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## Modulation of EAAC1-Mediated Glutamate Uptake by Addicisin

Mitsushi J. Ikemoto and Taku Arano

*Biomedical Research Institute*

*National Institute of Advanced Industrial Science and Technology (AIST)*

*Graduate School of Science, Toho University*

*Japan*

### 1. Introduction

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS). In addition to functioning as a neurotransmitter at the majority of brain synapses, it is the substrate for synthesis of the major inhibitory transmitter  $\gamma$ -aminobutyric acid (GABA). However, glutamate is also a neurotoxin, and a number of molecular control mechanisms are responsible for maintaining extracellular glutamate below excitotoxic levels.  $\text{Na}^+$ -dependent excitatory amino acid transporters (EAATs) are crucial regulators of extracellular glutamate and also act to control the dynamics of excitatory transmission in the CNS (Danbolt, 2001). The  $\text{Na}^+$ -dependent excitatory amino acid carrier 1 (EAAC1) is expressed in the somata and dendrites of many neuronal types, including pyramidal cells of the hippocampal formation and cortex, and many subtypes of GABAergic inhibitory neurons (Rothstein et al., 1994). The physiological significance of EAAC1 is unclear because the subcellular distribution and kinetic properties of this transporter would not allow for a substantial contribution to glutamate clearance from the synaptic cleft; rather, these functions are mediated by glial EAATs (EAAT1 and EAAT2) located in the perisynaptic region. Recent studies have demonstrated multiple functions for EAAC1 distinct from clearance of glutamate from CNS synapses (Kiryu-Seo et al., 2006; Levenson et al., 2002; Peghini et al., 1997; Sepkuty et al., 2002). For example, decreased EAAC1 expression in the CNS impairs neuronal glutathione (GSH) synthesis, leading to oxidative stress and age-dependent neurodegeneration (Aoyama et al., 2006), suggesting that aberrant EAAC1 expression contributes to the pathogenesis of neurodegenerative diseases.

Studies conducted over the past decade on the kinetics of EAAC1 and regulation of transporter expression and function have lead to a greater appreciation of the physiological and pathophysiological relevance of EAAC1 (Aoyama et al., 2008b; Danbolt, 2001; Kanai & Hediger, 2004; Nieoullon et al., 2006), but there are many issues to be resolved for a thorough understanding of the significance of EAAC1 in normal brain function and disease. In particular, the regulatory mechanisms of EAAC1-mediated glutamate uptake are largely unknown. The recent discovery of addicisin (glutamate transporter-associated protein 3-18, GTRAP3-18) as an EAAC1 binding protein has contributed greatly to our understanding of the regulatory mechanisms of EAAC1 activity (Lin et al., 2001). Furthermore, we recently

proposed a regulatory model of EAAC1-mediated glutamate uptake by adducin complexes (Akiduki & Ikemoto, 2008). In this chapter, we describe the regulation of EAAC1-mediated glutamate uptake based on our recent results. To better understand this regulatory mechanism, we first explain three key molecules involved in this regulatory pathway—EAAC1, adducin, and ADP-ribosylation factor-like 6 interacting protein 1 (Arl6ip1).

### 1.1 EAAC1

The EAAC1 protein was first identified as a Na<sup>+</sup>-dependent high-affinity glutamate transporter by expression cloning in *Xenopus* oocytes (Kanai & Hediger, 1992). Stoichiometric analysis demonstrates that EAAC1 transports L-glutamate, L-aspartate, and D-aspartate, accompanied by the cotransport of 3 Na<sup>+</sup> and 1 H<sup>+</sup>, and the countertransport of 1 K<sup>+</sup> (Kanai & Hediger, 2003). In mammalian tissues, there are five different subtypes of EAATs—EAAT1 (glutamate/aspartate transporter, GLAST), EAAT2 (glutamate transporter 1, GLT-1), EAAT3 (EAAC1), EAAT4, and EAAT5 (Danbolt, 2001). These EAATs are structurally similar; all have eight transmembrane domains and a pore loop between the seventh and eighth domain. Most EAATs play an important role in removing extracellular glutamate from the synaptic and extrasynaptic space (Kanai & Hediger, 2003), particularly GLAST and GLT-1. These two isoforms are primarily expressed in glial cells and play a major role in protecting neurons from glutamate-induced toxicity (Rothstein et al., 1994) as well as terminating glutamatergic transmission (Rothstein et al., 1993; Tong & Jahr, 1994). In contrast, EAAC1 is diffusely localized to the cell bodies and dendrites of neurons and is enriched in cortical and hippocampal pyramidal cells as well as in some inhibitory neurons (Conti et al., 1998; Rothstein et al., 1994). This subcellular localization and restricted distribution indicate that EAAC1 does not play a major role in glutamate clearance from the synaptic cleft (Rothstein et al., 1996). Recent studies suggest that EAAC1 contributes to multiple physiological functions distinct from glutamate clearance. Indeed, EAAC1 transport provides cysteine as a substrate of GSH synthesis (Y. Chen & Swanson, 2003; Himi et al., 2003; Watabe et al., 2008; Zerangue & Kavanaugh, 1996). Neurons cannot transport extracellular GSH and therefore must transport cysteine from the extracellular space for *de novo* GSH synthesis from cysteine (Aoyama et al., 2008b). In the CNS, the depletion of GSH is associated with neurodegenerative disorders, including Alzheimer's and Parkinson's diseases (Ramassamy et al., 2000; Sian et al., 1994). Consistent with these results, EAAC1 knockout mice show oxidative stress in neurons and age-dependent neurodegeneration, pathologies that are rescued by *N*-acetylcysteine, a membrane-permeable cysteine precursor (Aoyama et al., 2006). These mice also show alteration of zinc homeostasis and increased neural damage after transient cerebral ischemia (Won et al., 2010). Furthermore, in a knockin mouse model of Huntington's disease, in which human *huntingtin* exon 1 with 140 CAG repeats was inserted into the wild-type low CGA repeat mouse *huntingtin* gene, oxidative stress and cell death were caused by abnormal Rab11-dependent EAAC1 trafficking to the cell surface (X. Li et al., 2010). In addition, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice, an animal model of Parkinson's disease, show reduced EAAC1-mediated neuronal cysteine uptake, impaired GSH synthesis, and motor dysfunction (Aoyama et al., 2008a). These results indicate that dysfunctional EAAC1-mediated cysteine transport increases neural vulnerability to oxidative stress and could contribute to the pathogenesis of neurodegenerative diseases.

In addition to cysteine transport, EAAC1 has several other functions unrelated to removal of extracellular glutamate. For instance, EAAC1 promotes GABA synthesis by supplying the substrate glutamate (Mathews & Diamond, 2003; Sepkuty et al., 2002). Therefore, EAAC1 can strengthen inhibitory synapses in response to elevations in extracellular glutamate and contribute indirectly to GABA release (Mathews & Diamond, 2003). Indeed, a loss of EAAC1 function leads to epilepsy (Sepkuty et al., 2002), underscoring the importance of EAAC1 in GABAergic transmission. Furthermore, EAAC1 plays a crucial role in preventing neuronal death by suppressing glutamate excitotoxicity (Kiryu et al., 1995; Murphy et al., 1989) and has a mitochondria-mediated anti-apoptotic function in injured motor neurons (Kiryu-Seo et al., 2006). These studies and those discussed in Section 3.4 strongly suggest that EAAC1 contributes to multiple functions in the CNS distinct from glutamate clearance.

