Ubiquitination and the proteasome rather than caspase-3-mediated C-terminal cleavage are involved in the EAAT2 degradation by staurosporine-induced cellular stress

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Abstract

Diminished glutamate (Glu) uptake via the excitatory amino acid transporter EAAT2, which normally accounts for ~90% of total forebrain EAAT activity, may contribute to neurodegeneration via Glu-mediated excitotoxicity. C-terminal cleavage by caspase-3 (C3) was reported to mediate EAAT2 inactivation and down-regulation in the context of neurodegeneration. For a detailed analysis of C3-dependent EAAT2 degradation, we employed A172 glioblastoma as well as hippocampal HT22 cells and murine astrocytes over-expressing VSV-G-tagged EAAT2 constructs. C3 activation was induced by staurosporine (STR). In HT22 cells, STR-induced C3 activation-induced rapid EAAT2 protein degradation. The mutation of asparagine 504 to aspartate (D504N), which should inactivate the putative C3 cleavage site, increased EAAT2 activity in A172 cells. In contrast, the D504N mutation did not protect EAAT2 protein against STR-induced degradation in HT22 cells, whereas inhibition of caspases, ubiquitination and the proteasome did. Similar results were obtained in astrocytes. Phylogenetic analysis showed that C-terminal ubiquitin acceptor sites—but not the putative C3 cleavage site—exhibit a high degree of conservation. Moreover, C-terminal truncation mimicking C3 cleavage increased rather than decreased EAAT2 activity and stability as well as protected EAAT2 against STR-induced ubiquitination-dependent degradation. We conclude that cellular stress associated with endogenous C3 activation degrades EAAT2 via a pathway involving ubiquitination and the proteasome but not direct C3-mediated cleavage. In addition, C3 cleavage of EAAT2, described to occur in other models, is unlikely to inactivate EAAT2. However, mutation of the highly conserved D504 within the putative C3 cleavage site increases EAAT2 activity via an unknown mechanism.

Abbreviations: aC3, active caspase-3; AD, Alzheimer’s disease; ALS, Amyotrophic Lateral Sclerosis; ANOVA, analysis of variance; Ara-C, cytosine β-D-arabinofuranoside hydrochloride; C3, caspase-3; CHX, cycloheximide; CNS, central nervous system; DAPI, 4′,6-diamidino-2-phenylindol; DHK, dihydrocainic acid; DMEM, Dulbecco’s modified Eagle medium; Dox, doxycycline; DTT, DL-dithiothreitol; EAAT, Excitatory amino acid transporter; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; Glu, glutamate; HA, haemagglutinin; HBSS, Hank’s buffered salt solution; HD, Huntington’s disease; ICLAC, International Cell Line Authentication Committee; IR, immune reactivity; LME, L-leucine methyl ester; MS, Multiple Sclerosis; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ns, not significant; QVD, Q-VD-OPh; RT, room temperature; SÉM, standard error of mean; STR, staurosporine; TBOA, DL-threo-β-benzoxoaspartic acid; TBS(-T), tris-buffered saline (with Tween; TRIS, tris(hydroxymethyl)-aminomethane.

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INTRODUCTION

Chronic glutamate (Glu) excitotoxicity is thought to contribute to neuronal loss in several neurodegenerative and neuroinflammatory diseases including but not limited to Amyotrophic Lateral Sclerosis (ALS) (Rothstein et al., 1992), Multiple Sclerosis (MS) (González et al., 2008; Vercellino et al., 2007), Alzheimer’s (AD) (Sheldon & Robinson, 2007; Zádori et al., 2018) and Huntington’s disease (HD) (Faideau et al., 2010). In the central nervous system (CNS), the extracellular Glu concentration is tightly controlled by excitatory amino acid transporters (EAAT), of which in the mammalian forebrain EAAT2 contributes to ~90% of the total EAAT activity (Danbolt et al., 1992). In postmortem CNS tissues of all diseases mentioned above, EAAT2 protein levels were found to be prominently and consistently decreased, especially in those regions where neuronal loss is known to be most prominent, for example, in the ALS spinal cord (Rothstein et al., 1992), inflammatory cortical lesions in MS (Vercellino et al., 2007) and the HD striatum (Faideau et al., 2010), but also the frontal cortex of AD patients (Hoshi et al., 2018; Li et al., 2015). Thus, a plethora of evidence supports the view that pathophysiological relevance of EAAT2 down-regulation in neurodegeneration was underscored by the observation that transgenic astroglial EAAT2 over-expression ameliorated the disease phenotype in a mouse model for ALS (Guo et al., 2003; Takahashi et al., 2015). This, a plethora of evidence supports the view that EAAT2 down-regulation is a unifying mechanism that may contribute to chronic Glu excitotoxicity in diverse CNS diseases (Lewerenz & Maher, 2015). Thus, understanding the underlying mechanism(s) can be expected to impact future therapeutic strategies.

As in ALS spinal cord (Bristol & Rothstein, 1996; Rothstein et al., 1995) and in AD frontal cortex (García-Esparcia et al., 2018), EAAT2 mRNA levels remain normal, at least in these two diseases post-transcriptional mechanisms at the level of translation and/or protein degradation can be assumed to underly the observed decrease in EAAT2 protein. Indeed, fine-tuned, probably demand-dependent mechanisms regulate the translation of EAAT2 mRNA into protein in astrocytes and their processes (Tian et al., 2007; Foster et al., 2018), for example via neuron-derived exosomes and their cargo, the micro-RNA miR-124a (Morel et al., 2013) or cross-talk of neuronal Ephrin type-A receptor 4 and astroglial membrane-bound ligand ephrin-A3 (Carmona et al., 2009). In contrast, ubiquitination of EAAT2—a process frequently involved in targeting proteins for proteasomal degradation—rather determines the relative proportion of EAAT2 protein inserted into the plasma membrane and thereby its activity than affecting total EAAT2 protein levels (González-González et al., 2008; Ibáñez et al., 2016; Martinez-Villarreal et al., 2012); while EAAT2 ubiquitination of a stretch of C-terminal lysines initiates its internalization, efficient de-ubiquitination ensures rapid recycling to the cell surface in several cell lines. Similar observation was made in as in oocytes for recombinant EAAT2 (Boehmer et al., 2006) as well as for endogenous cerebral EAAT2 in mice (Zhang et al., 2017). Only pharmacological inhibition of de-ubiquitination was able to force ubiquitinated EAAT2 protein towards degradation (Martinez-Villarreal et al., 2012).

Of note, the role of EAAT2 ubiquitination and subsequent degradation has so far not been studied in the context of neurodegenerative diseases. In contrast, proteolytic cleavage by caspase-3 (C3), the consensus site for which, DTID, was identified in the EAAT2 C-terminus, was proposed as a pathway through which EAAT2 could be inactivated and degraded (Boston-Howes et al., 2006; Gibb et al., 2007). Injecting active C3 but not C7 inhibited the activity of recombinant EAAT2 expressed in oocytes (Boston-Howes et al., 2006). Correspondingly, incubating murine spinal cord extracts with active C3 but not C7 led to size shift and degradation of EAAT2 protein compatible with C-terminal cleavage (Boston-Howes et al., 2006). Mutating the aspartate 504 at position P1 of the DTID motif, which is absolutely required for caspase-mediated protease sensitivity (Fang et al., 2006), to asparagine (D504N) was shown to protect the activity of recombinant EAAT2 in oocytes against C3 as well as to prevent a C3-mediated EAAT2 protein size shift in lysates of EAAT2-over-expressing cells (Boston-Howes et al., 2006).

In vivo, changes in miR-124a microRNA expression indicative of decreased translation of EAAT2 in the spinal cord of the SOD1G93A transgenic mouse model for ALS were reported as putative molecular basis for the observed loss EAAT2 protein (Morel et al., 2013). Others identified a peptide immunologically resembling the putative C-terminal EAAT2 C3 cleavage product in SOD1G93A mouse spinal cord (Rosenblum et al., 2017). As homozygous knock-in of the EAAT2 D504N mutation ameliorated the disease phenotype in SOD1G93A mice (Rosenblum et al., 2017), it was concluded that C3-mediated cleavage of EAAT2 can be regarded as functionally important for EAAT2 degradation in the context of ALS in vivo.

