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Glutamate transporter expression and function in a striatal neuronal model of Huntington's disease

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Abstract

Excitotoxicity may contribute to the pathogenesis of Huntington's disease. High affinity Na⁺ dependent glutamate transporters, residing in the plasma membrane, clear glutamate from the extracellular space and are the primary means of prevention against excitotoxicity. Many reports suggest that Huntington's disease is associated with a decrease in the expression and function of glutamate transporters. We studied the expression and function of these transporters in a cellular model of Huntington's disease, *STHdh*^{Q111/Q111} and *STHdh*^{Q7/Q7} cells. We found that only GLT-1b and EAAC1 were expressed in these cell lines and only EAAC1 significantly contributed to the glutamate uptake. Surprisingly, there was an increase in Na⁺-dependent glutamate uptake in *STHdh*^{Q111/Q111} cells accompanied by an increase in surface expression of EAAC1. We studied the influence of the Akt pathway on EAAC1 mediated uptake, since EAAC1 surface expression is influenced by Akt and previous studies have shown increased Akt expression in *STHdh*^{Q111/Q111} cells. Glutamate uptake was inhibited by Akt pathway inhibitors in both the *STHdh*^{Q7/Q7} and the *STHdh*^{Q111/Q111} cell lines, and, in fact, we have found no difference in Akt activation between the two cell lines under our conditions of culture. Therefore a difference in Akt activation does not seem to explain the increase in EAAC1 mediated uptake in the *STHdh*^{Q111/Q111} cells.

Keywords

Huntington's disease; striatal; glutamate uptake; glutamate transporters; EAAC1

1. Introduction

Huntington's disease (HD) is a chronic neurodegenerative disorder characterized by abnormal movements, personality changes and psychiatric manifestations. It is caused by an

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expanded CAG repeat in the first exon of the gene huntingtin (Htt), whose function is unknown. The disease is expressed when a polyQ tract in the Htt gene product is longer than 35 repeats. The pathogenesis of HD is unknown, and the leading hypotheses are that the polyQ expansion causes transcriptional dysregulation (Cha, 2000; Cui et al., 2006), defects in axonal transport (Gauthier et al., 2004; Gunawardena et al., 2003; Velier et al., 1998) or failure of energy metabolism (Browne and Beal, 2004; Powers et al., 2007). How these defects lead to neuronal death is unclear.

Many lines of evidence converge to support the hypothesis that excitotoxicity plays an important role in the pathogenesis of HD (DiFiglia, 1990; Fan and Raymond, 2007). Excitotoxicity is cell death due to overactivation of glutamate receptors. Early studies by Schwarz and Coyle showed that injections of the excitatory amino acid agonist kainic acid into the striatum produced neuronal loss comparable to HD (Coyle and Schwarcz, 1976). Neuropathological examination of brains of HD patients suggests that neurons with the highest NMDA receptor density are most vulnerable to degeneration in HD (Albin et al., 1990; Young et al., 1988).

By controlling the concentration profile of glutamate in and around the synaptic cleft after its release (Huang & Bergles, 2004), glutamate transporters appear to play an important role in preserving the signaling functions of synapses (Katagiri et al., 2001; Stoffel et al., 2004); regulating the activation of nearby metabotropic receptors (Otis et al., 2004; Scanziani et al., 1997); and in some regions may shape the amplitude or decay kinetics of excitatory postsynaptic currents (EPSCs) (Barbour et al., 1994; Takahashi et al., 1995).

Na⁺-dependent glutamate transporters include five members: GLT-1 (Pines et al., 1992), whose human counterpart is EAAT2; GLAST, whose human counterpart is EAAT1 (Storck et al., 1992); EAAC1, whose human counterpart is EAAT3 (Kanai and Hediger, 1992); EAAT4 (Fairman et al., 1995), and EAAT5, expressed primarily in the retina (Arriza et al., 1997). GLAST and GLT-1 have been described as the major glial glutamate transporters (Rothstein et al., 1994), however, GLT-1 has also been found in neurons (Chen et al., 2002; Chen et al., 2004; Furness et al., 2008; Melone et al., 2009).

Impaired function of these transporters can cause accumulation of glutamate in the extracellular space and cell death due to overactivation of glutamate receptors. In this study, we used neuronal striatal cell lines that express full-length Htt protein with either 7 glutamine repeats (*STHdh*^{Q7/Q7} or Q7 for figures) or 111 glutamine repeat (*STHdh*^{Q111/Q111} or Q111 for figures) for our model. The Q111 cell line was established from E14 striatal primordia of the *Hdh*^{Q111} knock-in mice. They were immortalized with the tsA58 SV40 large T antigen (Trettel et al., 2000). Our aim was to investigate whether expansion of the glutamine repeats in *huntingtin* is associated with changes in glutamate transporter expression and function in this cell model.

