Table 1 (*continued*)

Description	Mascot score	Accession[a]	Confirm[b]
Band 13			
Actin, beta-like 2	12.18	157823033	ND
PREDICTED: similar to cellular repressor of E1A-stimulated genes 2	8.07	109485991	ND
Band 14			
Syntaxin 1B2	20.20	6981600	DN
Glyceraldehyde-3-phosphate dehydrogenase	40.20	8393418	DN
Apolipoprotein E	10.15	162287337	ND
PREDICTED: similar to glyceraldehyde-3-phosphate dehydrogenase	10.13	62657298	DN
Hypothetical protein LOC301563 (mitochondrial fission factor)	10.11	84781650	ND
Band 15			
G chain G, rat liver F1-Atpase	30.20	6729936	ND
Vacuolar H + ATPase E1	10.16	38454230	ND
14-3-3 epsilon	40.21	5803225	DN
Band 16			
AF370442_1 LEK1	14.14	14091667	Ref.

[a] Genbank Accession Number.
[b] NS, found non-specific in further experiments; CR, cross reactivity by anti-N-cadherin antibody; DN, binding was denied in further experiments; Ref, confirmed literally; ND, not determined.

Table 2
Proteins identified in shotgun analyses by subtracting IgG-bound items from anti-N-cadherin antibody-bound items.

Description	Mascot score	Accession[a]	Confirm[b]
Cadherins			
Cdh2 cadherin-2	140.28	12558	–
Cdh2 cadherin 2 precursor	20.21	12558	–
Cdh4 cadherin-4	10.12	12561	Ref.
Catenins			
Ctnnb1 catenin beta-1	210.35	12387	Ref.
Ctnna2 isoform 1 of catenin alpha-2	110.30	12386	Ref.
Ctnna2 isoform 2 of catenin alpha-2	10.22	12386	Ref.
Ctnnd2 isoform 1 of catenin delta-2	50.21	18163	Ref.
Jup junction plakoglobin	20.25	16480	Ref.
Cytoskeletal/membranous			
Actr3 actin-related protein 3	10.16	74117	Ref.
Spna2 spectrin alpha 2	10.18	20740	Ref.
Atp1b1 sodium/potassium-transporting ATPase subunit beta-1	10.19	11931	Ref.
Atp1a3 sodium/potassium-transporting ATPase subunit alpha-3	10.16	232975	Ref.
Unknown			
BC005561 cDNA sequence BC005561	10.12	100042165	ND
Non-specific			
Spag9 isoform 2 of C-jun-amino-terminal kinase-interacting protein 4	50.24	70834	CR
Trim33 isoform alpha of E3 ubiquitin-protein ligase TRIM33	10.18	94093	CR

[a] Genbank Accession Number.
[b] CR, cross-reactivity with anti-N-cadherin antibody; Ref, confirmed literally; ND, not determined.

between plakoglobin and intermediate filaments in desmosomes (Kowalczyk et al., 1997; Schmidt et al., 1994; Smith and Fuchs, 1998), was also found in band 10 (Table 1). In the case of chick optic tectum, β-catenin and plakoglobin are enriched at synapses and associated with N-cadherin, but they are differentially distributed forming mutually exclusive complexes (Miskevich et al., 1998). Taking this into account, we speculate that plakoglobin/desmoplakin complex might tether intermediate filaments to a certain subset of synaptic junctions whose adhesion is mediated by N-cadherin (Fig. 3B).

3.4. Proteins tethering microtubules

In our immunoprecipitate, there was abundant cytoplasmic dynein heavy chain 1 in band 1 (Table 1). In addition, band 16 included LEK1/CENPF/mitosin, which is known to associate with dynein bridged with NudE1/LIS1 (Soukoulis et al., 2005). It has been reported that cytoplasmic dynein binds to β-catenin at the cell cortex where E-cadherin forms adherens junction with the neighboring cell (Ligon et al., 2001). Microtubules (MT) are tethered by this cadherin-β-catenin-dynein complex at the adherens junction and facilitate junction assembly (Ligon and Holzbaur, 2007). It has also been proposed that LIS1 interacts with IQGAP1, a β-catenin-binding partner, and a Rho-family GTPase Cdc42 in a calcium-dependent manner (Kholmanskikh et al., 2006).

Transportation of N-cadherin to plasma membrane is consistently dependent on MT networks as well as MT-based motors (Mary et al., 2002; Teng et al., 2005). Recent studies have shown that MT plus-ends terminate at the adherens junctions of mammary tumor cells and that MT depolymerization causes

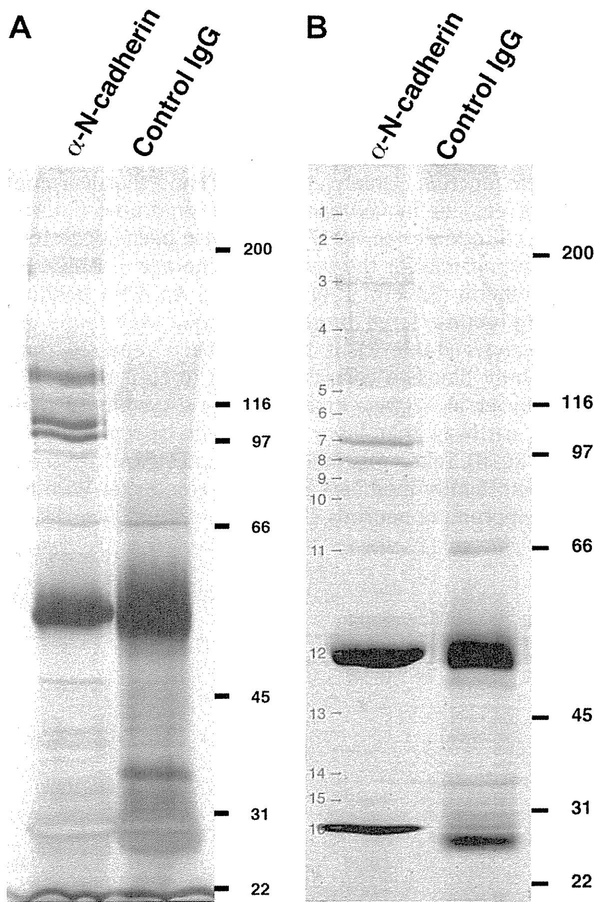

Fig. 2. Immunoprecipitation from the Triton X-100 extracts of cultured rat hippocampal neurons. (A) Silver staining. (B) Sypro Ruby staining. Numbers correspond to the bands excised and subjected to in-gel digestion–LC–MS/MS (Table 1).

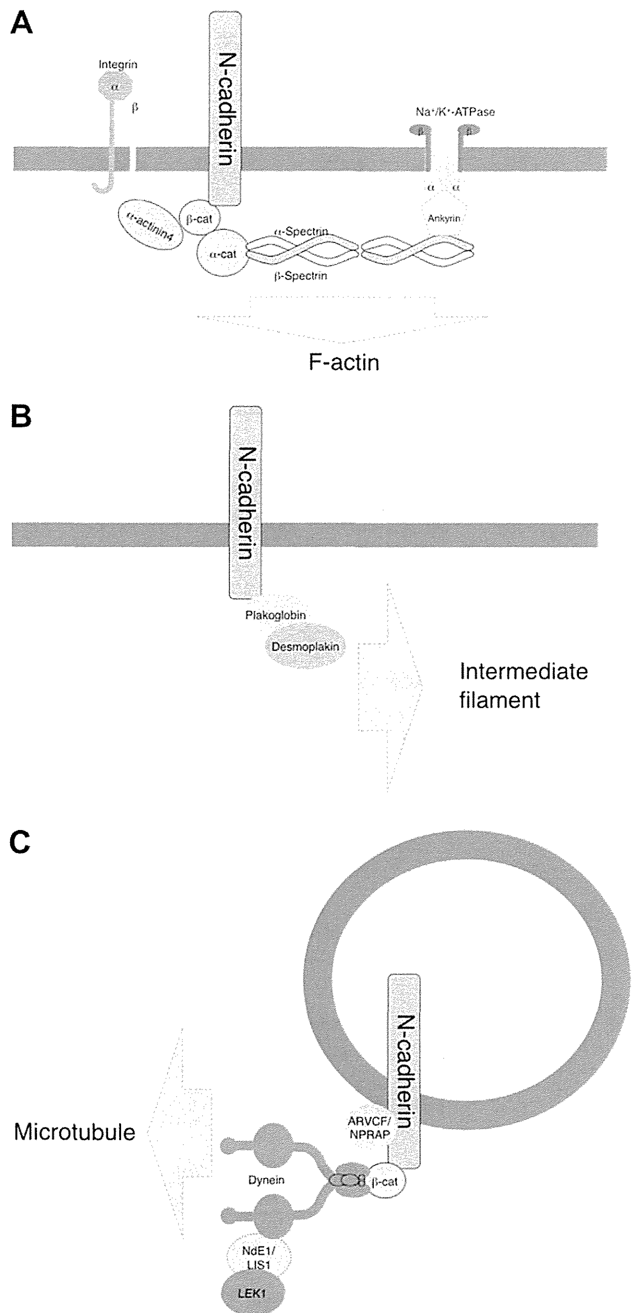

Fig. 3. Models of N-cadherin-associated cell machineries connected to cytoskeletal elements.

disorganized accumulation of E-cadherin in these cells (Stehbens et al., 2006), as observed earlier with other cells (Waterman-Storer et al., 2000). The M-cadherin–catenin complex also interacts with MTs (Kaufmann et al., 1999). Although most of these studies suggest that MT plus-ends interact with cell junctions, one study reported that N-cadherin-mediated adhesion stabilizes the minus-ends (Chausovsky et al., 2000). There is another study which showed that adherens junction is tethered via PLEKHA7/Nezha to MT minus-ends (Meng et al., 2008). Our data suggest that, in hippocampal neurons, N-cadherin is more dominantly tethered to MT plus-ends via β-catenin-dynein than to minus-ends.

