Functional Indicators of Glutamate Transport in Single Striatal Astrocytes and the Influence of Kir4.1 in Normal and Huntington Mice

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This study evaluates single-cell indicators of glutamate transport in sulforhodamine 101-positive astrocytes of Q175 mice, a knock-in model of Huntington's disease (HD). Transport-related fluorescent ratio signals obtained with sodium-binding benzofuran isophthalate (SBFI) AM from unperturbed or voltage-clamped astrocytes and respective glutamate transporter currents (GTCs) were induced by photolytic or synaptic glutamate release and isolated pharmacologically. The HD-induced deficit ranged from -27% (GTC maximum at -100 mV in Ba^{2+}) to -41% (sodium transients in astrocytes after loading SBFI-AM). Our specific aim was to clarify the mechanism(s) by which Kir4.1 channels can influence glutamate transport, as determined by either Na^+ imaging or transport-associated electrical signals. A decrease of Kir4.1 conductance was mimicked with Ba^{2+} ($200 \mu\text{M}$), and an increase of Kir4.1 expression was obtained by intravenous administration of AAV9–gfaABC1D–Kir4.1–EGFP. The decrease of Kir4.1 conductance reduced the sodium transients but increased the amplitudes of somatic GTCs. Accordingly, after genetic upregulation of Kir4.1, somatic GTCs were found to be decreased. In individual cells, there was a negative correlation between Kir4.1 currents and GTCs. The relative effect of the Kir4.1 conductance was higher in the astrocyte periphery. These and other results suggest that the Kir4.1 conductance affects glutamate transporter activity in a dual manner: (1) by providing the driving force (voltage dependency of the transport itself) and (2) by limiting the lateral charge transfer (thereby reducing the interference with other electrogenic transporter functions). This leads to the testable prediction that restoring the high conductance state of passive astrocytes will not only normalize glutamate uptake but also restore other astrocytic transporter activities afflicted with HD.

Key words: adenoviral transduction; glutamate uptake; neuron-glia; paired recording; photolytic uncaging; SBFI imaging

Significance Statement

Insufficiency of astrocytic glutamate uptake is a major element in the pathophysiology of neurodegenerative diseases. Considering the heterogeneity of astrocytes and their differential susceptibility to therapeutic interventions, it becomes necessary to evaluate the determinants of transport activity in individual astroglial cells. We have examined intracellular Na^+ transients and glutamate transporter currents as the most telling indicators of glutamate clearance after synaptic or photolytic release of glutamate in striatal slices. The results show that, in Huntington's disease, glutamate uptake activity critically depends on Kir4.1. These channels enable the high conductance state of the astrocytic plasma membrane, which ensures the driving force for glutamate transport and dumps the transport-associated depolarization along the astrocyte processes. This has significant implications for developing therapeutic targets.

Introduction

The efficacy of the principal neurotransmitter glutamate in excitatory synapses relies on the rapid switch between states of very

high (up to 3 mM; Raghavachari and Lisman, 2004; Greget et al., 2011; Kessler, 2013) and very low (25 nM; Herman and Jahr, 2007) glutamate concentration. Four types of high-affinity electrogenic transporters, two neutral amino acid transporters, and several splice variants are available in the mammalian brain for

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removal of glutamate from the extracellular space (Danbolt, 2001; Vandenberg and Ryan, 2013; Jensen et al., 2015). The bigger part of this job is performed by EAAT2 (GLT1 in rodents), the most abundant glutamate transporter in striatal astrocytes (Rothstein et al., 1996) in which glutamate uptake not only accounts for the efficient clearance of the neurotransmitter from extracellular space but also for the initiation of the glutamate–glutamine cycle to replenish the glutamate reserve in presynaptic terminals.

In the healthy brain, glutamate uptake operates with a sufficient functional reserve. It is hardly overwhelmed even under conditions of high-frequency activation of synaptic input (Diamond and Jahr, 2000). However, in some neurodegenerative conditions, such as Alzheimer's disease or amyotrophic lateral sclerosis (ALS), glutamate uptake might become insufficient (Lin et al., 1998; Scott et al., 2011). In postmortem material from Huntington's disease (HD) patients, the loss of glial GLT1 mRNA paralleled the progression of the disease (Arzberger et al., 1997; Faideau et al., 2010). Ceftriaxone-mediated stimulation of GLT1 expression alleviated symptoms of ALS in humans (Rothstein et al., 2005) and in mouse models of HD (Miller et al., 2008, 2012). Glutamate toxicity inversely correlated with the level of GLT1 (Estrada-Sánchez et al., 2009). However, in a rat model of spinal cord injury (Li et al., 2014), astrocyte-targeting GLT1 overexpression failed to produce functional recovery and even exacerbated the disease symptoms. This raises the possibility that, in the investigated disease models, loss of GLT1 activity represents a mechanism that helps to cope with the deficiency of one or several factors that are higher in the hierarchy of pathophysiological events, such as a deficiency of Kir4.1 conductance (Tong et al., 2014).

Most of the HD-related studies dealt with overall changes in the expression levels of GLT1 mRNA/protein in homogenized tissue or transport of radiolabeled amino acids in *ex vivo* preparations. Such an approach primarily excludes any chance to test for correlations between transporter activities and other functional indicators exhibited by the same cell. We aimed at filling this gap of information by examining the applicability of different indicators of glutamate transporter function in individual cells of a defined phenotype, i.e., "passive astrocytes" (Steinhäuser et al., 1992). These astrocytes were identified by vital staining with sulforhodamine 101 (SR101; Nimmerjahn et al., 2004). The availability of a caged form of the transport substrate (Fino et al., 2009) and an image-controlled application system (UGA-40; Rapp Optoelectronic) offered, in addition, an opportunity to address site- and dose-dependent aspects of transport activity.

Another aim of the present study was to clarify the role of Kir4.1-type potassium channels as a major factor implied in the regulation of glutamate transport (Djukic et al., 2007; Kucheravaykh et al., 2007; Sibille et al., 2014; Tong et al., 2014). Kir4.1 channels are likely to influence the strength of electrogenic glutamate uptake by setting the level of astrocytic membrane potential (Tzingounis and Wadiche, 2007; Olsen and Sontheimer, 2008; Seifert et al., 2009). In addition, they may limit the spread of transport-associated electrical signals by providing a large shunt conductance (Djukic et al., 2007). Much less is known on the reverse effect, i.e., the possibility that transporter-induced depolarization might activate potassium currents through Kir4.1 channels, thereby creating a compensation for the electrogenic charge produced by the transporter.

Because pharmacological tools for efficient and selective block of Kir4.1 are not yet available and Ba^{2+} is to be used to isolate Kir4.1 effects, it appeared necessary to introduce a contrasting

approach, i.e., we shall also examine the effects of genetic upregulation of Kir4.1 expression.

Materials and Methods

Ethics statement. Every precaution was taken to minimize stress and to reduce the number of animals used in each part of this project. The experiments were performed in accordance with the European Union Directive 2010/63/EU for animal experiments and complies with the requirements for manuscripts submitted to biomedical journals. The work was registered at the Office of Health Protection and Technical Safety of the regional government of Berlin (Landesamt für Arbeitsschutz, Gesundheitsschutz und Technische Sicherheit Berlin, T0448/12, G0006/13, and G0233/14).

Animals. The Z-Q175-KI mouse line was created by CHDI Foundation and provided by the The Jackson Laboratory (catalog #370437). It originated from the Q140 knock-in (KI) mouse described previously (Mennalled et al., 2012) and expresses a chimeric mouse/human exon 1 *mhtt*. Genotyping and analysis of CAG length was performed by Laragen. The average CAG length was 185.9 ± 0.4 ($n = 269$), and age ranged from 46 to 84 weeks. Experiments were performed in either Q175 wild types (WTs) or in homozygotes (HOMs). Although Q175 heterozygotes provide a more appropriate genetic model of human HD, homozygotes were chosen because preceding studies showed that single-cell indicators of HD were more prominent in HOMs (Heikkinen et al., 2012). A few additional experiments were performed in R6/2 mice. In this case, the comparison was made between WT and heterozygotes (HETs). These R6/2 mice were 10–11 weeks old and had 120 ± 1.2 ($n = 17$) CAG repeats. In any case, the groups used for comparison in any given test were carefully age matched. We shall refer to symptomatic mice carrying a mutant form of huntingtin as "HD mice" or just "HD."

Vector production and application. The adeno-associated virus serotype 9 (AAV9) was selected as shuttle for a plasmid containing an astrocyte-specific promoter sequence (gfaABC1D) and a sequence encoding the potassium channel Kir4.1 fused to EGFP. The plasmid was produced by Dr. Baljit Khakh (UCLA, Los Angeles, CA) and available for free use at Addgene (catalog #52925). The final vector construct (AAV9–gfaABC1D–Kir4.1–EGFP) was generated by the University of Pennsylvania Vector Core. Virus injections were performed in 12- to 13-month old male Q175 mice. Using a Broome restrainer (Föhr Medical Instruments), 100 μ l of virus solution containing 9.38×10^{12} gene copies/ml in sterile PBS was injected into the tail vein. After the injection, animals were returned to their cages, and a transduction time between 3 and 6 weeks was allowed before performing additional tests.

Immunohistochemistry. For histological processing, animals were anesthetized with isoflurane and perfused transcardially with physiological saline (0.9% sodium chloride), followed by 4% paraformaldehyde in PBS. Brains were harvested and immersion fixed in 4% paraformaldehyde for 24 h. To ensure cryoprotection, the tissue was then immersed for 72 h into 30% sucrose in PBS. Sagittal sections of 30 μ m were prepared using a Leica CM1950 cryostat (Leica Biosystems) and Tissue-Tek O.C.T. compound (Sakura Finetek). Until further use, the free-floating sections were stored at 4°C in 0.02% sodium azide–PBS.

Before immunostaining, the sections were washed two times for 10 min in PBS containing 0.2% Triton X-100 (Sigma-Aldrich). The primary antibodies were diluted in immuno-buffer consisting of PBS containing 4% horse serum and 0.04% thiomersal. They were applied overnight with light agitation, and the sections were maintained at room temperature (RT). Here we used the following primary antibodies (supplier and dilutions are shown): mouse anti-S100 β (Novus, NBP1-41373, 1:2000), rabbit anti-GFAP (Dako, Z033429-2, 1:3000), and goat anti-EGFP (Novus, NB100-1770, 1:2000). On the next day, after two 10 min washes in Triton X-100–PBS, appropriate fluorophore-conjugated secondary antibodies were diluted 1:800 in immuno-buffer and applied to the sections with light agitation at RT for 3 h. The secondary antibodies were donkey anti-goat Alexa Fluor 555, donkey anti-rabbit Alexa Fluor 488, and donkey anti-mouse Alexa Fluor 647 (all from Life Technologies). After incubation with the secondary antibodies, the sections were again washed twice in Triton X-100–PBS (10 min), mounted onto glass slides, and coverslipped.