2. Material and Methods

2.1. Antibodies and drugs

A polyclonal antibody against the C-terminal peptide of GLT-1b [α GLT-1b; 1:500 for Western Blot (WB); 1:400 for immunocytochemistry (ICC)] and a polyclonal antibody against transcripts that encode for the 15 amino acid sequence MASTEGANNMPKQVE at the N-terminus (α nGLT-1; 1:500 for WB; 1:400 for ICC) were generated in rabbits and previously characterized (Chen et al., 2002; Chen et al., 2004). The polyclonal antibody against the C-terminus of GLT-1a (α GLT-1a; 1:500 for WB; 1:5000 for ICC) was generously provided by Dr. Jeff Rothstein (Johns Hopkins University) and has been previously characterized (Rothstein et al., 1994). Polyclonal rabbit antibodies against

EAAC1 (1:200 for WB; 1:100 for ICC) and GLAST (1:200 for WB; 1:2000 for ICC) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Akt (1:1000 for WB) and phospho-Akt (Ser473; 1:500 for WB), both polyclonal rabbit antibodies, were obtained from Cell Signaling Technologies (Danvers, MA, USA). A polyclonal rabbit anti-GAPDH antibody, (1:2500 for WB; Abcam, Cambridge, MA, USA) was used as an internal control. All drugs were purchased from Tocris Bioscience (Ellisville, Missouri, USA). Dihydrokainate acid (DHK), a specific inhibitor of GLT-1, was used at concentrations of 300 μ M. Threo- β -Benzoylaspartic acid (TBOA) was used to inhibit EAAC1 and GLT-1 at low concentration (50 μ M) and to inhibit EAAC1, GLT-1 and GLAST at high concentration (250) (Shimamoto et al., 1998). In the uptake studies, the drug was added in the uptake solution without prior incubation time. The inhibitors of the Akt pathway, LY294002 and wortmannin, both block phosphatidylinositol 3-kinase (PI3K) and were used at concentrations of 25 μ M and 0.1 μ M. In experiments using Akt inhibitors, cells were exposed to drugs in high glucose DMEM without other supplements.

2.2. Culture and differentiation of STHdh cells

STHdh^{Q7/Q7} and *STHdh*^{Q111/Q111} were generously provided by Dr. Marcy McDonald (Massachusetts General Hospital, Boston) and have been described previously (Trettel et al., 2000). Cells were grown in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 4.5 g/L glucose, 4mM L-glutamine, 1mM sodium pyruvate and antibiotics (all Invitrogen, Carlsbad, CA, USA). Cells were cultured on 10 cm-diameter cell culture dishes at 33°C in an atmosphere containing 5 % CO₂.

For differentiation, cells were washed once with PBS followed by the addition of the differentiation medium consisting of 10 ng/ml aFGF, 250 μ M IBMX, 200 nM PMA, 50 μ M forskolin, and 20 μ M dopamine in serum-free DMEM (Trettel et al., 2000).

2.3. Genotyping of STHdh cells

Cells from a 10 cm plate were washed with PBS and trypsinized. Growth medium was added and cells were pelleted for 3 min by centrifugation at 800 $\times$ g. The pelleted cells were lysed in 300 μ L PCRDIRECT (Viagen Biotech, Los Angeles, USA) with 20 μ g/ml proteinase K. The vials were incubated overnight at 56°C and heated to 85°C for 1 hour. The DNA was precipitated by adding 300 μ L isopropanol and pelleted by centrifugation. The pellet was washed in 250 μ L 70 % (v/v) ethanol and dried. The DNA was resuspended in 300 μ L 10 mM Tris-Cl, (pH 8.0) 1 mM EDTA buffer. 2 μ L (1 mg/ml) of DNA was used in the PCR for the cell line identification as described previously (Trettel et al., 2000). The PCR product was run on a 2% agarose gel.

2.4. Immunostaining

STHdh cells were grown for 24 hours on poly-D-lysine covered glass cover slips. The next day, half of the cells were differentiated for one hour. The medium was aspirated, and the cells were washed with PBS on ice. After fixation with paraformaldehyde in phosphate-buffered saline (4 % (w/v) on ice for 7 min, the cells were washed three times with PBS, incubated for 20 min with blocking solution (5 % (v/v) goat serum and 1 % (w/v) bovine serum albumin in PBS) supplemented with 0.1 % (v/v) Triton X-100. The cells were then incubated for 90 min with primary antibodies against EAAC1, nGLT-1, GLT-1a, GLT-1b, and GLAST diluted in blocking solution. Next, the cells were washed three times with PBS for 7 min and then incubated with Alexa-conjugated secondary antibodies for 30 min prior to washing and mounting in Fluoromount-G (Southern Biotech, Birmingham, USA). A Hoechst staining was used to visualize the nucleus. Images were collected at a 40 $\times$ magnification on a Zeiss LSM510 2-photon confocal scanning microscope (Zeiss, Thornwood, USA).