MTs have not been considered as being able to enter dendritic spines or to play a role in spine development or dynamics, probably because of dynamic instability at the plus-end within spines. Recent advances in imaging techniques for visualizing MTs in living neurons, however, have revealed that MTs transiently invade into and, therein, are involved in activity-induced spine dynamism and spine development (Gu et al., 2008; Hu et al., 2008). These transient invasions of MTs are correlated with the transient emergence of protrusions on spine heads (transient spine head protrusion: tSHP) (Hu et al., 2008). Interestingly, inactivation of N-cadherin activity in dendritic spines drastically enhances the protrusions of activity-induced tSHPs, as we have previously observed (Okamura et al., 2004). This seems to be consistent with the idea that the N-cadherin-β-catenin-dynein complex tether MT plus-ends and regulate the growth of MTs in the close proximity to synaptic junctions. Taken together, the co-ordination of F-actin and MT at the N-cadherin-mediated synaptic junction is important for activity-induced remodeling of synaptic junctions (Fig. 3C) (Okamura et al., 2004).

3.5. p120ctn family at the juxta-membrane domain

p120ctn family proteins, which bind to the juxta-membrane cytoplasmic domain of classic cadherins, serve as another pathway connected to MTs in non-neuronal tissues (Chen et al., 2003; Franz and Ridley, 2004; Ichii and Takeichi, 2007; Yanagisawa et al., 2004). Among various p120ctn family members, p120ctn/δ1-catenin is involved in synapse development (Elia et al., 2006) and is expressed in cultured hippocampal neurons (Fig. 4). However, N-cadherin-immunoprecipitate did not include

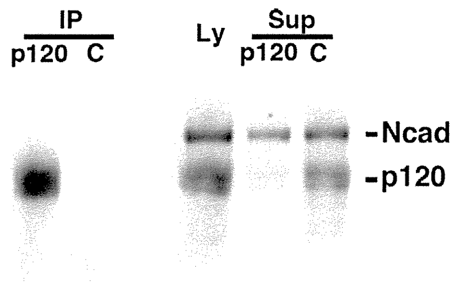

Fig. 4. Immunoprecipitation of p120ctn. Neuronal lysate was immunoprecipitated with anti-p120ctn antibody (p120) or control IgG (C). Ten percent of the lysate (Ly) and flow through (Sup) were also electrophoresed. The transferred membrane was probed with both anti-N-cadherin and anti-p120ctn antibodies.

p120ctn in either in-gel digestion or shotgun analyses (Tables 1 and 2). Western blot following the immunoprecipitation of p120ctn also failed to detect N-cadherin (Fig. 4). On the other hand, ARVCF and NPRAP (neurojungin, δ2-catenin) were found in bands 5 and 7, and in the results of shotgun analysis (Tables 1 and 2). Other family members, plakophilins 1–3 and p0071 (plakophilin 4) were not found. These results are reasonable in that δ2-catenin has been shown to be important for cognitive and synaptic function (Israely et al., 2004) and that learning disabilities are evident in velo-cardio-facial syndrome (Shprintzen et al., 1978; Sirotkin et al., 1997). It has also been suggested that NPRAP is responsible for the synaptic anchorage of AMPA receptor to N-cadherin (Silverman et al., 2007). An actin polymerization protein, actin-related protein 3 (Actr3) was found in the shotgun analysis (Table 2). It has also been reported that the p120ctn family binds to cortactin, which in turn binds to Arp3 (Boguslavsky et al., 2007). In the Xenopus system there is an alternative pathway that links ARVCF with spectrin via kazrin (Cho et al., 2010). Taken together, ARVCF and NPRAP are the dominant p120ctn family members that form complexes with N-cadherin in hippocampal neurons (Fig. 3C).

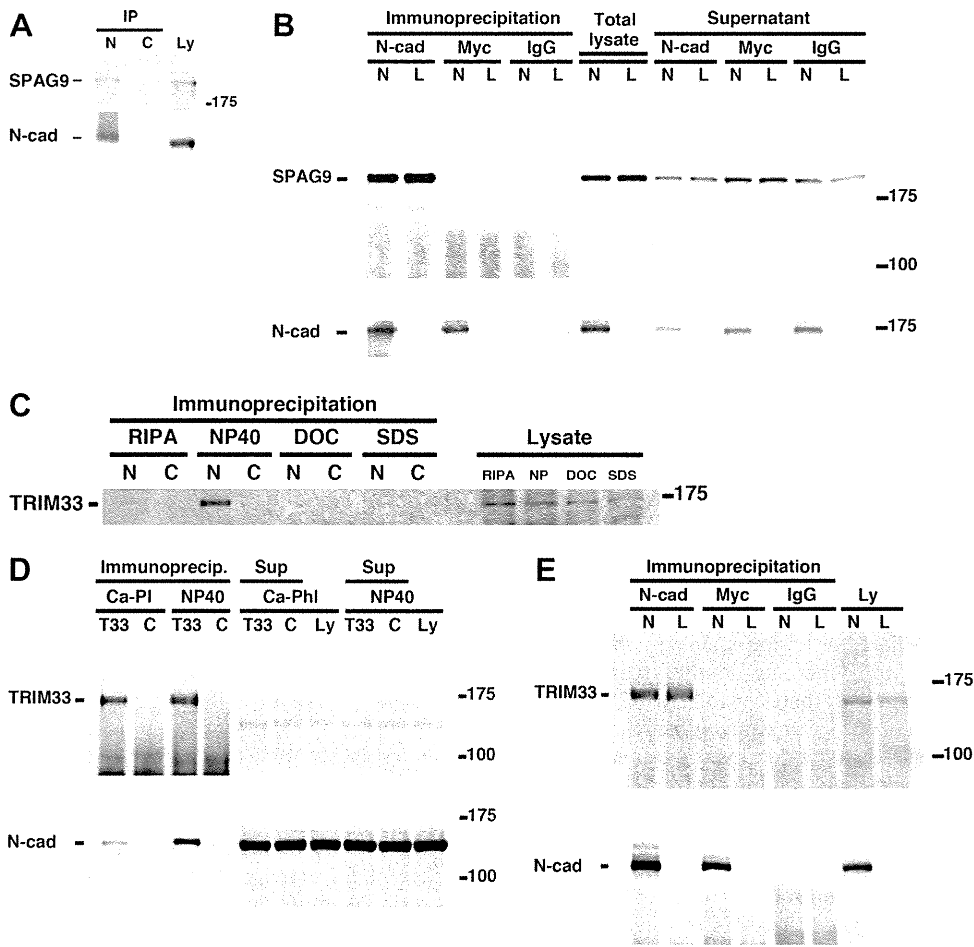

Fig. 5. False positive cases due to the cross-reactivity of anti-N-cadherin antibody were ruled out by rigorous examination. (A) N-cadherin was immunoprecipitated from neuronal lysate and immunoblotted for SPAG9 (top) and N-cadherin (bottom). Immunoprecipitate with anti-N-cadherin antibody (N), with control IgG (C), and 10% of lysate (Ly) were applied. (B) L929 fibroblast cells (L) and those stably transfected with myc-tagged N-cadherin (N) were extracted and immunoprecipitated with anti-N-cadherin (N-cad), anti-myc-tag (myc) antibodies, and control IgG (IgG). The immunoprecipitates, total cell lysates and flow through (supernatant) were immunoblotted with anti-SPAG9 (top) and anti-N-cadherin (bottom) antibodies. (C) Neurons were extracted and immunoprecipitated in the indicated buffer conditions: RIPA (20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 1 μg/ml aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride); NP40 (same without sodium deoxycholate or SDS); DOC (without SDS); SDS (without sodium deoxycholate). The immunoprecipitate with anti-N-cadherin antibody (N), with control IgG (C), and 10% of total lysate were immunoblotted with anti-TRIM33 antibody. (D) Neurons were extracted and immunoprecipitated with anti-TRIM33 antibody in Ca-PI-lysis buffer or NP40 buffer. The immunoprecipitate with anti-TRIM33 antibody (T33), with control IgG (C), flow through (Sup), and 10% of total lysate (Ly) were immunoblotted with anti-TRIM33 antibody (top) and anti-N-cadherin antibody (bottom). E, L929 fibroblast cells (L) and those stably transfected with myc-tagged N-cadherin (N) were extracted and immunoprecipitated with anti-N-cadherin (N-cad), anti-myc-tag (myc) antibodies, and control IgG (IgG). The immunoprecipitates and total cell lysates (Ly) were immunoblotted with anti-TRIM33 antibody (top) and anti-N-cadherin antibody (bottom).

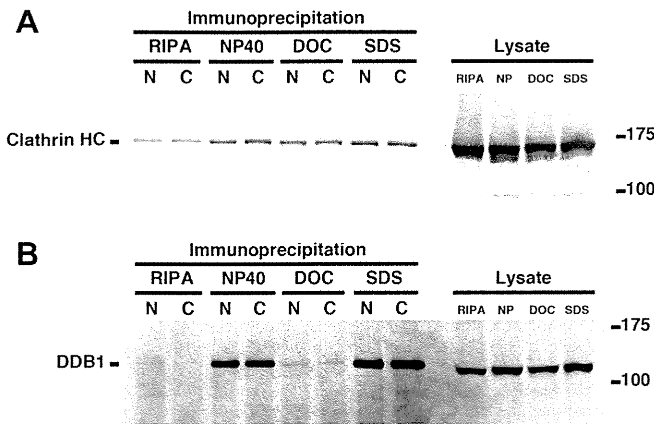

Fig. 6. Non-specific binding cases revealed by immunoblotting. Neurons were extracted and immunoprecipitated in the indicated buffer conditions. The immunoprecipitate with anti-N-cadherin antibody (N), with control IgG (C), and 10% of total lysate were immunoblotted with anti-clathrin heavy chain (A) and anti-DDB1 (B) antibodies.

3.6. Implications for the interactions with specific membrane compartments

Band 10 contained annexin A2, a calcium-dependent membrane-associated protein complexed with p11 (Table 1). In HUVEC cells, the annexin A2/p11 heterotetramer complex, which accumulates underneath the plasma membrane at cholesterol raft sites, is associated with VE-cadherin, although there was no association with N-cadherin (Heyraud et al., 2008). This interaction is probably involved in the stabilization of cadherin–catenin complex at the forming adherens junction. The binding of annexin A2 to N-cadherin found in the present setting therefore suggests that synaptic dynamism involves the stabilization and destabilization of the synaptic junction via interaction with the lipid raft-associated cell machinery.

Furthermore, the inclusion of amphiphysin, a BAR domain protein to form membrane curvature, may reflect the involvement of N-cadherin in the curved membrane such as that of the endosome (Table 1, band 5). A *Caenorhabditis elegans* F-BAR domain protein SRGP-1 is also reported to co-localize with cadherin–catenin complex (HMR-1 and HMP-1) in the adherens junction and SRGP-1 loss-of-function results in a compromised cadherin–catenin complex (Zaidel-Bar et al., 2010).