Table 1. Cell counts in immunostained sections and in vital slices

Line	Immunostained sections	WT		HD		<i>p</i>	Test	% Difference	Meaning <i>n</i>
		Mean ± SE	<i>n</i>	Mean ± SE	<i>n</i>				
1	Motorcortex								
	#S100β/VF ^a	18.7 ± 1.1	27	20.8 ± 1.1	27	ns	tt		VFs
2	#S100β w GFAP/#S100β (%)	20.2 ± 1.4	27	17.2 ± 1.4	27	ns	tt		VFs
3	Striatum								
	#S100β/VF	20.6 ± 0.8	45	23.7 ± 0.9	45	<0.05	tt	+21	VFs
4	#S100β w GFAP/#S100β (%)	19.1 ± 1.0	45	22.1 ± 1.3	45	0.073	tt	+16	VFs
5	Vital sections								
	#SR101/VF	4.0 ± 0.2	136	4.8 ± 0.3	128	<0.05	MWT	+20	VFs

MWT, Mann–Whitney *U* test; tt, *t* test.
^aVF size, 200 × 200 μm.

Figure 1. Mild astrogliosis in the HD striatum of Q175 homozygotes. **A**, Fluorescent images of a VF in the dorsal striatum of an HD mouse after double immunostaining for S100β and GFAP. **B**, **C**, Counts of S100β-positive cells. **D**, **E**, Percentage fraction of S100β-positive cell staining with GFAP immunoreactivity in the cortex (**D**) and striatum (**E**). Unpaired *t* tests.

Fluorescence microscopy and cell counts. Immunofluorescence images were acquired using a 40× objective and a standard wide-field microscope (Axiovert 100 TV; Zeiss), a SPOT Insight 2.0 monochrome digital camera, and SPOT Advanced 5.0 software (Visitron Systems). Cells were counted within view fields (VFs) of 200 × 200 μm (pixel size, 0.18 × 0.18 μm). Any test group contained the material from three animals, each contributing three sagittal sections, 150 μm apart, with five adjacent VFs in the dorsal striatum and three VFs in the cerebral cortex.

Preparation of brain slices. The animals were deeply anesthetized by inhalation of a mixture of isoflurane and carbogen (95% O₂ and 5% CO₂) and transcardially perfused with 60 ml of ice-cold (~4°C) saline solution containing the following (in mM): 92 *N*-methylglucamine chloride (NMDG),

2.5 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 20 glucose, 0.5 CaCl₂, 10 MgCl₂, 3 Na pyruvate, and 5 ascorbic acid, pH 7.3 (303 mOsm/L). The brains were removed quickly (~1 min), separated into two hemispheres, and transferred to ice-cold oxygenated saline of the same composition. Sagittal slices of 300–400 μm were prepared with a vibration-controlled VT1200 microtome (Leica Biosystems) and then maintained for at least 1 h in artificial CSF (ACSF) containing the following (in mM): 125 NaCl, 3 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 2 CaCl₂, 1 MgCl₂, 10 glucose, 3 Na pyruvate, 0.005 glutathione, and 2.8 ascorbic acid, pH 7.35 (303 mOsm/L). In some of the experiments, 1 μM (+)-MK 801 maleate (MK801) was added to the slice recovery solution.

Patch-clamp recording. For electrophysiological tests, slices were submerged into a perfusion chamber with a constant flow of oxygenated

ACSF. The flow rate was set to 1–2 ml/min. During the recordings, the chamber temperature was maintained at 26–27°C. The preceding experiments at various temperatures (range of 23–30°C) showed that, under the given conditions, slices from animals older than 1 year were best maintained at this temperature, the quality criterion being the level of astrocytic membrane potentials at break-in, in addition to the visual appearance of the slices. Pipette resistance was 3–6 M Ω when filled with the following saline (in mM): 100 K-gluconate, 50 KCl, 5 NaCl, 0.5 CaCl₂, 5 EGTA, 25 HEPES, 2 Mg-ATP, and 0.3 GTP. Electrophysiological signals were acquired using an EPC-8 amplifier (List), a 16-bit analog-to-digital board (ITC-16; HEKA Elektronik), and the software TIDA 4.11 (HEKA Elektronik). The signals were sampled at a rate of 10 kHz and filtered at 3 kHz. Liquid junction potentials were not corrected. In most cases, the holding potential was set to –80 mV (astrocytes) and –70 mV (neurons), which is close to the respective resting membrane potentials recorded in WT cells immediately after break in. Input resistance (R_{in}) and series resistance (R_s) were monitored by regularly applying pulses of –10 mV. Cell capacitance and R_s values were obtained by fitting a mono-exponential function to the capacitance transients. In astrocytes, only recordings with R_s below 12 M Ω were accepted. On average, R_s of astrocytes amounted to 10.1 ± 0.6 M Ω . Cells exhibiting a >20% change of R_s during an experiment were discarded. Genotype-dependent differences were absent. The membrane resistance (R_m) refers to the difference between R_{in} and R_s .

The records were limited to the dorsal striatum. Astrocytes were identified by staining with SR101 after incubating the slices for 5 min at 37–39°C in a solution of ACSF with 12 μ M SR101. SR101 was excited at 573 nm, and its emission was collected at 600–650 nm using filters from Omega Optical (catalog #QMAX/EM600-650/25). Staining striatal slices with SR101 is not entirely indifferent because it may affect the selection and functional characteristics of labeled astrocytes. Our SR101 staining protocol was adjusted to ensure that the resting membrane potential (V_m) values of SR101⁺ cells were not different from the V_m values obtained from unstained astrocytes transduced by systemically applied AAV9–gfaABC1D–td–tomato (A.D. and R.G., unpublished observations). However, td–tomato was only expressed in a subset of astrocytes, namely astrocytes with a sufficiently high level of GFAP promoter activity (~20% of S100 β ⁺ cells). Likewise, considering the different numbers of SR101⁺ and S100 β ⁺ cells (Table 1), we concluded that our SR101 protocol only marked a subset of passive astrocytes, and the latter might not be identical with the population of td–tomato-expressing cells. Furthermore, measurement of R_m in the HOM striatum (A.D. and R.G., unpublished observations) showed significantly lower mean values in unstained astrocytes transduced by AAV9–gfaABC1D–td–tomato than in age- and genotype-matched astrocytes stained with SR101 (tomato, 2.7 ± 0.3 M Ω , $n = 18$ vs SR101, 8.0 ± 0.8 M Ω , $n = 39$, $p < 0.001$, unpaired t test). At the end, we opted for the SR101 identification as a method granting consistency of astrocyte identification throughout all tests in WT and HOM. In contrast to Schnell et al. (2012) and Kang et al. (2010), staining of neurons or changes in neuron excitability were never observed.

Striatal projection neurons (SPNs) were recognized by their inward rectification and the long latency to the first spike (Kita et al., 1984; Nisenbaum and Wilson, 1995). To elicit EPSCs in parallel with synaptically induced glutamate transporter currents (GTCs), bipolar platinum–iridium electrodes (Science Products, catalog #PI2ST31.0A10) were inserted into the adjacent cerebral cortex. The electrodes had a tip distance of 250 μ m and a resistance of 1 M Ω . Pulse duration was 0.02 ms, and repetition frequency of pulses pairs was 0.1 Hz.

Recording of glutamate-induced spine currents. Glutamate-induced spine currents (GISCs) were recorded from SPNs filled with 50 μ M Alexa Fluor 568 hydrazide (Thermo Fisher Scientific). Dendrites running in parallel and no deeper than 50 μ m to the slice surface were selected for identification of suitable spines. To improve the image quality obtained under condition of conventional wide-field optics, stacks of 100 fluorescence images covering an object area of 40×80 μ m (pixel size, 0.1×0.1 μ m) were acquired using a PIFOC piezo-driven z-positioner (Physik Instrumente, catalog #P-725.1CD) attached to a Zeiss Plan-Apochromat

Figure 2. Basic properties of SR101⁺ astrocytes in the striatum and effect of Ba²⁺. **A**, SR101⁺ astrocytes (bright spots) in 400- μ m-thick slices at 20 $\times$ magnification and conventional fluorescent optics. **B**, **C**, Results of astrocyte counts in vital slices and comparison of soma size. Evaluation at 40 $\times$; VF size, 200×200 μ m. **D**, Resting membrane potential at break in. No added drugs. **E**, Same as in **D** but in the presence of Glu receptor blockers (CTRL) and after addition of Ba²⁺ 200 μ M. **F**, Membrane resistance of SR101⁺ astrocytes in the absence of Ba²⁺. **G**, Same as in **F** but in the presence of Glu receptor blockers (CTRL) and after addition of Ba²⁺ 200 μ M. Statistics: **B**, **C**, unpaired t test; **D**, **F**, MWT; **E**, two-way RM-stacked ANOVA. Genotype: $F_{(1,74)} = 7.761$, $p = 0.007$. With or without Ba²⁺: $F_{(1,74)} = 430.4$, $p < 0.0001$. Group comparison: Wilcoxon's matched-pairs signed-rank test. **G**, Two-way RM-stacked ANOVA. Genotype: $F_{(1,23)} = 0.18$, $p = 0.67$. With or without Ba²⁺: $F_{(1,23)} = 45.17$, $p < 0.0001$. Group comparison: Wilcoxon's test. Open circles, WT; gray circles, HD.

63 $\times$ water-immersion objective [numerical aperture (NA) 1.0] and controlled by a subroutine of the Live Acquisition software of TILL Photonics. Noise deconvolution was performed by Autoquant X3 running under Image-Pro Plus from Media Cybernetics.