The hypothesis that C3 activation might regulate EAAT2 levels in the context of neurodegeneration becomes even more attractive when considering that C3 activation has repeatedly been reported in CNS tissues in several neurodegenerative diseases where EAAT2 down-regulation has been observed, including ALS spinal cord (Ranganathan & Bowser, 2010), MS lesions (Vercellino et al., 2005), cerebral cortex of AD (Zhao et al., 2003), and HD caudate nuclei (Vis et al., 2005). Again, this C3 activation was found to be recapitulated in mouse models of these diseases including SOD1G93A mice (Chi et al., 2007; Dahlke et al., 2015; Li et al., 2000), a mouse model for MS (Guyton et al., 2010), a transgenic mouse model for AD (Gu et al., 2016) as well as the R6/2 mouse HD model (L’Episcopo et al., 2016).
et al., 2016). Attenuation of the disease phenotypes was reported upon pharmacological inhibition of C3 in models for ALS, AD as well as HD (D’Amelio et al. 2011; Gu et al., 2016; Guyton et al., 2010; Li et al., 2000; Toulmond et al. 2004).

In summary, it seems tempting to conclude that cleavage by C3 directly initiates EAAT2 down-regulation in numerous neurodegenerative diseases and thereby fosters neuronal demise by excitotoxicity. In this study, we thus set out to identify and apply mammalian cell systems of neuroectodermal origin for detailed analysis of EAAT2 degradation. Lack of endogenous EAAT activity was considered to be a prerequisite for detailed structure-activity analyses upon transient over-expression of modified EAAT2 constructs. Using such cellular models allowed a detailed characterization of the molecular pathways that regulate activity and protein levels of EAAT2 upon cellular stress associated with endogenous C3 activation in vitro.

2 | MATERIALS AND METHODS

No ethical approval was required for the presented exploratory study, no exclusion criteria were pre-determined and it was not pre-registered. Sacrificing new-born mice for the preparation of cortical astrocytes was covered by the licence o.225 of the animal facility of the Ulm University in accordance with the §4 of the German TierSchG. We will share all constructs generated by us upon request.

2.1 | Materials

Tissue culture dishes used were the following: T75 TC flask (Sarstedt, Nümbrecht, Germany), Corning™ Falcon 24-well plates, Corning™ 96-well plates from Thermo Fisher Scientific, white and black Corning® 96-well solid polystyrene microplates from Greiner Bio-One International, and Hard-Shell® 96-well PCR plates from BioRad Laboratories; the Dulbecco’s modified Eagle medium (DMEM; #41966-029), Dulbecco’s phosphate-buffered saline (DPBS; #14190-094), penicillin/streptomycin (#15070-067), Trypsin-EDTA (0.05%; #25300-054), Trypsin (2.5%; #15090046), Opti-MEM I (#31985-062) were from Gibco-Thermo Fisher Scientific, Lipofectamine 2000® (#11668-027), Lipofectamine 3000® (#L3000008), Pierce™ BCA protein assay kit (#23225) and the Pierce™ Coomassie Protein-Assay (#23200) were from Thermo Fisher Scientific, fetal bovine serum (FBS; #S0615) superior from Biochrom-Merck and Bio&Sell (#S0615), DL-threo-β-benzoyloxyspartic acid (TBOA; #1223) and dihydrocanic acid (DHK; #0111) were from Tocris. Methylthiazoltetrazolium (MTT; #M2128), cytosine β-D-arabinofuranoside hydrochloride (Ara-C; #C6645), L-leucine methyl ester hydrochloride (LME; #L1002), pyrazone-41 (PYR-41; #N2915) and 4′,6-diamidino-2-phenylindol (DAPI; #32670) were obtained from Sigma-Aldrich. Staurosporine (STR; #1285/100U) was obtained from R&D Systems, doxycycline (Dox; #A2951,0025), DL-dithiothreitol (DTT; #A2948,0025) and β-mercaptoethanol (β-ME; #A1108,0100) were from AppliChem. MG-132 (#474787) was from Merck-Millipore. Succ-Leu-Leu-Val-Tyr-AMC (Succ-LLVY-AMC; #ab142120) was obtained from Abcam. DNase I (#L5002139) was obtained from Worthington Biochemical Corporation. Oligonucleotides were synthesized by Thermo Fisher Scientific, biomers.net or Eurofins Scientific SE and reconstituted in nuclease-free H₂O at 100 µM. The EAAT2 Taqman probe was from biomers.net and reconstituted in TE (10 mM Tris pH 8.0, 1 mM ethylenediaminetetraacetic acid (EDTA)) buffer at 100 µM. All other Taqman probes were obtained from Eurofins Scientific SE. Endonucleases were obtained from Thermo Fisher Scientific or New England Biolabs. Radiolabelled ³H-L-Glu was obtained from Hartmann Analytic (#0103). All other chemicals were obtained from Sigma-Aldrich.

Antibodies: anti-actin (#A5441; RRID:AB_476744) and anti-HA (H6908; RRID:AB_260070) were from Sigma-Aldrich; anti-active-caspase-3 (#ab2302; RRID:AB_302962) and anti-VSV-G (#ab1874; RRID:AB_302646) were from Abcam; anti-ubiquitin (#171219; RRID:AB_619963) was from Bio-Rad Laboratories; anti-EAAT2 (#3838S; RRID:AB_2190743) was from Cell Signaling Technology. Horseradish peroxidase-conjugated secondary goat-anti-ti-rabbit (#172-1019; RRID:AB_11125143) and goat-anti-mouse (#172-1011; RRID:AB_11125936) antibodies were from Bio-Rad Laboratories and Clean-Blot™ IP Detection Reagent (#21230) was from Thermo Fisher Scientific.

The pcDNA3.1 myc-His(-) A (#V855-20) was from Thermo Fisher Scientific, the RIKEN FANTOM™ 3 set containing the Slc1a2 variant 1 (NCBI accession # NM_001077514) cDNA from Source BioScience. The pB1-5 (#631005) vector was from TET Systems, the pTet-Off® Advanced Tet transactivator 2° (tTA²; #630934) and pEGFP-C1 (#6084-1) were from Clontech Laboratories. The lentiviral expression vector pUltra-Hot (#24130, http://n2t.net/addgene:24130; RRID: Addgene_24130, kind gift from Malcolm Moore), the packing vectors pSAX2 (#12260; http://n2t.net/addgene:12260; RRID: Addgene_12260) and pMD2.G (#12259; http://n2t.net/addgene:12259; RRID: Addgene_12259; both kind gifts from Didier Trono, Lausanne, France) were from Addgene.

MTT, DNAse I and Ara-C were reconstituted in DPBS at concentrations of 5 mg/ml, 2 U/µl and 80 mM, respectively, DTT in H₂O at 2 mM, DAPI in H₂O at 1 mg/ml, Dox in H₂O at 200 µg/ml and DHK in DPBS at 100 mM, respectively. LME was reconstituted in DMEM at 750 µM and pH adjusted to 7.4. TBOA, MG-132, PYR-41, STR and CHX were dissolved in DMSO (#D2650) at concentrations of 100 mM, 10 mM, 1 mM, 1 mM and 10 mg/ml, respectively.

