reseded into 6-, 12-, or 24-well dishes, and then grown for an additional 18 h prior to experimentation.

**Primary Neuronal Cell Culture, Immunostaining, and Transfection**—Neuronal cultures were prepared from cortex obtained from embryonic day-20 rat embryos. All animal procedures were approved by the University of Western Ontario Animal Care Committee. Cells were plated on poly-D-lysine-coated 50-mm glass coverslips in isolation medium for 4 h at 37 °C and 5% CO₂ in a humidified incubator to permit cell attachment. Isolation medium was subsequently replaced with serum-free culture medium, and the cells were cultured with medium replenished every 3 days. Isolation medium consisted of minimum Eagle’s medium with Earle’s salts supplemented with 2 mM glutamate/glutamine, 10% heat-inactivated horse serum, 0.5 units/ml penicillin, 0.05 μg/ml streptomycin, 10 μM MK-801, and 25 mM KCl. Culture medium consisted of neurobasal medium supplemented with B-27, 2 mM glutamate/glutamine, 0.5 units/ml penicillin, 0.05 μg/ml streptomycin, 10 μM MK-801, 25 mM KCl. Before experimentation, neurons were washed two times with HEPES-buffered saline solution (HBSS), and all of the experiments were performed using HBSS, which does not contain glutamate. Primary neuronal cultures 4 days in vitro were transfected with Effectene (Qiagen, Hilden, Germany) following the manufacturer’s instructions (25). Confocal microscopy was performed using a Zeiss LSM-510 META laser scanning microscope with a Zeiss 63X or 100X NA 1.4 oil immersion lens and filters with emission wavelengths of 488 and 514 nm as described previously (26).

**Inositol Phosphate Formation**—Inositol lipids were radiolabeled by incubating the cells overnight with 1 μCi/ml [3H]myo-inositol in Dulbecco’s modified Eagle’s medium. Unincorporated [3H]myo-inositol was removed by washing the cells with HBSS (116 mM NaCl, 20 mM HEPES, 11 mM glucose, 5 mM NaHCO₃, 4.7 mM KCl, 2.5 mM CaCl₂, 1.3 mM MgSO₄, 1.2 mM KH₂PO₄, pH 7.4). The cells were preincubated for 1 h in HBSS at 37 °C and then preincubated in 500 μM of the same buffer containing 10 mM LiCl for an additional 10 min at 37 °C. The cells were then incubated in either the absence or the presence of increasing concentrations (0–30 mM) of quisqualate for 30 min at 37 °C. The reaction was stopped on ice by adding 500 μl of 0.8 M perchloric acid and then neutralized with 400 μl of 0.72 M KOH, 0.6 M KHCO₃. The total [3H]inositol incorporated into the cells was determined by counting the...
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FIGURE 1. Identification of OPTN as a negative regulator of Group I mGluR signaling. A, yeast two-hybrid backcrosses between the mGluR1α/Ct fusion and OPTN (amino acid residues 202–246). SNF1/SNFR4 interactions served as a positive control, and SNF1/mGluR1α/Ct and SNF4/OPTN crosses served as negative controls. Positive interactions display blue β-galactosidase staining. B, representative immunoblot from three experiments showing the co-precipitation of HA-OPTN with a GST fusion encoding the mGluR1α/Ct (GST-mGluR-Ct) but not with either GST or GST fusion encoding the angiotensin II type 1A receptor tail (GST-ATAR-Ct) from COS7 cells. Lower panel shows HA-OPTN expression in corresponding 50-µg aliquots of COS7 cell lysates. C, representative immunoblot (IB) from three independent experiments showing the co-immunoprecipitation (IP) of HA-OPTN from HEK 293 cells co-transfected with (+) and without (−) either FLAG-mGluR1α or FLAG-mGluR5a. NT, nontransfected. Lower panel shows HA-OPTN expression in corresponding 50-µg aliquots of HEK 293 cell lysates. D, micrographs show the co-localization (yellow) of green fluorescent protein-OPTN (green) and Alexa Fluor 546-conjugated monoclonal anti-HA (red) in transfected rat primary cortical neurons 5 days in vitro. Scale bar, 10 µm. Arrows point to colocalized green fluorescent protein-OPTN and FLAG-mGluR1α.