The regulatory mechanisms of EAAC1 have been widely investigated *in vitro*. Cumulative evidence demonstrates that glutamate uptake by EAAC1 is facilitated by cell signaling molecules and accessory proteins that promote the redistribution of EAAC1 from the endoplasmic reticulum (ER) to the plasma membrane. First, several reports demonstrate that several kinase signaling cascades regulate EAAC1 activity. In C6BU-1 glioma cells and primary neuronal cultures, phorbol 12-myristate 13-acetate (PMA), a protein kinase C (PKC) activator, rapidly increases EAAC1-mediated glutamate uptake (Dowd & Robinson, 1996). This effect is regulated by mechanisms that are independent of *de novo* synthesis of new transporters but is related to the redistribution of EAAC1 from subcellular compartments to the plasma membrane (Davis et al., 1998; Fournier et al., 2004; Sims et al., 2000). Pharmacological analyses demonstrate that PKC $\alpha$  regulates EAAC1 translocation from intracellular compartments to the cell surface, and that PKC $\epsilon$  increases EAAC1 functional activity (Gonzalez et al., 2002). PKC $\alpha$  interacts with EAAC1 in a PKC-dependent manner and phosphorylates EAAC1 (Gonzalez et al., 2003). Platelet-derived growth factor (PDGF) increases the delivery of EAAC1 to the cell surface through phosphatidylinositol 3-kinase (PI3K) activity (Fournier et al., 2004; Sheldon et al., 2006; Sims et al., 2000). Consistent with this result, wortmannin, a PI3K inhibitor, decreases cell surface expression of EAAC1 and inhibits EAAC1-mediated glutamate uptake (Davis et al., 1998). In addition, PKC and PDGF have different effects on trafficking and internalization of EAAC1; PMA, but not PDGF, reduces internalization of EAAC1 (Fournier et al., 2004). Thus, EAAC1 trafficking is regulated by two independent signaling pathways. In contrast, PKC negatively regulates EAAC1-mediated glutamate uptake in *Xenopus* oocytes (Trotti et al., 2001) and in Madin-Darby canine kidney (MDCK) cells (Padovano et al., 2009) by inhibiting cell surface expression through calcineurin-mediated internalization (Padovano et al., 2009; Trotti et al., 2001), suggesting that the regulatory mechanisms of EAAC1 surface expression and function by PKC are specific to cell type and depend on specific PKC isozymes. Second, accessory proteins regulate EAAC1 activity. For instance,  $\delta$  opioid receptor interacts with EAAC1 and inhibits EAAC1-mediated glutamate uptake in *Xenopus* oocytes and rat hippocampal neurons (Xia et al., 2006). In addition, *N*-methyl-*D*-aspartate receptors containing NR1, NR2A, and/or NR2B interact with EAAC1 and facilitate the cell surface expression of EAAC1 in C6BU-1 cells and rat hippocampal neurons (Waxman et al., 2007). Moreover, the cell surface expression of EAAC1 is controlled by interactions with Na<sup>+</sup>/H<sup>+</sup>-exchanger regulatory factor 3 (NHERF-3, also called PDZK1) and adaptor protein 2 (AP-2). While NHERF-3 promotes the delivery of EAAC1 to the plasma membrane, AP-2 regulates constitutive endocytosis of EAAC1 in MDCK cells (D'Amico et al., 2010). Furthermore,

reticulum 2B (RTN2B) interacts with EAAC1 and addicsin/GTRAP3-18, and promotes intracellular trafficking of EAAC1 in HEK293 cells and cultured cortical neurons (Liu et al., 2008). Addicsin/GTRAP3-18 interacts with EAAC1 and inhibits EAAC1 trafficking in HEK293 cells (Ruggiero et al., 2008). Thus, multiple regulatory mechanisms control EAAC1 trafficking and membrane expression, but the molecular details are generally unclear. In this study, we focus on the regulation of EAAC1 trafficking by addicsin.

## 1.2 Addicsin

In many papers, human addicsin and rat addicsin are called JWA and GTRAP3-18, respectively. Addicsin, GTRAP3-18, and JWA have been independently identified by several research groups (Ikemoto et al., 2002; Lin et al., 2001; Zhou et al., GeneBank, AF070523, unpublished observations). We first identified *addicsin* as a novel mRNA encoding a 22-kDa hydrophobic protein that is highly expressed in the basomedial nucleus of the mouse amygdala following repeated morphine administration (Ikemoto et al., 2002). Meanwhile, *GTRAP3-18* cDNA was identified as encoding an EAAC1 binding protein by yeast two-hybrid screening of a rat brain cDNA library using the C-terminal intracellular domain of EAAC1 as bait (Lin et al., 2001). The *JWA* gene was identified as an all-*trans* retinoic acid (RA)-responsive factor from human tracheobronchial epithelial cells (Zhou et al., GeneBank, AF070523, unpublished observations). Bioinformatic analysis demonstrates that JWA has a prenylated Rab acceptor 1 (PRA1) domain and 62% similarity with Jena-Muenchen 4 (JM4), a protein recently identified as PRA1 domain family member 2 (PRAF2) (Schweneker et al., 2005). Proteins containing a large PRA1 domain form a new family of PRA1 domain family proteins (PRAFs) that regulate intracellular protein trafficking. Thus, addicsin is a new member of the PRAF family, PRAF3.

The *addicsin* cDNA is approximately 1.4 kbp and consists of a 564-bp single open reading frame (Ikemoto et al., 2002). The *addicsin* gene contains three exons separated by two introns, and the sequence is highly conserved among vertebrates (Butchbach et al., 2002). Furthermore, *addicsin* is located on mouse chromosome 6, a location corresponding to human chromosome 3p (Butchbach et al., 2002; Ikemoto et al., 2002).

Mouse addicsin is a 22-kDa protein of 188 amino acids with putative transmembrane segments (Butchbach et al., 2002; Ikemoto et al., 2002). Mouse addicsin is 98% identical to rat GTRAP3-18 and 95% similar to human JWA (Butchbach et al., 2002; Ikemoto et al., 2002). Moreover, addicsin has two putative PKC phosphorylation motifs (amino acids 18–20 and 138–140) as well as two putative cAMP-dependent protein kinase and calcium/calmodulin-dependent protein kinase II phosphorylation motifs (amino acids 27–31 and 35–39) (Butchbach et al., 2002; Ikemoto et al., 2002) (Fig. 1). However, there is no evidence that these phosphorylation sites are phosphorylated by protein kinases *in vitro* and *in vivo*.