2.2 | Cell culture

A172 (RRID:CVCL_0131) glioblastoma cells (Giard et al., 1973), a kind gift from Dr Mike Andrew Westhoff, Department of Pediatrics, University Hospital Ulm, Germany. HT22 (RRID:CVCL_0321) cells (Maher & Davis, 1996) were a kind gift from Dr Pamela Maher, Salk Institute for Biological Studies. HEK293 (RRID:CVCL_0045) cells (Graham et al., 1977) were a kind gift from Dr Bernd Baumann, Physiological Chemistry, Ulm University. The HEK293 subclone...
LentiX 293 T was from Takara Clontech. All cell lines were cultured in DMEM supplemented with 10% FBS and 100 units/ml penicillin and 100 µg/ml streptomycin (growth medium) at 37°C, 5 ppm CO₂ and > 95% relative humidity. All cell lines are not listed as commonly misidentified cell lines by the International Cell Line Authentication Committee (ICLAC). Cell status is regularly checked and cell line authentication (for human cell lines) and multi contamination test (A172, HEK293, HT22) was performed in August 2019 by Multiplexion GmbH. After starting cultures from frozen stocks, A172 cells were passaged twice and both HT22 and HEK293 cells three times per week. After starting cultures from frozen stocks, A172 cells were passaged up to 15 and HT22 and HEK293 cells up to 25 times. For passaging, cells were dissociated using 0.05% trypsin-EDTA. For experiments A172 cells were plated as follows: 5 \times 10^5 cells/6 cm dish for transient transfection, 10^5 cells/well in 24-well plate for Glu uptake assays. HT22 cells were plated as follows: 5 \times 10^3 cells/10 cm dish for transient transfection, 5 \times 10^3 or 10^4 cells/well in 96-well plate for cell viability assays, 10^5 cells/6 cm dish for STR, PYR-41 and MG-132 treatments. HEK293 cells were plated for Glu uptake assays at a density of 10^5 cells/well in 24-well plates.

Murine primary cortical astrocytes were prepared as described previously (Bayer et al., 2017). All animals were housed within the animal facility of the Ulm University (German Red Cross, Deutsches Rotes Kreuz, DRK, 89081 Ulm) in accordance with § 11 Abs. 1 Nr. 1a und b of the German TierSchG and the ethics guidelines of the Ulm University and regional council of Baden-Württemberg (Referat 39, Konrad-Adenauer-Straße 20, 72072). Male and female (60–200 days old) C57BL/6J (Jackson Laboratories, No. 000664; RRID:IMSR_JAX:000664) mice were used for all experiments. Breeders (one male with one female) were housed in type II polysulfone long cages (428 cm³; ZOONLAB GmbH) with standard bedding under temperature-controlled conditions (23 ± 1°C) with 12-hr reversed light/dark cycles (6 a.m.–6 p.m.). Rodent diet (#V1124-300; Ssniff Spezialdiäten GmbH) and water (HCl-acidified, pH =3) were provided ad libitum. Mouse litter size varied between breeders and pregnancies. Therefore, glial cultures were prepared from the brains of two C57BL/6J neonatal mice per experiment (in total eight). New-borns (day 0–4) were decapitated early in the morning without sex determination or discrimination in accordance with §4 of the German TierSchG. The brains were dissected to obtain the cortices. Subsequently, the meninges and plexus of four cortices (i.e. two brains) were removed and the cortices were washed thrice with DPBS. The supernatant was removed completely, and the cortices were resuspended in 5 ml growth medium (DMEM + 10% fetal calf serum + antibiotics), cells were pelleted by centrifuged at 250 g for 10 min and the supernatant was discarded.

Cortical cells were again resuspended in 10 ml growth medium and plated on T75 flasks pre-coated using 5 ml 0.1 mg/ml poly-L-ornithine for 60 min at 37°C. After 3 days, medium was changed to new growth medium after washing the cells twice with medium. After another 3 days, most microglia were removed by vigorous shaking. The remaining astrocytes were washed with DPBS and detached with trypsin-EDTA. Detached astrocytes were resuspended in 50 ml growth medium and plated onto two 24-well plates per T75 flask (1 ml/well) followed by a medium change after 24h. Upon confluence (typically after 7–14 days), residual microglia were removed by adding 8 µM Ara-C for 4 days followed by a 1 hr-treatment with LME at a concentration 75 mM as described previously (Hamby et al., 2006). Finally, LME-containing medium was carefully aspirated and replaced by new growth medium without damaging the astrocyte monolayers. Cultures were allowed to recover for 2–3 days before being transduced.

2.3 Cloning
cDNA of the EAAT2 open reading frame (ORF) derived from the RIKENFANTOM™ 3 set containing the Scl1a2 variant 1 (NCBI accession # NM_001077514) cDNA from Source BioScience encoding for EAAT2a as indicated in Figure S6a was subcloned into pcDNA3.1 myc-His(+) A vector using BamHI (#R0136S) (pcDNA3.1-EAAT2). EAAT2 with a N-terminal VSV-G-tag (MAYTDIEMNRLGK) was generated amplification of the EAAT2 ORF from pcDNA3.1-EAAT2 using primers 1 and 3 (Table 1) and subcloning of the PCR product into pBl-S using Nhel (#FD0973) and SalI (#FD0644) (pBl-5-VSV-G-EAAT2). A construct expressing VSV-G-tagged EAAT2 truncated C-terminally of aspartate 504 (D504) by introducing a stop codon (pBl-5-VSV-G-EAAT2Δ505-572) was generated similarly using primer 4 instead of 3 (Table 1). The D504 to asparagine mutation (D504N) was generated by introducing a point mutation corresponding to G2106A in NM_001077514.4 into pcDNA3.1-EAAT2 using the QuikChange® II XL Site-Directed Mutagenesis Kit (Agilent Technologies; #200521) using primers 5 and 6 (Table 1, pcDNA3.1-mEAAT2 D504N). The mutated VSV-G-tagged inducible EAAT2 construct (pBl-5-VSV-G-EAAT2D504N) was generated similarly as pBl-5-VSV-G-EAAT2 following PCR amplification of pcDNA3.1-mEAAT2D504N using primers 1 and 3. A haemagglutinin (HA)-tagged EAAT2 was generated by amplification of the EAAT2 ORF using primers 2 and 3 and subcloning into the pcDNA3.1 vector after digestion with Nhel and BsrGI (pcDNA3.1-HA-EAAT2). VSV-G-EAAT2vt was cut out of the pBl5-construct using Nhel and SalI and subcloned into the lentiviral expression vector pUtra-Hot digested with XbaI and SalI. The lentiviral expression vector pUtra-Hot is devoid of all genes associated with packaging or replication of the virus; just the information for bacterial replication, the terminal recombination sequences and the packaging signal still remain. Thus, this third-generation vector represents a very safe lentiviral system as the virus particles are not able to replicate.
TABLE 1  List of primers used for polymerase chain reaction (PCR). The underlined sequences correspond to the nucleotides of the Slc1a2 reference sequence (NM_001077514.4) given in the right column. Grey background indicates the sequence encoding the VSV-G (1) or HA (2) tag, white letters with black background indicate the point mutations, underlined italic letters indicate the Kozak sequence.

| Primer | Sequence (5'−3') | NM_001077514.4 (base pair) |
|--------|-----------------|---------------------------|
| 1 | GCCGCTAGCGCCAGCTGCCGATTCATACGTGATGAAATGGACCGCCTGGGTAGATGCCATCAACAGGGGTGCC | 597-617 |
| 2 | GCCGCTAGCGCCAGCTGTTGCCATTGCCAGATTAATGGACCGCCTGGGTAGATGCCATCAACAGGGGTGCC | 597-617 |
| 3 | GCCGCCAGCTGGAATATTGTGCTTCTATCCTCAGTTCAGTTCAGCAGCTGG | 2288-2322 |
| 4 | GCCGTCGACGGGTCATTAGTCAATGGTGTCCAGCTCAGACTTGG | 2083-2109 |
| 5 | GTGTTGGGAGTTATCCATGTCAGTTCATGTCAGGTTCTTCC | 2087-2118 |
| 6 | GTGTGCTGGACACCCTTACTCCCAAACACC | 2087-2118 |

2.4 | Transient transfection

Twenty-four hours after seeding, A172 cells in 3 ml fresh growth medium were transfected using 500 µl transfection mixture (Opti-MEM I, 4% Lipofectamine 2000®, 1 µg expression plasmid, 1.5 µg tTA2 plasmid). After 5 hr, medium was changed to 5 ml fresh growth medium. After a medium change with 5 ml fresh growth medium, HT22 cells seeded 24 hr earlier were transfected by adding 200 µl transfection mixture (Opti-MEM I, 5% Lipofectamine 2000®, 2 µg expression plasmid, 4 µg tTA2 plasmid). After 6 hr, medium was changed to 10 ml fresh growth medium. Twenty-four hours after transfection, cells were re-plated for experiments. To repress transcription, Dox was added to the medium at a final concentration of 200 ng/ml. Viral transduction of astrocytes is described in detail in the Supplementary Methods section.