Sodium imaging. Our methods for sodium imaging in striatal astrocytes mostly the techniques described by C. R. Rose and colleagues (see Langer et al., 2012; Kleinhans et al., 2014). Astrocytes were incubated 20–30 min in

Table 2. Astrocytes and neurons in WT and HOM Q175 mice and effects Ba²⁺ 200 μ M

Line		WT		HD		<i>p</i>	Test	% Difference	Meaning <i>n</i>
		Mean $\pm$ SE	<i>n</i>	Mean $\pm$ SE	<i>n</i>				
Astrocytes									
Basic properties									
1	Membrane potential without drugs (mV)	−80.7 $\pm$ 0.2	129	−77.5 $\pm$ 0.5	103	<0.0001	MWT	−4	Cells
2	Membrane potential with GluR blockers (mV)	−79.4 $\pm$ 0.5	140	−75.8 $\pm$ 0.6	122	<0.0001	MWT	−5	Cells
3	Membrane potential with GluR blockers + Ba 200 μ M (mV)	−62.3 $\pm$ 1.5	30	−57.5 $\pm$ 1.4	46	<0.05	tt	−8	Cells
4	Membrane resistance ($R_m = R_{in} - R_s$) without drugs (M Ω)	6.1 $\pm$ 0.4	55	7.2 $\pm$ 0.6	37	NS	MWT		Cells
5	Membrane resistance with GluR blockers + Ba 200 μ M (M Ω)	9.1 $\pm$ 1.2	14	10.0 $\pm$ 2.4	17	NS	MWT		Cells
6	Ba-induced change of membrane resistance (% CTRL)	97.4 $\pm$ 13.3	14	43.6 $\pm$ 9.2	17	<0.01	tt	−55	Cells
[Na ⁺] _i transients									
MP free (SBFI-AM, Rubi-Glu 3 mM)									
7	Asp-induced Na transient (% baseline)	1.92 $\pm$ 0.12	73	0.84 $\pm$ 0.05	34	<0.0001	MWT	−56	Cells
8	Rubi-Glu-induced Na transient (% baseline)	1.39 $\pm$ 0.06	21	0.82 $\pm$ 0.07	28	<0.0001	tt	−41	Cells
9	Ba-induced change of Rubi-Glu-induced Na transient (% CTRL)	−14.9 $\pm$ 4.7	21	1.3 $\pm$ 9.4	28	NS	tt		Cells
MP controlled (SBFI ic, Rubi-Glu 3 mM, focal stimulation of soma, $V_h = -160$ mV)									
10	Rubi-Glu-induced Na transient (% baseline)	4.42 $\pm$ 0.32	16	3.32 $\pm$ 0.27	11	<0.05	tt	−25	Cells
11	Ba-induced change of Rubi-Glu-induced Na transient (% CTRL)	−35.3 $\pm$ 5.7	10	−30.7 $\pm$ 9.8	10	NS	tt		Cells
Kir4.1-related VAC and GTCs at [Rubi-Glu] 3 mM and V_h of −100									
12	Amplitude of Ba-sensitive VAC at 100 mV (pA)	1595 $\pm$ 189	21	793 $\pm$ 92	26	<0.001	MWT	−50	Cells
13	Ba-induced GTC increment at V_h of −100 mV (pA)	675 $\pm$ 45	21	404 $\pm$ 38	26	<0.0001	tt	−40	Cells
14	Calculated GTC maximum in Ba V_h of −100 mV (pA)	1959 $\pm$ 165	38	1432 $\pm$ 111	39	<0.05	<i>F</i>	−27	Cells (pooled)
15	GTC centroid in Ba at V_h of −100 mV (ms)	299 $\pm$ 12	11	341 $\pm$ 12	25	<0.05	tt	12	Cells
Neurons									
Synaptically induced GTC (cortical stimulation 1 mA)									
16	Mean GTC amplitude (pA)	220 $\pm$ 42	18	217 $\pm$ 60	16	NS	MWT		Cells
17	GTC/EPSC ratio	0.13 $\pm$ 0.02	10	0.04 $\pm$ 0.01	10	<0.05	tt	−69	Cells
Corticoatrial EPSCs									
18	Half decay time (ms)	3.58 $\pm$ 0.44	17	5.12 $\pm$ 0.33	14	<0.05	tt	+43	Cells
Glu-induced spine currents (GISCs, Rubi-Glu 1 mM)									
19	Ba-induced change of GISC decay time constant (% CTRL)	52.5 $\pm$ 5.9	9						Cells
Action potential-dependent sPSC frequency									
20	Frequency TTX-sensitive sIPSCs (s)	0.39 $\pm$ 0.22	6	1.95 $\pm$ 0.41	3	<0.05	MWT		Cells

MWT, Mann–Whitney *U* test; tt, *t* test.

oxygenated ACSF containing 222 μ M of the membrane-permeable form of SBFI [bis(acetyloxymethyl)4-[6-[13-[2-[2,4-bis(acetyloxymethoxycarbon-yl)phenyl]-5-methoxy-1-benzofuran-6-yl]-1,4,10-trioxo-7,13-diazacyclopentadec-7-yl]-5-methoxy-1-benzofuran-2-yl]benzene-1,3-dicarboxylate] (Thermo Fisher Scientific, catalog #S-1264) or individually filled with an intracellular solution containing 1.25 mM SBFI tetraammonium salt (Thermo Fisher Scientific, #S-1262). Wide-field fluorescence imaging of SBFI-AM-stained slices was performed using a digital Live Acquisition imaging system (TILL Photonics) and an Andor Clara CCD camera (TILL Photonics) attached to an upright microscope (Axioskope 2; Zeiss). Images were collected with either a 20 $\times$, NA 1.0 water-immersion objective (Olympus) or a 63 $\times$, NA 1.0 water-immersion Plan Apochromat objective (Zeiss). SBFI fluorescence was excited by a variable scan system (Polychrome V; TILL Photonics). Ratiometric sodium imaging was performed by alternating excitation of SBFI at 340 nm (weakly sodium-sensitive wavelength) and 380 nm (sodium-sensitive wavelength). SBFI emission was collected at >510 nm (dichroic mirror XF2002 and emission filter XF3086 from Omega Optical). Regions of interest (ROIs) with a size of 3.2 $\times$ 3.2 μ m were placed on either the cell body (experiments with SBFI-AM) or dendrites (experiments with membrane-impermeant SBFI). Binning was 16 $\times$ 16. Spatial resolution was then 1.6 μ m/pixel. Images were acquired at a frequency of 0.2/s or 14.3/s. After background correction, a fluorescence ratio (*R*) was calculated for individual ROIs and analyzed offline using an in-house written software routine (NaPro2.4; A.D., Charité Berlin, Berlin, Germany), followed by statistical analysis with Prism 6.0 software from GraphPad Software. To improve the signal-to-noise ratio of the traces, we typically averaged 5–10 traces and smoothed the signals with a five-point moving average. The aspartate (Asp)- or glutamate-induced Na responses were defined as $\Delta R/r = 100\% \times (\text{SBFI RatioGLT1 substrate} - \text{SBFI RatioControl}) / \text{SBFI RatioControl}$. Under the following slightly different experimental conditions, the *R* values were defined as follows: experiments with L-Asp in R6/2 mice, $-F(335 \text{ nm})/F(383 \text{ nm})$; experiments with L-Asp in Q175 mice, $F(340 \text{ nm})/F(380$

nm); and experiments with Rubi-glutamate in Q175 mice, $F(345 \text{ nm})/F(380 \text{ nm})$. When SBFI imaging was combined with glutamate uncaging, the images acquired during the laser flash were discarded, along with the first image after the flash (see Fig. 6C). The $\Delta R/R$ values were then based on the first five data points of the [Na⁺]_i tail and comprised a time period of 350 ms. At this time, the recorded Na response lagged the GTC maximum by 130–480 ms. To prevent the spread of Na⁺ through gap junctions (Langer et al., 2012), the gap junction blocker carbenoxolone (CBX; 100 μ M) was added to the superfusion solution, along with blockers of ionotropic and metabotropic glutamate receptor blockers.

Photolytic glutamate uncaging. The caged compound Rubi-glutamate was applied through a local superfusion system, thereby minimizing the drug expenditure. The tested concentrations ranged from 0.1 to 3 mM. Because Rubi-glutamate is light sensitive, the experiments were performed in complete darkness, and the computer screens and video monitors were covered with two layers of Rosco #27 medium red filters (Roscolab). A 473 nm laser from Rapp OptoElectronic was used for photolytic uncaging. The position of the light spot was defined on the basis of VF coordinates fed into the system for laser positioning (UGA-40; Rapp OptoElectronic). The area of glutamate application was varied by changing the laser intensity from 0 to 10 mW. Most of the data were acquired at laser intensities of 3 or 10 mW. To obtain a spatial estimate of the stimulated region, we determined the area of half-maximal fluorescence intensity obtained at a laser intensity of 3 mW in a planar layer of Lucifer yellow (100 mg/ml) on a coverslipped glass slide. The diameters were 4 and 1 μ m for the 20 $\times$ (Olympus) and 63 $\times$ (Zeiss) objectives, respectively. Of course, the effective concentration of glutamate at any given site could only be determined with a glutamate sensor in combination with a super-fast detection system. This was not done here. A larger illumination area could be produced by a rapid sequence of laser flashes at slightly different positions. The application frequency of the UGA-40-controlled laser

Figure 3. The intracellular sodium transient as an indicator of glutamate transport in SR101⁺ astrocyte from the WT and HD striatum. **A**, Fluorescent images from acute slices of WT to illustrate colocalization of SR101 and SBFI. **B**, In the experiments with L-Asp, the substrate for the glutamate transporter was delivered by bath application in the presence of DNQX (10 μ M), APV (50 μ M), LY341495 (40 μ M), BMI (20 μ M), and TTX (0.5 μ M). DL-TBOA (100 μ M) was added at the end of recordings to block the Asp- or glutamate-induced $[Na^+]_i$ elevation. SBFI fluorescence was recorded from ROIs placed on the somata of individual cells, as marked by an arrowhead in **A**. The traces in **B** and the results in **C** were derived from symptomatic R6/2 WT and HETs. In these experiments, the Na response was defined on the basis of $\Delta R/R = 100\% \times (R_{Asp} - R_{TBOA})/R_{TBOA}$, where $r = F_{335}/F_{385}$. The experiments with Q175 WT and HOMs were performed in similar ways, but for the construction of the graph, we determined $r = F_{340}/F_{380}$ and used the baseline before the Asp application. In **E–G**, the substrate for the glutamate transporter was delivered by photolytic uncaging of Rubi-glutamate (3 mM) with a laser intensity of 10 mW. The illuminated region comprised the soma and at least part of the dendritic field. Again, astrocytes were loaded with SBFI-AM. The SBFI fluorescence was acquired immediately after the 473 nm laser flash. **F**, The black traces (CTRL) were obtained in the presence of glutamate receptor blockers (DNQX at 10 μ M and MK801 at 1 μ M) and the gap junction blocker CBX (100 μ M). Red trace, The same but in the presence of 200 μ M Ba^{2+} . For definition of $\Delta R/R$, see Materials and Methods. The shaded interval comprises the first five data points after the flash from ROIs of $5 \times 5 \mu$ m, as illustrated in **E** (yellow square). **G**, Quantification of results from photolytically applied glutamate shows significant differences between WT and HD mice. **H**, The experiments with Ba^{2+} revealed a significant Ba^{2+} -mediated reduction of the glutamate-induced elevation of $[Na^+]_i$ in WT but not HD mice. Statistics: paired *t* tests.

spot could be as high as 1 kHz. Therefore, in a typical $63 \times$ VF, approximately three astrocytes could be tested almost simultaneously by applying glutamate to the somata of SR101⁺ cells. If not mentioned otherwise, the duration of the glutamate flash was 100 or 200 ms, and the frequency of application did not exceed $\frac{1}{5}$ s.