Radioactivity present in 50 µl of the cell lysate. Total inositol phosphate was purified from the cell extracts by anion exchange chromatography using Dowex 1-X8 (formate form) 200–400 mesh anion exchange resin. [3H]Inositol phosphate formation was determined by liquid scintillation using a Beckman LS 6500 scintillation system.

Co-immunoprecipitation—Co-immunoprecipitation of endogenous proteins from HdhQ7/Q7 striatal cells was performed using lysis buffer (25 mM HEPES, pH 7.5, 300 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.1% Triton X-100, 20 mg/ml leupeptin, 20 mg/ml aprotinin, and 20 µg/ml phenylmethylsulfonyl fluoride). mGluR5 was immunoprecipitated from striatal cell supernatant (500 µg of total protein) using 1.5 mg of mGluR5 rabbit polyclonal antibody (Upstate Biotechnology) and 1:75 OPTN rabbit polyclonal antibody. Co-immunoprecipitation experiments were also performed using 500 µg of total cell lysate protein solubilized from HEK 293 cells transiently transfected with the various cDNA constructs as described in the legends to Figs. 1–8. The cells were solubilized in lysis buffer. FLAG-mGluR1α and FLAG-mGluR5α were immunoprecipitated with FLAG-Sepharose beads from cell lysates by overnight rotation at 4 °C. The beads were washed four times with lysis buffer, and proteins were eluted in 3X SDS sample buffer and then separated by SDS-PAGE. The membranes were blocked with 10% milk in wash buffer (TBS-T) (150 mM NaCl, 10 mM Tris-HCl, pH 7.0, 0.05% Tween 20) and then incubated with rabbit GRK2-, OPTN-, or mGluR5-specific antibodies, as well as Htt-, myc-, FLAG-, or HA-specific monoclonal antibodies diluted 1:1000 in wash buffer containing 3% skim milk. The membranes were rinsed three times with wash buffer and then incubated with secondary horseradish peroxidase-conjugated donkey anti-rabbit IgG or anti-mouse IgG (Amersham Biosciences) diluted 1:2500 in wash buffer. The membranes were washed two times with wash buffer and then incubated with secondary horseradish peroxidase-conjugated donkey anti-rabbit IgG or anti-mouse IgG (Amersham Biosciences) diluted 1:2500 in wash buffer containing 3% skimmed milk. Membranes were rinsed twice with TBS-T and twice with Tris-buffered saline and were incubated with ECL Western blotting detection reagents. Intensities of immunoblot signals were determined with the ImageJ 1.44p software from Scion Corp. (Frederick, MD).

Flow Cytometry Analysis of FLAG-mGluR1α Expression—Cells were placed on ice and washed twice in HBSS. Cells were incubated in a 1:500 solution of mouse anti-FLAG (Sigma) for 45 min. Antibody was washed off and replaced with 1:500 fluorescein isothiocyanate (Sigma) for 45 min followed by two washes in HBSS. Cells were incubated in PBS containing 0.5 mM EDTA for 5 min. Cells were removed from the plates and fixed in 1.6% formaldehyde in PBS. Cell surface immunofluorescence was measured by flow cytometry on a BD Biosciences FACScalibur.

Determination of Cell Viability by MTT Reaction—40 h post-transfection cells were incubated in fresh medium containing 5 mg/ml thiazolyl blue (MTT) (Sigma) for 3 h at 37 °C. Viable cells convert the MTT to an insoluble purple formazan. Medium was replaced with dimethyl sulfoxide to solubilize cells, lysates were transferred to cuvettes, and cell viability of transfected cells was analyzed by spectrophotometry at 570 nm. Percentage cell viability was calculated as compared with that of the nontransfected control.

Hoechst Staining—40 h post-transfection cells were washed twice in PBS and incubated for 20 min in 3.6% paraformaldehyde. Cells were washed two times in PBS and incubated in 1:200 Hoechst stain (Sigma) for 45 min and washed twice with PBS. Cells were viewed using an Olympus IX70 inverted fluorescence microscope using a 20X lens with 0.40 NA. Apoptotic cells were defined as having pyknotic nuclei and exhibiting condensed chromatin. We used Northern Eclipse software to process the data presented as the number of apoptotic cells compared with the total number of cells.