For virus production, LentX 293 T cells were co-transfected with psPAX2, pMD2.G as well as pUH-VSV-G-EAAT2 (pUH-EAAT2) using the CalPhos™ mammalian transfection kit (#631312; Clontech® Laboratories, Inc.). Six hours after transfection, the medium was changed to remove the transfection reagent. Forty-eight hours after transfection, the conditioned medium containing virus particles was collected, filtered using a 0.2 µm sterile filter (Sarstedt, Nümbrecht, Germany) and transferred to a 38.5 ml Beckman Ultra-Clear™ tube containing 3 ml 20% sucrose and spun for 2.5 hr at 4°C and 24,000 rpm (~70,000 g) in a Beckman SW32Ti swinging bucket rotor. Supernatant was discarded and pelleted virus was resuspended in DPBS, aliquoted and stored at −20°C until use.

2.5 | Viral transduction

In a first step, the minimal amount of virus needed for maximal expression of the vector-encoded proteins in astrocytes was determined. To this end, aliquoted virus suspension was thawed and 0.5–10 µl were added per well to microglia-free primary murine cortical astrocytes grown in 24-well plates. Five days later, cells were detached using 0.05% trypsin-EDTA without phenol red. The number of mCherry expressing cells was analysed via flow cytometry (Cytomax, Beckman Coulter) using 488 nm for excitation and 610 nm for emission with non-transduced astrocytes as negative control. This experiment was repeated for each virus preparation. Transduction efficacy was calculated as the percentage of mCherry expressing cells of all cells analysed. Using 3 µl of the initial virus suspension induced ~95% mCherry expression and was thus used for the subsequent experiments. To analyse the effect of STR and inhibitors of ubiquitination and the proteasome on recombinant EAAT2 protein levels, microglia-free astrocytes grown on 24-well plates were transduced with pUH-VSV-G-EAAT2 as optimized.

2.6 | RNA isolation and quantitative Taqman multiplex PCR

A172 cells were re-seeded on 6 cm dishes 24 hr post-transfection. Twenty-four hours later, the cells were washed once with DPBS and scraped into 350 µl RLT buffer containing 20 µM DTT from the RNeasy Mini Kit (QIAGEN; #74134) and RNA was isolated following the manufacturer’s protocol using 30 µl H₂O for elution.

For complementary DNA (cDNA) synthesis, 1 µg RNA and the iScrip™ cDNA synthesis Kit (Bio-Rad Laboratories; #170-8891) were used following the manufacturer’s protocol. Reverse transcriptase quantitative real-time PCR (RT-qPCR) to analyse mRNA levels of the VSV-G-tagged EAAT2, mEAAT2Δ505 or EAAT2Δ505-572 in comparison to the expression of four reference genes (Primer/probes: Table 2) was performed in duplicates. To this end, 3 µl cDNA was added to a reaction mix containing the reference gene primer pairs, the respective DNA Taqman probes and SsoAdvanced® universal probes supermix (Bio-Rad Laboratories; #172-5280) in a total volume of 15 µl. RT-qPCR was performed and analysed with the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories) with the following conditions: denaturation 95°C for 45 s, 45 cycles amplification (denaturation 8 s at 95°C, annealing/elongation 20 s at 60°C).
2.7 | MTT assay

Twenty-four hours after adding of staurosporine, cell survival was judged by phase contrast microscopy and assayed by the MTT method as described previously (Hansen et al., 1989). Briefly, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the cells to a final concentration of 1 mg/ml. After 2 hr, cells were lysed and the formazan precipitates solubilized by adding 100 μl lysis buffer containing 50% dimethyl formamide, 20% sodium dodecyl sulphate, 2.5% acetic acid, adjusted to pH 4.7. Absorbance was measured at 570 nm after incubation for 24–48 hr at 37°C in a humid chamber using a microplate reader (BioTek).

2.8 | Radiolabelled Glu uptake

Uptake assays were performed as described previously (Lewerenz & Maher, 2009). Briefly, cells seeded onto 24-well plates were washed thrice with 37°C warm Hank’s buffered salt solution without chloride (140 mM sodium D-glucuronate, 5 mM D-glucose, 10 mM HEPES, 0.4 mM KH₂PO₄, 1 mM MgSO₄, 2.5 mM calcium D-glucuronate, 5.4 mM potassium D-glucuronate, pH 7.4; HBSS-Cl⁻). Uptake was performed by incubating the cells with 2 nCi/μl ³H-L-Glu diluted in 10 μM unlabelled Glu in HBSS-Cl⁻ in the absence or presence of EAAT inhibitors (1 mM DHK or 100 μM TBOA) for 20 min at 37°C. Uptake was stopped by adding ice-cold HBSS-Cl⁻ and two washing with ice-cold DPBS. Cells were lysed with 0.1 M NaOH at 37°C overnight. Radioactivity in the lysate was determined by liquid scintillation counting (Tri-Carb 2810 TR; Perkin Elmer) and normalized to cellular protein quantified by the Pierce™ Coomassie protein assay. EAAT activity was calculated by subtracting radioactivity per mg protein in the presence in inhibitors from activity taken up in the absence of inhibitors.

2.9 | Immunoblotting

Twenty-four hours after transfection, HT22 cells were re-seeded at a density of 10⁶ cells/6 cm dish. After another 24 hr, the cells were treated with 250 nM STR, 250 μM PYR-41, 5 μM pan-caspase inhibitor Q-VD-OPh (QVD), 10 μM MG-132, vehicle (DMSO) or a combination thereof for 6 hr. After 6 hr, cells treated with STR already showed some rounding, indicative of reduced adhesion. Thus, they were harvested directly by scraping into their growth medium without preceding washings. Cells were then pelleted by centrifugation and washed twice with ice-cold DPBS. Finally, the cells were lysed into either 100 μl luciferase assay buffer (25 mM Tris-HCl pH 8.0, 150 mM NaCl, 25 mM disodium pyrophosphate, 50 mM β-glycerophosphate, 2 mM ethylene glycol-bis(2-aminoethyl ether)-N,N′,N′-tetraacetic acid, 2 mM EDTA, 5% glycerol, 1% Triton-X) or 100 μl cell lysis buffer (50 mM HEPES, 50 mM NaCl, 50 mM NaF, 10 mM sodium pyrophosphate, 5 mM EDTA, 1% Triton, 100 mM sodium orthovanadate) supplemented
with 1:100 protease inhibitor cocktail (Sigma-Aldrich; #P8340) and phosphatase inhibitor cocktail (Sigma-Aldrich; #P0044) for 30 min on ice with repetitive mixing. Nuclei were removed by centrifugation (15 min at 14,000 g and 4°C) and protein concentration was determined using the Pierce™ BCA protein assay kit. For gel loading, lysates were mixed with loading dye (1:5, final concentration: Tris 10 mM, pH 8.0, sodium dodecyl sulphate 30 mM, 4% glycerol, 0.008% bromphenol blue, 300 μM β-ME). Per lane 30 μg or 100 μg protein were separated using a precast 10% gels or 4%-12% gradient gels (Bio-Rad Laboratories, respectively), and separated using a XT 4-morpholinepropanesulfonic acid buffer (Bio-Rad Laboratories; #161-0788). Proteins were transferred to polyvinylidene fluoride membranes, which were blocked with 5% skim milk in tris-buffered saline with Tween (TBS-T; 20 mM Tris buffer pH 7.5, 0.5 M NaCl, 0.1% Tween 20) and incubated with a rabbit polyclonal primary antibodies (anti-active-caspase-3 1:1,000, anti-VSV-G 1:40,000, anti-EAAT2 1:1,000) or an mouse monoclonal anti-actin antibody (1:10,000) overnight. Blots were washed three times in TBS-T and incubated for 2 hr with the respective horseradish peroxidase-conjugated secondary antibodies goat-anti-rabbit (1:10,000) or goat-anti-mouse (1:3,000). Bound antibodies were visualized as described previously (Bayer et al., 2017). Each experiment from transfection to immunoblotting was performed at least three times. The optical band densities were quantified using the ImageStudio Lite software (vers. 5.2; LI-COR Biosciences). To correct for protein loading per lane, the ratio of the optic density of the band representing the protein of interest to the optic density of the band representing Actin was calculated. Thereafter, for each immunoblot the ratios of the protein of interest to Actin for each lane were normalized by the mean ratio for that individual blot. For activated C3 (aC3), only the p17 (17 kDa) band was quantified (Han et al., 1997).