The amplitudes of photolytically induced GTCs refer to the amplitude maximum reached during the laser flash. The time constants of decay (τ Decay) were obtained by fitting a monoexponential function to the trace starting at the end of the flash. The GTC centroid values ($\langle t \rangle$) were calculated for the entire trace (on and off current) as described previously (Diamond, 2005), i.e., the sum of all GTC amplitude values multiplied by the corresponding time was divided by the sum of the GTC amplitude values alone.

Chemicals, drugs, and other compounds. Most chemicals were from Sigma-Aldrich. In addition, we used the following materials: from Tocris Bioscience (R&D Systems, MK801, (3S)-3-[[[4-(trifluoromethyl)benzoyl]amino]phenyl]methoxy]-L-aspartic acid (TFB-TBOA); from Thermo Fischer Scientific, Alexa Fluor 488 hydrazide, Alexa Fluor 568 hydrazide, SBFI tetraammonium salt cell impermeant, SBFI AM cell permeant; from Abcam, bicuculline methiodide (BMI), cyclothiazide (CTZ), DL-AP-5 sodium salt,

2-(3-carboxypropyl)-3-amino-6-(4-methoxyphenyl)-pyridazinium (gaba-zine), LY341495 disodium salt [(2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid], Rubi-glutamate, tetrodotoxin (TTX).

Data evaluation and statistics. All data were evaluated offline using TIDA 4.11 (HEKA Elektronik), Microsoft Excel, Prism 6.01 (GraphPad Software), and SPSS 21 (SPSS). The quantitative results are presented as mean $\pm$ SEM. Normality of data distributions was evaluated by the Kolmogorov–Smirnov or the Shapiro–Wilk test. Differences between means were determined by paired or unpaired *t* test (normally distributed data) or Mann–Whitney *U* test (MWT, not normally distributed data). Bonferroni's correction was implemented in case of multiple comparison tests. One-way ANOVA (normally distributed data) or Kruskal–Wallis (KW) statistics (not normally distributed data) was used for differences between three groups of data from different cells. Two-way ANOVA with *post hoc* Bonferroni's correction was performed for experiments in the classical 2×2 design, with two independent factors (for instance, genotype and position of glutamate application). Time course data were subjected to a repeated-measures (RM) ANOVA test, followed by Tukey's multiple comparisons test. The asterisks in the figures indicate the fol-

Figure 4. Astrocytic GTCs after photolytic uncaging of glutamate from Rubi-glutamate applied by local superfusion. **A**, Specimen traces of GTCs at V_h of -80 mV after photolytic uncaging of 1 mM Rubi-glutamate by 473 nm laser pulses of different duration. To reach their maximum, the GTCs required pulse durations between 100 and 250 ms. However, to obtain reasonable current amplitudes without toxic side effects of glutamate, impulse durations were limited to 100–200 ms. **B**, Interval-dependent paired pulse ratio (PPR) of GTCs in response to 200 ms pulses. **C**, Quantification of results from paired-pulse uncaging as shown in **A** and **B**. Stimulation with frequencies higher than 0.5/s induced depression. Therefore, in most of the following experiments, GTC activation frequency was set to 0.17/s (intervals of 6 s). **D–G**, I – V relationships of GTCs and effects of the Kir4.1 channel blocker Ba^{2+} , the gap junction blocker CBX, and the blocking substrate TFB-TBOA. [Rubi-glutamate] of 1 mM, pulse duration of 100 ms, laser intensity of 10 mW. In view of the limited voltage control in astrocytes, the depolarization actually achieved by variation of V_h is likely to be underestimated. Note the strong potentiation of GTCs by Ba^{2+} and decrease by CBX in the presence of Ba^{2+} . TFB-TBOA completely blocked the photolytically induced GTCs. The trace obtained in TFB-TBOA was used for subtraction. Statistics: two-way RM-stacked ANOVA. **E**, Genotype, $F_{(1,30)} = 4.79$, $p = 0.037$; V_h , $F_{(20,600)} = 150.7$, $p < 0.0001$. **F**, Genotype, $F_{(1,22)} = 9.64$, $p = 0.005$; V_h , $F_{(20,440)} = 108.0$, $p < 0.0001$. **G**, Genotype, $F_{(1,14)} = 4.72$, $p = 0.048$; V_h , $F_{(20,280)} = 70.75$, $p < 0.0001$.

lowing: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. The numbers in parentheses refer to cells, if not mentioned otherwise.

Results

Astrocytes in HD

Present knowledge on the characteristics of astrocytes and the HD-related dynamics of astrocyte numbers and reactivity is still incomplete. To provide a reference point for a comparison with other studies, we first evaluated the number of astrocytes in immunostained sections and vital brain slices (Fig. 1A; Table 1, lines 1–4). These counts revealed a slightly increased number of

S100 β + astrocytes per VFs in the dorsal striatum of Q175 HOMs aged 1 year and older (Fig. 1C). Moreover, there was a tendency of a mild increase in the fraction of S100 β cells staining for GFAP (Fig. 1E), but in this case, a larger sample size would probably be needed to reach significance. Under the given staining conditions, the number of S100 β cells detected in fixed sections was larger than the number of stained SR101 cells (cf. Nimmerjahn et al., 2004), but, again, the number of labeled astrocytes was larger in vital sections from HD mice (Fig. 2A,B; Table 1, line 5). Soma size was unaffected (Fig. 2C).

SR101 $^{+}$ astrocytes in the striatum belong to the glia phenotype of “passive astrocytes.” The membrane potentials of SR101 $^{+}$ of Q175 WT and HOM astrocytes were similar to the age group of 10–12 months described previously by Tong et al. (2014). Compared with WT preparations, HD astrocytes were more depolarized (Fig. 2D). Addition of the Kir4.1 blocker Ba^{2+} (200 μ M) depolarized both the WT and the HD astrocytes (Fig. 2E). The R_m tended to be higher in HD, notably in the presence of Ba^{2+} (Fig. 2F,G). However, the relative Ba^{2+} -induced increase of astrocyte membrane resistance $[(R_{m(Ba)} - R_{m(CTRL)})/R_{m(CTRL)}]$ was smaller in HD (Table 2, line 6). This is in line with the previous notion (Tong et al., 2014) that the Kir4.1 conductance is weaker in HD mice.

Estimation of glutamate transport activity by SBFI imaging of sodium transients in individual astrocytes

Na^{+} influx is closely associated with the transport cycle itself (Danbolt, 2001; Vandenberg and Ryan, 2013). Therefore, the elevation of $[Na^{+}]_i$ should be a reliable indicator of glutamate uptake. Indeed, in organotypic hippocampal slice cultures, Asp-induced $[Na^{+}]_i$ was used to estimate lesion-induced changes in glutamate transport activity (Schreiner et al., 2013). Application of the membrane-permeant compound SBFI-AM (Fig. 3A,E) leaves the most important variables of glutamate transport activity free, i.e., the membrane potential and the Na^{+}/K^{+} concentration gradients. TFB-TBOA (Shimamoto et al., 2004) identifies the joint contribution of GLT1 (EAAT2) and GLAST (EAAT1; Fig. 3B). Initially, glutamate transport was activated by bath application of L-Asp in the presence of glutamate receptor blockers. In both R6/2 and Q175, the L-Asp-induced Na^{+} transients were smaller in HD mice (Fig. 3B–D, Table 2, line 7). Later, we succeeded in combining focal photolytic glutamate application with SBFI imaging (Fig. 3E–H) and confirmed the results with L-Asp. In the case of whole-cell uncaging of Rubi-glutamate, the HD-related reduction of glutamate transport measured in cells with

Figure 5. HD-related deficiency of the Ba²⁺-sensitive voltage-activated conductance (presumably Kir4.1) and the Ba²⁺-induced enhancement of GTCs. Evidence for an effect of Ba²⁺ on the GTC charge transfer. Experiments with [Rubi-glutamate] 3 mM. **A, B**, Differential traces obtained by subtraction of control currents (in DNQX at 10 mM, MK801 at 1 mM, and LY341495 at 40 mM) and in the presence of additional Ba²⁺ (200 μM). V_h of -140 to +100 mV. **A** presents Ba²⁺-sensitive voltage-activated currents, and **B** shows the GTC gain in Ba²⁺. **C**, I-V plot of the Ba²⁺-sensitive VAC, presumably generated by Kir4.1 channels. Statistics: two-way RM-stacked ANOVA. Genotype, $F_{(1,24)} = 6.08$, $p = 0.02$; V_h , $F_{(12,288)} = 52.3$, $p < 0.0001$. Group differences (WT vs HD), unpaired t test. **D**, The effect of Ba²⁺-sensitive VACs at different holding voltages. Statistics: two-way RM-stacked ANOVA. Genotype, $F_{(1,24)} = 7.65$, $p = 0.011$; V_h , $F_{(12,288)} = 98.9$, $p < 0.0001$. Group differences, unpaired t test. **E**, Sample traces of somatic currents and potentials evoked by photolytic uncaging of 3 mM Rubi-glutamate in CTRL (black) and in 200 μM Ba²⁺ (red) at V_h of -60 mV. Records were obtained sequentially from the same WT astrocyte. **F**, Ba²⁺-induced increment of GTCs in dependence on the position of the photolytic spot. [Rubi-glutamate] at 1 mM, laser intensity of 3 mW. 0 μm refers to the center of the soma. In WT but not in HD, the Ba²⁺-mediated potentiation of somatically recorded GTCs increased with the distance of the photolytic glutamate spot.

uncontrolled membrane potential amounted to -41% (Table 2, line 8, results in the presence of glutamate receptor blockers and CBX). The latter approach was also used to estimate the possible effect of Kir4.1 (Fig. 3H). Considering the depressant action of Ba²⁺ on the Kir4.1 channels, we interpreted the results of Figure 3H as evidence that, in WT, and to a lesser extent in HD, Kir4.1 conductance could influence the efficacy of glutamate uptake by astrocytes. These findings further validate the sensitivity of [Na⁺]_i imaging for the quantification of GLT1-dependent glutamate transport in single astrocytes and underline the usefulness of the SBFI-AM approach for analysis of HD-related alterations of astrocyte function.