Data Analysis—The means ± S.D. or S.E. are shown for the number of independent experiments indicated in the legends to Figs. 1–8. GraphPad Prism software was used to analyze data for statistical significance, as well as to analyze and fit curves for quisqualate dose-response curves. Statistical significance was determined by either the Student’s t test or the one-way analysis of variance with the post-hoc Tukey’s multiple comparison test.

RESULTS

OPTN Interacts with Group I mGluRs—To identify novel signaling proteins that might interact with Group I mGluRs, we screened a human brain cDNA library using the C-tail domain of mGluR1α (amino acid residues 841–1199) as bait in the yeast two-hybrid system. We identified OPTN as a potential Group I mGluR interacting protein, and the specificity of the interaction was confirmed by growth on −Leu/
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FIGURE 2. Inhibition of mGluR1a signaling by OPTN. A, quisqualate dose responses for FLAG-mGluR1a-stimulated inositol phosphate formation (IP Formation) in HEK 293 cells cotransfected with either empty vector (Control) or OPTN. B, basal mGluR1a activity in the presence and absence of OPTN expression in HEK 293 cells. Data represent the mean ± S.E. for five experiments.

OPTN Expression Inhibits mGluR1a Signaling—Having established that OPTN binds to Group I mGluRs, we assessed whether OPTN interactions with mGluR1a might influence the ability of the receptor to couple to phospholipase Cβ and stimulate increases in intracellular inositol phosphate concentrations. We find that the overexpression of OPTN in HEK 293 cells reduces the maximum velocity ($V_{\text{max}}$) for quisqualate-stimulated inositol phosphate formation to $61 ± 8\%$ of control without affecting the half-maximal effective concentration (EC$_{50}$) for the agonist (Fig. 2A). The prevailing mechanism considered to underlie the attenuation of GPCR signaling involves phosphorylation by G protein-coupled receptor kinases (GRKs) (27, 28). However, GRK2 attenuates both agonist-stimulated and basal mGluR1a signaling in the absence of phosphorylation (21, 22). Unlike what is observed for GRK2, OPTN expression in HEK 293 cells leads to increased basal mGluR1a activity (Fig. 2B). Thus, OPTN represents an alternative and novel regulator of mGluR signaling that, similar to GRK2, mediates the phosphorylation-independent inhibition of agonist-stimulated mGluR activity.

GRK2 and OPTN Compete for mGluR1a Binding—To test whether GRK2 and OPTN attenuate mGluR1a signaling by similar mechanisms, we examined whether the proteins might compete for mGluR1a binding. We found that, when expressed with OPTN, GRK2 displaces OPTN binding to mGluR1a and is preferentially co-precipitated with the receptor (Fig. 3A). In addition, $V_{\text{max}}$ for quisqualate-stimulated mGluR1a inositol phosphate formation is attenuated by GRK2 to the same extent, independently of whether GRK2 is expressed either with or without OPTN (Fig. 3B). This observation suggests that at maximum expression levels GRK2 and OPTN have nonadditive effects on the inhibition of mGluR1a function. The observation that OPTN expression increases basal mGluR1a activity is likely related to the competition between endogenous GRK2 and OPTN for mGluR1a binding. Interestingly, although endogenous OPTN is expressed at similar levels in cerebellar, cortical, striatal, and hippocampal tissues, GRK2 is expressed at relatively low levels in the striatum (Fig. 3C), the brain region that is susceptible to neuronal degeneration in HD. GRK2 expression levels in the striatum are equivalent to GRK2 expression levels in HEK 293 cells (Fig. 3C). Thus, it is possible that OPTN replaces GRK2 to regulate mGluR signaling in striatal neurons.
Htt Is Associated with mGluR1a—Because OPTN binds to Htt, we tested whether Htt could be co-precipitated in a complex with mGluR1a C-tail in an OPTN-dependent manner. We found that the amino-terminal domain (amino acid residues 1–704) of wild-type Htt (N-HttQ15) can be precipitated with the mGluR1a C-tail in the absence of OPTN overexpression, presumably as a complex with endogenous OPTN that is coprecipitated with the mGluR1a C-tail (Fig. 4A). The overexpression of OPTN increases N-HttQ15 co-precipitation with the mGluR1a C-tail by 2.7 ± 0.7-fold (Fig. 4A). Full-length wild-type HttQ15 also co-precipitates with FLAG-mGluR1a from HEK 293 cells (Fig. 4B). However, unlike what is observed for mGluR1a C-tail interactions, OPTN expression does not significantly enhance the co-precipitation of either N-HttQ15 or polyglutamine-expanded mutant Htt (N-HttQ138) with full-length FLAG-mGluR1a (Fig. 4C).