After 5 days after transduction, the medium of microglia-free astrocytic cultures was exchanged by 1 ml fresh growth medium. Twenty-four hours later, the astrocytes were treated with STR, PYR41 and MG132 for 6 hr, as described for HT22 cells. Astrocytes were then collected in immunoprecipitation buffer (50 mM HEPES, 50 mM NaCl, 50 mM NaF, 10 mM sodium pyrophosphate, 5 mM EDTA, 1% Triton, 100 mM sodium orthovanadate supplemented with 1:100 protease inhibitor cocktail and phosphatase inhibitor cocktail) and cellular protein was used for immunoblotting using the anti-VSV-G antibody and anti-Actin as loading control as described for HT22 cells.

2.10 | Immunoprecipitation

HT22 cells were plated at a density of 2.5 × 10^6 cells per 10 cm dish in 10 ml growth medium. Forty hours after seeding, HT22 cells were transfected by adding 1.8 ml transfection mixture per plate (900 μl Opti-MEM I, 900 μl Lipofectamine 3000®, 18 μg pcDNA3.1-HA-EAAT2 and 36 μl P3000™ Reagent; mixed and incubated for 20 min at 20°C). After 6 hr, medium with transfection mixture was removed and cells were grown in 10 ml fresh growth medium for another 40 hr. Then, one pair of dishes each were either exposed to h 250 nM STR or vehicle (DMSO). After 3 hr, MG-132 at a final concentration of 10 μM was added to one of the two dishes with cells treated similarly. After another hour, the medium of each dish was collected separately to pellet detached cells by centrifugation at 400 g for 5 min at 4°C. The pellets were stored on ice. The remaining cells on the dishes were lysed by adding 1 ml lysis buffer (μMACS HA Isolation Kit; #130-091–122; Miltenyi Biotec, Bergisch Gladbach, Germany) containing 1x HALT Protease Phosphatase inhibitor cocktail (#78446; Thermo Fisher Scientific), followed be incubation on ice for 15 min. Lysates were scraped into the tubes containing the corresponding pellets and the mixture was incubated for another 15 min on ice. Then, insoluble material was pelleted by centrifugation at 15,000 g for 15 min at 4°C. The supernatant was kept at −20°C prior immunoprecipitation (IP). IP using beads coated with anti-HA antibodies was performed according to the μMACS HA Isolation Kit protocol. Immunoblot analysis of precipitated protein was carried out as described above using antibodies against ubiquitin (1:1,000) and HA (1:1,000) and Clean-Blot™ IP Detection Reagent.

2.11 | Proteasome activity assay

The proteasome activity in HT22 cells was determined as described previously using Suc-LLVY-AMC as substrate (Maher, 2014). Briefly, 10^6 cells/6cm dish seeded 24 hr before were treated with or without STR and simultaneously with or without 250 μM PYR-41, 10 μM MG-132 for 6 hr. Both cell lysis and the assay were performed as reported, the latter using a VICTOR Multilabel Plate Reader (PerkinElmer Inc.) for 1 s per sample. Chymotrypsin-like proteasome activity was calculated as relative fluorescent units per sec per mg protein were determined using the Pierce™ BCA protein assay kit.

2.12 | Luciferase activity assay

To 20 μl lysate prepared as for caspase activity per well in white 96-well plates, 100 μl Beetle-Juice (PJK Biotech GmbH, Kleinbittersdorf, Germany; #102-511) were added. Luminescence was analysed at RT using the VICTOR Multilabel Plate Reader for 1 s. Luciferase activity was calculated as light units per sec normalized to the protein concentration determined by Pierce™ BCA protein assay kit.

2.13 | Caspase-3/7 activity assay

The Caspase-Glo® 3/7 Assay (Promega Corporation, Fitchburg, WI, US; #G811C) was used according to the manufacturers protocol.
Briefly, HT22 seeded onto black Corning® 96-well solid polystyrene microplates cells and treated with STR with or without 250 µM PYR-41, 10 µM MG-132 or 5 µM QVD for 6 hr were washed once with DPBS and then lysed using 100 µl of the ready-to-use Caspase-Glo® 3/7 Assay buffer (#G810C). The plate was incubated at RT under constant agitation for 30 min. Subsequently, luminescence indicating release of luciferin by cleavage of the substrate peptide DEVD-luciferin was determined using the VICTOR Multilabel Plate Reader for 0.1 s per sample, protein concentrations using the Pierce™ BCA protein assay kit. Caspase-3/7 activity was calculated as light units per sec per mg protein.

2.14 | Statistics

Statistics were performed using GraphPad Prism (vers. 5.00 or 6.01). No sample size calculation, data randomization or blinding were performed. Data are shown as mean ± standard error of mean (SEM). Proper statistics are stated in the figure legends. Briefly, two-tailed Student’s t-test was performed to compare parametric means of two unmatched groups, one-way ANOVA to compare three or more unmatched groups with Bonferroni post-tests or two-way ANOVA with Bonferroni post-tests to compare how a result is affected by two factors. No test for normality was conducted on the data. Grubb’s test for outliers was conducted on data with high SEM, however, no value was excluded. Values of p less than 0.05 were considered as statistically significant: *p < .05, **p < .01 and ***p < .001.

3 | RESULTS

3.1 | The human glioblastoma cell line A172 is EAAT-deficient and thus suitable for functional analysis of recombinant EAAT2 variants

In order to identify a mammalian cell system with low or absent endogenous EAAT activity suitable for studying structure-activity relationships of recombinantly expressed EAAT2 variants, we examined three cell lines, two of neuroectodermal origin, human glioblastoma A172 cells (Giard et al., 1973) and murine hippocampal HT22 cells (Maher & Davis, 1996), as well as one cell line of mesenchymal origin, HEK293 cells (Graham et al., 1977), for endogenous EAAT activity. In A172 cells, endogenous EAAT activity measured as TBOA-inhibitable Glu uptake was >50-fold lower (~2 pmol·mg protein⁻¹·min⁻¹) compared to the two other cell lines (HT22: ~130 pmol·mg protein⁻¹·min⁻¹, HEK293: ~150 pmol·mg protein⁻¹·min⁻¹, Figure 1a). Measurement of EAAT2 activity defined as DHK-inhibitable Glu uptake indicated that A172 cells are devoid of active EAAT2, whereas ~25% and ~10% of the total EAAT activity was attributable to EAAT2 in HT22 and HEK293 cells, respectively (Figure 1a).