Time- and voltage-dependent GTCs and the effect of Kir4.1 and GAP junction blockers

The reported stoichiometry of GLT1 implies that each transport cycle of glutamate is directly coupled to the cotransport of three Na⁺ ions and one proton, followed by the outflux of one K⁺ ion (Danbolt, 2001; Reyes et al., 2013; Vandenberg and Ryan, 2013; Jensen et al., 2015). In contrast to the key significance assigned to Na⁺, the role of K⁺ gradients/fluxes/binding is less well understood. It has been suggested that K⁺ binding to the inward-facing conformation of the transport protein enables the relocation of the substrate-free conformation of glutamate transporters (Verdon et al., 2014). High extracellular [K⁺] affects the efficacy of glutamate uptake, but there are mechanisms by which Na⁺-dependent transport is protected against the fluctuations of [K⁺]_o (Longuemare et al., 1999). In any case, glutamate transport is strongly electrogenic and therefore associated with large electrical signals that can be recorded with whole-cell patch-clamp techniques (Bergles and Jahr, 1997; Diamond, 2005; Goubar et al., 2011; Unichenko et al., 2012; Afzalov et al., 2013; Campbell et al., 2014; Sibille et al., 2014). However, in view of the high conductance state of individual astrocytes and the electrical coupling between them results obtained with GTC, recording must be treated with some caution. Under what additional conditions and to what extent GTC recordings can actually be regarded as suitable to quantify genotype-related differences in glutamate uptake activity still needs to be clarified.

The following experiments were performed to better understand the nature of current flow associated with glutamate transport in striatal astrocytes and to determine the use of GTC recordings for measurement of the HD-related deficits in GLT1. To this end, GTCs were activated photolytically by uncaging Rubi-glutamate at a nominal concentration of 1, 3 or 10 mM. At first, we regarded the temporal requirements for recording maximal GTC amplitudes (Fig. 4A–C). It was found that laser flashes with a duration of 200 ms and a frequency below 0.25 s⁻¹ were suitable for multiple testing. Varying the holding potential (V_h) between -100 and +100 mV rendered I–V relationships with small but significant differences between the genotypes (Fig. 4D,E). However, in the presence of Ba²⁺, the absolute values of GTCs were much larger, and the differences between WT and HD became more obvious (Fig. 4D,F). In view of the slight astrocytosis seen in HD, we also applied the gap junction blocker CBX. In the presence of Ba²⁺, it decreased the size of GTCs in a genotype-dependent manner (Fig. 4D,G). Therefore, all following GTC recordings were performed in the presence of CBX, in addition to glutamate receptor block. Also in the presence of Ba²⁺, the GLT1/GLAST-preferring glutamate uptake blocker TFB-TBOA completely abolished the current response to exogenous glutamate (Fig. 4D). The respective traces were used for subtraction to estimate amplitude and time course of photolytically induced GTCs.

To isolate the Kir4.1-related components within the compound voltage-activated current (VAC; Fig. 5A,C) and to determine the degree of GTC potentiation obtained by Ba²⁺-induced block of Kir4.1 (Fig. 5B,D) we performed a similar subtraction of Ba²⁺ traces. At low concentrations, Ba²⁺ preferentially blocks Kir4.1 channels (IC₅₀ for Ba²⁺ in isolated astrocytes <10 μM; Seifert et al., 2009). At higher concentrations, as used here, Ba²⁺ may in addition block other K⁺ channels. Nonetheless, VACs isolated with Ba²⁺ have helped to estimate the level of Kir4.1 in two mouse models of HD (Tong et al., 2014). There was a strong deficit of Ba²⁺-sensitive VACs in HD mice, a result now reproduced in older Q175 HOMs (Table 2, line 12). The related but

Figure 6. Contrasting site dependency of exogenous glutamate effects on GLT1-related $[\text{Na}^+]_i$ transients and somatic GTCs. Simultaneously recorded traces of $[\text{Na}^+]_i$ transients and somatic GTCs of SBFI-loaded astrocytes. [Rubi-glutamate] at 3 mM, laser intensity of 10 mW, pulse duration of 100 ms, nominal V_h of 160 mV. Experiment in CBX at 100 μM , DNQX at 10 μM , MK801 at 1 μM . **A, B**, Data from 10 cells was pooled together and averaged for illustration purposes. **C**, Aligned traces of the Rubi-glutamate-induced somatic $[\text{Na}^+]_i$ transients in response to glutamate application centered to the soma. **D, E**, Graphs showing the responses to increasingly eccentric positions of the laser spot. Note that $[\text{Na}^+]_i$ had its maximum at the proximal dendrites. The amplitudes of GTCs significantly decreased with increasing excentricity of the glutamate application. Statistics: two-way RM ANOVA for 10 matched pairs in CTRL and in Ba^{2+} . **D**, With or without Ba^{2+} , $F_{(1,9)} = 28.01$, $p < 0.0005$; distance, $F_{(3,27)} = 18.45$, $p < 0.0001$. **E**, With or without Ba^{2+} , $F_{(1,9)} = 96.01$, $p < 0.0001$; distance, $F_{(3,27)} = 30.24$, $p < 0.0001$. **F**, Increase of the relative Ba^{2+} effect on GTCs elicited with glutamate spots at increasing excentricity. Mean values from 10 matched pairs. One-way RM ANOVA. Difference between distance groups: $F_{(3,36)} = 4.876$, $p < 0.01$.

new finding is that HD also affected the Ba^{2+} -dependent increment of GTCs (Table 2, line 13).

These results raise the possibility that, at least under the condition of strong photolytic stimulation with glutamate, the depolarization associated with the electrogenic glutamate transport may activate a compensatory outward current through Kir4.1 channels until the membrane is sufficiently repolarized and K^+ ions can flow back into the astrocytes. The weakly inward rectifying nature of Kir4.1 permits a bidirectional movement of K^+ ions, and Kir4.1-mediated release of K^+ ions into the extracellular space has been shown in a variety of preparations (Olsen and Sontheimer, 2008). If applicable, the hypothetic K^+ outward current through Kir4.1 would represent a functionally rather relevant mechanism of charge compensation (Grewer et al., 2012). However, an outward current of approximately half the size of the measured GTC increment in Ba^{2+} would require a substantial depolarization. Could such a depolarization be induced at the site of transport? Under the admittedly artificial situation of glutamate uncaging, we found that a GTC of 100 pA can only produce a somatic depolarization between 1.75 and 2 mV (Fig. 5E). Although in peripheral dendrites the depolarization could be larger, we consider the available evidence as insufficient to further pursue this idea. A detailed two-electrode study of passive membrane properties in combination with morphological reconstruction and multicompartment modeling is necessary to make additional progress along this line.

More promising at this point seems an alternative hypothesis that implies that the high conductance state of passive astrocytes, in general, and the Ba^{2+} -sensitive Kir4.1 conductance, in partic-

ular, is required to limit the charge spread along the astrocytes dendrites. The effect of membrane conductance on the spread of synaptic signals in neurons has been widely acknowledged and was subject to numerous studies on the cable properties of neuronal dendrites. On theoretical grounds (for a concise summary of background information, see Spruston et al., 1994), the lateral spread of any signal generated by astrocytes should heavily affect the activity of other transporters or voltage-dependent ion channels. The “dendritic filtering” effect may differ in WT and HD because of the reduced expression of Kir4.1. More prominent charge loss along WT dendrites could then lead to an underestimation of HD-related differences. To test this possibility, we performed Rubi-glutamate experiments with smaller light spots centered to sites located at variable distance to the soma. If correct, our hypothesis predicts that the Ba^{2+} -induced increment of GTCs should increase with increasing excentricity of the laser spot. Figure 5F provides first evidence in support of this idea. Indeed, in WT astrocytes, the Ba^{2+} -induced potentiation of GTCs was stronger at more distal sites of glutamate application. In HD astrocytes, this correlation was weaker and failed to reach significance, presumably because of lower expression of Kir4.1 channels and,

accordingly, lower amplitudes of the Ba^{2+} -induced GTC increments.

To conclude, it seems possible that one of the functions of Kir4.1 might be to limit the spread of charge generated by electrogenic transport activity, i.e., to reduce the length constant of astrocytic processes. As a helpful side effect of the decreased membrane conductance enabled by Ba^{2+} , GTCs of distal origin are better resolved. Indeed, in both WT and HD, GTC amplitudes were much larger than in control (Fig. 4, compare E, F). At the same time, the Ba^{2+} -induced increment of GTC amplitude was significantly smaller in HD (Table 2, line 13), consistent with the lower values of Ba^{2+} -sensitive VACs.

Contrasting site dependency of exogenous glutamate effects on GLT1-related $[\text{Na}^+]_i$ transients and somatic GTCs

The issue of dendritic filtering is not just of methodical interest. The hypothesis emerging from the above results is that the function of Kir4.1 might not be limited to the maintenance of a sufficiently negative membrane potential, and Kir4.1 might also be needed to isolate the action of electrogenic transporters along the processes of an astrocyte. A wider spread of transporter currents attributable to a weaker Kir4.1 shunt may lead to an overall loss of transporter function by mutual interference in the case that neighboring synapses are active simultaneously.

To further pursue this idea, we repeated the experiments with focal glutamate uncaging but using higher (3 mM) concentrations of Rubi-glutamate for better resolution of re-

Figure 7. Dose–response curves for whole-cell GTCs. **A, B**, Sample traces of GTCs evoked by photolytic uncaging in control solution (CTRL, **A**) and Ba^{2+} (100 μM , **B**). Traces from HD mice are drawn with thinner lines. The current maxima obtained in WT versus HD with 3 mM Rubi-glutamate are indicated by horizontal lines. Experiments at V_h of -80 mV in MK801 (1 μM), DNQX (10 μM), LY341495 (20 μM), and CBX (100 μM). **C, D**, Dose–response curves for GTCs at V_h of -100 mV. The lines represent exponential fitting curves. The stars in **B** and **C** refer to the comparison of the mean values obtained in WT and HD at the given concentration. A significant difference between WT and HD is only seen at higher Rubi-glutamate concentration. The EC_{50} (calculated from the fitting curves in CTRL and Fig. 7). Dose–response curves for whole-cell GTCs. **A, B**, Sample traces of GTCs evoked by photolytic uncaging in control solution (CTRL, **A**) and Ba^{2+} (100 μM , **B**). Traces from HD mice are drawn with thinner lines. The current maxima obtained in WT versus HD with 3 mM Rubi-glutamate are indicated by horizontal lines. Experiments at V_h of -80 mV in MK801 (1 μM), DNQX (10 μM), LY341495 (20 μM), and CBX (100 μM). **C, D**, Dose–response curves for GTCs at V_h of -100 mV. The lines represent exponential fitting curves. The stars in **B** and **C** refer to the comparison of the mean values obtained in WT and HD at the given concentration. A significant difference between WT and HD is only seen at higher Rubi-glutamate concentration. The EC_{50} (calculated from the fitting curves in CTRL and Ba^{2+}) did not differ, but there was a significant HD-related deficit in the calculated response maximum. Statistics: two-way ordinary ANOVA. **C**, Genotype, $F_{(1,93)} = 6$, $p = 0.02$; [Rubi-glutamate], $F_{(3,93)} = 147.9$, $p < 0.0001$; interaction, $p = 0.019$. The genotype-related difference within groups (same [Rubi-glutamate]) was tested with unpaired t tests. Comparison of fit parameters with extra sum-of-squares F test: maximum, $F_{(1,97)} = 6.37$, $p = 0.013$; EC_{50} , $F_{(1,97)} = 0.59$, $p = 0.45$. **D**, Genotype, $F_{(1,69)} = 19.65$, $p < 0.001$; [Rubi-glutamate], $F_{(3,69)} = 194.4$, $p < 0.0001$; interaction, $p < 0.001$. The genotype-related difference within groups (same [Rubi-glutamate]) was tested with unpaired t tests. Comparison of fit parameters with extra sum-of-squares F test: maximum, $F_{(1,73)} = 6.1$, $p = 0.016$; EC_{50} , $F_{(1,73)} = 0.12$, $p = 0.73$. The total number of tested astrocytes is given in parentheses above and below the fitting curves. Note different scaling of y-axes in **C** and **D**. **E, F**, Maximal GTC values calculated for