2.5. Western blotting

STHdh cells were washed twice with ice-cold PBS and then lysed in T-Per (Pierce, Rockford, USA), containing protease inhibitor cocktail (Roche, Heidelberg, Germany). The protein content was determined using a Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein in a Laemmli buffer were applied to each lane on 4-20% gradient SDS polyacrylamide gels (25-50 µg per lane) and then transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, USA) by electroblotting. The gels were stained with Ponceau S Red to check for equal loading. Blots were incubated with primary antibodies overnight at 4°C in 5% non-fat milk, TBS with 0.01% Tween 20 (TBST), and then washed 3 times with TBST buffer, incubated for 1 hour with horseradish peroxidase-conjugated secondary IgG antibody (Amersham Biosciences, Piscataway, NJ, USA) at 1:10,000 dilutions and washed again. Immunoreactive proteins were detected using enhanced chemiluminescence (Pierce, Rockford, IL, USA).

2.6. Uptake measurement

Previously published procedures were followed for measuring the uptake of glutamate (Wang et al., 1998) in cultured cells. Cells were seeded at a density of 0.15×10^5 cells/well in a poly-D lysine covered 48-well plate or 2×10^6 cells/well in a poly-D-lysine coated 24-well plate. The next day, the cells were washed with a warmed washing buffer [(in mM): 140 NaCl or 140 choline chloride, 2.5 KCl, 1.2 CaCl₂, 1.2 MgCl₂, 1.2 K₂HPO₄, 10 glucose, 5 Tris base, and 10 HEPES] and incubated for 10 min at 37°C. The washing buffer was removed and replaced by washing buffer containing glutamate [0.5 µM L-glutamate and 0.022 µM [³H]-L-glutamate (PerkinElmer, Boston, MA, USA)] either in the presence (sodium uptake buffer) or absence (choline uptake buffer) of sodium for 10 min at 37°C. L-glutamate concentrations for the saturation analysis were: 2.5, 5, 10, 25, 50, 100, 150, and 300 µM. The uptake assay was terminated by aspirating the radioactive solution followed by three washes in ice-cold stopping buffer. The stopping buffer contained choline washing buffer plus 1% BSA (Garlin et al., 1995). The cells were then solubilized in 250 µL 0.1 M NaOH. Aliquots of the lysate were analyzed for protein with the Bradford protein assay (Bio-Rad, Hercules, CA, USA) and radioactivity was measured by liquid scintillation counting (TRI-CARB 2200CA, ΔPACKARD, Long Island Scientific, Inc.). The radioactivity taken up by the cultures in the absence of sodium was subtracted from that taken up in the presence of sodium to isolate sodium-dependent transport. If differentiated cells were used, the differentiation medium was added 1 hour prior to the uptake experiment.

2.7. Electrophysiological measurements

Recordings of voltage-dependent potassium currents were obtained with the whole-cell configuration of the patch-clamp technique. The extracellular solution contained (in mM): 115 NaCl, 2.5 KCl, 2.0 MgCl₂, 10 HEPES, 10 D-glucose; pH was adjusted to 7.2 with concentrated KOH; 0.250 mM TTX was added to inhibit voltage gated sodium channels. The intracellular (electrode) solution contained (in mM): 100 K-Gluconate, 11 EGTA, 10 KCl, 1 MgCl₂, 1 CaCl₂ × 2H₂O, 10 HEPES; pH was adjusted to 7.2 with concentrated KOH; 0.22 mM ATP was added and osmolarity was adjusted to 280 mOsm with sucrose. All measurements were obtained under voltage clamp with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) and pClamp software (Molecular Devices) using 2-3 MOhm electrodes. Partial compensation (80%) for series resistance was provided in all instances. Currents were filtered at 2 kHz and digitized at 10 kHz (Digidata; Axon Instruments). K⁺ currents were evoked with a series of voltage steps from a holding potential of -80 mV to +50 mV in 10 mV increments. Current densities were calculated by dividing the peak steady state current obtained at +50 mV by the whole-cell capacitance.

2.8 Biotinylation of surface proteins

We followed a previously published procedure to determine the fraction of EAAC1 associated with the plasma membrane (Gonzalez et al., 2007). *STHdh* cells were plated at 150,000 cells/ml in 10 cm dish and incubated for 48 hours. Cells were rinsed twice with ice-cold PBS with 1mM Ca²⁺/0.1mM Mg²⁺ (PBS Ca/Mg), incubated with Sulfo-NHS-Biotin (1mg/ml; Thermo Scientific) for 30 minutes; rinsed twice and incubated with PBS Ca/Mg + 100mM glycine for 30 minutes; then rinsed twice with PBS Ca/Mg, incubated in RIPA/lysis buffer with protease and phosphate inhibitors (150mM NaCl, 1mM EDTA, 100mM Tris, pH7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1μg/ml leupeptin, 250μM PMSF, 1μg/ml aprotinin, 1mM iodoacetamide, 10mM NaF, 30mM Na pyrophosphate and 1mM Na₃VO₄) for 1 hour. Cells were scraped and centrifuged. 300ul of each sample was taken for “lysate” fraction and added to an equal amount 4× sample buffer; 100ul of each sample was taken for protein assay; 300ul of each sample was added to same volume of UltraLink Immobilized Monomeric avidin beads (Thermo Scientific), incubated at room temperature for 1 hour and centrifuged. 200ul of each sample of avidin supernatant was taken for “intracellular” fraction and added to an equal amount 4× sample buffer. Avidin beads were resuspended in 1ml RIPA buffer with protease and phosphate inhibitors and centrifuged. Supernatant was discarded and beads were washed twice in high-salt wash buffer (0.1% Triton X-100, 500 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.5) and once in no-salt wash buffer (50 mM Tris pH 7.5), then beads were incubated in 600μl of 2× sample buffer for 10 minutes at room temperature and 30 minutes at 37°C. Beads were centrifuged and 500μl supernatant was removed for “biotinylated” fraction.