To test whether functional EAAT2 can be expressed in A172 cells, these were transiently transfected with pcDNA3.1-EAAT2. This resulted in high EAAT activity (~60 pmol·mg protein⁻¹·min⁻¹, ~30-fold) compared to endogenous EAAT activity when transfected with empty vector (Figure 1b). Thus, EAAT activity in EAAT2-transfected A172 cells is hereinafter referred to as EAAT2 activity. In order to facilitate immunodetection, evaluate transfection efficacy

![Figure 1](https://example.com/figure1.png)

**FIGURE 1** Human A172 glioblastoma cells are a suitable model for the functional characterisation of recombinant EAAT2 variants. (a) Endogenous total EAAT and EAAT2 activities in A172, HT22 and HEK293 cells were measured as [³H]-glutamate (Glu) uptake sensitive to 100 µM DL-threo-β-benzoyloxyaspartic acid (TBOA, black dots) or to 1 mM dihydrokainic acid (DHK, black triangles), respectively, and normalized to cellular protein. (b) EAAT activity in A172 cells 3 days after transient transfection with either pcDNA3.1 (empty) or pcDNA3.1-EAAT2 (EAAT2). (c) EAAT activity measured as in (b) in A172 cells co-transfected with or without tTA2S and VSV-G-tagged wild-type EAAT2 (EAAT2) sub-cloned into the pBI-5 vector and cultured in absence of doxycycline (empty background) or the presence of 200 ng/ml doxycycline (Dox, grey) for 2 days starting 24 hr after transfection. (d) A172 and HT22 cells were co-transfected as in (c). After one day, cells were re-seeded with or without Dox for 24 hr. Then cells were harvested for immunoblotting using anti-VSV-G (upper panel) and anti-EAAT2 antibodies (upper panel) to detect recombinant EAAT2. The height of EAAT2 dimer band faintly detectable in A172 cells in the absence of Dox (lanes 2–3) is indicated by an arrowhead. Anti-Actin served as loading control. Untransfected cells (lanes 1 and 4) were used to analyse background immunoreactivity. The approximate mass of the protein bands is indicated on the right of the blots. (a–c) The graphs show the mean of each individual experiment performed in duplicates or triplicates (circles, triangles) as well as the mean ± SEM of the means of all independent experiments (bars, error bars; a: N = 4–5, b and c: N = 3). The uncropped immunoblots of (d) can be found in the Supplementary Data (Figure S7a). Statistical analysis was performed by (b) two tailed student’s t-test comparing pcDNA3.1-EAAT2 with empty vector or (c) one-way ANOVA with Bonferroni post-tests as indicated using the GraphPad Prism software; **p < .001.
and to manipulate EAAT2 expression after transfection, we sub-cloned a N-terminally VSV-G-tagged EAAT2 construct into the tetracycline-regulatable pBI-5 vector, in which a bidirectional promoter drives the expression of recombinant EAAT2 as well as firefly luciferase as control (Baron et al., 1995). Upon co-transfection of pBI-5-VSV-G-EAAT2 with tTA2S, A172 cells exhibited robust EAAT2 activity in the absence but not in the presence of Dox (Figure 1c). Attempts to detect the recombinant EAAT2 protein in co-transfected A172 cells by immunoblotting yielded only faint VSV-G and no detectable EAAT2 immunoreactivity (IR; Figure 1d). In contrast, HT22 cells, which did not show neither VSV-G nor EAAT2 IR when not transfected, exhibited robust Dox-suppressible VSV-G and EAAT2 IR after co-transfection (Figure 1d). These findings were explained by the more than threefold lower transfection efficacy in A172 cells compared to HT22 cells (Figure S1). As a consequence, we decided to employ A172 cells to study EAAT2 activity, whereas protein analysis was performed in HT22 cells.

3.2 | Site-directed mutagenesis (D504N) increases EAAT2 activity

Activated C3 has been reported to cleave EAAT2 c-terminally of aspartate 504 thereby inactivating EAAT2, while this is prevented by mutating this aspartate at position P1—crucial for protease sensitivity of the caspase cleavage site motif (Fang et al., 2006)—to asparagine (D504N) (Boston-Howes et al., 2006). Upon expression of the variant mEAAT2D504N in A172 cells, EAAT2 activity was found to be significantly higher (~40%) compared to wild-type EAAT2 (EAAT2wt, Figure 2a). To explore whether the increased

![FIGURE 2](image-url)
EAAT2 activity in cells transfected with mEAAT2\textsuperscript{D504N} is associated with higher EAAT2 protein levels, EAAT2 protein levels were analysed by immunoblotting of similarly transfected HT22 cells using the anti-VSV-G antibody. However, expression levels of mEAAT2\textsuperscript{D504N} were not different to those of EAAT2\textsuperscript{wt} (Figure 2b).

In addition, luciferase activity expressed upon transfection with both constructs in A172 cells proved to be identical (Figure 2c). As the peptide that was used for immunisation to generate the EAAT2 antibody included the caspase cleavage site (Cell Signaling Technology, personal communication), the presence of the D504N mutation could be confirmed by the substantially impaired binding of the anti-EAAT2 antibody to the mutant recombinant EAAT2 protein (Figure S2a and b).

3.3 | Stauroporine-induced C3 activation is associated with a time-dependent decrease in EAAT2 protein levels independent of direct C3-mediated EAAT2 cleavage

In order to induce C3 activation, we capitalized on the fact that C3 activation occurs in cells destined to undergo apoptosis (Porter & Janicke, 1999). We therefore exposed our mammalian cell models to the classical apoptosis inducer stauroporine (STR). While A172 cells proved to be resistant against STR (Figure S3a), STR-induced dose-dependent cell death in HT22 cells within 24 hr when quantified using the MTT assay (Figure S3b). As expected, in HT22 cells transiently co-transfected with tTA\textsuperscript{2} and EAAT2\textsuperscript{wt} or mEAAT2\textsuperscript{D504N} an increase in the C3 cleavage products p19 and p17 was detected in up to 6h STR treatment (Figure 2d), as was an increase in caspase-3 and/or −7 activity measured as cleavage of an artificial substrate containing their common canonical target peptide DEVD (Figure S3c).

Conversely, recombinant EAAT2 protein levels in these cells markedly decreased to approximately a half within 4 hr and one-third within 6 hr of STR irrespective of the presence or absence of the C-terminal C3 cleavage site (Figure 2e). However, no shift in protein size as expected for C3 cleavage (see Figure 4b) was observed. Nevertheless, caspase inhibition by 5 µM QVD (Figure S3c) largely diminished the EAAT2 down-regulation by STR (Figure 2f). Under basal conditions, the ubiquitin-activating enzyme E1 inhibitor PYR-41 (Yang et al., 2007) had no effect on EAAT2 activity in transiently transfected STR-resistant A172 cells (Figure S4a). In contrast, in HT22 cells expressing EAAT2\textsuperscript{wt} PYR-41 more that reversed STR-induced EAAT2 down-regulation, resulting EAAT2 protein levels in the presence of PYR-41 and STR that were even −2.2-fold higher than those in control cells not treated with STR (Figure 2f). Thus, at least in our neuroectodermal mammalian cell system, the observed C3-dependent EAAT2 protein degradation upon cellular stress evoked by STR is not because of direct C3-mediated cleavage at aspartate 504, but rather involves ubiquitination. To test the relevance of our observation, murine cortical astrocytes were virally transduced with VSV-G-tagged EAAT2. As in HT22 cells, the STR-induced a reduction in EAAT2 protein levels. This loss was rescued by PYR-41 (Figure S5).

3.4 | Lysine residues reported to act as ubiquitin acceptor sites in the EAAT2 C-terminus are phylogenetically highly conserved, whereas the C-terminal C3 cleavage site is not

Most parts of the N- and C-termini are shared between the different EAAT2 isoforms described, whereas only the most N- or C-terminal amino acids of the N- and C-terminus, respectively, are splice variant-specific (Figure S6a). C-terminal lysine residues were suggested to be important ubiquitin acceptor sites governing membrane trafficking (González-González et al., 2008; Ibáñez et al., 2016; Martínez-Villarreal et al., 2012). As the degree of phylogenetic conservation may also provide insights into the importance of C-terminal ubiquitination as well as C3 cleavage for the physiological function of EAAT2, we performed a phylogenetic analysis by comparing the murine EAAT2 C-terminus to the corresponding amino acid sequence in several species of mammals, marsupials, birds, amphibians and finally fish (Figure 3). The functionally relevant lysine residues K517, K526, K550 and K570 in rat EAAT2 (González-González et al., 2008; Ibáñez et al., 2016; Martínez-Villarreal et al., 2012) correspond to K517, K526 and K549 and K569 in murine EAAT2a (Figure 3) and all but K569, which is lacking in Xenopus tropicalis, showed complete phylogenetic conservation across the species tested (Figure 3). In addition, the UbPred algorithm (Radvivoc et al., 2010) predicted a high likelihood for the completely conserved K517 and K526, in addition to the EAAT2a-specific K557, only absent in the bony fish Xiphophorus couchianus, to represent functionally relevant ubiquitin acceptor sites. However, with exception of D504 in position P1 the part of the EAAT2 protein where the putative caspase cleavage motif DTID is located showed a much lesser degree of amino acid similarity. Most importantly, within the DTID motif a mutation of the aspartate in P4 position to glutamate was identified to have developed independently in amphibians and ungulates. This aspartate to glutamate mutation has been shown to reduce protease sensitivity towards caspase-3 to <10% (Thornberry et al., 1997). In contrast to the C-terminus, the N-terminus of EAAT2 is much less evolutionarily conserved (Figure S6b) with only one lysine, K12, found in all species tested. However, UbPred also predicted K12 as a functionally relevant ubiquitin acceptor site (Figure S6c). Of note, no tetrapeptide even remotely resembling a caspase cleavage motif is present in the N-terminus (Figure S6b).