3.7. Assessment of unreported proteins

In addition to the previously reported N-cadherin-binding partners, there were several proteins that were apparently specific for N-cadherin compared to IgG control (Fig. 2A). For example, band 3 showed highly specific affinity for N-cadherin and included sperm associated antigen 9 (SPAG9) (Table 1). SPAG9 were also found in band 4 (Table 1) and in shotgun analysis (Table 2). This apparently specific binding of SPAG9 to N-cadherin was reproduced by immunoblotting following immunoprecipitation (Fig. 5A). However, immunoprecipitation of SPAG9 was observed from L929 cells with or without the expression of N-cadherin. Moreover, anti-myc tag antibody did not precipitate SPAG9 from the cells transfected with recombinant N-cadherin fused with myc-tag (Fig. 5B). These data indicate that the anti-N-cadherin antibody directly cross-reacts with SPAG9, so there is no intrinsic association of SPAG9 with N-cadherin.

Cross-reactivity was also the case for tripartite motif protein 33 (TRIM33) found in band 4 and in shotgun analysis (Tables 1 and 2). In immunoblot analyses, the apparent binding of TRIM33 was specific and sensitive to ionic detergent treatment, such as sodium deoxycholate and SDS (Fig. 5C and D). Immunoprecipitation of L929 cells with or without N-cadherin-myc revealed that the anti-N-cadherin antibody was directly bound to TRIM33 (Fig. 5E).

Clathrin heavy chain was abundant in band 3 (Table 1). In the immunoblot analysis, this binding was found to be non-specific (Fig. 6A). Similarly, DNA damage-binding protein 1 (DDB1)/damage-specific DNA-binding protein 1 found in band 6 turned out to be non-specific (Fig. 6B). These non-specific signals were successfully ruled out in shotgun analysis by subtracting the non-specific binders (control IgG) from the specific binders.

Band 14 included syntaxin 1B2 and glyceraldehyde-3-phosphate dehydrogenase, and band 10 included TNF receptor associated protein 1 (TRAP1) (Table 1). The co-immunoprecipitation of these proteins has not been detectable in our immunoblottings so far (Fig. 7).

3.8. Dynamism of the adhesion complex upon synaptic stimulation

N-cadherin expressed in developed brain is distributed to specific synaptic contacts and has been implicated in the formation and maintenance of the CNS synaptic junction (Benson and Tanaka, 1998; Fannon and Colman, 1996; Togashi et al., 2002). However, N-cadherin in CNS is now appreciated as a dynamic regulator of synaptic structure and function, responding instantly to synaptic activity (Bozdagi et al., 2004, 2010; Jungling et al., 2006; Mendez et al., 2010; Okamura et al., 2004; Tanaka et al., 2000). The activation of the NMDA receptor results in the dephosphorylation of β-catenin at Tyr-654. The dephosphorylated β-catenin is recruited to the cytoplasmic domain of N-cadherin and stabilizes it on the cell surface (Murase et al., 2002; Tai et al., 2007). At the same time, N-cadherin itself shows stabilized conformation as demonstrated by its super-resistance to extracellular trypsin and its stable dimer in SDS-gels (Tanaka et al., 2000). We reasoned that such a change

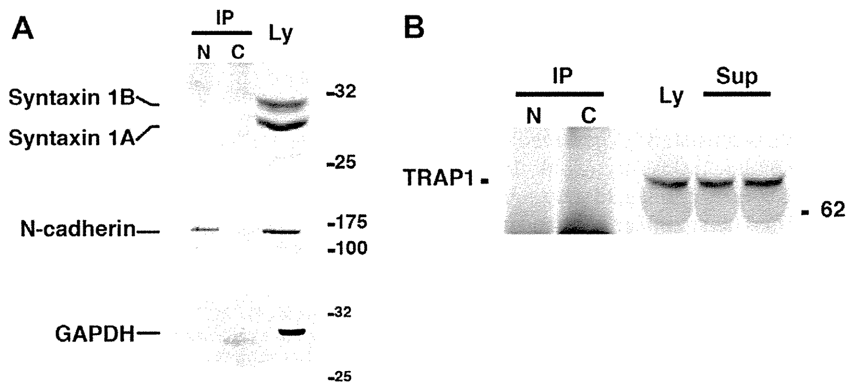

Fig. 7. Non-reproducible cases in immunoblotting. Neurons were extracted and immunoprecipitated with anti-N-cadherin antibody. The immunoprecipitate with anti-N-cadherin antibody (N), with control IgG (C), 10% of total lysate (Ly), and flow through (Sup), were immunoblotted with anti-syntaxin 1 (A, top), anti-N-cadherin (A, middle), anti-GAPDH (A, bottom), and anti-TRAP1 (B) antibodies.

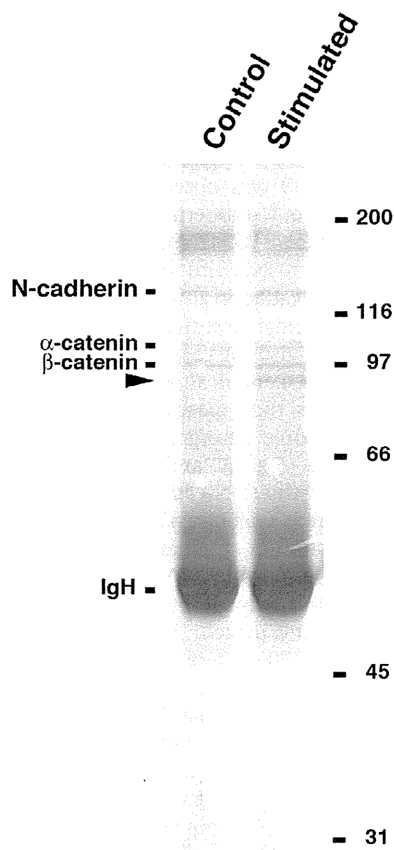

Fig. 8. Synaptic stimulation induces a change in the profile of N-cadherin immunoprecipitates. Control (left) or depolarized (50 mM K^+, 15 min; right) rat hippocampal neurons were extracted with Ca-PI lysis buffer and immunoprecipitated with anti-N-cadherin antibody. IgH: Immunoglobulin heavy chain. Arrowhead indicates a band specifically intensified in the stimulated neurons.

in N-cadherin conformation accompanies alterations of N-cadherin-binding proteins.

The immunoprecipitation of N-cadherin from neurons depolarized for 15 min showed a stronger 93-kDa band than from non-stimulated neurons (Fig. 8). This band corresponded to band 9, which included a shorter variant of α2-catenin and a fragment of β-catenin (Table 1). It has been reported that NMDA-receptor activation yields an increase in shorter β-catenin immunoreactivity around this molecular size (85 kDa) via the cleavage of full-length β-catenin by activated calpain (Abe and Takeichi, 2007). Although the calpain-cleaved β-catenin fragment is released from N-cadherin, the 93-kD β-catenin maintained its association with N-cadherin under our experimental conditions, probably because it maintains the binding domain to N-cadherin. Our immunoprecipitation buffer contained 1% Triton X-100, but without any ionic detergent, and was mild enough to maintain relatively weak protein–protein interactions. N-cadherin-binding domain is consistently preserved in the N-terminal-truncated fragment (Kemler, 1993; Sacco et al., 1995); calpain cleaves the N-terminal 28–30 amino acid fragment of β-catenin (Abe and Takeichi, 2007). The significant enhancement of truncated β-catenin observed in silver-stained gel suggests that the regulation of N-cadherin adhesivity by this modification of β-catenin is a major mechanism underlying synaptic remodeling.

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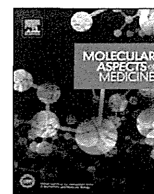

Review

The SLC1 high-affinity glutamate and neutral amino acid transporter family ☆

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ABSTRACT

Glutamate transporters play important roles in the termination of excitatory neurotransmission and in providing cells throughout the body with glutamate for metabolic purposes. The high-affinity glutamate transporters EAAC1 (SLC1A1), GLT1 (SLC1A2), GLAST (SLC1A3), EAAT4 (SLC1A6), and EAAT5 (SLC1A7) mediate the cellular uptake of glutamate by the co-transport of three sodium ions (Na^+) and one proton (H^+), with the counter-transport of one potassium ion (K^+). Thereby, they protect the CNS from glutamate-induced neurotoxicity. Loss of function of glutamate transporters has been implicated in the pathogenesis of several diseases, including amyotrophic lateral sclerosis and Alzheimer disease. In addition, glutamate transporters play a role in glutamate excitotoxicity following an ischemic stroke, due to reversed glutamate transport. Besides glutamate transporters, the SLC1 family encompasses two transporters of neutral amino acids, ASCT1 (SLC1A4) and ASCT2 (SLC1A5). Both transporters facilitate electroneutral exchange of amino acids in neurons and/or cells of the peripheral tissues. Some years ago, a high resolution structure of an archaeal homologue of the SLC1 family was determined, followed by the elucidation of its structure in the presence of the substrate aspartate and the inhibitor L-*threo*-benzyloxy aspartate (L-TBOA). Historically, the first few known inhibitors of SLC1 transporters were based on constrained glutamate analogs which were active in the high micromolar range but often also showed off-target activity at glutamate receptors. Further development led to the discovery of L-*threo*-β-hydroxyaspartate derivatives, some of which effectively inhibited SLC1 transporters at nanomolar concentrations. More recently, small molecular inhibitors have been identified whose structures are not based on amino acids. Activators of SLC1 family members have also been discovered but there are only a few examples known.

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1. Introduction

In mammals, the SLC1 family encompasses five high-affinity glutamate transporters and two neutral amino acid transporters. In humans, the five glutamate transporters possess 44–55% amino acid sequence identity with each other, whereas the two amino acid transporters exhibit 57% identity with each other. For the seven human SLC1 family members, Table 1 summarizes their type of transport mechanism and predominant transport substrates, as well as their localization of expression and links to disease. Fig. 1 depicts their phylogenetic relationships.

The first three members of the SLC1 family – *SLC1A1* (EAAC1), *SLC1A2* (GLT1) and *SLC1A3* (GLAST) – were identified in 1992. Kanai and Hediger (1992) used expression cloning in *Xenopus* oocytes to isolate a cDNA encoding EAAC1 (Kanai and Hediger, 1992). Independently, Kanner et al. purified a synaptosomal glycoprotein (GLT1) that was shown to transport

Table 1
SLC1: the high-affinity glutamate and neutral amino acid transporter family. For detailed information about the SLC gene tables, please visit: http://www.bioparadigms.org/.