3. Results

3.1. A subset of glutamate transporters are expressed in *STHdh*^{Q7/Q7} and *STHdh*^{Q111/Q111} cell lines

First, we established which glutamate transporters are expressed in *STHdh* cells. Cell lysates of undifferentiated cells were separated by SDS-Page and incubated with antibodies against EAAC1, GLT-1 (nGLT-1, GLT-1a, GLT-1b), and GLAST. We found expression of only EAAC1 (Fig 1A), GLT-1 (nGLT-1 antibody; Fig 1B), and GLT-1b (Figure 1C), however, GLT-1a and GLAST were not expressed in these cell lines (see Supplemental Figure 1 for GLT-1a). Blots from three different experiments were scanned and the band intensity was measured. The relative expression in the *STHdh*^{Q111/Q111} cells compared to the *STHdh*^{Q7/Q7} cells is shown in the histograms. Glutamate transporter expression was normalized to β-actin expression (Figure 1). EAAC1 and GLT-1b expression were observed to be significantly decreased in the *STHdh*^{Q111/Q111} when compared to *STHdh*^{Q7/Q7} cells (69 ± 7 and 22 ± 15%, respectively). In contrast, no significant difference was observed between *STHdh*^{Q7/Q7} and *STHdh*^{Q111/Q111} cells for GLT-1 expression, using an antibody directed against the N-terminus of GLT-1, recognizing isoforms of GLT-1 that encode for the amino acid sequence MASTEGANNMPKQVE.

3.2. Localization of glutamate transporters is mostly cytoplasmic in both cell lines

Most of EAAC1 immunoreactivity was detected in the cytoplasm in both differentiated and undifferentiated cell lines with little detectable near-membrane expression (Figure 2, top row). Detection using nGLT-1 antibody also showed most of the immunoreactivity in the cytoplasm in both cell lines, except in differentiated *STHdh*^{Q7/Q7} cells, where increased near-membrane enrichment was seen (Figure 2, second row). GLT-1b protein was also mostly cytoplasmic in both lines, in both states (Figure 2, third row). In agreement with Western blot results, GLT-1a and GLAST immunoreactivity was absent in both cell lines (Figure 2, fourth row). In conclusion, immunoreactivity of glutamate transporters is

observed mainly in cytoplasm, in both *STHdh*^{Q7/Q7} and *STHdh*^{Q111/Q111} cells, with increased near-membrane localization of nGLT-1 in differentiated *STHdh*^{Q7/Q7} cells.

3.3. Glutamate uptake is significantly increased in *STHdh*Q111/Q111 cells

After establishing which glutamate transporters are expressed in *STHdh* cells, we assayed for glutamate uptake. First, we performed a saturation analysis in *STHdh*^{Q7/Q7} and *STHdh*^{Q111/Q111} undifferentiated cells. From three different experiments, the Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) were calculated for *STHdh*^{Q7/Q7} and *STHdh*^{Q111/Q111} cells. V_{max} of sodium-dependent glutamate transport was 3.6-fold higher ($p < 0.0001$, t-test) in *STHdh*^{Q111/Q111} cells (2349 ± 19 pmol/mg/min) compared to *STHdh*^{Q7/Q7} (659 ± 10 pmol/mg/min) (Figure 3). The K_m values were 41 ± 2 μ M and 71 ± 2 μ M for the *STHdh*^{Q7/Q7} and *STHdh*^{Q111/Q111} cells, respectively (Figure 3). In conclusion, *STHdh*^{Q111/Q111} cells have a higher uptake capacity when compared to the *STHdh*^{Q7/Q7} cells.