sponses to peripheral sites (Fig. 6). Individual tested astrocytes were filled with SBFI for additional recording of the $[\text{Na}^+]_i$ transients at the site of stimulation. Figure 6, **A** and **B**, shows simultaneously recorded traces of $[\text{Na}^+]_i$ and GTCs for a case of photolytic glutamate application centered to the soma. Although in general the acquired Na^+ signals lag the respective GTCs, there is still sufficient overlap to compare the Ba^{2+} effects (Fig. 6C). Again, and as a possible consequence of incomplete voltage control and the spread of glutamate in the tissue, Ba^{2+} reduced the size of the $[\text{Na}^+]_i$ transients, although the recordings were made in the voltage-clamp mode. Figure 6D further illustrates that maximal Na^+ elevations were achieved at sites between 10 and 30 μm from the soma, whereas the GTC values steadily decreased with increasing eccentricity of the glutamate spot (Fig. 6E). Again, there was a clear site dependency of the Ba^{2+} -induced GTC increment (Fig. 6F).

These results reinforce our position that $[\text{Na}^+]_i$ transients might be better suited to quantify glutamate uptake activity in individual astrocytes. GTC-based comparison between the genotypes requires the block of Kir4.1, in addition to glutamate receptor and gap junction block, to gain access to signals generated in the processes.

Dose–response relationships for photolytically induced GTCs in single astrocytes

Therefore, the following dose–response relationships were determined both under control conditions (CTRL) and in the presence of Ba^{2+} . Our aim was to explore the possibility that GTC recordings might provide an electrophysiological equivalent to the maximal glutamate transport activity of single astrocytes and to test for genotype-dependent differences. Figure 7, **A** and **B**, shows transporter currents elicited by photolytic uncaging of various concentrations of Rubi-glutamate in CTRL and in Ba^{2+} . The dose–response curves of Figure 7, **C** and **D**, are derived from the pooled data obtained from 48 WT and 53 HD astrocytes for a glutamate concentration range between 0.1 and 3 mM. The GLT1 transport maxima calculated from the fitting curves of WT and HD mice in CTRL and Ba^{2+} correspond to glutamate concentrations as high as 30 mM. Both the saturating values of [Rubi-glutamate] and the EC_{50} values are likely to be overestimated (compare the K_m values reported for EAAT2 in various expression systems in the study by Zhou and Danbolt, 2013). Nonetheless, the data obtained in Ba^{2+} appear to be suitable to compare the amount of HD-related depression of glutamate uptake activity. Under condition of Kir4.1 block with Ba^{2+} , the HD-related decrease of the GTC maxima at V_h of -100 mV amounted to $\sim 27\%$ (Table 2, line 14). Under the given experimental conditions, the genotype-related differences reached significance at [Rubi-glutamate] equal or larger than 1 mM. The corresponding I – V plots of the calculated GTC maxima demonstrate a genotype-dependent difference throughout the entire range of tested voltages (Fig. 7E, F).

Slowing of glutamate uptake activity in single HD astrocytes

A reduction in the number of transport sites may reveal itself as a slowing of glutamate uptake activity after prolonged exposure to

different levels of V_h . The mean values of GTC (maximum) in WT and HD are different in CTRL (**D**) and Ba^{2+} (**E**). Statistics: two-way ordinary ANOVA. (**E**, genotype) - $F(1, 2079) = 63.9$, $p < 0.001$. (**E**, V_h) - $F(20, 2079) = 56.4$, $p < 0.001$. (**F**, genotype) - $F(1, 1575) = 84.2$, $p < 0.001$; V_h , $F(20, 1575) = 63.8$, $p < 0.001$; interaction, NS. Differences between groups, F test. The difference between WT and HD does not depend on V_h (no interaction).

Figure 8. HD-related slowing of glutamate uptake activity in astrocytes. **A**, Traces of GTCs elicited by whole-cell uncaging of Rubi-glutamate. Records under control conditions and in the presence of $200 \mu\text{M Ba}^{2+}$. V_h range: 0–160 mV. [Rubi-glutamate] at 3 mM. **B**, Amplitude-scaled traces for comparison of GTC decay in HD and WT mice under control conditions (black, gray) and in the presence of $200 \mu\text{M Ba}^{2+}$ (red, orange). **C**, Time constants of GTC decay in $200 \mu\text{M Ba}^{2+}$ at different V_h . Note genotype-related difference in the GTC decay kinetics under condition of Kir4.1 block. Statistics: two-way RM-stacked ANOVA. Genotype, $F_{(1,24)} = 12.53$, $p = 0.0017$; V_h , $F_{(8,192)} = 0.53$, $p = 0.83$; comparison within groups, unpaired t test. **D**, GTC centroid values in $200 \mu\text{M Ba}^{2+}$ at different V_h . Statistics: two-way RM-stacked ANOVA. Genotype, $F_{(1,24)} = 6.06$, $p = 0.02$; V_h , $F_{(8,192)} = 0.89$, $p = 0.53$; comparison within groups, unpaired t test. **E**, Mean GTC centroid values for WT and HD at lower and higher concentrations of Rubi-glutamate. The slowing of glutamate uptake activity is significant at [Rubi-glutamate] at 3 mM. Data shown for V_h of -80 mV. Statistics: ordinary two-way ANOVA. Genotype, $F_{(1,64)} = 3.16$, $p = 0.08$; [Rubi-glutamate], $F_{(1,64)} = 13.63$, $p = 0.0005$; comparison within groups, unpaired t test.

glutamate at high concentrations, as envisaged previously (Diamond, 2005). In line with this hypothesis, the responses to photolytically induced GTCs lasted longer, with a larger off-component of the GTCs, i.e., the currents generated after the glutamate flash (Fig. 8A,B). At first, we examined the kinetics of the GTC decay. Figure 8C shows a genotype-dependent but voltage-independent increase of the time constant of GTC decay. Calculation of the corresponding centroid values revealed an HD-related deficit in the speed of the entire glutamate uptake response at a glutamate concentration of 3 mM (Fig. 8D,E). An increase of the time period required to complete clearance of glutamate from the extracellular space is consistent with the as-

sumed HD-related decrease in the number of GLT1 transporters. Both should affect the temporal resolution of glutamatergic signals in corticostriatal and thalamostriatal pathways.

Evidence for HD-related deficits in astrocytic glutamate transport after synaptic release of glutamate by electrical stimulation in the motor cortex

Photolytic focal application of exogenous glutamate simplifies the quantification of GLT1-related electrical signals. At the same time, these results need verification under the condition of synaptic glutamate release. In the present experiments, synaptic glutamate release was induced by electrical stimulation of the motor cortex. Figure 9A illustrates the experimental settings for parallel recording from astrocytes and SPNs at an intersoma distance of $<50 \mu\text{m}$. The traces from astrocytes show a biphasic current comprising the TBOA-sensitive GTC itself, followed by a slow inward current of smaller amplitude. The latter was reduced by Ba^{2+} (Fig. 9B) and completely abolished by Kir4.1 knock-out (Sibille et al., 2014). As in the case of photolytically induced GTCs, the synaptically evoked GTCs increased in Ba^{2+} (Fig. 9B).

The corresponding neuronal traces showed a substantial amplitude decrease and prolongation of EPSC decay by TBOA (Fig. 9B). According to the modeling data obtained by Zheng et al. (2008) for the hippocampal area CA1, such effects were to be expected in cases of massive failure of extrasynaptic glutamate transporters and an involvement of NMDA receptors. Interestingly, the EPSC decay was also slowed by addition of Ba^{2+} , a finding further substantiated by recording single-spine responses to exogenous glutamate (Fig. 10, compare C–G).

When we evaluated the GTC and EPSC traces obtained for cortical stimulation at various sites, we encountered a substantial variability in the GTC amplitudes (Fig. 9D) that might reflect variability not only in the spatial interrelations between the tested neurons and astrocytes but also the variability in the activation of glutamate release from the glutamatergic afferents (Fig. 9E). Therefore, we calculated the GTC/EPSC ratio as a more telling indicator of HD-related deficits in the astroglial responses (Fig. 9F). The GTC/EPSC ratio showed HD-related differences at any intensity tested. For responses obtained with 1 mA, the GTC/EPSC ratio amounted to 0.13, which is similar to the previously reported value for striatal recordings from rat slices (Goubard et al., 2011). The HD-related deficit was -69% , i.e., even higher than the difference obtained for $[\text{Na}^+]_i$ in nonperturbed astrocytes (SBFI-AM experiments) with somatic glutamate application (-41%). Note that the values of GTC/EPSC amplitude presented in the graph of Figure 9F and Table 2, line 17, apply to GTCs in the absence of Ba^{2+} and therefore are likely to be underestimated, especially in WT.

HD- and Ba^{2+} -related effects on EPSC kinetics

In view of the abovementioned Ba^{2+} effect on the EPSC kinetics, we also expected an HD-related difference in the mean values of the half decay time of corticostriatal EPSCs. This was the case (Fig. 10A,B; Table 2, line 18).