Expression profiles of addicsin and *addicsin* mRNA were investigated in the developing and mature brain. In the developing rat brain, the expression levels of addicsin decrease significantly from embryonic day 17 to post-natal day 0 (Maier et al., 2009). Meanwhile, *addicsin* mRNA levels increase gradually during early maturation, peaking around post-natal day 5, and then declining by about 50% by post-natal day 14 (Inoue et al., 2005). This developmental expression pattern corresponds to periods of elevated synaptogenesis,



suggesting that addicisin is involved in synapse formation. Indeed, later in this chapter, we discuss evidence that addicisin participates in intracellular protein trafficking of neurotransmitter receptors. Addicisin is widely distributed in the brain (Akiduki et al., 2007; Butchbach et al., 2002). In the mature CNS, addicisin is expressed in the cerebral cortex, amygdala, striatum, hippocampus (CA1–3 fields), dentate gyrus, and cerebellum. Addicisin is expressed in the somata of glutamatergic and GABAergic neurons and exhibits presynaptic localization in restricted regions such as CA3 stratum lucidum (Akiduki et al., 2007). *In situ* hybridization analysis reveals that *addicisin* mRNA is widely distributed in the brain, predominantly expressed in principal neurons, including glutamatergic and GABAergic neurons in the mature CNS (Inoue et al., 2005). However, the precise subcellular localization of addicisin remains controversial. Recent reports found that addicisin is an integral ER membrane protein that prevents EAAC1 maturation and function by inhibiting ER trafficking (Ruggiero et al., 2008). However, our protein fractionation analysis using mouse whole brain lysates prepared in PBS, NaCl, or Na<sub>2</sub>CO<sub>3</sub> buffer, all indicate that addicisin is predominantly present in the S1 soluble fraction, while the ER transmembrane protein calnexin is present in the P2 pellet fraction (Ikemoto et al., 2002). Our subcellular fractionation analysis with highly purified synaptic fractions prepared from mouse forebrain also support the notion that addicisin is present in the cytoplasmic and presynaptic membrane fractions (Akiduki et al., 2007). Furthermore, immunocytochemical studies reveal that addicisin is present in both the plasma membrane and the intracellular compartments, including the ER (Ikemoto et al., 2002; Watabe et al., 2007, 2008). Consistent with these findings, bioinformatic analysis demonstrates that the  $\alpha$ -helix is not long enough for a transmembrane domain; nevertheless, addicisin is predicted to be a hydrophobic protein composed of 62%  $\alpha$ -helix and 8%  $\beta$ -sheet (Butchbach et al., 2002), suggesting that it is membrane-associated. Further investigations are needed to clarify the subcellular localization of addicisin, but it is apparent that this protein can exist in both soluble and membrane-associated forms.

Addicisin easily forms homo- and heteromultimers (Ikemoto et al., 2002; Lin et al., 2001) and many reports demonstrate that addicisin can associate with a multitude of proteins (Akiduki & Ikemoto, 2008), including Arl6ip1 (Akiduki & Ikemoto, 2008), ARL6 (Ingley et al., 1999),  $\delta$  opioid receptor (Wu et al., 2011), EAAC1 (Lin et al., 2001), Rab1 (Maier et al., 2009), and RTN2B (Liu et al., 2008). Moreover, recent studies using the yeast two-hybrid system revealed many potential addicisin-binding proteins (M.J. Ikemoto et al., unpublished data), strongly suggesting that addicisin exerts multiple physiological functions by forming various molecular complexes. It is vital to catalog these interacting proteins and to determine the presence and location of these molecular complexes.

These potential functions remain largely speculative, but molecular studies have provided several intriguing candidates (Fig. 2). First, addicisin is involved in apoptosis induced by 12-*O*-tetradecanoylphorbol-13-acetate, all-*trans* RA, *N*-(4-hydroxyphenyl) retinamide, arsenic trioxide, and cadmium (Mao et al., 2006; Zhou et al., 2008). Knockdown of addicisin attenuates all-*trans* RA-induced and arsenic trioxide-induced apoptosis (Mao et al., 2006; Zhou et al., 2008). Therefore, addicisin serves as a pro-apoptotic molecule. Second, addicisin acts as an environmental stress sensor to protect cells from oxidative stress and subsequent genomic damage. Addicisin is also involved in cellular responses to environmental stresses, including oxidative stress and heat shock, and in the differentiation of leukemia cells under

nonphysiological conditions (Cao et al., 2007; Huang et al., 2006a, 2006b; T. Zhu et al., 2005). Addicisin is upregulated after exposure to the pro-oxidants benzo[ $\alpha$ ]pyrene and hydrogen peroxide through activation of the nuclear transcription factor I (NFI) (R. Chen et al., 2007). Addicisin facilitates DNA repair by interacting with X-ray cross-complementing group 1 protein, a regulator of the DNA base excision repair processes that translocates to the nucleus in response to oxidative stress (R. Chen et al., 2007; Wang et al., 2009). Thus, NFI-mediated addicisin upregulation protects against DNA damage induced by benzo[ $\alpha$ ]pyrene and hydrogen peroxide. Third, addicisin also inhibits cancer cell migration as was observed in HeLa, B16, and HCCLM3 cancer cells. (H. Chen et al., 2007). Addicisin has an important role in maintaining the stability of F-actin and in the initiation of actin cytoskeletal rearrangements. Moreover, knockdown of addicisin results in the inactivation of the MEK-ERK signaling cascade. Thus, addicisin inhibits cell migration by activating the mitogen-activated protein kinase (MAPK) cascade and regulating the rearrangement of the F-actin cytoskeleton (H. Chen et al., 2007). Fourth, addicisin participates in the regulation of GSH synthesis; the association of addicisin with EAAC1 at the plasma membrane inhibits the uptake of cysteine for GSH synthesis and thus determines the intracellular GSH content *in vitro* and *in vivo* (Watabe et al., 2007, 2008). This suggests that addicisin is a therapeutic target for enhancing GSH levels in patients with neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases, associated with oxidative stress. Fifth, addicisin significantly inhibits neurite growth in differentiated CAD cells by inactivating Rab1, a positive regulator of ER-to-Golgi trafficking (Maier et al., 2009). Finally, addicisin participates in the regulation of EAAC1-mediated glutamate uptake (Akiduki & Ikemoto, 2008) and ER protein trafficking (Liu et al., 2008; Ruggiero et al., 2008). We discuss these latter two physiological functions in detail (Section 2).

### 1.3 Arl6ip1

The "ADP-ribosylation factor-like 6 interacting protein 1 (Arl6ip1)" is the new name assigned to three independently described factors: the original Arl6ip, apoptotic regulator in the membrane of the ER (ARMER), and protein KIAA0069. The Arl6ip1 protein was first identified by yeast two-hybrid screening using mouse ARL6 as bait (Ingley et al., 1999) and as a negative regulatory factor during myeloid differentiation by differential display (Pettersson et al., 2000). Moreover, a novel protein, designated ARMER, initially discovered as a false-positive clone by yeast two-hybrid screening using Bcl-xL as bait, is also Arl6ip1 (Lui et al., 2003). In addition, Arl6ip1 has more than 96% homology with the human protein KIAA0069, the product of a cDNA isolated from the human myeloblast cell line KG-1 during a systematic effort to characterize complete cDNAs (Nomura et al., 1994). Amino acid analysis of Arl6ip1 demonstrates that it is composed of 203 amino acids and encodes a 23-kDa protein with four putative transmembrane segments (Pettersson et al., 2000). Several studies indicate that Arl6ip1 is an integral membrane protein localized to the ER (Lui et al., 2003; Pettersson et al., 2000). Furthermore, computational analysis of the topology of Arl6ip1 demonstrates that the N- and C-terminal ends are both exposed to the cytoplasm (Lui et al., 2003). Consistent with these results, Arl6ip1 has two putative casein kinase II phosphorylation motifs (amino acids 18–21 and 128–131), three putative PKC phosphorylation motifs (amino acids 94–96, 115–117, and 128–130), a *N*-glycosylation motif (amino acids 6–9), a prenyl group-binding motif (amino acids 72–75), and an ER retention signal in the C-terminal cytoplasmic region (amino acids 200–203) (Akiduki &

Ikemoto, 2008; Lui et al., 2003) (Fig. 1). Thus, Arl6ip1 function may be controlled by diverse intracellular cell signals, but it is unknown whether these motifs are physiologically functional.