Human gene name	Protein name	Aliases	Predominant substrates	Transport type/ coupling ions	Tissue distribution and cellular/ subcellular expression	Link to disease	Human gene locus	Sequence accession ID	Splice variants and their specific features
SLC1A1	EAAC1, EAAT3	System X^-_{AG}	L-Glu, D/L-Asp	C/Na^+, H^+ and K^+	Brain (neurons), intestine, kidney, liver, heart, placenta	Huntington's disease, Epilepsy, Ischemia, Alzheimer's disease, Niemann-Pick disease, Obsessive-compulsive disorder	9p24	NM_004170	
SLC1A2	GLT-1, EAAT2	System X^-_{AG}	L-Glu, D/L-Asp	C/Na^+, H^+ and K^+	Brain (astrocytes, Bergmann glia, neurons), liver, pancreas	Amyotrophic lateral sclerosis, Alzheimer's disease, Huntington's disease, Epilepsy, Ischemia, Schizophrenia	11p13-p12	NM_004171 NM_001195728 NM_001252652	3 Splice variants differ in their C-terminus
SLC1A3	GLAST, EAAT1	System X^-_{AG}	L-Glu, D/L-Asp	C/Na^+, H^+ and K^+	Brain (astrocytes, Bergmann glia), heart, skeletal muscle, placenta	Alzheimer's disease, Huntington's disease, Epilepsy, Cerebellar ataxia type 7, Schizophrenia	5p13	NM_004172 NM_001166695 NM_001166696	3 Splice variants differ in their 3′ UTR, coding sequences, C-terminus
SLC1A4	ASCT1, SATT	System ASC	L-Ala, L-Ser, L-Cys, L-Thr	C/Na^+, E/ amino acids	Widespread		2p15-p13	NM_003038 NM_001193493	2 Splice variants differ in their 5′ UTR, coding sequences, start codon
SLC1A5	ASCT2, AAAT	System ASC	L-Ala, L-Ser, L-Cys, L-Thr, L-Gln, L-Asn	C / Na^+, E/ amino acids	Lung, skeletal muscle, large intestine, kidney, testis, adipose tissue		19q13.3	NM_005628 NM_001145144 NM_001145145	3 Splice variants differ in their 5′ UTR, coding sequences, N-terminus
SLC1A6	EAAT4	System X^-_{AG}	L-Glu, D/L-Asp	C/Na^+, H^+ and K^+	Cerebellum (Purkinje cells)	Spinocerebellar ataxia type 5	19p13.12	NM_005071	
SLC1A7	EAAT5	System X^-_{AG}	L-Glu, D/L-Asp	C/Na^+, H^+ and K^+	Retina (rod photoreceptors and bipolar cells		1p32.3	NM_006671	

C: cotransporter, *E*: exchanger, *F*: facilitated transporter, *O*: orphan transporter.

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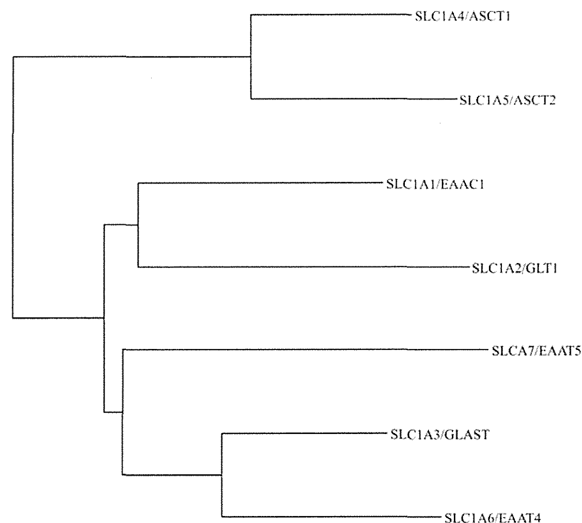

Fig. 1. Phylogenetic tree of the seven human SLC1 family members. The tree was generated using the ClustalW program for multiple protein sequence alignment and SeaView 4 software (distance measurement method using BioNJ algorithm with Poisson parameter) for computation of the phylogenetic tree (Gouy et al., 2010).

glutamate with high affinity when reconstituted into proteoliposomes (Danbolt et al., 1992; Pines et al., 1992). Stoffel et al. co-purified a hydrophobic glycoprotein (GLAST) and identified its cDNA through partial amino acid sequencing (Storck et al., 1992). Later on, the two glutamate transporters EAAT4 (*SLC1A6*) and EAAT5 (*SLC1A7*), as well as the two transporters of neutral amino acids, ASCT1 (*SLC1A4*) and ASCT2 (*SLC1A5*), were identified because of their sequence homology to the previously cloned SLC1 family transporters (Arriza et al., 1997; Arriza et al., 1993; Fairman et al., 1995; Kekuda et al., 1996; Shafqat et al., 1993; Storck et al., 1992; Utsunomiya-Tate et al., 1996).

2. Functional properties

Glutamate transporters couple glutamate uptake to the transport of inorganic ions. It is now generally accepted that 3 Na^+ ions and 1 H^+ are co-transported while 1 K^+ is counter-transported with each glutamate molecule (Zerangue and Kavanaugh, 1996b). Based on this stoichiometry, it was calculated that glutamate transporters could concentrate glutamate 5×10^6-fold inside cells under physiological conditions (Zerangue and Kavanaugh, 1996b). According to the stoichiometry, glutamate transport is electrogenic. Detailed analysis of the steady-state and pre-steady state currents displayed by glutamate transporters in response to step changes of the membrane voltage has provided clues to their dynamics and structure.

Glutamate transporters mediate two distinct types of substrate-induced steady-state currents. The first is the inward-rectifying current due to the stoichiometric movement of two positive charges through the membrane accompanied by each transported glutamate molecule. The second is the "uncoupled" current carried by Cl^- and activated by substrates of the transporter (Arriza et al., 1997; Fairman et al., 1995; Wadiche, 1995a). Cl^- is mainly translocated in the presence of glutamate or related substrates. Cl^- movement is not thermodynamically coupled to the substrate transport. Cl^- is therefore not necessary for the substrate translocation (Fairman et al., 1995; Wadiche, 1995a). The anion permeability of glutamate transporters decreases in the order EAAT4/5 > GLAST > EAAC1 ≫ GLT1. The anion conductance has the characteristics of a substrate-gated anion channel with a selectivity order $NO_3^- > I^- > Br^- > Cl^- > F^-$ (Fairman et al., 1995; Wadiche et al., 1995a). By applying glutamate rapidly to outside-out patches excised from transfected human embryonic kidney 293 cells, it was demonstrated that both anion currents and stoichiometric currents display similar kinetics, suggesting that anion channel gating and stoichiometric charge movement are linked to early transitions in the transport cycle (Otis and Kavanaugh, 2000). Consistent with this, human GLAST with the mutation or modification of ^{449}Val, which likely limits the closure of helical hairpin 2 (HP2, see Fig. 3) after substrate binding, is unable to transport substrates, whereas it retains substrate-elicited anion currents, supporting the idea that the anion conducting state occurs at an early stage after substrate binding (Jiang et al., 2011). It was shown that EAAT5 exhibits a prominent chloride conductance compared with its amino acid fluxes (Arriza et al., 1997). Such intrinsic glutamate-gated anion conductance is proposed to prevent depolarization of the membrane caused by electrogenic glutamate uptake via glutamate transporters. When the membrane voltage is changed in the absence of a substrate, sodium-dependent pre-steady state transient currents are observed. With respect to the glutamate transporter GLT1, kainate, a non-transported inhibitor, was used to "freeze" the transporter-inhibitor complex in a non-transporting form, in order to isolate pre-steady state currents (Wadiche et al., 1995b). Based on the Na^+-dependence of the pre-steady state currents, it was concluded that the currents reflect the voltage-dependent binding and unbinding of Na^+ (Wadiche et al., 1995b).

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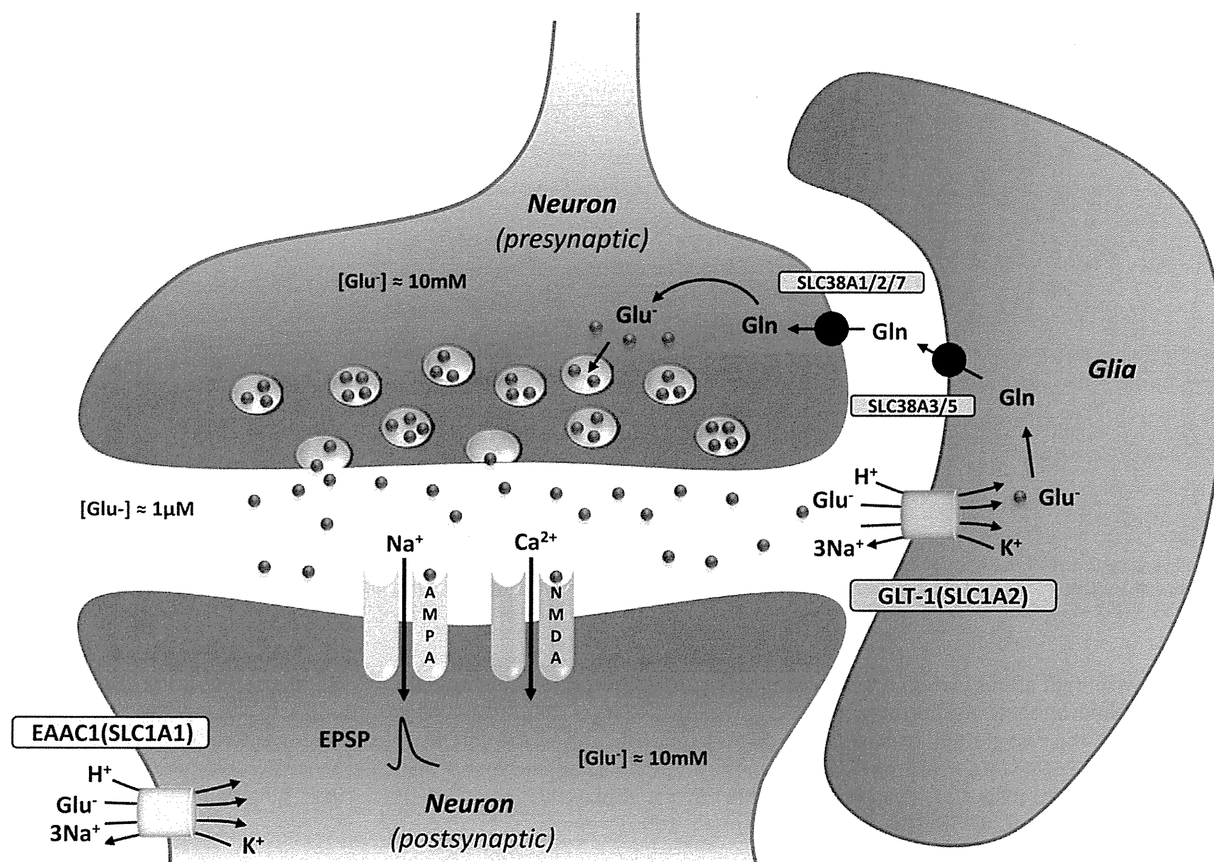