3.5 | C-terminal EAAT2 truncation mimicking C3 cleavage at D504 increases EAAT2 transport activity and prolongs transporter half-life

It has been proposed that EAAT2 cleaved at aspartate 504 is functionally inactive (Boston-Howes et al., 2006; Foran & Trotti, 2009). In order to study the functional properties of the EAAT2 variant mimicking the predicted C3 cleavage product in our cell models, we generated and transiently over-expressed a truncated EAAT2 lacking the 67 amino acids located C-terminally of aspartate 504 (EAAT2\textsuperscript{Δ505\textendash572}). qPCR using primers specifically amplifying all
recombinant EAAT2 constructs (Table S2) showed that recombinant EAAT2 mRNA levels in A172 cells transfected with both full-length and truncated EAAT2 were not different (Figure 4a). Upon immunoblotting with the VSV-G tag antibody, expression of the EAAT2Δ505-572 in HT22 cells yielded a band with the expected size shift of ~16 kDa for the dimer and ~8 kDa for the monomer (Figure 4b). Semiquantitative densitometry indicated comparable EAAT2 protein levels in HT22 cells expressing truncated EAAT2 compared full-length EAAT2 (Figure 4b). Unexpectedly, EAAT2 activity measured when expressing truncated EAAT2 in A172 cells was slightly but significantly higher (21 ± 6%, mean ± SEM) when compared with full-length EAAT2 (Figure 4c). Thus, truncation of EAAT2 mimicking C3 cleavage neither inactivates EAAT2 activity nor destabilizes the EAAT2 protein in our cell models.

As the truncated EAAT2 lacks all C-terminal lysine residues reportedly functionally relevant for EAAT2 trafficking, we characterized changes in stability in response to C-terminal truncation in the absence of cellular stress. To this end, we repressed transcription of recombinant EAAT2wt and EAAT2Δ505-572 after being expressed for 24 hr in A172 cells using Dox. Compared to similarly transfected cells not treated with Dox, 24 hr of Dox-mediated transcriptional repression led to a 39 ± 3% and 28 ± 5% decrease in EAAT2 activity in cells transfected with EAAT2wt and EAAT2Δ505-572, respectively, whereas the respective decrease for EAAT2wt and EAAT2Δ505-572 was 67 ± 3% and 59 ± 2% after 48h of Dox (Figure 4d). EAAT2 activity decayed moderately but significantly less in cells expressing truncated EAAT2 upon Dox compared to those expressing wild-type EAAT2 (two-way-ANOVA, F(1,14) = 11, p < .0046). Thus, at least in the cell systems under study, mimicking putative C3-mediated cleavage rather increases transfection-induced EAAT2 activity and decreases its decay over time after transcriptional repression of the transgene in the absence of stress.

3.6 The C-terminus signals proteasomal degradation of EAAT2 in response to staurosporine-induced cellular stress in HT22 cells

FIGURE 3 C-terminal lysine residues reportedly targeted by ubiquitination are phylogenetically more conserved than the caspase-3 cleavage site. Alignment of the C terminus of murine EAAT2 isoform 1 (NCBI accession number: NP_001070982.1; EAAT2a) with the respective EAAT2 amino acid sequences of diverse vertebrates from primates (Homo sapiens, NP_004162.2), carnivores (Canis lupus, NP_001003138.2), even toed ungulates (Sus scrofa, XP_003480756.2; Bos taurus, XP_010811096.1), marsupials (Monodelphis domestica, XP_007497388.1), birds (Gallus gallus, XP_015142476.1), amphibians (Xenopus tropicalis, XP_002937340.1; Nanorana parkeri, XP_018412969.1) to bony fish (Xiphophorus couchianus, XP_002937340.1) in the order of the phylogenetic distance to rodents. Completely conserved amino acid residues are indicated by bold letters. Light grey background marks the putative caspase-3 (C3) cleavage site with letters in green for the three N-terminal amino acid residues and D504 in red. Yellow letters with red background mark mutations predicted to eradicate C3 cleavage. Darker grey background indicates two pairs of closely related species with and without the critical aspartate in position P1 of the putative C3 cleavage site, the names of which are marked by a grey background at the end of the sequence. All lysine residues are indicated by red letters on white background, the number of those experimentally shown to be involved in ubiquitination-induced EAAT2 internalisation are boxed in red above the sequence (González-González et al., 2008). Red boxes within the sequence indicate high likelihood of ubiquitination predicted by UbPred algorithm (Radijovac et al., 2010), grey boxes indicate low likelihood. The arrowhead marks the completely conserved aspartate in the P1 position of the putative C3 cleavage site. The number of the first amino acid residue on the left for each line is shown in brackets; the total number of amino acids of the EAAT2 protein at the end of the sequence. The alternative C-terminus starting C-terminally of threonine 550 found in the murine splice variants 3 (NP_035523.1; EAAT2B) and 4 (NP_001347947.1; EAAT2b) is indicated by the arrow. The part of the sequence representing the transmembrane region 8 (TM8) according to the model by (Ye et al., 2010) is indicated by the black box. Sequences were compared using the CLC Main Workbench 7 software by CLC bio
lysine residues reportedly relevant of ubiquitination-dependent EAAT2 internalisation (González-González et al., 2008; Ibáñez et al., 2016; Martinez-Villarreal et al., 2012) will impact EAAT2 protein degradation upon STR-induced cellular stress. To exclude that STR directly activates the proteasome and PYR-41 acts as a proteasome inhibitor, thereby explaining our results, we first established that the total chymotrypsin-like proteasomal activity in HT22 cells is neither affected by STR (Figure 4e) nor by additional PYR-41 (Figure S4b). In addition, the proteasome inhibitor MG-132, which at a concentration of 10 µM completely inhibited chymotrypsin-like proteasomal activity (Figure 4e), also blocked the STR-induced degradation of EAAT2 protein in HT22 cells co-transfected with EAAT2wt and tTA2S (Figure 4f) as well as in astrocytes virally transduced to express EAAT2wt (Figure S5). Of note, MG-132 alone did not increase EAAT2 protein levels within 6 hr (Figure S4c). In contrast, EAAT2Δ505-572 protein levels neither decreased in response to STR nor increased upon additional treatment with MG-132 in HT22 cells (Figure 4g). Thus, we conclude that, at least in the system under study, sites functionally relevant for proteasomal degradation upon STR-induced cellular stress can be localized to the EAAT2 C-terminus.

DISCUSSION

Multiple lines of evidence suggest that glutamate dysregulation plays a role in neurodegenerative diseases, especially cellular glutamate transport capacity appears to be reduced, primarily as a...
consequence of an impairment of the EAAT2 transporter function (Lewerenz & Maher, 2015). Enhanced inactivation by proteolytic cleavage and accelerated degradation was suggested to be a particularly important mechanism in ALS and AD (Bristol & Rothstein, 1996; Garcia-Esparcia et al., 2018; Rothstein et al., 1995). C3-mediated proteolytic cleavage of EAAT2 has been proposed as a mechanism for EAAT2 degradation and inactivation (Boston-Howes et al., 2006; Gibb et al., 2007; Rosenblum et al., 2017). Consistent with this idea, knock-in mutagenesis of the critical aspartate at the P1 position to asparagine (D504N) conferred protection to the SOD1<sup>G93A</sup>-mediated neurotoxicity in mice (Rosenblum et al., 2017).