The functions of Arl6ip1 remain largely unknown, but culture studies have provided several intriguing possibilities. For example, Arl6ip1 protects HT1080 fibrosarcoma cells from apoptosis induced by serum starvation, doxorubicin, UV irradiation, tumor necrosis factor  $\alpha$ , and ER stressors by inhibiting caspase-9 activity (Lui et al., 2003). In addition, Arl6ip1 suppresses cisplatin-induced apoptosis in CaSki human cervical cancer cells by regulating the expression of apoptosis-related proteins caspase-3, caspase-9, p53, NF- $\kappa$ B, MAPK, Bcl-2, Bcl-xL, and Bax (Guo et al., 2010a). Furthermore, Arl6ip1 is involved in cell growth, cell cycle progression, and invasion of cancer cells. Downregulation of Arl6ip1 suppresses cell proliferation and colony formation, arrests cell cycling at the G0/G1 phase, and inhibits migration of CaSki human cervical cancer cells (Guo et al., 2010b). Most relevant to the present discussion, Arl6ip1 is involved in the regulation of EAAC1. Recently, we demonstrated that Arl6ip1 is a novel addicisin-associating factor that indirectly promotes PKC-dependent EAAC1-mediated glutamate uptake by decreasing the number of addicisin molecules available for suppression of EAAC1 (Akiduki & Ikemoto, 2008).

## 2. Regulation of EAAC1 function by addicisin

The mechanisms by which addicisin regulates EAAC1 activity have not been definitively established. However, the discovery of addicisin/GTRAP3-18 has contributed greatly to our understanding of EAAC1 function. Recent evidence demonstrates two major mechanisms of addicisin-mediated regulation of EAAC1 activity. One regulatory pathway is dependent on the dynamic competition for free addicisin molecules by other addicisin molecules to form the homocomplex and by Arl6ip1 to form a heterocomplex. This addicisin-Arl6ip1 complex sequesters addicisin molecules and blocks the interaction of addicisin with EAAC1 in the plasma membrane, thereby reducing the inhibitory effect of addicisin on EAAC1-mediated glutamate uptake (Akiduki & Ikemoto, 2008; Lin et al., 2001) (Fig. 3). Second, addicisin functions as a negative regulator of EAAC1 trafficking through the ER and inhibits the cell surface expression of EAAC1 (Liu et al., 2008; Ruggiero et al., 2008). In this section, we discuss these two mechanisms in detail.

### 2.1 Modulation of EAAC1-mediated glutamate uptake by addicisin

As an introduction to addicisin/GTRAP3-18-mediated regulation of EAAC1 activity, we discuss two early papers in detail. Lin et al. demonstrated that addicisin/GTRAP3-18 binds to EAAC1 and inhibits EAAC1-mediated glutamate uptake by this direct interaction (Lin et al., 2001). The second is our study showing that addicisin inhibits EAAC1-mediated glutamate uptake in a PKC activity-dependent manner while Arl6ip1 promotes glutamate uptake (also in a PKC activity-dependent manner) by inhibiting the interaction of addicisin with EAAC1 (Akiduki & Ikemoto, 2008). Lin et al. first identified addicisin/GTRAP3-18 as an EAAC1-interacting protein by yeast two-hybrid screening of a rat brain cDNA library. To evaluate whether addicisin/GTRAP3-18 modulates EAAC1 function, they examined the effect of increasing addicisin/GTRAP3-18 expression on EAAC1-mediated glutamate uptake *in vitro* and *in vivo*. First, they showed that glutamate uptake decreased progressively with



increasing expression of addicsin/GTRAP3-18 in HEK293 cells. Subsequent kinetic analyses in HEK293, C6BU-1, and COS7 cells revealed that elevated expression of addicsin decreased the glutamate affinity of EAAC1 without altering the maximal transport velocity (correlated with expression). Furthermore, HEK293 cells coexpressing addicsin/GTRAP3-18 and a truncated EAAC1 missing the addicsin/GTRAP3-18 association region showed higher glutamate uptake than cells expressing wild-type EAAC1. In addition, this truncated EAAC1 had a higher affinity for glutamate, suggesting that addicsin/GTRAP3-18 normally reduces EAAC1-mediated glutamate uptake by binding to this association region and reducing transporter glutamate affinity. Next, they evaluated the effect of intraventricular injection of an addicsin/GTRAP3-18 antisense mRNA on EAAC1-mediated glutamate uptake *in vivo*. The antisense treatment resulted in reduced addicsin/GTRAP3-18 expression, a significant increase in cortical EAAC1-mediated glutamate uptake, and an increase in glutamate affinity compared to saline-treated or sense mRNA-treated control animals. In conclusion, addicsin/GTRAP3-18 can negatively modulate EAAC1-mediated glutamate uptake by a direct interaction with EAAC1.

We first isolated addicsin as a novel protein richly expressed in the amygdala of mice under chronic morphine treatment. Addicsin has a tendency to form the multimeric complex *in vitro* (Ikemoto et al., 2002; Lin et al., 2001). The initial discovery of addicsin prompted us to perform yeast two-hybrid screening of an amygdala cDNA library constructed from chronic morphine-administered mice. From this screen, we identified Arl6ip1 as a candidate addicsin-interacting protein. As described in section 1.3, Arl6ip1 is an anti-apoptotic protein located in the ER. As previously described, addicsin inhibits EAAC1-mediated glutamate uptake by direct association at the plasma membrane (Lin et al., 2001), so we speculated that Arl6ip1 upregulates EAAC1-mediated glutamate transport by inhibiting the interaction between addicsin and EAAC1 (Fig. 3).