We then measured the sodium-dependent glutamate uptake in the presence of different glutamate transporter inhibitors, DHK and TBOA. These drugs allowed us to discern specific contribution of transporter subtypes to the total sodium-dependent glutamate uptake in these cell lines. The K_i values of DHK and TBOA for the EAAC1, GLT-1 and GLAST transporters are listed in Table 1. The total Na⁺-coupled uptake in *STHdh*^{Q111/Q111} cells was 6.7 times higher compared to *STHdh*^{Q7/Q7} (Figure 4A inset). In the presence of 300 μ M DHK, which inhibits almost all GLT-1 mediated uptake, the overall uptake was not significantly changed in either cell line, in both states (Figure 4A). Thus, GLT-1 does not play a major role in glutamate uptake observed in these cells. In contrast, in the presence of 50 μ M TBOA, which blocks GLT-1 and EAAC1 transporters, uptake was inhibited by more than 85 % (88% in *STHdh*^{Q7/Q7} and 87% in *STHdh*^{Q111/Q111} cells) in both undifferentiated cell lines (Figure 4A). Therefore, we concluded that the majority of sodium-dependent glutamate uptake in both cell lines was mediated by EAAC1. Increasing the concentration of TBOA to 250 μ M reduced the uptake only slightly, most likely due to a more complete block of EAAC1, since GLAST is not expressed in these cells (Figure 4A).

The *STHdh* cell lines were established from neuronal striatal progenitor cells and may be induced to differentiate into neuron-like cells. Since it is neurons that are lost in HD, we wanted to determine if the increase in EAAC1-mediated glutamate uptake observed in undifferentiated Q111 cells was also observable in cells with a more neuronal phenotype. Therefore, we also measured uptake in cells that were differentiated for 1 hour. As with undifferentiated cells, the uptake was significantly increased in the *STHdh*^{Q111/Q111} cells (6.4 fold higher) compared to *STHdh*^{Q7/Q7} (Figure 4B inset). Similar to undifferentiated cells, 300 μ M DHK had a statistically insignificant effect on glutamate uptake in both cell lines (Figure 4B). However, 50 μ M TBOA blocked 82% of total glutamate uptake in *STHdh*^{Q111/Q111} and 66% of glutamate uptake in *STHdh*^{Q7/Q7} cells, indicating that most of the sodium-dependent uptake in the differentiated cells was also mediated by EAAC1 (Figure 4B). There was a decrease in the total uptake observed in differentiated cells when compared to the undifferentiated cells. Due to very small uptake in differentiated *STHdh*^{Q7/Q7} cells, no statistically significant inhibition was observed with any inhibitor used (Figure 4B). In conclusion, differentiation of the cells has no major impact on the contribution of specific transporters mediating Na⁺-coupled glutamate uptake.

The increase in uptake capacity in the *STHdh*^{Q111/Q111} cells might be due to increase in surface expression of EAAC1. Therefore, we used biotinylation to isolate proteins expressed on the cell surface to determine whether there is an increase in EAAC1 expression (Figure 5). Protein from the total lysate, intracellular fraction and biotinylated fractions were

separated by SDS-Page and incubated with an antibody against EAAC1). We found that the surface expression of EAAC1 was greatly increased in the *STHdh*^{Q111/Q111} cells.

In summary, total glutamate uptake was significantly increased in undifferentiated and differentiated *STHdh*^{Q111/Q111} cells when compared to *STHdh*^{Q7/Q7} cells and, in both cell lines, sodium-dependent glutamate uptake was mediated primarily by EAAC1.

3.4. Inhibition of Akt pathway decreases glutamate uptake in both cell lines

It has been reported in the literature that the amount of EAAC1 on the cell surface is increased upon activation of the Akt pathway (Krizman-Genda et al., 2005). In addition, an increase in activity of the Akt pathway has been reported in *STHdh*^{Q111/Q111} cells (Gines et al., 2003). To determine if the increased activation of the Akt pathway contributed to the observed increase in EAAC1-mediated uptake, we measured glutamate uptake in the presence and absence of a potent, irreversible PI3 kinase inhibitor, wortmannin (Powis et al., 1994; Vlahos et al., 1994). We expected that inhibition of the Akt pathway in *STHdh*^{Q111/Q111} cells would decrease EAAC1 mediated glutamate uptake back to levels observed in the *STHdh*^{Q7/Q7} cells. However, this was not the case, as wortmannin inhibited glutamate uptake in both cell lines (Figure 6). Because of these results, we sought to confirm the previously published results showing increased Akt phosphorylation in Q111 cells. However, western blot analysis demonstrated that there was no change in the levels of phosphoAkt in the *STHdh*^{Q111/Q111} cells compared to the *STHdh*^{Q7/Q7} cells, indicating that the activity of the Akt pathway is unchanged in the *STHdh*^{Q111/Q111} cells (Figure 7) under conditions of culture we employed.