To further explore the effect of astrocytic Kir4.1 channels on the kinetics of the postsynaptic responses in SPNs but bypassing eventually existing effects of Ba^{2+} block on synaptic glutamate release, we applied the new approach of activating GISCs in SPNs, as illustrated in Figure 10, C and D. Over the distance examined (0–80 μm from the soma periphery), there was no site-dependent difference in the average amplitude or decay time constants of GISCs, nor was there

Figure 9. Parallel recording of astrocytic and neuronal responses to electrical stimulation of the cerebral cortex. **A**, Experimental scheme and overlay image comprising an SR101⁺ astrocyte after injection of SBFI and an SPN filled with Alexa Fluor 488. Both cells were located at close distance (typically <50 μ m between the soma centers) and characterized by parallel recording of whole-cell currents and, in some cases, $[Na^+]_i$ transients (data not illustrated). Synaptic glutamate release was activated by stimulation of the motor cortex via a bipolar platinum–iridium electrode, tip distance of 250 μ m, pulse duration of 20 μ s. **B**, The sample records were performed at V_h of -80 mV (astrocyte) and -70 mV (neuron). The traces illustrate the effect of Ba^{2+} (200 μ M) and TFB-TBOA (1 μ M). The TFB-TBOA traces were used to calculate the GLT1-related GTCs in response to cortical stimulation at variable intensity. The EPSCs recorded from SPNs were used to normalize the corresponding astrocytic GTCs, because there was considerable site-dependent variability of synaptically evoked responses. The inset in **B**, shows the full trace of a synaptically induced GTC and illustrates the depressant effect of Ba^{2+} on the slow component of the complex astroglial current response observed after cortical stimulation. **C**, GTC traces of an SR101⁺ astrocyte (top) and an SPN (bottom). Responses to cortical stimulation at increasing current intensities. EPSCs were recorded in the presence of gabazine (20 μ M) and LY341495 (40 μ M). GTCs were acquired afterward, in the presence of MK801 (1 μ M), DNQX (10 μ M), and LY341495 (40 μ M). The recording pipettes contained QX314 (500 μ M). **D–F**, Quantification of results. Note significant HD-related differences in the GTCs normalized to the EPSC amplitudes. Statistics: two-way RM-stacked ANOVA. **D**, Genotype, $F_{(1,19)} = 2.98$, $p = 0.10$; stimulation intensity, $F_{(6,114)} = 19.59$, $p < 0.0001$. **E**, Genotype, $F_{(1,32)} = 0.099$, $p = 0.76$; stimulation intensity, $F_{(6,192)} = 44.22$, $p < 0.0001$. **F**, Genotype, $F_{(1,17)} = 12.62$, $p = 0.0025$; stimulation intensity, $F_{(6,102)} = 0.48$, $p = 0.82$.

any genotype-related difference at Rubi-glutamate concentrations up to 1 mM. This suggests that at least in WT perisynaptic glutamate uptake is not overwhelmed by glutamate up to this concentration, but it became clear that Ba^{2+} block of Kir4.1 can produce a significant prolongation and even amplitude decrease of the GISCs (Fig. 10E–G). This intervention thus mimics the HD-related prolongation of EPSCs and underlines the importance of Kir4.1 for the time course of glutamate clearance at synaptic sites.

GTCs after genetic upregulation of Kir4.1 in GFAP-expressing HD astrocytes

Tong et al. (2014) presented evidence that enhanced expression of Kir4.1 in Q175 HD astrocytes not only rescues astrocytic membrane potentials and Kir4.1 currents but also increases the amount of GLT1 protein in the striatum. Thus, upregulation of Kir4.1 in HD was beneficial for the affected animals. In line with this assumption, we observed a return to more normal values of EPSC time constants of decay (Table 3, line 15) and a reduction of action-potential-dependent PSC frequencies (Table 3, line 16). Both indicators exhibited a significant alteration in HD (Dvorzhak et al., 2013).

However, the main aim of the present experiments with AAV9–Kir4.1 was to verify the results obtained with Kir4.1 block and to make additional progress in differentiating between HD-related functional changes resulting from Kir4.1 as opposed to GLT1 deficiency. Specifically, we aimed to clarify the effects of enhanced Kir4.1 expression in striatal astrocytes. If the hypothesis of charge-isolating function of Kir4.1 conductance is correct, genetically induced upregulation of Kir4.1 should influence the GTCs in a manner opposed to Ba^{2+} .

We used the novel approach of intravenous application of an AAV9 vector construct to elicit transduction of Kir4.1 under the control of a GFAP promoter sequence. Counts were performed to estimate the transduction yield on the basis of immunostained sections (Fig. 11A). In the striatum, approximately one-third of the astrocytes with immunoreactivity for S100 β and GFAP stained with an antibody against EGFP (Fig. 11B), suggesting a successful expression of the transgene.

First, we compared the mean values of the Ba^{2+} -sensitive VACs obtained from nontreated and treated HD mice and found a significant increase in the injected cohort (Fig. 11C–F;

Figure 10. HD-related differences in the EPSC kinetics and effects of Ba^{2+} on GISCs in WT SPNs. **A**, Scaled traces of EPSCs (average from 10) in response to electrical stimulation of the motor cortex. Conditions as in Figure 9. Records in the absence of NMDA receptor block. Note slower EPSC decay in HD mice. **B**, Quantification of half decay time of corticostriatal EPSCs. **C**, Single spines were visualized by filling SPNs with Alexa Fluor 568 hydrazide. For these experiments, we chose dendrites running in parallel with the slice surface. Appropriate spine images were obtained by deconvolution of z-stacks comprising 100 wide-field $63\times$ images. **D**, Series of GISCs elicited by stimulating different spines, as indicated in **C**. The flash duration was adjusted to produce a response mimicking the amplitude of unitary EPSC in SPNs (Rothe et al., 2015). [Rubi-Glu] at 1 mM, pulse duration of 1 ms, laser intensity of 3 mW; presumed spot size, $\sim 1\ \mu\text{m}$. **E**, Specimen traces of a WT GISC in CTRL (black) and Ba (red). Right, Same but scaled to the current maximum. Experiment in the presence of CTZ ($50\ \mu\text{M}$), after NMDA receptor block with MK101 ($1\ \mu\text{M}$). **F**, **G**, Significant effects of Ba ($200\ \mu\text{M}$) on the GISC time constants of decay (GISC Tau) and amplitudes in WT SPNs. Statistics: **B**, two-way RM-ANOVA. Genotype, $F_{(1,30)} = 5.73$, $p = 0.023$; stimulation intensity, $F_{(6,180)} = 3.75$, $p = 0.0015$. **F**, **G**, Paired *t* tests.

Table 3, line 8) across the voltage range in which the genotype-dependent differences were different in the comparison between WT and HD (cf. Fig. 5C). The HD-related differences in the astrocytic membrane potential and the Ba^{2+} -mediated increase of the membrane resistance were alleviated (Fig. 11G,H; Table 3, lines 6 and 7). Finally, we looked at the influence of enhanced Kir4.1 expression on the mean amplitudes of GTCs in CTRL and in Ba^{2+} and found a significant treatment-related decrease of GTC amplitudes at negative V_h (Fig. 11I; Table 3, lines 9 and 10).

In vital slices, transduced astrocytes could not be reliably distinguished from nontransduced astrocytes, because the EGFP fluorescence was rather low. We then considered the possibility that the transgene expression in part of the HD astrocyte population might have expanded the inter-individual range of Kir4.1 conductance levels. This would be an opportunity to test for correlations between the variables of interest. Such correlations indeed existed. Figure 11, K and L, presents significant evidence supporting the conclusion that, in individual astrocytes, the GTC

amplitudes were smaller in cells with larger Ba^{2+} -sensitive VACs or larger Ba^{2+} -induced changes in the membrane resistance.

Together, these results render additional support to the viewpoint that the level of Kir4.1 conductance affects both the electrical signals generated by glutamate transport and the measurement of these signals.

Discussion

We examined indicators of glutamate transport activity in adult astrocytes under four different conditions: (1) WT; (2) HD; (3) (partial) block of Kir4.1 with Ba^{2+} ; and (4) AAV9-Kir4.1-mediated upregulation of Kir4.1 expression. The results provide a basis for identification of new biomarkers of HD. They hopefully also advance current knowledge on the mechanisms of glutamate uptake and the interrelations between Kir4.1 and GLT1. In addition, they may shed some light on a yet insufficiently explored aspect of astrocyte physiology that will, in analogy to neurons, be referred to as “signal integration.”

Glutamate uptake in HD

Parallel recordings from neurons and astrocytes or recordings from single astrocytes in slices provide a variety of indicators that illuminate different facets of GLT1 transporter activity in advanced HD (Q175 mice older than 1 year). At this age, homozygote Q175 mice are hypokinetic and generate pathological gamma oscillations, and they present with a massive deficit in striatal dopamine release and a reduction in the number of corticostriatal synapses (Rothe et al., 2015). Q175 HOMs also suffer from a massive loss of brain and body weight (Heikkonen et al., 2012), but they are not yet moribund. According to Menalled et al. (2012), Q175 HOMs die much later, between weeks 75 and 105.

Because insufficiency of astrocytic glutamate uptake can affect the processing of glutamatergic and GABAergic signals in neurons, one may expect HD-induced changes in the patterns of spontaneous and evoked network activity in the striatum (Miller et al., 2011; Dvorzhak et al., 2013; Rothe et al., 2015). For instance, it had been shown that HD mice experience an enhanced tonic activation of group I mGluRs (Dvorzhak et al., 2013) and a reduced efficacy of nonsynaptic astrocytic GABA release resulting from the functional coupling of GAT3 to GLT1 (Wójtowicz et al., 2013). Here we report that HD mice exhibit slower EPSC decay kinetics (Table 2, line 18), a phenomenon consistent with the results of partial GLT1 block with TBOA.