Fig. 2. High-affinity glutamate transporters in the glutamatergic synapses. L-glutamate is stored in synaptic vesicles in the presynaptic terminals. Glutamate is released into the synaptic cleft and acts on NMDA or AMPA glutamate receptors that are permeable for Ca^{2+} or Na^+, respectively, leading to membrane depolarization and generation of action potentials. High-affinity glutamate transporters regulate the glutamate concentration and thereby the neurotransmission by removing the excitatory neurotransmitter from the synaptic cleft. Glutamate transporters are also essential to maintain extracellular glutamate concentration below neurotoxic levels. The five high-affinity glutamate transporters (EAAT1 to 5) exhibit different regional and cellular localizations. GLAST (EAAT1) (blue) and GLT1 (EAAT2) (green) are mainly expressed in glia throughout the central nervous system and are responsible for 90% of the glutamate uptake. EAAC1 (EAAT3) (yellow) is a postsynaptically localized neuronal glutamate transporter widely distributed within the central nervous system. EAAT4 (red) is also a postsynaptic transporter mostly expressed in Purkinje cells of the cerebellum.

The glutamate transporter EAAC1 can also facilitate substrate exchange, in addition to electrogenic glutamate uptake, although under normal conditions the contribution of the exchange component is small when compared to the uptake component (Zerangue and Kavanaugh, 1996b,c). In contrast, the ASC transporters ASCT1 and ASCT2 exclusively mediate Na^+-dependent exchange of substrate amino acids (Broer et al., 2000; Zerangue and Kavanaugh, 1996a). Mutation of Glu 404 or Tyr 403 resulted in loss of K^+-coupling in rat GLT1 (Kavanaugh et al., 1997; Zhang and Kanner, 1999). Those GLT1 mutants displayed only the exchange mode without regular uptake, similar to ASC transporters. Thus, it was concluded that these Glu and Tyr residues are crucial for the K^+-coupling which drives the relocation step of glutamate transporters.

3. Physiological roles

3.1. Glutamate transporters

3.1.1. Glutamatergic synapses

Glutamate is the principal excitatory neurotransmitter in the central nervous system. After glutamate release in the synaptic cleft, the neurotransmitter will bind ionotropic or metabotropic receptors. There are two classes of ionotropic glutamate receptors, the NMDA receptors and the non-NMDA receptors including the AMPA receptors. AMPA receptors allow a Na^+ influx inducing fast excitatory postsynaptic potentials in contrast to NMDA receptors which are permeable to Ca^{2+}. The glutamate concentration in the synaptic clef is tightly regulated and depends on glutamate release and glutamate clearance (Kanai and Hediger, 2003). The glial glutamate transporter GLT1 (encoded by *SLC1A2*) plays a crucial role in removing glutamate from the synaptic cleft, terminating the glutamatergic transmission (Fig. 2). After being released into the synaptic

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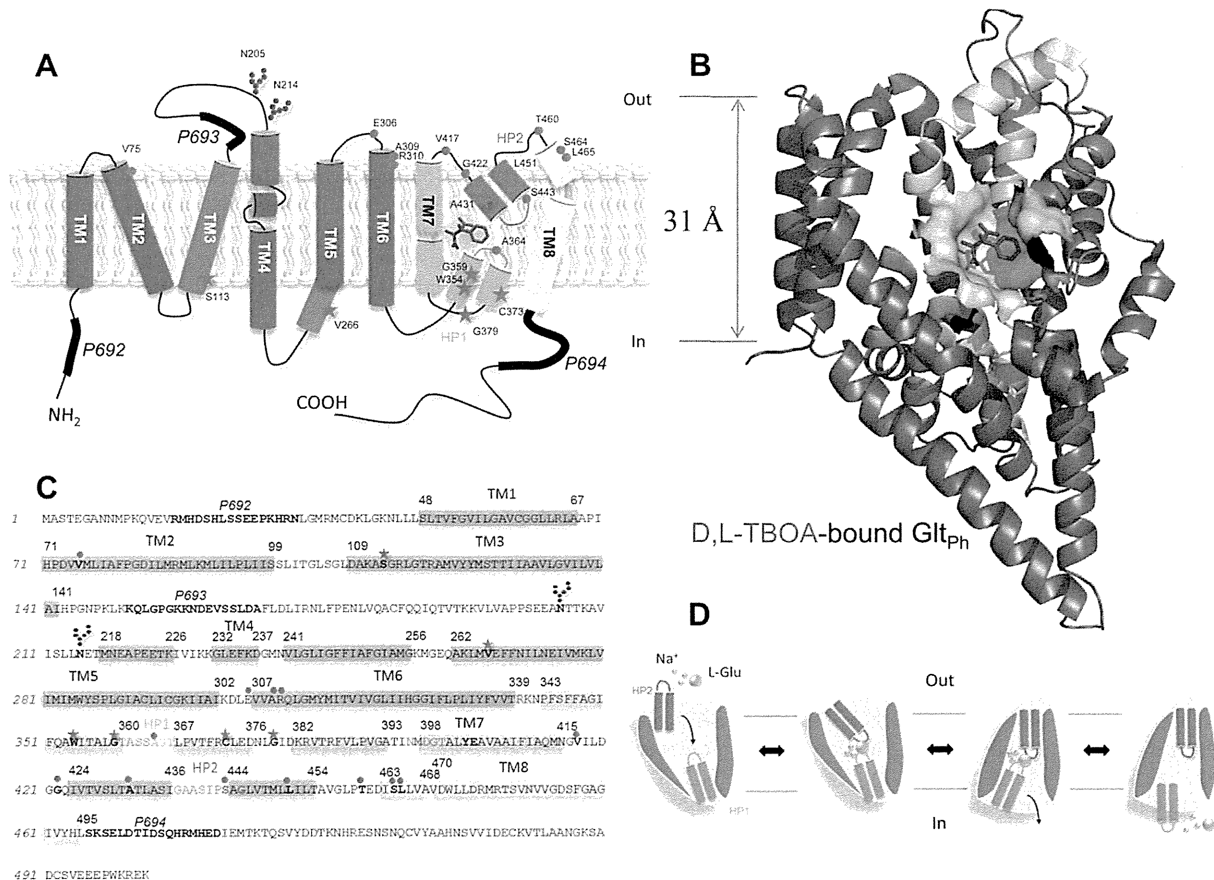

Fig. 3. Membrane topology and transport mechanism model of mammalian glutamate transporters. (A) This schematic topology representation is based on a putative 3D-structure generated by PHYRE server (Kelley and Sternberg, 2009) with the structure of the archaeal Glt_{Ph} as a template. The obtained topology model of mammalian GLT1 is similar to that of Glt_{Ph} (not shown), and also depicts results of experiments published in the literature involving labeling with a biotinylation agent in the absence of permeabilization *(purple full circles)* or labeling after permeabilization (*purple stars*) [14, 15]. There are eight transmembrane (TM) domains and two hairpins (HP1, HP2) that differ in length. The binding site domains are indicated in green (HP1), yellow (TM7), blue (HP2) and orange (TM8) in contrast to the rest of the protein (in grey). The results of published accessibility studies with antibodies targeting *P692*, *P693* and *P694* domains are also shown. The putative position of D,L-TBOA is indicated. (B) Protomer Glt_{Ph} (PDB ID: 2NWW) in complex with D,L-TBOA (in red) viewed in the plane of the membrane at 31 Å by PyMOL v0.99 software (adapted from Yernool et al., Nature, 2004). The structure includes a surface modeling of the buried chamber (in silver) at 6 Å around D,L-TBOA. The D,L-TBOA binding cavity is formed by HP1, TM7, HP2 and TM8. (C) Amino acid sequence of GLT1 (P31596.2) using the legend code as in Fig. 3A. Conserved amino acids in the binding site are colored in red. (D) Hypothetical model of glutamate transport mechanism across the plasma membrane from the extracellular space (Out) to the cytosol (In) (adapted from (Reyes et al., 2009)). For the sake of clarity, only the protomer shapes with its hairpins are drawn. L-glutamate (L-Glu) and sodium ions (Na^+) are represented as purple and blue balls, respectively. In the "open" conformation that is closed from the cytosol side, L-glutamate is trapped by HP2 and HP1 in the cavity. After an intermediate state, where the cavity is closed on both sides, the repositioned HP2 finally releases L-glutamate. This mechanism is coupled to the transport of 3 Na^+ ions.

cleft, glutamate is rapidly taken up by nearby astrocytes via GLT1, where it is converted to glutamine by glutamine synthetase. Glutamine is transported into presynaptic terminals of the neurons, where phosphate activated glutaminase (PAG) converts it back to glutamate for loading into synaptic vesicles. Because high extracellular glutamate concentrations cause excitotoxicity, glutamate transporters are important in protecting neurons from glutamate toxicity.

Studies *in vitro* and *in vivo* using chronic administration of antisense oligonucleotides targeting each glutamate transporter isoform demonstrated that under normal conditions, glial isoforms (GLT1 and GLAST), but not the neuronal isoform (EAAC1), are critical to maintain the extracellular glutamate concentration in a non-neurotoxic range (Rothstein et al., 1996). A similar neuroprotective effect of the glial glutamate transporters has been observed in GLT1 and GLAST knockout mice (Tanaka et al., 1997; Watanabe et al., 1999; Watase et al., 1998). Another glutamate transporter belonging to the SLC1 family, EAAT4, is a neuronal high affinity glutamate transporter like EAAC1, but its expression is restricted to Purkinje cells (Fairman et al., 1995; Nagao et al., 1997), limiting the spillover of released glutamate to neighboring synapses and thereby preventing undesired neurotransmission (Takayasu et al., 2009). EAAT5 is the last glutamate transporter known so far and is selectively expressed at photoreceptor and bipolar cell terminals in the retina (Arriza et al., 1997; Eliasof et al., 1998; Pow and Barnett, 2000). Like EAAT4, EAAT5 exhibits a glutamate-gated chloride conductance (Fairman et al., 1995; Nagao et al., 1997) that

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could provide a feedback mechanism controlling neuron depolarization and further glutamate release and thereby limiting signal transmission (Picaud et al., 1995).