Mutant Htt Enhances OPTN-mediated Inhibition of mGluR1a Signaling—Expression of either N-HttQ15 or N-HttQ138 in the absence of OPTN does not lead to a significant change in either the Vmax or the EC50 for quisqualate-stimulated mGluR1a inositol phosphate formation.
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**FIGURE 6. Effect of wild-type and mutant Htt on mGluR1a cell surface expression and cell viability.** A, the mean cell surface expression of mGluR1a in HEK 293 cells following transfection with and without OPTN, myc-N-HttQ15, and myc-N-HttQ138 constructs. The data represent the mean ± S.D. of four independent experiments. B, quantification of the fraction of cells exhibiting evidence of apoptosis following transfection of cells with empty vector, FLAG-mGluR1a alone and with OPTN, myc-N-HttQ15, and myc-N-HttQ138 constructs. Apoptosis was measured by Hoechst stain and was defined by pyknotic nuclei exhibiting condensed chromatin. The data represent the mean ± S.D. of three independent experiments. C, quantification of cell viability by MTT assay. The viability of HEK 293 cells transfected with FLAG-mGluR1a alone and with OPTN, myc-N-HttQ15, and myc-N-HttQ138 constructs was compared with nontransfected cells. The data represent the mean ± S.D. of three independent experiments.

(Fig. 5A). However, N-HttQ138 expression increases the OPTN-dependent attenuation of the V_{max} for quisqualate-stimulated mGluR1a inositol phosphate formation and shifts the E_{50} for quisqualate-stimulated inositol phosphate formation from 255 to 2.5 μM when compared with cells expressing OPTN alone (Fig. 5B). In contrast, the expression of either wild-type or mutant N-Htt prevents the OPTN-dependent increase in basal mGluR1a activity (Fig. 5C). It is possible that the observed alterations in mGluR1a signaling in the presence of OPTN and N-HttQ138 are not the consequence of either reduced cell surface receptor expression or increased cell death under the conditions in which we did the inositol phosphate formation experiments. When tested, we found that the overexpression of mGluR1a alone or with either HttQ15 or N-HttQ138 in the presence and absence of OPTN had no effect on cell surface FLAG-mGluR1a expression, apoptosis, or cell viability (Fig. 6). Taken together, these results suggest that the increase in the association of N-HttQ138 and OPTN with mGluR1a augments OPTN-mediated antagonism of mGluR1a signaling rather than altering either cell expression or cell viability.

**Impaired mGluR Signaling in Striatal Cells Derived from HdhQ111/Q111 Mice**—Because the co-expression of N-HttQ138 augmented the attenuation of mGluR1a activity by OPTN, we examined quisqualate-stimulated inositol phosphate responses in striatal neuronal cell lines generated from mice homozygous for the knock-in of a polyglutamine expansion Htt mutant (HdhQ111/Q111) and wild-type Htt (HdhQ7/Q7) littermate embryos (29). Initial experiments demonstrated that both cell lines express equivalent levels of both OPTN and mGluR5 and do not express detectable levels of mGluR1 by immunoblot (Fig. 7A). In contrast, endogenous OPTN expression is not detectable in either HEK 293 cells or COS7 cells (data not shown). However, OPTN expression in both striatal cell lines was higher than COS7 cells transfected to overexpress OPTN (Fig. 7A). We were able to co-immunoprecipitate endogenous mGluR5 with endogenous OPTN from HdhQ7/Q7 striatal cells (Fig. 7B). Consistent with what we observed in HEK 293 cells, the quisqualate dose response for endogenous mGluR-stimulated inositol phosphate formation was severely impaired in the HdhQ111/Q111 striatal cells when compared with control HdhQ7/Q7 striatal cells (Fig. 7C). Specifically, the V_{max} for quisqualate-stimulated inositol phosphate formation was reduced by 39 ± 3% in HdhQ111/Q111 versus HdhQ7/Q7 striatal cells, and the E_{50} obtained for quisqualate-stimulated inositol phosphate formation in HdhQ111/Q111 striatal cells was increased 33-fold to 1.4 mM. The inositol phosphate responses following the activation of two other G_{q}-coupled GPCRs (muscarnic acetylcholine or serotonin receptors) were not altered between the two cell lines, suggesting that this does not represent a generalized impairment of phospholipase C signaling (Fig. 7D). Taken together, these observations indicate that mutant Htt overexpression leads to attenuation of Group I mGluR coupling to phospholipase C and inositol phosphate formation. Because this observation is only recapitulated in HEK 293 cells following the overexpression of OPTN with mutant Htt and HdhQ111/Q111 striatal cells express relatively high levels of endogenous OPTN we suggest the possibility that OPTN may synergize with mutant Htt to attenuate mGluR signaling.