3.5. Potassium channel currents are decreased in the *STHdh*Q111/Q111 cells

Interestingly, an alternative inhibitor of the Akt pathway, LY294002, that also affects the potassium channel Kv2.1, decreased EAAC1 mediated glutamate uptake in *STHdh*^{Q111/Q111} cells to levels observed in the *STHdh*^{Q7/Q7} cells (Figure 6). In fact the inhibitory effect of LY294002 was significantly increased in the *STHdh*^{Q111/Q111} cells compared to its effect in the *STHdh*^{Q7/Q7} cells (Figure 6 inset). As the activation of the Akt pathway is unaltered between the cell lines, this increased inhibition in the *STHdh*^{Q111/Q111} cell line by LY294002, might be attributed to increased activity of the potassium channels in these cells. Recordings of voltage-dependent potassium currents were obtained from the *STHdh*^{Q7/Q7} and *STHdh*^{Q111/Q111} cells with the whole-cell configuration of the patch-clamp technique (Figure 8). Contrary to our expectations, we determined that the potassium currents in the *STHdh*^{Q111/Q111} cells were in fact decreased compared to the *STHdh*^{Q7/Q7} cells.

4. Discussion

To determine if glutamate transporter dysfunction has a role in Huntington's disease, we studied the expression and function of high affinity, Na⁺-dependent glutamate transporters in striatal neuronal cell lines. First, we demonstrated that GLT-1 and EAAC1 are the only glutamate transporters expressed in these cell lines. We also demonstrated that the protein expression of EAAC1 in the *STHdh*^{Q111/Q111} cells is modestly decreased compared to *STHdh*^{Q7/Q7}. For GLT-1, using an antibody against the N-terminal amino acid sequence MASTEGANNMPKQVE, expression was similar in both cell lines. Using a C-terminus antibody to detect GLT-1a, we found no expression of GLT-1a in either cell line. With an alternative C-terminus antibody that detects GLT-1b, we determined that there was a decrease in GLT-1b expression in the *STHdh*^{Q111/Q111} cells. This decrease in GLT-1b expression is not detected with the N-terminus antibody, possibly because the most abundant isoform is detected by the anti-nGLT-1 antibody and is not GLT-1b. We know that GLT-1a is not expressed in these cells, but other isoforms of GLT-1, such as GLT-1c, do exist

(Rauen et al., 2004). However, an antibody specific for this isoform of GLT-1 is not currently available. Furthermore, alternative splice variants of GLT-1 encoding a different N-terminus also exist. These variants include a form that encodes the C-terminus of GLT-1b (DIETCI) and is the least abundant GLT-1 transcript present in the mouse cortex (Peacey et al., 2009). A loss in this specific transcript in the Q111 cells may account for the detected decrease in GLT-1b expression, while immunoreactivity detected by our anti-nGLT-1 antibody remains unchanged. Yet another explanation is that GLT-1b in the *STHdh*^{Q111/Q111} cells may have undergone post-translational modification such as dephosphorylation or palmitoylation that renders it undetectable by the C-terminus specific antibody (Huang et al., 2010; Wilson et al., 2003).

Characterization of the functional properties of glutamate transporters in the *STHdh* cells demonstrated that, surprisingly and contrary to expectation, glutamate uptake into the differentiated and undifferentiated *STHdh*^{Q111/Q111} cells is almost 4 fold higher compared to *STHdh*^{Q7/Q7} cells. GLT-1 does not contribute significantly to the uptake in either cell line. In both cell lines EAAC1 is the primary glutamate transporter (88% in Q7 and 87% in Q111). The increase in uptake in *STHdh*^{Q111/Q111} cells is mediated by EAAC1. The increase in glutamate uptake in the *STHdh*^{Q111/Q111} cells is not due to an overall increase in the protein level of EAAC1 because *STHdh*^{Q111/Q111} cells have actually decreased levels of total EAAC1. Using immunocytochemistry, very little EAAC1 is detected at the surface of *STHdh*^{Q7/Q7} and the *STHdh*^{Q111/Q111} cells; previously it has been noted that EAAC1 is mainly localized in the cytoplasm (Conti et al., 1998; He et al., 2000; Rothstein et al., 1994). Furthermore, using immunocytochemistry, there is no evident difference in the localization of EAAC1 to the cell surface or in the soma between the two cell lines. However, there is likely to be an increase in the plasma membrane expression of EAAC1 in the *STHdh*^{Q111/Q111} cells as there is a significant increase in uptake in these cells. In fact, we found that a significant increase in surface EAAC1 was demonstrable in the *STHdh*^{Q111/Q111} cells, consistent with an increase in trafficking of EAAC1 to the cell surface. These findings are not consistent with recent studies that have suggested that in primary cortical neurons from HD^{140Q/140Q} mice there is a deficit in EAAC1 trafficking to the membrane, due to impaired Rab11 trafficking to the cell surface (Li et al., 2010). The discrepancy between our results and those of Li et al. may be due to differences between primary cortical cells used by Li and colleagues and the immortalized striatal cell lines used in this study.