3.1.2. Cell metabolism

In addition to the very well established expression of SLC1 family members in the central nervous system, studies demonstrated expression of EAAC1, GLAST and GLT1 also in the peripheral nervous system, where they play important roles in cell metabolism (Berger and Hediger, 2000; Carozzi et al., 2008; Tao et al., 2004). GLAST has been found in epithelial cells, macrophage-lineage cells, lymphocytes, fat cells, interstitial cells and salivary gland acini (Berger and Hediger, 2006). A different distribution has been shown for GLT1, which is expressed in glandular tissues including mammary glands, lacrimal gland, salivary gland ducts and acini. GLT1 has also been detected in perivenous hepatocytes, follicular dendritic cells in spleen and lymph nodes, and recently in α and β cells of the pancreas (Berger and Hediger, 2006; Cadoret et al., 2002; Meabon et al., 2011). Expression of glutamate transporters in these peripheral organs is important to support glutamine synthesis. In the liver, perivenous hepatocytes take up glutamate, presumably via GLT1. This is coupled to glutamine synthesis and release, supporting interorgan glutamine supply. The glutamate transporter EAAC1 that is present in muscle and lung takes up glutamate, which is also coupled to glutamine synthesis, followed by its release into the blood. EAAC1 is also expressed in kidney proximal tubules S3 segments where it mediates absorption of filtered glutamate as well as glutamate formed in the renal tubule by intraluminal glutamine hydrolysis, catalyzed by the phosphate-independent glutaminase (PIG). Once again, absorbed glutamine is converted into glutamine by glutamine synthetase, followed by its release into the blood.

3.2. Neutral amino acid transporters

The SLC1 family also possesses two ASC transporters, ASCT1 and 2, which transport neutral amino acids such as L-serine, L-alanine, L-cysteine and L-threonine (Arriza et al., 1993; Shafqat et al., 1993). Additional to these neutral amino acids, ASCT2 has been found to transport L-glutamine and L-asparagine at high affinity and other neutral amino acids with lower affinity (Broer et al., 2000; Shafqat et al., 1993; Utsunomiya-Tate et al., 1996). In the brain, ASCT1 and ASCT2 are preferentially expressed in astrocytes where their function has been detected, but they are also present in neurons (Broer et al., 1999; Deitmer et al., 2003; Gliddon et al., 2009; Weiss et al., 2001; Yamamoto et al., 2003; Yamamoto et al., 2004). ASCT1 seems to be the main uptake system of L-serine in neurons (Yamamoto et al., 2004). In contrast, ASCT2 might contribute to the glutamine homeostasis of neuronal and glial cells (Broer and Brookes, 2001; Deitmer et al., 2003; Gliddon et al., 2009). Both ASC transporters have been identified in non-neuronal tissue. ASCT1 has been immunolocalized in the digestive system on the basolateral membrane of the basal cells of stratified squamous epithelia from oral parietes to the nonglandular region of the stomach, chief cells of the glandular stomach, acinar cells of the salivary gland and exocrine pancreas, and Paneth's cells of the small intestine (Hashimoto et al., 2004). ASCT1 was also detected at the plasma membrane of hepatocytes in the liver, of epithelial cells of the proximal tubule of the kidney and of supporting cells in the medulla of the adrenal gland (Hashimoto et al., 2004). ASCT2 expression in the kidney and intestine has been described but is restricted to the brush border of proximal tubular cells and enterocytes (Avissar et al., 2001). These findings suggest a physiological role of ASC transporters in peripheral tissues contributing to the amino acid transport (Avissar et al., 2001).

4. Pathological relevance

As noted above, glutamate transporters in the central nervous system play a critical role in maintaining extracellular glutamate concentrations below excitotoxic levels. Glutamate transporter dysfunction is therefore associated with neurodegenerative diseases (e.g. amyotrophic lateral sclerosis, Alzheimer disease) and ischemic damage (e.g. after a stroke). These observations suggest glutamate transporters may be important drug targets. Initial work by Rothstein revealed the involvement of the glial glutamate transporter GLT1, encoded by the *SLC1A2* gene, in the pathogenesis of amyotrophic lateral sclerosis (ALS) (Rothstein, 2009). Recently, studies using knockout mice deficient of glutamate transporters, as well as analyses of clinical cases, have indicated roles for each glutamate transporter isoform in glaucoma, autism and schizophrenia as well as neurodegenerative diseases.

Because GLT1 is the predominant glial glutamate transporter in forebrain and hippocampus, where it covers approximately 95% of glutamate uptake, the decrease of its expression or function is involved in the pathogenesis of a variety of diseases. GLT1 protein level is decreased in the lesions in the brain and spinal cord of patients with ALS and also in the ventral horn of the spinal cord of transgenic rats overexpressing Cu^{2+}/Zn^{2+} superoxide dismutase 1 (SOD1) harboring an ALS-linked familial genetic mutation, suggesting a role for GLT1 in the events leading to neuronal cell death in ALS (Howland et al., 2002; Rothstein et al., 1995).

Slc1a2-knockout mice showed lethal spontaneous epileptic seizures and selective neuronal degeneration in the hippocampal CA1 region and in layer II of the neocortex (Kiryk et al., 2008; Tanaka et al., 1997). Deficits in glutamate transporter function are supposed to contribute to the pathogenic processes associated with Alzheimer disease (AD).

GLT1 protein is found to be reduced in the brain of patients with AD (Mookherjee et al., 2011). In the AD model transgenic mice expressing mutations of amyloid-β protein precursor and presenilin-1 (AβPPswe/PS1ΔE9), partial loss of GLT1 by cross-

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ing with GLT1(+/−) mice exacerbated spatial memory deficits accompanied by an increase in the accumulation of insoluble Aβ42/Aβ40 (Mookherjee et al., 2011).

In addition to neurodegenerative diseases, recent large-scale genetic analyses for disease-associated genes have suggested a link between the *SLC1A2* gene and autism (Szatmari et al., 2007; Xu et al., 2008).

The distribution of GLAST in the CNS is distinct from that of GLT1. GLAST, encoded by the *SLC1A3* gene, is strongly expressed in Bergmann glia of the cerebellum and in Muller cells in the retina. At lower levels, GLAST is also expressed ubiquitously in glial cells throughout the central nervous system. *Slc1a3*-knockout mice as well as EAAC1/*Slc1a1*-deficient mice exhibit spontaneous degeneration of retinal ganglion cells and the optic nerve without elevated intraocular pressure; thus these mice can be regarded as models of normal tension glaucoma (Harada et al., 2007). In the *Slc1a3*-knockout mice, glutathione in Muller cells was decreased, and the retinal ganglion cells became more vulnerable to oxidative stress in *Slc1a1*-deficient mice. This suggests the role of glutamate transporters in the synthesis of glutathione, an antioxidant tripeptide of glutamate, cysteine and glycine. GLAST has furthermore been liked with the pathogenesis of schizophrenia. In the genome of patients with schizophrenia, a microdeletion involving the *SLC1A3* gene was identified (Walsh et al., 2008). Consistent with this, *Slc1a3*-knockout mice show behavioral abnormalities relevant to the positive, negative and cognitive symptoms of schizophrenia (Karlsson et al., 2008; Karlsson et al., 2009). *Slc1a3*-knockout mice have also been studied in the context of noise trauma. Exposure to intense, loud noise causes dendrite swelling below the inner hair cells and physical damage to the outer hair cells of the ear and it has been proposed that glutamate excitotoxicity may be in part responsible for hearing loss. In the GLAST deficient mice, excess glutamate was observed in the perilymphs and resulted in exacerbation of hearing loss (Hakuba et al., 2000).

Neuronal type EAAC1 possesses a high capacity for transporting L-cysteine, a substrate of glutathione synthesis, although the contribution to maintain extracellular glutamate concentration is less than that of GLAST and GLT1 (Rothstein et al., 1996; Zerangue and Kavanaugh, 1996c). Thus, the role of EAAC1 to protect neurons against oxidative stress by providing neurons with L-cysteine has been proposed. *Slc1a1*-knockout mice in fact show vulnerability to oxidative stress. As described above, degeneration of retinal ganglion cells resembling that in normal tension glaucoma was reported in the retina of *Slc1a1*-knockout mice (Harada et al., 2007). *Slc1a1*-knockout mice furthermore show reduced neuronal glutathione levels and develop brain atrophy and behavioral changes with aging, which were reversed by the treatment with *N*-acetylcysteine (Aoyama et al., 2006; Berman et al., 2011). In addition, dopaminergic neurons in the substantia nigra pars compacta are degenerated age-dependently in *Slc1a1*-knockout mice, with more than 40% of these neurons lost by age 12 months, a defect which is also rescued by *N*-acetylcysteine (Rossi et al., 2000).

The role of glutamate transporters in neuronal damage in brain ischemia has long been proposed. Because glutamate transport is driven by the free energy stored in the form of electrochemical gradients of the coupling ions across the plasma membrane, its disruption due to insufficient energy supply during ischemia results either in decreased glutamate uptake or reversed glutamate transport, which causes a rise in the extracellular glutamate concentration above neurotoxic levels (Grewer et al., 2008; Rossi et al., 2000). Given the high intracellular glutamate concentration in neurons, the neuronal glutamate transporter EAAC1 is more likely to run in reverse in ischemia compared to GLT1 in astrocytes (Kanai and Hediger, 2001; Nishida et al., 2004a). Therefore, blockage of "reversal glutamate transport" by EAAC1 under ischemic conditions may lead to a possible therapeutic strategy. Due to the electrogenicity of glutamate transport, membrane depolarization during ischemia will result in a reversal of the transport direction of glutamate, resulting in release of glutamate and causing excitotoxicity. Thus, a selective inhibitor for EAAC1 would prevent glutamate excitotoxicity under ischemic conditions. In support of this hypothesis, during short ischemia (5 min) *Slc1a2* knockout mice show higher extracellular glutamate levels in the hippocampal CA1 region than wild-type mice, probably due to the reversal of neuronal glutamate transport and the loss of glial glutamate uptake caused by knockout of GLT1 (Mitani and Tanaka, 2003).