As a first step to verify this hypothesis, we investigated whether addicsin could bind Arl6ip1 *in vitro* and *in vivo*. To eliminate the possibility of false-positive clones, reconfirmation tests using a full length mouse Arl6ip1 as prey or bait were performed. This tests revealed the specific interaction with addicsin in the yeast AH109 strain. We next examined the reproducibility of this screening result by yeast two-hybrid screening using a different cDNA library prepared from whole brains of 7-week-old mice. We obtained 20 positive clones that clearly displayed  $\alpha$ -galactosidase activity (the gene driven by the protein-protein interaction in the two-hybrid screen). Among these positive clones, 11 were identical to *Arl6ip1* cDNA (M.J. Ikemoto et al., unpublished data), confirming the interaction with addicsin and Arl6ip1 in the yeast AH109 strain. We then performed immunoprecipitation analysis, glycerol gradient analysis, and immunocytochemical analysis to directly test the interaction between Arl6ip1 and addicsin *in vitro*. For this purpose, we prepared cell lysates from NG108-15 cells expressing FLAG-tagged Arl6ip1 (Arl6ip1-FLAG), Myc-tagged addicsin (addicsin-myc), or both. Immunoprecipitation analysis of these cell lysates demonstrated that Arl6ip1-FLAG specifically interacted with addicsin-myc in the cell lysates prepared from coexpressing cells, but not from cells expressing Arl6ip1-FLAG or addicsin-myc alone. Glycerol gradient analysis revealed that the elution profile of Arl6ip1-FLAG was similar to that of addicsin-myc. The elution peaks of both proteins were observed in the fraction with a deduced molecular mass of 24 kDa. Moreover, the elution peak of the addicsin homodimer was present in the 44-kDa fraction,

suggesting that addicisin forms Arl6ip1–addicisin heterodimers and addicisin–addicisin homodimers *in vitro*. Immunocytochemical analysis in NG108-15 cells overexpressing Arl6ip1-FLAG and addicisin-myc demonstrated subcellular colocalization (M.J. Ikemoto et al., unpublished data). To examine the interaction of both proteins *in vivo*, we performed *in vivo* immunoprecipitation assays of whole brain lysates using an anti-Arl6ip1 polyclonal antibody (generated from a synthetic peptide spanning amino acids 185–199 of mouse Arl6ip1) that again revealed a specific interaction between Arl6ip1 and addicisin. Western blot analysis demonstrated that Arl6ip1 was widely expressed in the mature brain and showed substantial regional overlap with addicisin. In addition, immunohistochemical staining confirmed that Arl6ip1 was widely expressed in the mature brain and localized in neuron-like cells. The neural expression pattern of Arl6ip1 was the same as addicisin, suggesting that Arl6ip1 is colocalized with addicisin in the mature CNS. We concluded that addicisin specifically interacted with Arl6ip1 *in vitro* and *in vivo*.

As a second step, we then determined the Arl6ip1- and addicisin-binding regions on addicisin. If Arl6ip1 does regulate EAAC1 activity by competitively binding to addicisin molecules and thus preventing the formation of addicisin homodimers that downregulate EAAC1 activity, the Arl6ip1- and addicisin-binding regions on addicisin should be located close enough for such a competitive interaction. Immunoprecipitation assays using several addicisin truncation mutants indicated that Arl6ip1 associated with full length addicisin (wt), a truncation lacking the C-terminal region at amino acids 145–188 (d1), a deletion mutant of the N-terminal domain at amino acids 1–102 (d2), and a mutant missing the region containing the C-terminal phosphorylation motif at amino acids 136–144 (d3). However, Arl6ip1 could not interact with a mutant lacking a portion of the hydrophobic region at amino acids 103–117 (d4). As expected, addicisin was able to associate with the wt, d1, d2, or d3 mutant, but not the d4 truncation mutant, indicating that the hydrophobic region at amino acids 103–117 of addicisin is a crucial domain for the formation of addicisin–addicisin homodimers and addicisin-Arl6ip1 heterodimers (Fig. 1). These results strongly support our hypothesis that Arl6ip1 antagonizes addicisin-mediated downregulation of EAAC1 activity by sequestering free addicisin.

As a third step, we investigated whether Arl6ip1 had a positive effect on EAAC1-mediated glutamate uptake. For this purpose, we selected C6BU-1 glioma cells that expressed EAAC1 as the principal or only EAAT (Palos et al., 1996). We created two stably expressing C6BU-1 cell lines, designated C6BU-1-pSw-addicisin and C6BU-1-pSw-Arl6ip1. In these cell lines, we could strictly control the expression levels of V5-tagged addicisin (addicisin-V5) or V5-tagged Arl6ip1 (Arl6ip1-V5) by exposure to 10 nM mifepristone (11 $\beta$ -[4-dimethylamino]phenyl-17 $\beta$ -hydroxy-17-[1-propynyl]estra-4,9-dien-3-one), a synthetic 19-norsteroid. In addition, a cell viability assay demonstrated that upregulation of Arl6ip1-V5 or addicisin-V5 by exposure to 10 nM mifepristone was not cytotoxic, making these cell lines excellent models to evaluate the effects of changing Arl6ip1 and addicisin expression on the functional activity of EAAC1. Compared to control cells untreated with mifepristone or the PKC agonist PMA, the upregulation of Arl6ip1-V5 or addicisin-V5 by 10 nM mifepristone alone did not change EAAC1-mediated glutamate uptake. When these cells were stimulated with 100 nM PMA alone, the glutamate uptake activity in C6BU-1-pSw-addicisin cells and C6BU-1-pSw-Arl6ip1 cells increased about two-fold compared to untreated controls. EAAC1-mediated glutamate uptake was significantly lower in C6BU-1-pSw-addicisin cells stimulated with both

mifepristone and PMA compared to C6BU-1-pSw-addicsin cells treated with PMA alone, indicating that activation of addicsin expression inhibited PKC-dependent EAAC1 activity. In contrast, C6BU-1-pSw-Arl6ip1 cells treated with PMA and mifepristone exhibited a three-fold increase in glutamate uptake compared to the same line treated with PMA alone, indicating that Arl6ip1 overexpression enhanced PKC-dependent EAAC1 activity. On the other hand, the nonstimulating PMA analog 4 $\alpha$  phorbol did not increase glutamate uptake relative to controls.

To further support these conclusions, we performed a knockdown experiment by transient transfection of double-stranded siRNAs into C6BU-1-pSw-Arl6ip1 cells to investigate the effect of decreased addicsin expression on EAAC1-mediated glutamate uptake. As expected, cells transfected with either of two alternative addicsin siRNAs showed about a two-fold increase in glutamate uptake in response to PMA exposure compared to cells treated with control scrambled siRNA. The elevated glutamate uptake concomitant with addicsin knockdown strongly supported the proposed mechanism for EAAC1 regulation by addicsin and Arl6ip1.

To investigate the molecular mechanisms for altered EAAC1-mediated glutamate uptake in C6BU-1-pSw-Arl6ip1 cells, we performed kinetic analysis of glutamate flux across C6BU-1-pSw-Arl6ip1 cell membranes. When Arl6ip1 was conditionally overexpressed using mifepristone, PMA treatment increased the glutamate affinity but not the maximal velocity compared to vehicle-treated controls (PMA:  $K_m = 647 \mu\text{M}$ ,  $V_{max} = 1.5 \times 10^3 \text{ pmole/mg/min}$ ; vehicle:  $K_m = 824 \mu\text{M}$ ,  $V_{max} = 1.5 \times 10^3 \text{ pmole/mg/min}$ ) with no change of addicsin expression levels. Thus, Arl6ip1 promoted EAAC1-mediated glutamate uptake by increasing the catalytic efficacy of EAAC1. Specifically, Arl6ip1 blocked the addicsin-mediated reduction in EAAC1 glutamate affinity.