**Single Nucleotide Polymorphism OPTN Variant Defective in Htt Binding**—The OPTN gene was recently reported to possess both causal and risk-associated alleles for open angled glaucoma (20, 30, 31). We found that one missense single nucleotide polymorphism OPTN mutant, OPTN-H486R, demonstrated reduced association with N-HttQ138 (52 ± 20% of wild-type OPTN binding) but was unimpaired in either FLAG-mGluR1a or N-HttQ15 binding (Fig. 8A). Therefore, we tested whether the H486R mutation might influence OPTN-mediated antagonism of mGluR1a signaling. OPTN-H486R reduced FLAG-mGluR1a-stimulated inositol phosphate formation in response to treatment with 30 μM quisqualate to the same extent as wild-type OPTN when expressed either alone or with N-HttQ15 (Fig. 8B). The expression...
of N-HttQ138 increased wild-type OPTN-dependent inhibition of FLAG-mGluR1a-stimulated inositol phosphate formation from 49 ± 2% to 27 ± 1% of control inositol phosphate responses obtained in the absence of OPTN expression (Fig. 8B). However, OPTN-H486R expression significantly reduced the ability of N-HttQ138 to synergistically antagonize FLAG-mGluR1a signaling (Fig. 8B). On the basis of these observations, we suggest that intact Htt/OPTN interactions are required for the synergistic antagonism of mGluR1a signaling.

**DISCUSSION**

In the present study, we provide evidence that OPTN can substitute for GRK2 to mediate the attenuation of mGluR signaling. To our knowledge, OPTN represents the first mGluR-interacting protein other than GRK2 that mediates the phosphorylation-independent uncoupling of mGluRs from heterotrimeric G proteins. OPTN is an Htt-interacting protein suggesting that Htt may be involved in the regulation of mGluR signaling. However, expression of wild-type or mutant huntingtin alone has no effect on mGluR1a coupling to (Gq-mediated) increases in inositol phosphate formation. In contrast, when wild-type and mutant Htt are coexpressed with OPTN, only mutant Htt expression leads to an augmentation in OPTN binding to mGluR1a and increased antagonism of mGluR1a signaling. Surprisingly, in cells derived from the striatum of knock-in mice homozygous for the expression of a polyglutamine-expanded Htt mutant, mGluR signaling is severely impaired. Mutant Htt binding to OPTN appears to be essential in mediating the synergistic increase in OPTN-dependent G protein-uncoupling of mGluR1a. Taken together, these observations suggest that not only does OPTN have the capacity to substitute for GRK2 to attenuate mGluR signaling in striatal tissues but mutant Htt protein may further antagonize mGluR signaling in HD.