Dicarboxylic aminoaciduria (DA) is a rare, autosomal recessive disorder characterized by the excessive loss of aspartate and glutamate in urine. Detailed clinical features of the disorder remain to be elucidated, because only a few cases have been reported so far. Of the four independent cases described, two report an association with neurological dysfunction such as mental retardation (Swarna et al., 1989; Teijema et al., 1974). DA is caused by *SLC1A1* mutations leading to substitution of arginine to tryptophan at position 445 (R445W) and deletion of isoleucine at position 395 (I395del) in EAAC1 (Bailey et al., 2011). Structurally, arginine 445 is located within transmembrane domain 8 and thus predicted to be in close proximity to the substrate binding site (see subsequent Section 5 and Fig. 3), whereas isoleucine 395 lies in the hairpin loop 2 (HP2), reported to resemble the ion-permeating region of ion channels (Kanai and Hediger, 2004). Functional effects of those mutations on substrate transport activity were examined in *Xenopus* oocytes. Transport conductance decreased to 20% of that of wildtype oocytes expressing the EAAC1 R445W mutant and was even abrogated in oocytes expressing EAAC1 I395del (Bailey et al., 2011).

Concerning the above described roles of glutamate transporters, the drugs increasing glutamate transporter function and/or expression would have clinical advantages. Ergo the derivatives bromocriptine and nicergoline were reported to increase the functional activity of glutamate transporter proteins (Nishida et al., 2004b; Yamashita et al., 1995). Nicergoline reduces extracellular glutamate by enhancing the uptake of glutamate without affecting reversed transport of glutamate, which could be beneficial to prevent ischemic neuronal damage (Nishida et al., 2004b). Drugs increasing glutamate transporter expression were identified by screening of FDA-approved drugs (Rothstein et al., 2005). Many β-lactam antibiotics are found as potent stimulators of GLT1 expression, probably increasing transcription of the *SLC1A2* gene. In fact ceftriaxone, a β-lactam antibiotic, was confirmed to be neuroprotective *in vitro* and *in vivo* (Rothstein et al., 2005).

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5. Structure–function relationship

In 2004, twelve years after the cloning and expression of a glutamate transporter from rat brain (Pines et al., 1992), Gouaux and colleagues determined the high-resolution structure of an archaeal homologue of the SLC1 family, Glt_{Ph}, by X-ray crystallography (Yernool et al., 2004). Glt_{Ph} is an archaeal glutamate transporter from *Pyrococcus horikoshii* that shares about 36% amino acid identity with human EAAT glutamate transporters, and co-transports L-aspartate with 2 Na^+. With its trimeric architecture, Glt_{Ph} forms a large open cavity facing the extracellular side (Yernool et al., 2004). Each protomer itself represents an independent transport unit.

Two structures of Glt_{Ph} in the presence of the substrate L-aspartate and the inhibitor D,L-TBOA (D,L-threo-β-benzyloxyaspartate) were obtained in 2007 by the same group at a resolution of about 3 Å (Boudker et al., 2007). D,L-TBOA is a unspecific, competitive and non-transportable inhibitor of EAATs (Shimamoto et al., 1998) and represents a promising starting point for rational drug design (Esslinger et al., 2005; Kim et al., 2011; Shimamoto et al., 2004; Takaoka et al., 2004).

Each individual protomer is composed of eight transmembrane (TM) domains (Fig. 3A) as was previously predicted by hydropathy plots (Grunewald et al., 2002), as well as two helix-turn-helix motifs or hairpin loops (HP1, HP2). HP1 was previously described as an unstructured "reentrant cytosolic loop" with a serine (S440) closing the glutamate binding site and playing an important role in ion selectivity (Zarbiv et al., 1998). HP2 corresponds to the "extracellular reentrant loop" between TM7 and TM8 and was reported to resemble the ion-permeating region of ion channels (Kanai and Hediger, 2004). The structure of Glt_{Ph} reveals a long glycosylated extracellular loop between TM3 and TM4, and the C-terminal half of the protein (TM7, TM8, HP1 and HP2) is involved in the transport of substrate (Boudker et al., 2007). The D,L-TBOA bound Glt_{Ph} structure (PDB ID: 2NWW) differs from that obtained in the presence of L-aspartate (PDB ID: 2NWL) primarily in the position of HP2. After binding of D,L-TBOA, its slight relocation to the extracellular space leads to contact with the loop between the TM3 and TM4 segments.

To better integrate all published data in the literature, the putative structure of a mammalian glutamate transporter was generated using the Protein Homology/Analogy Recognition Engine (PHYRE, Version 0.2) server (Kelley and Sternberg, 2009), with the structure of Glt_{Ph} (PDB ID: 2NWL) as a template (Fig. 3B). The structure obtained correlates well with the results of previous biochemical studies analyzing the solvent accessibility of GLT1 by the use of antibodies targeting mutagenesis specific protein domains (Grunewald and Kanner, 1995), and experiments involving biotinylation of single cysteine mutagenesis before and after permeabilization (Grunewald et al., 1998) and cysteine-scanning mutagenesis (Grunewald and Kanner, 2000) (Fig. 3A and C). There are many amino acids in the regions HP1, TM7, HP2 and TM8 that are potentially involved in substrate binding. However, among them, only a few appear to be conserved in mammalian homologues, namely G445 (HP2), D474/R477/T478/N481 (TM8) and M395 (TM7) (Figure C, amino acids in red). M395 is the NMDGT motif corresponding to one of the conserved sequences in all human EAATs. In addition, it is suggested that M395 may interact with the benzyl group of D,L-TBOA (Boudker et al., 2007). Another conserved motif, T(A/S)SS, is composed of three or four hydroxyl amino acids and seems to be part of the substrate binding site. Based on all these observations, a transport mechanism for Glt_{Ph} has been proposed involving regions HP1 and HP2 (Reyes et al., 2009). However, this model was criticized by another study suggesting that smaller movements are affecting HP1 and HP2, based on a comparative study (L-aspartate vs. D,L-TBOA) using limited trypsin proteolysis combined with site-directed cysteine-scanning mutagenesis and fluorescein-5-maleilide labeling (Compton et al., 2010). From this evidence, a hypothetical model of glutamate transport mechanism across the plasma membrane is proposed in Figure D.

6. Pharmacological aspects

The study of the pharmacological specificity of the SLC1 family started long before the transporters were fully identified and characterized. Using different tissue preparations several groups have described selectivity and inclination of the glutamate transporters using radio-labeled substrates since the 1970s. These results were carefully reviewed in 2005 by (Bridges and Esslinger (2005)). A recent comprehensive and dedicated review highlighted the role of the high-affinity glutamate transporters as potential drug targets. Apart from consideration of their structure, function and distribution, the authors presented highlights of published work on the discovery of inhibitors, enhancers and substrates of the different subtypes (Bunch et al., 2009).

The increase in resources involved in the search for small molecules as inhibitors or modulators of glutamate transporters started with the selective cloning of the different subtypes of the SLC1 family in oocytes or mammalian cell lines. The conceptual origin for the design of inhibitors was the structure of known substrates. It is not unexpected that the first targets included conformationally constrained analogs of natural transport substrates or the introduction of isosteric groups, like azole-based rings, to mimic the acid group in the side chain of glutamate (Bridges and Esslinger, 2005).

Bridges' group reported their significant results on a series of synthetic cyclic glutamate analogs, such as L-*trans*-2,4-PDC (Fig. 4), and their inhibitory activity against EAAC1, GLT1 and GLAST, discriminating between substrate and non-substrate inhibitors of the glutamate uptake (Koch et al., 1999). The ancestor of this class of molecules can be identified as dihydrokainic acid, a known weakly potent inhibitor of glutamate uptake (K_i = 28 μM). The suitability of this strategy was confirmed with the publication of WAY-855, a conformationally restricted glutamate analog (Dunlop et al., 2003). The molecule attracted attention during a screening campaign aimed at identifying positive modulators of GLT1. WAY-855 was identified

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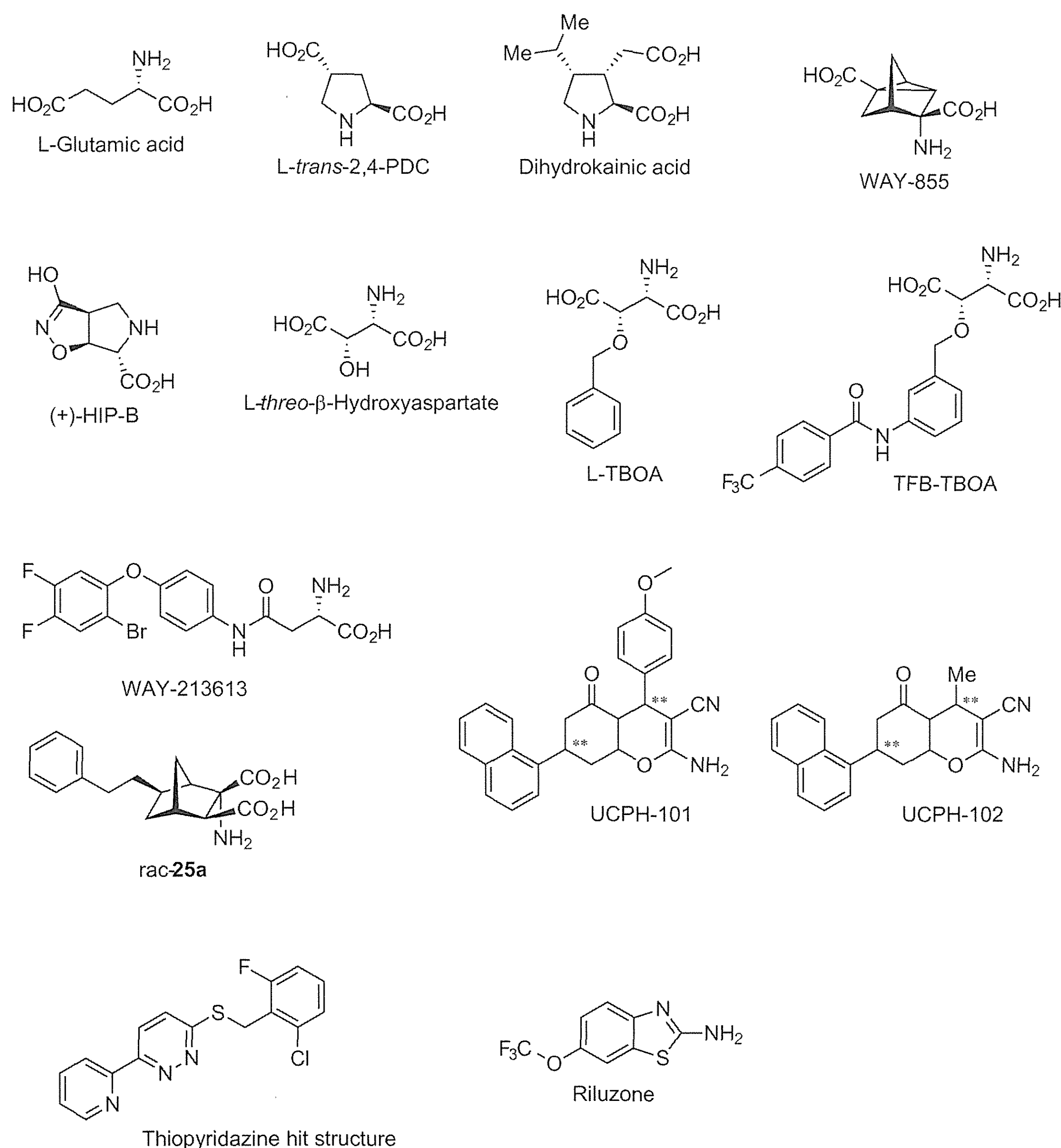