As a fourth step, we then examined the subcellular localization of Arl6ip1 in C6BU-1-pSw-Arl6ip1 cells. Western blot analysis revealed that Arl6ip1-V5 expression levels were unaffected by 100 nM PMA exposure. Immunocytochemical analysis demonstrated that Arl6ip1-V5 was predominantly localized to cytoplasmic structures such as the ER and that this subcellular expression pattern was not changed by PMA. Furthermore, cell biotinylation analysis indicated that Arl6ip1 did not interact with the plasma membrane, consistent with our previous result that Arl6ip1 failed to interact with EAAC1 by immunoprecipitation. Therefore, Arl6ip1 was localized to the ER under all conditions tested and acted to “trap” addicsin molecules in Arl6ip1-addicsin heterodimers, thus preventing the direct interaction of addicsin with EAAC1. To confirm our hypothesis, we produced an addicsin mutant that lacked interaction with Arl6ip1 but not with other addicsin molecules. Fine mutational analysis was used to separate the Arl6ip1- and addicsin-binding regions within the addicsin d4 region. We compared addicsin sequences among various species and noted that two amino acids at positions 110 and 112 of mouse addicsin were completely conserved from fruit fly to human. We created a double-mutated form of addicsin that substituted both the native tyrosine at amino acid 110 and the leucine at amino acid 112 with alanine. The mutant, designated addicsin Y110A/L112A (or addicsinYL), showed markedly less binding to Arl6ip1 (40% of wild-type addicsin) but normal wild-type binding to addicsin, as revealed by immunoprecipitation. In addition, a cell biotinylation assay indicated that addicsinYL was unable to localize to the plasma membrane, suggesting that addicsinYL lost EAAC1-binding activity. To evaluate the effect of addicsinYL on EAAC1-mediated

glutamate uptake, we created a conditional C6BU-1 cell line, designated C6BU-1-pSw-addicisinYL. This cell line exhibited mifepristone-dependent upregulation of V5-tagged addicisinYL and increased glutamate uptake in response to PMA that was unchanged by mifepristone-induced upregulation of addicisinYL. That is, glutamate uptake was not reduced by induced addicisinYL expression. These data strongly suggest that addicisin is a key negative regulator of EAAC1 in the plasma membrane and that Arl6ip1 is a negative regulator of addicisin.

As a final step, we examined the effect of addicisin PKC phosphorylation sites on EAAC1-mediated glutamate uptake in C6BU-1 cells. Addicisin has putative PKC phosphorylation motifs at amino acids 18-20 and 138-140, and PKC activation increases EAAC1-mediated glutamate uptake. We established conditional C6BU-1 cell lines, designated C6BU-1-pSw-addicisinS18A and C6BU-1-pSw-addicisinS138A. C6BU-1-pSw-addicisinS18A cells expressed a V5-tagged addicisin point mutant that substituted native serine 18 for alanine in the N-terminal motif in response to mifepristone, while C6BU-1-pSw-addicisinS138A cells expressed a V5-tagged addicisin point mutant that substituted native serine 138 for alanine in the C-terminal motif. These cells showed no cytotoxicity in response to 10 nM mifepristone. In contrast to cells expressing wild-type addicisin, expression of addicisinS18A did not suppress the PMA-induced increase in EAAC1-mediated glutamate uptake. Moreover, increased expression of addicisinS18A caused a significant increase in glutamate uptake even without PMA stimulation by a dominant negative effect. Similarly, addicisinS138A expression did not suppress the PMA-induced increase in EAAC1-mediated glutamate uptake. Thus, these mutations abolished the inhibitory effect of addicisin. However, in contrast to addicisinS18A, addicisinS138A expression had no influence on EAAC1-mediated glutamate uptake activity in the absence of PMA stimulation. Both serine 18 and serine 138 within the putative PKC phosphorylation motifs are critical for the negative regulation of EAAC1-mediated glutamate uptake and suggest that the PKC phosphorylation site at serine 138 is functional under physiological conditions.

Based on these data, we proposed the regulatory model of EAAC1-mediated glutamate uptake illustrated in Fig. 3. If addicisin expression is high enough relative to Arl6ip1 to form many more addicisin homodimers than addicisin-Arl6ip1 heterodimers, EAAC1-mediated glutamate uptake is reduced. Furthermore, activation of the PKC isozyme that phosphorylates addicisin at S18 or S138 may further potentiate this negative regulation. On the other hand, if addicisin expression is low enough or Arl6ip1 expression high enough that formation of heterodimers predominates, fewer addicisin homodimers are available to suppress EAAC1 activity. The resulting decrease in addicisin-EAAC1 binding will enhance the catalytic efficacy of EAAC1, in a PKC-activity dependent manner. In sum, Arl6ip1 acts as a positive regulator of EAAC1-mediated glutamate uptake (Fig. 3) and may therefore possess significant neuroprotective efficacy against neurodegenerative diseases linked to excitotoxicity and oxidative stress.

## 2.2 Modulation of ER protein trafficking by addicisin

Addicisin is a member of the PRAF protein family with homology to PRA1 and PRAF2 (JM4) (Schweneker et al., 2005). PRA1 is associated with the Golgi membrane and interacts with Rab, a member of the Ras superfamily of small GTP-binding proteins, which regulates intracellular protein trafficking (Bucci et al., 1999; Liang & Li, 2000; Martincic et al., 1997).



Immunocytochemical studies reveal that mature addicisin is present in both the plasma membrane and the intracellular compartment, including the ER (Ikemoto et al., 2002; Watabe et al., 2007, 2008). Thus, addicisin may also be involved in intracellular protein trafficking. To investigate this possibility, we examined EAAC1 oligosaccharide residues under conditions of varying addicisin expression. The oligosaccharide residues on EAAC1 are an excellent indicator of the extent of ER-to-Golgi trafficking and plasma membrane localization because the newly synthesized EAAC1 is N-glycosylated with high mannose oligosaccharide chains that are subsequently processed into more complex sugar chains by resident Golgi enzymes (Yang & Kilberg, 2002). In HEK293T cells coexpressing addicisin, EAAC1 is predominantly modified by high mannose oligosaccharides, suggesting that EAAC1 proteins are largely confined to the ER. Furthermore, addicisin delays oligosaccharide maturation of EAAC1 but does not induce EAAC1 degradation (Ruggiero et al., 2008). These data suggest that addicisin delays ER-to-Golgi trafficking of EAAC1. Moreover, addicisin inhibits ER-to-Golgi trafficking of dopamine transporter, GABA transporter 1, and several G-protein-coupled receptors, including  $\beta_2$ -adrenergic receptor,  $\alpha_1$ - $\beta$  receptor, and D<sub>2</sub> receptor (Ruggiero et al., 2008). Furthermore, addicisin inhibits the function of RTN2B, a member of the reticulon protein family localized in the ER, which enhances ER-to-Golgi trafficking of EAAC1 (Liu et al., 2008). As addicisin, RTN2B, and EAAC1 are coexpressed in neurons, they may interact in one complex. Indeed, addicisin and EAAC1 can interact with RTN2B by binding to different regions of the protein. In addition, coexpression of RTN2B and EAAC1 in HEK293 cells increases EAAC1 cell surface expression, while increasing addicisin expression blocks this effect. Thus, EAAC1 trafficking is inhibited by addicisin and facilitated by RTN2B (Liu et al., 2008). Based on these data, Liu et al. proposed a model in which the regulation of ER trafficking governs the activity and density of EAAC1 at the plasma membrane. Under normal conditions, RTN2B facilitates EAAC1 trafficking from the ER because basal expression of addicisin is too low to have an inhibitory effect. Under stressful conditions, such as oxidative and chemical stress, addicisin expression is upregulated and the inhibitory effect on EAAC1 trafficking predominates over the facilitating effect of RTN2B (Liu et al., 2008). Addicisin can delay ER-to-Golgi trafficking of structurally and functionally distinct proteins in addition to EAAC1. Thus, addicisin is a stress-induced multifunctional protein that participates in various physiological and pathological functions by regulating ER trafficking of many membrane effector proteins, including receptors and transporters.