GRK2 is a ubiquitously expressed protein that is an essential GPCR regulatory protein that protects all cells against receptor overactivation...
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(27, 28). However, GRK2 is most abundantly expressed in hematopoietic and brain tissues, and expression is particularly enhanced at synapses (32). Relative GRK2 expression levels are notably lower in striatal tissue when compared with other brain regions, suggesting that this important GPCR regulatory mechanism may be of limited effectiveness in striatal neurons. We find that GRK2 expression in striatal tissue is equivalent to GRK2 expression in HEK 293 cells, and we have demonstrated previously that mGlur expression leads to HEK 293 cell apoptosis (33). Thus, the observation that OPTN can substitute for GRK2 to mediate the attenuation of mGlur signaling is particularly relevant in the striatum in which OPTN may play a primary role in preventing pathophysiologically release of Ca^{2+} from intracellular stores in response to constitutive and agonist-induced mGlur signaling. Moreover, because OPTN is unlikely to promote β-arrestin binding to mGlurS, it is possible that the resensitization of mGlur signaling in the striatum is delayed, especially in the presence of mutant Htt.

The observation that mutant Htt facilitates OPTN activity and mGlur desensitization was surprising because the prevailing expectation was that Group I mGlur signaling via IP_3 release, leading to intracellular Ca^{2+} release might be expected to enhance mutant Htt neurotoxicity (8, 9, 34–36). Htt and Htt-associated protein 1 form a ternary complex with the IP_3 receptor (14), and upon transfection of these proteins into medium spiny striatum neurons Ca^{2+} release from intracellular stores is significantly increased in response to treatment with threshold levels of the mGlur agonist 3,5-dihydroxyphenylglycine. Consistent with this observation, repetitive application of glutamate to medium spiny neurons derived from YAC128 transgenic mouse model of HD but not control mice also increased intracellular Ca^{2+} concentrations and induced apoptosis (15). The excitotoxic actions of glutamate in these experiments were mediated by both Group I mGlurs and NR2B glutamate receptors. Thus, it is possible that the uncoupling of mGlur signaling represents an adaptive cellular response that protects against increased IP_3 receptor sensitivity in HD.

Recent studies have demonstrated that single nucleotide polymorphism OPTN variants are associated with hereditary forms of normal tension glaucoma (20, 30, 31). Alterations in mGluR signaling are also implicated in contributing to the etiology of glaucoma (37). However, it is unknown whether Htt might be involved in regulating retinal degeneration associated with glaucoma. Nonetheless, retina degeneration has been reported in both mouse and Drosophila models of HD, as well as in a small cohort of HD patients (38 – 40). Consistent with a potential link between the molecular pathology of HD and glaucoma, we show here that a single nucleotide polymorphism OPTN variant linked to heritable glaucoma, OPTN-H486R, specifically exhibits reduced ability to bind to mutant Htt and antagonize mGlur signaling. Thus alterations in both mGlur signaling and OPTN function may be linked to both HD and glaucoma. Whether a link exists between glaucoma and HD remains to be determined.

Group 1 mGlurs play a dual role in regulating neurotranscyt and neuroprotection (41, 42). Thus, as suggested above, the discordance between our observations and previous studies examining the role of polyglutamine-expanded mutant Htt in facilitating IP_3-stimulated Ca^{2+} from intracellular stores might be explained by the specific adaptation of mGlur signaling in HD models. It is possible that the uncoupling of mGlur5 from phospholipase C in striatal cells derived from Hdh_{Q111/Q111} mice represents a compensatory mechanism to protect against altered Ca^{2+} homeostasis in striatal neurons. Unlike what might be expected for the dysregulation of Ca^{2+} release in cells transfected with mutant Htt, the reduction in IP_3 formation in Hdh_{Q111/Q111} striatal cells is specific to mGlur signaling and was not observed for other G_{q12}-coupled GPCRs. Alternatively, the increased G protein uncoupling in Hdh_{Q111/Q111} striatal cells may abrogate the neuroprotective actions that have been described for Group I mGlurs. Thus, a loss of neuroprotective activity may exacerbate excitotoxic signaling by ionotropic glutamate receptors in HD. In summary, understanding the relative role of OPTN and mutant Htt in regulating the neurotoxic and neuroprotective signaling of mGlurs will be essential for the development of new therapeutic targets for the treatment of HD. Our data provide further evidence that mGlur5 is a potential target for pharmacological treatment of HD.

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OPTN and Htt Attenuate mGluR Signaling

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Inhibition of Metabotropic Glutamate Receptor Signaling by the Huntingtin-binding Protein Optineurin
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