Fig. 4. Structural formulas of the endogenous substrate L-Glu and of known EAAT inhibitors and activators. Their biological activities and isoform selectivities are described in the text.

as a strong inhibitor of glutamate transport in the low μM range with a slight selectivity for GLT1. Several other examples of analogs were synthesized and investigated for efficacy and selectivity (Bunch et al., 2006). The last example of this class of compound was reported recently (Callender et al., 2012). The inhibition of EAAC1 by (+)-HIP-B was found to exhibit a mixed mechanism. A pure competitive mechanism was excluded and the existence of a regulatory site was proposed.

A landmark in the recent story of the SLC1 family was the exploration of a class of inhibitors, derived from an early analog, L-*threo*-β-hydroxyaspartate (Kanai and Hediger, 1992). Several generations of analogs were synthesized and tested in the late 1990s and subsequent years. The major breakthrough was the discovery of L-*threo*-benzyloxyaspartate (L-TBOA), one of the most effective inhibitors at that time (Lebrun et al., 1997; Shimamoto et al., 1998; Shimamoto et al., 2000). Subsequent optimizations identified more potent and selective inhibitors. (2*S*,3*S*)-3-(3-[4-(trifluoromethyl)-benzoylamino]benzyloxy)aspartate or TFB-TBOA belongs to this second generation of compounds and exhibits an IC_{50} value in the low nM range, which is about 100 times more potent than L-TBOA (Shimamoto et al., 2004). The adaptation of this strategy to the glutamate core exemplified by the synthesis of β- and γ-benzyloxy glutamic acid derivatives was less successful; the inhibitory activity was markedly reduced with respect to L-TBOA (Tamborini et al., 2009).

The same industrial group that first described the inhibitory effects of WAY-855 published the results of a screening campaign with aspartamides and diaminopropionic acid analogs in 2005 (Dunlop et al., 2005; Greenfield et al., 2005). The com-

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pounds attained activity in the high nM range and in the best case (WAY-213613) selectivity for GLT1 *versus* EAAC1 and GLAST of 45- and 59-fold, respectively, was achieved.

Recently, different strategies have been applied to the challenging objective of discovering new inhibitors of glutamate transport. The screening of a small commercially-available compounds library allowed the identification of a completely new class of inhibitors featuring a 2-amino-5-oxo-5,6,7-tetrahydro-4H-chromene-3-carbonitrile skeleton (Jensen et al., 2009). The most potent analog synthesized on the original core, UCPH-101, achieved activity in high nM range (IC_{50} = 0.66 μM) with more than 400-fold selectivity for GLAST over EAAC1 and GLT1. The same group investigated this class of compounds further by synthesizing a new series of analogs. As a result, the knowledge of the structure–activity relationship was enhanced considerably; however, only a small improvement of the inhibitory potency (e.g. UCPH-102, IC_{50} = 0.42 μM) was achieved (Erichsen et al., 2010; Huynh et al., 2012).

To expand the search for inhibitors, an *in silico* approach was recently attempted. A virtual library of conformationally constrained aspartate and glutamate analogs was generated and screened by high-throughput docking to the glutamate binding site of the glutamate transporter homologue from *Pyrococcus horikoshii* (PDB ID: 1XFH). The best hits identified were synthetized and showed selective inhibition of GLT1 in the low μM range (e.g. rac-25a displayed an IC_{50} value of 1.4 μM) (Luethi et al., 2010).

A different but very interesting category of compounds are glutamate transporter enhancers. Two different modes of action have been identified. For instance, there are compounds acting on the expression levels of the transporters. The interaction can occur at any of the several steps of the process, i.e. the compounds can enhance transcriptional, translational or post-translational processes. Numerous examples were reported in the literature and where possible a mode of action was investigated (Bunch et al., 2009). As expected from the heterogeneous targets involved in the potentiating effect, marginal similarity was found between activator and endogenous substrates or between activators themselves. Recently a new class of molecules, thiopyridazines, was identified from high-throughput screening of a library of approximately 140,000 compounds (Colton et al., 2010). A hit-to-lead effort was undertaken with resulting enhancement of GLT1 levels by more than 6-fold at a compound concentration of less than 5 μM (Xing et al., 2011).

There are some rare examples of compounds belonging to a second class of activators, which can interact with the transporter directly and enhance the ability of glutamate transport (positive allosteric modulators). Riluzole, the only drug approved for the treatment of Amyotrophic Lateral Sclerosis (ALS), is considered to exert a neuroprotective effect through different mechanisms. Riluzone was found to significantly increase glutamate uptake in a dose-dependent manner in cell lines stably expressing EAAC1, GLT1 and GLAST by enhancing the affinity of glutamate to the transporter (Fumagalli et al., 2008). A second such compound is Parawixin 1, a substance with unknown structure extracted from the venom of the Brazilian spider, Parawixia bistriata. Parawixin 1 is selective for GLT1 *versus* EAAC1 and GLAST (Fontana et al., 2003; Torres-Salazar and Fahlke, 2007). The mechanism of action is related to the turnover of the process, i.e. the compound accelerates the activity of the glutamate transporter (Fontana et al., 2007).

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Lysyl 5-Hydroxylation, a Novel Histone Modification, by Jumonji Domain Containing 6 (JMJD6)*

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Background: JMJD6 hydroxylates U2AF65, but its role in histone modification has been obscure.
Results: Our analysis of histones purified from JMJD6 knock-out mouse embryos reveals that JMJD6 hydroxylates histone lysyl residues.
Conclusion: JMJD6 mediates histone lysyl 5-hydroxylation, which is a novel histone modification.
Significance: Our study identifies a new function for Jumonji family proteins in epigenetic modification of histones.

JMJD6 is reported to hydroxylate lysyl residues of a splicing factor, U2AF65. In this study, we found that JMJD6 hydroxylates histone lysyl residues. *In vitro* experiments showed that JMJD6 has a binding affinity to histone proteins and hydroxylates multiple lysyl residues of histone H3 and H4 tails. Using JMJD6 knock-out mouse embryos, we revealed that JMJD6 hydroxylates lysyl residues of histones H2A/H2B and H3/H4 *in vivo* by amino acid composition analysis. 5-Hydroxylysine was detected at the highest level in histones purified from murine testis, which expressed JMJD6 at a significantly high level among various tissues examined, and JMJD6 overexpression increased the amount of 5-hydroxylysine in histones in human embryonic kidney 293 cells. These results indicate that histones are additional substrates of JMJD6 *in vivo*. Because 5-hydroxylation of lysyl residues inhibited *N*-acetylation and *N*-methylation by an acetyltransferase and a methyltransferase, respectively, *in vitro*, histone 5-hydroxylation may have important roles in epigenetic regulation of gene transcription or chromosomal rearrangement.

Jumonji domain containing 6 (JMJD6),[2] which possesses high binding affinity to single-stranded RNA, is reported to hydroxylate lysyl residues of an RNA splicing factor, U2AF65 (1, 2). JMJD6 contains a JmjC domain that catalyzes lysyl hydroxylation of proteins in the presence of 2-oxoglutarate, Fe(II), and ascorbate. Proteins belonging to the JmjC family are classified into 2-oxoglutarate oxygenases (3). Among the known 2-oxoglutarate oxygenases, PLOD3 (procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3) mediates hydroxylation of unmodified lysyl residues, yielding 5-hydroxylysine (4). Most JmjC family members catalyze hydroxylation of *N*-methyl groups at the ε-amino group of lysyl residues and generate hydroxymethyl groups, which are immediately processed to formaldehyde molecules, resulting in demethylation of methylated lysyl residues (5). However, JMJD6 does not add a hydroxyl group to the *N*-methyl group but adds it to one of the backbone carbons in a lysyl side chain and generates a stable 5-hydroxylysine (1). JMJD6 knock-out mice exhibited severe anemia, growth retardation, and a delay in terminal differentiation of the kidney, intestine, liver, and lung during embryogenesis, resulting in perinatal lethality (6, 7).

In this study, we first identified JMJD6 as a novel UHRF1 (ubiquitin-like with PHD and RING finger domains 1) interacting protein. UHRF1 has important roles in transferring DNA methylation status and recognizes histone modification status (8). Therefore, we thought that JMJD6 might hydroxylate histone molecules through interaction with UHRF1. Using JMJD6 knock-out mice, we revealed that JMJD6 hydroxylates histone lysyl residues and generates 5-hydroxylysine *in vivo*. 5-Hydroxylation is a novel histone lysyl modification. Because it interfered with *N*-acetylation and *N*-methylation by an acetyltransferase and a methyltransferase, respectively, the modification may regulate transcription through these interactions with other histone modifications.

EXPERIMENTAL PROCEDURES

JMJD6 Wild-type and Knock-out Mice—Details of the JMJD6 knock-out mice were described elsewhere (6). C57BL/6 mice

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[2] The abbreviations used are: JMJD6, Jumonji domain containing 6; qRT-PCR, quantitative RT-PCR; HAT, histone acetyltransferase; E14.5, embryonic day 14.5; AdoMet, S-adenosyl-L-methionine.