Depending on the technique used, the estimated GLT1-related deficits reported for HD mice differ quite substantially. For striatal GLT1 mRNA in ~ 10 -month-old Q175 HOMs, it ranged from -23 to -53% in the studies by Tong et al. (2014) and Menalled et al. (2012), respectively. The protein level was reduced by 33% (Tong et

Table 3. Effects of systemic application of AAV9–Kir4.2–EGFP

Line		HD noninjected		HD injected		<i>p</i>	Test	% Change	Meaning <i>n</i>
		Mean ± SE	<i>n</i>	Mean ± SE	<i>n</i>				
	Animals								
1	Age at death (d)	362.9 ± 5.5	9	349.6 ± 2.9	10	NS	tt		Animals
2	Final body weight (g)	19.64 ± 0.33	9	20.22 ± 0.39	10	NS	tt		Animals
3	Number of CAG repeats	188.1 ± 1.8	9	186.6 ± 1.6	10	NS	tt		Animals
	Transduction yield (3 weeks)								
	Motor cortex								
4	#S100β with Kir4.1-EGFP/#S100β with GFAP (%)			87.7 ± 13.6	27				VFs
	Striatum								
5	#S100β with Kir4.1-EGFP/#S100β with GFAP (%)			35.5 ± 4.7	45				VFs
	Astrocyte physiology								
	Basic properties								
6	Membrane potential at break in with GluR blockers (mV)	−75.8 ± 0.6	122	−79.3 ± 0.4	38	<0.01	MWT	+5	Cells
7	Ba-induced changed of <i>R</i> _m (% CTRL)	43.6 ± 9.2	17	76.4 ± 12.6	13	<0.05	tt	+75	Cells
	Kir4.1-related VAC and GTCs in response to [Rubi-Glu] 3 mM								
8	Ba-sensitive VAC at <i>V</i> _h of 100 mV (pA)	793 ± 92	26	1388 ± 130	15	<0.001	tt	+75	Cells
9	GTC amplitude at <i>V</i> _h of −120 mV in CTRL (pA)	543 ± 20	30	447 ± 22	33	<0.05	tt	−18	Cells
10	GTC amplitude at <i>V</i> _h of −120 mV in Ba (pA)	960 ± 61	19	732 ± 46	19	<0.01	tt	−24	Cells
	Neuron physiology								
	Basic properties of SPNs								
11	Membrane potential at break in (mV)	−73.1 ± 0.5	17	−74.2 ± 1.2	21	NS			Cells
12	Input resistance (MΩ)	232.6 ± 13.2	17	269.5 ± 21.8	21	NS			Cells
13	Rheobase (pA)	8.8 ± 0.5	11	7.25 ± 0.4	12	NS			Cells
14	Maximal firing frequency (action potentials/s)	34.8 ± 1.6	11	36.5 ± 3.1	12	NS			Cells
	Cortico-striatal input to SPNs								
15	Decay time constant of EPSCs	6.43 ± 0.31	4	4.63 ± 0.48	4	<0.05	MWT	−28	Cells
	Spontaneous activity								
16	Average frequency of action potential-dependent (TTX-sensitive) events (s)	1.85 ± 0.47	15	0.63 ± 0.10	21	<0.05	MWT	−66	Cells

MWT, Mann–Whitney *U* test; tt, *t* test.

al., 2014). We found deficits of −27% for the estimated GTC maxima obtained from dose–response curves in Ba²⁺, −41% for the photolytically induced elevation of [Na⁺]_i in the soma region of unperturbed astrocytes, and −69% for the GTC/EPSC ratio after synaptic glutamate release. It is not surprising that the HD-related functional deficits determined on the basis of GTC recordings in voltage-clamped and Ba²⁺-treated astrocytes were smaller than those obtained with the same stimulus by recording [Na⁺]_i transients in SBFI-AM-loaded astrocytes. The former condition would eliminate much of the Kir4.1 influence, but it does not fully reflect the physiological situation in which membrane potential and membrane conductance are free and depolarization can influence the final outcome of glutamate transporter activity, the glutamate uptake itself. In the case of synaptic glutamate release, the genotype-dependent difference was the biggest, which suggests that, under this condition, the challenge of glutamate transport is possibly the strongest.

It is noteworthy that the saturating concentrations of photolytically applied glutamate were well above the 1–3 mM usually assumed in model synapses with included glutamate uptake (Greget et al., 2011; Kessler, 2013). Although maximal care was taken in the handling of Rubi-glutamate, a compound that is uncaged by visible light (Fino et al., 2009), we could not entirely exclude that the effective glutamate concentration at the site of transport was less than assumed by the presented dose–response relations, attributable to unavoidable partial uncaging and dilution in the tissue. Nonetheless, these dose–response curves are quite telling because they show substantial genotype-related differences in the GTC maxima (Table 2, line 14).

In the present study, treatment with AAV9–Kir4.1–EGFP has been applied as an experimental tool rather than being analyzed as a potential therapeutic measure for HD. Nonetheless, our results shed

some light on the possible benefits of systemically applied AAV9–Kir4.1. In treated HD mice, the astrocytes recovered normal membrane potential and input resistance values, exhibited a significant shortening of EPSC decay time constants, and showed a reduction of the action potential-dependent spontaneous activity (Table 3, line 16).

Interrelations between Kir4.1 and GLT1

What are the mechanisms underlying the probably beneficial effects of Kir4.1 upregulation in HD? In general, evaluation of the physiological role of Kir4.1 channels suffers from the lack of good blocking tools. The complete knock-out of Kir4.1 cannot be survived very long and has not yet been performed in adult mice, but the available conditional *Kir4.1*^{−/−} mice (<25 d; Djukic et al., 2007) validated the use of Ba²⁺ as a blocker of Kir4.1 in passive astrocytes. Varying the extracellular concentration of Ba²⁺, we determined that 200 μM was sufficient to reach 85% of the maximal potentiation of photolytically induced GTC (our unpublished result). The maximum was reached with 3.3 mM, in which Ba²⁺ can block other voltage-activated K⁺ channels (K_A , K_{DR}) that also exist in passive astrocytes (Bordey and Sontheimer, 2000).

In a colorimetric assay of glutamate clearance in primary astrocyte cultures, 100 μM Ba²⁺ exerted a similar depression, as did treatment with Kir4.1 siRNA (Kucheryavykh et al., 2007). Sibille et al. (2014) used *Kir4.1*^{−/−} mice to prove that the inward current dominating the slow phase of the composite astroglial response evoked by activation of Schaffer collateral input actually represented a K⁺ inward current through Kir4.1. Ba²⁺ at 200 μM reduced this slow current by 60%, a result similar to what was found here in striatal astroglia after synaptic glutamate release after cortical stimulation.

The Ba²⁺-induced enhancement of GTCs was initially interpreted to be at variance with the idea that Kir4.1 channels support

Figure 11. Transduction of Kir4.1 in symptomatic Q175 homozygotes after intravenous injection of AAV9–Kir4.1–EGFP. **A**, Colocalization of the astrocyte marker S100 β with Kir4.1–EGFP. Wide-field acquisition of fluorescent images at $40\times$. **B**, Percentage fraction of S100 β + cells expressing Kir4.1–EGFP in relation to the number of S100 β + cells expressing GFAP. In parentheses are shown the number of view fields. Data from three injected mice per condition. **C**, **D**, Traces of the Ba $^{2+}$ -sensitive component of voltage-activated currents, i.e., presumed Kir4.1 currents in astrocytes from nontreated (CTRL) and treated (AAV9–Kir4.1–EGFP) HD mice. Single sweeps. **E**, Voltage dependence of VACs obtained by calculating the difference between the control currents and the currents obtained in the presence of 200 μM Ba $^{2+}$. Pipette solution: 50 mM KCl, 100 mM K gluconate, and EGTA 5 mM (for the full composition, see Materials and Methods). Statistics: two-way RM-stacked ANOVA. With and without AAV9–Kir4.1, $F_{(1,42)} = 5.427$, $p = 0.025$; V_h , $F_{(13,546)} = 229.6$, $p < 0.0001$. **F**, The values of Ba $^{2+}$ -sensitive currents apply to V_h of +100 mV. Statistics: KW test. KW value = 8.8, $p = 0.012$. **G**, **H**, AAV9–Kir4.1-induced recovery of the membrane potential and the values of Ba $^{2+}$ -sensitive R_m , respectively. Statistics: **G**, KW test. KW value = 42.8, $p < 0.0001$. Group comparison, MWT. **H**, One-way ANOVA. $F_{(2,40)} = 6.073$, $p = 0.005$. Group comparison, unpaired t test. **I**, Quantification of results for GTCs. Note significant (Figure legend continues.)

GLT1 activity. This issue was specifically addressed by Afzalov et al. (2013) who considered a role of dendritic filtering. However, probing this idea by using Cs-filled electrodes failed to resolve the role of dendritic filtering, most likely because Cs is not as effective as Ba^{2+} in blocking dendritic K^+ channels on passive astrocytes (Ransom and Sontheimer, 1995). Therefore, it was important to extend the applied approach by performing genetic upregulation of Kir4.1. The results indicate contrasting effects of Kir4.1 block with Ba^{2+} as opposed to AAV9-mediated enhancement of Kir4.1 expression, which supports the idea that the Kir4.1 conductance counterbalances the effect of electrical signals associated with glutamate transport. In addition, it is a requirement for sufficient glutamate uptake because it maintains the negative membrane potential needed as a driving force.

In case of a complex disease like HD, in which astrocytes may have an impairment of both Kir4.1 and GLT1 activity, it is particularly important to isolate the involved variables and to identify the element that is higher in the hierarchy of pathophysiological events. Our results point to an impairment of both Kir4.1 and GLT1. Additional experiments comparing the effects of AAV9–Kir4.1 and AAV9–GLT1 will hopefully resolve the issue of hierarchy.

Signal integration in astrocytes

The question remains as to what extent interaction of electrical signals matters in an unexcitable cell that lacks a defined site of signal integration. There certainly is no “initial segment” as a point of reference for all membrane potential changes generated in a single astrocyte, but the spread of depolarization could make a difference at the site(s) of gliotransmitter release that appear to be concentrated at specific locations (Schreiner et al., 2014; Gundersen et al., 2015). GLT1, too, is not evenly distributed along the astrocyte membrane but has a tendency to aggregate in spots (Schreiner et al., 2014), presumably in the vicinity of glutamatergic synapses (Murphy-Royal et al., 2015). Interactions between neighboring sites could amplify/modify the electrogenic effects contributed by individual transporters at any given site of glutamate uptake. Therefore, it would be important to estimate the actual voltage change produced by glutamate transport in peripheral dendritic branches and to determine the role of Kir4.1 role in neutralizing such effects. According to Higashi et al. (2001), Kir4.1 channels are also more abundant on astrocyte processes wrapping synapses and blood vessels.

Large transporter-induced depolarizations, if uncompensated by an outward flux of positively charged ions or shunted by a high membrane conductance, could interfere with the activity of other transporters and ion currents. Thus, we already see sufficient ground to predict that insufficiency of Kir4.1 may not only compromise the uptake of glutamate but also afflict other electrogenic transporter functions. In short, the high conductance state should be essential for normal transporter activity of passive astrocytes.

←

(Figure legend continued.) differences between treated (green) and nontreated (red) mice at membrane potentials >120 mV. Statistics: two-way RM-stacked ANOVA. With and without AAV9–Kir4.1, $F_{(1,61)} = 1.95$, $p = 0.170$; V_h , $F_{(8,488)} = 84.7$, $p < 0.0001$; interaction, $F_{(8,488)} = 3.82$, $p = 0.0002$. Group comparison, unpaired t tests. **K**, Significant negative correlation between GTC amplitudes and amplitudes of Ba^{2+} -sensitive VACs in a set of astrocytes pooled from treated and nontreated HD mice. V_h of 120 mV. **L**, Significant negative correlation between GTC amplitudes and the Ba^{2+} -induced increase in input resistance. V_h of 120 mV. Symbols: open circles with bars, WT; red circles with bars, HD; green circles with bars, HD treated with intravenous injection of AAV9–Kir4.1.

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