Activation of the Akt pathway increases EAAC1 surface expression and function (Krizman-Genda et al., 2005). We did not observe any difference in activation of Akt, in contrast to results reported previously (Colin et al., 2005; Gines et al., 2003). This may be attributed to different sub clones of the *STHdh* cells that were used. Furthermore, wortmannin, a strong and irreversible inhibitor of the Akt pathway equally suppressed glutamate uptake in both cell lines suggesting that differences in Akt function between *STHdh*^{Q7/Q7} and *STHdh*^{Q111/Q111} cells could not be responsible for the increase in glutamate transport in *STHdh*^{Q111/Q111} cells. Another reversible inhibitor of Akt, LY294002 had greater inhibitory activity on glutamate uptake in *STHdh*^{Q111/Q111} cells than *STHdh*^{Q7/Q7}. In fact, levels of glutamate uptake in the *STHdh*^{Q111/Q111} were reduced to basal levels normally observed in the *STHdh*^{Q7/Q7} cells. Furthermore, the inhibitory effect of LY294002 on the glutamate uptake activity in the *STHdh*^{Q111/Q111} cells was greater than wortmannin. Both LY294002 and wortmannin are known to have inhibitory effects on other targets such as mammalian target of rapamycin (mTOR) and DNA-dependent Protein Kinase (DNA-PK), indicating that the effects of LY294002 on glutamate uptake in the *STHdh*^{Q111/Q111} cells are via an independent mechanism. LY294002 is also known to inhibit potassium channels (El-Kholy et al., 2003). However, we found that potassium channel currents in *STHdh*^{Q111/Q111} cells were smaller, not larger than in *STHdh*^{Q7/Q7} cells. The basis for this difference between

potassium permeability in *STHdh*^{Q7/Q7} and *STHdh*^{Q111/Q111} cells is unclear at this time, but nonetheless, the effects of LY294002 on potassium channels currents fails to explain the observed increased inhibitory effects in the *STHdh*^{Q111/Q111} cells.

In summary, we have demonstrated a marked increase of glutamate uptake activity in an immortalized Huntington's disease striatal cell line model. The primary mediator of the increased uptake was EAAC1, a Na-coupled glutamate transporter whose expression is unchanged in both cells. Activated and non-activated Akt expression was found to be the same in both cells. Therefore an increase in Akt activations cannot explain the increase in EAAC1 mediated uptake in *STHdh*^{Q111/Q111} cells.

It is unclear how the observed change in glutamate uptake might be related to the pathogenesis of HD. It is possible that the increase in the observed EAAC1 mediated glutamate uptake in the *STHdh*^{Q111/Q111} cells may be closely linked to its role as a cysteine transporter (Aoyama et al., 2006; Zerangue and Kavanaugh, 1996). EAAC1 has a high affinity for cysteine (Shanker et al., 2001), which contributes to the synthesis of glutathione, a major anti-oxidant agent (Dringen, 2000). Indications of oxidative stress, due to an overproduction of reactive oxygen species and/or a reduction in the antioxidant capacity, are apparent in HD (Li et al., 2010; Wytenbach et al., 2002). In the *STHdh*^{Q111/Q111} cells, an increase in oxidative stress may lead to increased trafficking of the EAAC1 transporter to the membrane surface in order to increase cysteine uptake and elevate glutathione levels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- EAAC1 is the primary sodium dependent glutamate transporter in *STHdh*^{Q7/Q7} and *STHdh*^{Q111/Q111} striatal neuronal cell lines expressing wildtype and mutant *huntingtin* genes.
- Glutamate uptake in *STHdh*^{Q111/Q111} cells is 3.6-fold higher than in *STHdh*^{Q7/Q7} cells.
- Surface expression of EAAC1 is increased in *STHdh*^{Q111/Q111} cells.
- The increase in glutamate uptake in *STHdh*^{Q111/Q111} cells is mediated by EAAC1.

Figure 1. The glutamate transporters GLT-1b and EAAC1 are expressed in *STHdh* cell lines
A, Expression of EAAC1 is decreased in *STHdh*^{Q111/Q111} cells compared to *STHdh*^{Q7/Q7}.
B, There is no difference in immunoreactivity with anti-nGLT-1 between *STHdh*^{Q7/Q7} and *STHdh*^{Q111/Q111} cells. **C**, GLT-1b expression is stronger in *STHdh*^{Q7/Q7} cells compared to *STHdh*^{Q111/Q111} cells;* P<0.05, two-way ANOVA; data from 3 separate experiments. Error bars indicate SEM. GLT-1a and GLAST specific antibodies did not detect any signal in *STHdh* cells (Supplemental Figure 1 and data not shown).

Figure 2. Localization of glutamate transporters in *STHdh* cell lines

Expression of both GLT-1b and EAAC1 was predominantly in the cytoplasm and not in the plasma membrane. A small fraction of near-membrane staining for EAAC1 was observed in both cell lines in both states. In differentiated *STHdh*^{Q7/Q7} cell lines, more prominent near-membrane staining for nGLT-1 was observed. Only weak near-membrane staining was observed for GLT-1b. There was no GLT-1a or GLAST expression. Small inset boxes display higher magnification. “Q7D” and “Q111D” designate differentiated phenotypes of respective cell lines. Scale bar is 20μm. Bisbenzamide (blue) was used to visualize nuclei.