### 3. Addicisin & neurological disorders

Recent studies have also linked addicisin to the pathophysiology of several neurological diseases, including drug addiction, schizophrenia, and epilepsy. In this section, we focus on these diseases and review the putative pathophysiological functions of addicisin in the mammalian CNS.

#### 3.1 Drug abuse

Several studies demonstrate that addicisin is involved in drug abuse, the development of morphine dependence (Ikemoto et al., 2002; Wu et al., 2011), and ethanol tolerance (C. Li et al., 2008). In an effort to clarify the molecular mechanism of opiate addiction, we performed subtractive hybridization of mRNA expressed in the amygdala of mice treated

with repeated doses of morphine and identified *addicisin* mRNA as a factor selectively upregulated relative to drug-naïve mice (Ikemoto et al., 2000, 2002). Upregulation of *addicisin* mRNA was specifically induced by chronic, but not acute, morphine administration and was completely inhibited by coadministration of naloxone, an opiate receptor antagonist (Ikemoto et al., 2002). In that study, we used a morphine administration protocol that had been previously shown to induce morphine dependence and tolerance (Kaneto et al., 1973). Thus, our data strongly suggested that addicisin was involved in the development of morphine dependence in this animal model. Later reports have confirmed our findings by directly demonstrating that addicisin is directly involved in the development of morphine dependence (Wu et al., 2011). Chronic morphine treatment upregulated addicisin in prefrontal cortex, nucleus accumbens, and amygdala, which are regions known to be critical for the development of morphine dependence and other addictive behaviors. Furthermore, addicisin knockdown by infusion of addicisin antisense nucleotides into the cerebral ventricles significantly decreased withdrawal behaviors following chronic morphine treatment in rats (Wu et al., 2011). Addicisin knockdown suppressed the upregulation of  $\delta$  opioid receptors, the activation of the dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32), and MAPK activation normally induced by chronic morphine treatment. Furthermore, addicisin knockdown enhanced the degradation of  $\delta$  opioid receptors through the ubiquitin-proteasome pathway (Wu et al., 2011). These data suggest that addicisin directly contributes to the regulation of  $\delta$  opioid receptor stability and the development of morphine dependence by suppressing  $\delta$  opioid receptor expression and the activation of DARPP-32 and MAPK. The  $\delta$  opioid receptor knockout mice do not develop analgesic tolerance to morphine without affecting the development of physical dependence (Kieffer & Gaveriaux-Ruff, 2002; Nitsche et al., 2002; Y. Zhu et al., 1999). Thus, further investigations are needed to clarify whether addicisin is involved in analgesic tolerance.

Ethanol-induced cellular responses are analogous to those elicited by heat shock stresses (Piper, 1995; Wilke et al., 1994). Similarly, addicisin expression is enhanced in response to various environmental stressors, such as oxidative stress and heat shock stress (R. Chen et al., 2007). Furthermore, our study demonstrated that addicisin plays an important role in the development of morphine dependence and tolerance (Ikemoto et al., 2002). In the light of these observations, addicisin is considered to be essential for the development of ethanol tolerance. To address this issue, addicisin knockdown flies were generated. To estimate ethanol tolerance objectively, the inebriation test was performed (Bellen, 1998). Flies were exposed to ethanol vapor, and the mean elution time (MET) was measured three times after inebriation. The addicisin knockdown flies showed no difference between the first MET and third MET, while wild-type flies exhibited a significant higher third MET (C. Li, et al., 2008), indicating that addicisin knockdown flies failed to acquire ethanol tolerance.

### 3.2 Schizophrenia

Glutamatergic neurotransmission and plasticity are disrupted in patients with schizophrenia (Javitt, 2010; Kantrowitz & Javitt, 2010; Paz et al., 2008). This has led some researchers to speculate that EAATs and EAAT-interacting proteins that regulate glutamate transport efficacy or transporter expression may be abnormal in patients with schizophrenia (Bauer et al., 2008; Huerta et al., 2006). Indeed, addicisin/JWA transcripts were

overexpressed in the thalamus (Huerta et al., 2006) and the anterior cingulate cortex of schizophrenics as shown by *in situ* hybridization (Bauer et al., 2008). In these studies, the protein expression levels of addicisin/JWA were not determined. In addition, expression of EAAT3, the human homolog of EAAC1, was also upregulated in the anterior cingulate cortex of schizophrenic patients (Bauer et al., 2008). Furthermore, a microarray study of multiple human brain regions demonstrates that the anterior cingulate cortex is more vulnerable to these aberrant gene expression patterns (Katsel et al., 2005), and hypofrontality is a key feature of schizophrenia. Addicisin is thus a promising target for further research focusing on the role of glutamate transporters in schizophrenia. Moreover, addicisin regulates trafficking of a plethora of other membrane proteins, including dopamine receptors, suggesting another pathway through which addicisin participates in the pathogenesis of schizophrenia.

### 3.3 Epilepsy

Anatomical analysis of EAAT expression reveals that EAAC1 is enriched in neurons and particularly localized to inhibitory GABAergic neurons (Conti et al., 1998; He et al., 2000; Rothstein et al., 1994). Cerebroventricular injection of EAAC1 antisense oligonucleotides caused no elevation of extracellular glutamate in the rat striatum but did produce mild neurotoxicity and epileptiform activity (Rothstein et al., 1996). Furthermore, epilepsy in EAAC1 knockdown rats is caused by decreased GABA synthesis (Sepkuty et al., 2002). Glutamate is a precursor for GABA synthesis, so molecules that alter the intracellular availability of glutamate in GABAergic interneurons, including addicisin/GTRAP3-18, may have an important role in epileptogenesis or ictogenesis. In a recent study of the antiepileptic drug levetiracetam (LEV), changes in the expression of addicisin/GTRAP3-18, glutamate transporters, and GABA transporters were examined in a rat post-traumatic epilepsy model induced by FeCl<sub>3</sub> injection into the amygdala. Administration of LEV increased expression of EAAC1 and GABA transporter 3 (GAT-3) but decreased expression of addicisin/GTRAP3-18 in the rat hippocampal formation (Ueda et al., 2007). These results suggest that both the suppression of glutamatergic excitation and the enhancement of GABAergic inhibition induced by chronic LEV administration are due to the upregulation of EAAC1 and GAT-3 subsequent to downregulation of addicisin/GTRAP3-18. A long-lasting suppression of addicisin/GTRAP3-18 expression was observed in the rat pentylenetetrazole (PTZ)-induced kindling model of epilepsy (Ueda et al., 2006). Similarly, antisense-mediated knockdown of addicisin/GTRAP3-18 decreases seizure threshold and promotes PTZ kindling. In addition, addicisin/GTRAP3-18 knockdown increases basal release of glutamate and GABA in the rat hippocampal formation, indicating that knockdown of addicisin/GTRAP3-18 promotes GABA synthesis (Ueda et al., 2006). These studies, demonstrating that addicisin can increase GABA synthesis by increasing the substrate (i.e., glutamate) supply, define addicisin as a novel therapeutic target in epilepsy.