Our study demonstrates that C-terminally truncated EAAT2 mimicking the C3 cleavage product expressed in neuroectodermal cells almost devoid of endogenous EAAT activity rather increases than decreases EAAT2 activity and C-terminal truncation mimicking C3 cleavage confers protection against STR-induced EAAT2 protein degradation. This finding is in line with data published for human EAAT2, showing preserved activity of similarly truncated EAAT2 (Leinenweber et al., 2011).

As the inhibitory effect of C3 depending on the presence of an intact C-terminal C3 cleavage site was identified in oocytes injected with active C3 (Boston-Howes et al., 2006), not in our mammalian cells upon activation of endogenous C3 activity, it seems reasonable to assume that differences in the experimental models explain the divergent results. Nevertheless, in our neuroectodermal cell model the D504N mutation still increased EAAT2 activity by ~40%. In contrast, the presence of the mutation had no effect on recombinant EAAT2 protein levels nor did the mutation-induced resistance to direct C-terminal C3 cleavage protect against EAAT2 protein degradation by endogenous C3 activation following STR-induced cellular stress. These findings exclude a relevant role of direct C3-mediated cleavage in STR-induced EAAT2 degradation in our experimental set-up.

Of note, D504, the mutation of which to asparagine has been investigated by us and others (Boston-Howes et al., 2006; Gibb et al., 2007; Rosenblum et al., 2017), is the only completely conserved amino acid residue residing within a stretch of 12 amino acid residues of rather low phylogenetic conservation in which the C3 consensus site is located (Figure 3). Here, we report that even the critical aspartate in position P4 of the C3 cleavage consensus site (Thornberry et al., 1997) is lost in some species. In our opinion, this observation in synopsis with the reported effects of the D504N mutation by us and others (Boston-Howes et al., 2006; Gibb et al., 2007; Rosenblum et al., 2017) strongly indicates that D504 might be structurally crucial for EAAT2 with regard to functions other than C3 cleavage or degradation of EAAT2 upon cellular stress.

In our cell systems, the D504N mutation robustly increased the EAAT2 transport activity without detectable changes in protein levels. It is known that the number of negatively charged amino acid in the C-terminus modulates gating properties of EAAT2 (Leinenweber et al., 2011). Thus, it is seems possible that the underlying mechanism of the increased activity because of the D504N mutation, which changes the net charge of the C-terminus, might be a higher transport velocity per transporter molecule. As degradation upon STR treatment is not affected by the D504N mutation, it seems less likely that conformational changes of the C-terminus because of the D504N mutation affect C-terminal ubiquitination and thereby increase the relative number of EAAT2 molecules inserted in the plasma membrane. In summary, based on our data it seems tempting to speculate whether knock-in of the D504N mutation in vivo also increases EAAT2 activity via a mechanism that still remains to be defined, thereby contributing to the protective effect reported in mouse models of ALS (Rosenblum et al., 2017).

Of note, our in vitro findings neither exclude that C3 cleavage of EAAT2 occurs in vivo nor that the C3 cleavage mutation in the context of mutant SOD1 toxicity might protect by prohibiting the generation of toxic C-terminal cleavage products of EAAT2 by activated C3 as proposed by others (Gibb et al., 2007; Rosenblum et al., 2017). However, replacing the glutamate at position P3 of the canonical DEVD caspase-3/7 consensus site by threonine, as in the DTID motif within the EAAT2 C-terminus, reduces its protease sensitivity towards C3 by 75%, whereas the substitution of the valine at P2 by the isoleucine can be predicted to induce an additional 25% loss (Lien et al., 2004). These two effects combined, in addition to our data, support the idea that the DTID motif in EAAT2 has a rather low sensitivity towards caspase-3/7-mediated cleavage. However, the leucine at position P5 (Figure 3), which should shift protease sensitivity from C7 to C3 (Boston-Howes et al., 2006; Fang et al., 2006), might explain the reported resistance of EAAT2 against cleavage even by high amounts of recombinant active C7, whereas cleavage by recombinant active C3 reached detectable levels (Boston-Howes et al., 2006; Fang et al., 2006).

Taken together, our data did not support the conclusion that direct C3 cleavage underlies the observed rapid degradation of the EAAT2 protein upon cellular stress induced by STR. Thus, we asked whether ubiquitination, an ubiquitous process for flagging proteins for degradation, might be involved, as it was well known that EAAT2 is subject to ubiquitination (González-González et al., 2008; Ibáñez et al., 2016; Martínez-Villarreal et al., 2012). However, it is generally assumed that rapid ubiquitination/de-ubiquitination cycles regulate the demand-dependent EAAT2 membrane insertion rather than changing total EAAT2 protein levels (González-González et al., 2008; Ibáñez et al., 2016; Martínez-Villarreal et al., 2012).

Of note, our phylogenetic analysis shows that three of the four C-terminal lysine residues experimentally shown to represent functionally relevant ubiquitin acceptor sites for ubiquitination-induced EAAT2 internalisation in the EAAT2 protein (González-González et al., 2008; Ibáñez et al., 2016; Martínez-Villarreal et al., 2012) are completely conserved across vertebrates from fish to mammals (K517, K526 and K549, with K569 absent in <i>Xenopus tropicalis</i> only), whereas the C3 cleavage site—with exception of the C-terminal aspartate S04— is not. Therefore, ubiquitination may represent a better conserved regulatory mechanism in EAAT2 physiology than cleavage by C3 does. Indeed, it has been demonstrated that pharmacological inhibition of de-ubiquitination can direct EAAT2 to ubiquitination-dependent degradation (González-González et al., 2008). As the pharmacological
inhibition of both ubiquitination and the proteasome blocked the STR-induced EAAT2 protein degradation in HT22 cells and astrocytes, it can be concluded that in our model of STR-induced cellular stress the EAAT2 protein is shifted from the reported ubiquitination/de-ubiquitination cycle to proteasomal degradation. Conversely, we demonstrate that deletion of the C-terminal portion of the EAAT2 protein including all lysine residues identified as functionally important for ubiquitination-induced EAAT2 internalisation (González-González et al., 2008; Ibáñez et al., 2016; Martínez-Villarreal et al., 2012) has a similar protective effect against STR-induced EAAT2 degradation in HT22 and A172 cells as had the inhibition of ubiquitination and the proteasome. We also could exclude that STR induces EAAT2 degradation in HT22 cells by inducing the proteasomal activity in general. In our opinion, these observations strongly indicate that C-terminal EAAT2 ubiquitination and subsequent proteasomal degradation are involved in the rapid degradation of the EAAT2 protein upon STR-induced cell stress that is associated with C3 activation, although we did not demonstrate increased ubiquitination of the EAAT2 protein itself. However, we cannot exclude that other pathways that have been reported to be involved in the turn-over of membrane proteins upon ubiquitination, for example, lysosomal degradation as well as release in microvesicles (Foot et al., 2017), contribute to the decrease in EAAT2 proteins levels in response to STR in our cell models, especially as the inhibition of ubiquitination seems to be more effective that proteasome inhibition. However, proteasomal degradation similar to the pathway reported here has also been reported for the metabotropic glutamate receptor 7 (Lee et al., 2019).

In summary, our data strongly suggest that in response to STR—our in vitro paradigm of cell stress associated with C3 activation—the EAAT2 protein in neuroectodermal cells as well as in murine astrocytes is rapidly degraded following C-terminal ubiquitination—at least in part via degradation by the proteasome. We acknowledge that our in vitro model of cell stress cannot nearly reflect the complexity of the in vivo situation. However, an increase C3 activity in human CNS tissues from subjects with neurodegenerative diseases (Ranganathan & Bowser, 2010; Vercellino et al., 2007; Vis et al., 2005; Zhao et al., 2003) as well as a protective effect of C3 inhibition in animal models of these diseases have repeatedly been found (D’Amelio et al. 2011; Li et al., 2000; Toulmond et al. 2004). As non-apoptotic functions of C3 have been reported in astrocytes (Acarin et al., 2007; Aras et al., 2012), it seems reasonable to put forward the working hypothesis for future confirmatory studies that ubiquitination, possibly as a consequence of C3 activation, contributes to the down-regulation of astrocytic EAAT2 in neurodegenerative diseases.

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CONFLICT OF INTERESTS
The authors declare that they have no conflict of interests.

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