Figure 3. Na-dependent glutamate uptake capacity is higher in *STHdh*^{Q111/Q111} cell lines
 Saturation analysis was conducted on undifferentiated *STHdh*^{Q7/Q7} and *STHdh*^{Q111/Q111} cell lines. The K_m for *STHdh*^{Q7/Q7} was $41 \pm 2 \mu\text{M}$ and for *STHdh*^{Q111/Q111} $71 \pm 2 \mu\text{M}$, $p < 0.0001$. The V_{max} for *STHdh*^{Q7/Q7} was $659 \pm 10 \text{ pmol/mg/min}$ and for *STHdh*^{Q111/Q111} $2349 \pm 19 \text{ pmol/mg/min}$, $p < 0.0001$. Error bars represent standard error of the mean; pooled data from 3 experiments.

Figure 4. Na^+ -dependent glutamate uptake was increased in *STHDH*^{Q111/Q111} cell lines and was mainly mediated by EAAC1

A, There was a 6.7 fold higher Na^+ -dependent glutamate uptake in undifferentiated *STHdh*^{Q111/Q111} cells compared to *STHdh*^{Q7/Q7} (shown in the inset). Na^+ -dependent uptake in both cell lines was not decreased significantly in the presence of 300 μM DHK (inhibiting only GLT-1). In the presence of 50 μM TBOA (inhibits GLT-1 and EAAC1 at this concentration) or 250 μM TBOA (also inhibits GLAST at this concentration), glutamate uptake was almost completely inhibited in both cell lines. **B**, In the differentiated state, Na^+ -dependent glutamate uptake was 6.4 fold higher in *STHdh*^{Q111/Q111} cells compared to *STHdh*^{Q7/Q7} (shown in the inset). As in undifferentiated cells, 300 μM DHK did not inhibit uptake in differentiated *STHdh*^{Q111/Q111} cells. Both 50 μM and 250 μM TBOA significantly inhibited the uptake. No significant uptake inhibition was measured in differentiated *STHdh*^{Q7/Q7} cell lines. Error bars indicate standard error of the mean. Shown is pooled data from 5 experiments; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, analyzed by two-way ANOVA.

Figure 5. Surface expression of EAAC1 was increased in the *STHdh*^{Q111/Q111} cell line

A, We assayed distribution of EAAC1 protein between the intracellular compartment and the surface by biotinylating surface proteins and isolating them by avidin bound to beads. **B**, Consistent with results from western blotting of total lysates, there appeared to be decreased expression in the whole cell lysate and intracellular fractions in the *STHdh*^{Q111/Q111} cell line. However, whereas there was almost no detectable EAAC1 in the surface fraction in the wildtype cell line, in the *STHdh*^{Q111/Q111} cell line there was substantial expression of the transporter.

Figure 6. Inhibition of the Akt pathway decreased glutamate uptake in both cell lines

Glutamate uptake was measured in the absence (vehicle only, no drug), or presence of an Akt pathway inhibitor: 25 μ M LY294002 or 0.1 μ M wortmannin (both drugs inhibit PI3K). A significant decrease in glutamate uptake was observed in both cell lines in the presence of wortmannin and only in *STHdh*^{Q111/Q111} using LY294002. Note that LY294002 showed greater inhibitory effect compared to wortmannin in *STHdh*^{Q111/Q111} cells. Also, LY294002 showed higher inhibition in *STHdh*^{Q111/Q111} compared to *STHdh*^{Q7/Q7} cell lines (as shown in the inset). Pooled data of 3 experiments. * $p < 0.05$, *** $P < 0.001$, analyzed by two-way ANOVA.

Figure 7. There was no difference in Akt expression between *STHdh*^{Q7/Q7} and *STHdh*^{Q111/Q111} cells

In both cell lines Akt and phosphAkt expression is the same. Total brain lysate from P7 mouse brain served as a positive control.

Figure 8. Potassium current was decreased in *STHdh*^{Q111/Q111} cells

Top, representative whole-cell K⁺ currents evoked by a series of 10 mV incremental depolarizing steps from a holding potential of -80 mV in a *STHdh*^{Q7/Q7} (left) and *STHdh*^{Q111/Q111} (right). Bottom, mean (± SEM) peak steady state K⁺ current densities at +50 mV obtained from either *STHdh*^{Q7/Q7} (n=9) or *STHdh*^{Q111/Q111} (n=11) cells (**p<0.005; unpaired two-tailed t test).

Table 1

K_i values of DHK and TBOA for corresponding glutamate transporters. K_i values of DHK: for EAAC1, (Dowd et al., 1996); GLT-1, (Wang et al., 1998); and GLAST, (Arriza et al., 1994). K_i values of TBOA for EAAC1, (DeSilva et al., 2009); GLT-1 and GLAST (Shimamoto et al., 1998).

	EAAC1	GLT1	GLAST
DHK	1000	8	>3000
TBOA	6	6	70