### 3.4 Other neurological disorders

Addicisin directly modulates glutamate and cysteine uptake by EAAC1, suggesting that addicisin participates in the pathogenesis of neurological disorders associated with excitotoxicity and oxidative stress. Here we briefly discuss some representative EAAC1 functions relevant to CNS pathology. A recent study demonstrated that EAAC1-deficient

mice developed age-dependent brain atrophy and behavioral abnormalities in the cognitive and motivational domains. In addition, EAAC1 knockout mice displayed impaired GSH homeostasis and age-dependent neurodegeneration, and these pathologies were rescued by treatment with the membrane permeable cysteine precursor *N*-acetylcysteine (Aoyama et al., 2006). These EAAC1 knockout mice also display dicarboxylic aminoaciduria and significant motor impairments (Peghini et al., 1997). These results indicate that EAAC1 functions as a cysteine transporter in neurons and sustains intracellular GSH to ameliorate oxidative stress *in vivo*. Furthermore, neuronal glutamate uptake can also regulate memory formation (Levenson et al., 2000; Maleszka et al., 2000). The increase of EAAC1-mediated neuronal glutamate uptake is associated with the induction and expression of early phase long-term potentiation (LTP) in the CA1 area of the hippocampal formation and with contextual fear conditioning, a form of hippocampus-dependent memory thought to depend on induction of LTP (Levenson et al., 2002). These results suggest that regulation of glutamate uptake by EAAC1 is a physiologically important mechanism for the modulation of synaptic strength during long-term changes in synaptic efficacy (plasticity). Thus, dysfunction of EAAC1 induced by aberrant addicsin expression may lead to neurodegeneration and cognitive decline. Of particular interest is the role of addicsin in the pathogenesis of neurodegenerative diseases such as Alzheimer’s and Parkinson’s diseases. These questions warrant further research.

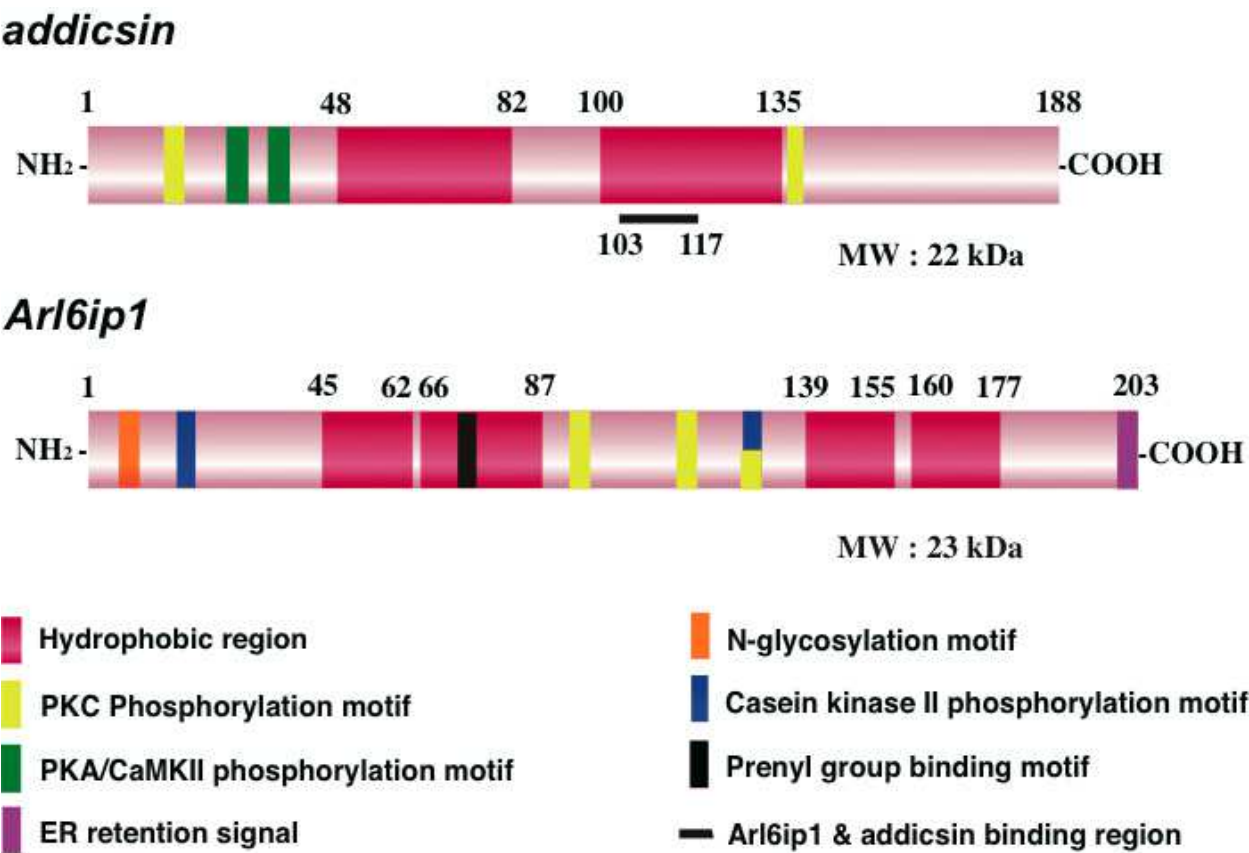


Fig. 1. A schematic presentation of addicsin and Arl6ip1



#### 4. Future research perspective

Despite these advances, our understanding of the regulatory mechanisms of addicisin expression and the range of addicisin functions is far from complete. The elucidation of the regulatory mechanism of addicisin expression under basal and pathological conditions is essential for understanding the physiological and pathological roles of addicisin. For instance, while addicisin has consensus PKC phosphorylation sequences, it is unclear whether PKC actually phosphorylates addicisin and controls addicisin functions *in vivo*. It is also unknown whether or how PKC phosphorylation affects the interaction between addicisin and Arl6ip1. To overcome these challenges, it is crucial to clarify whether PKC phosphorylation sites of addicisin are physiologically controlled by PKC signaling and by which PKC isoforms. Furthermore, it remains controversial whether addicisin is an integral membrane protein. Our results strongly support the notion that addicisin is a membrane-associating protein with a soluble and membrane-localized form. Thus, it is important to clarify the different molecular features and functions of the soluble and membrane-localized forms of addicisin.

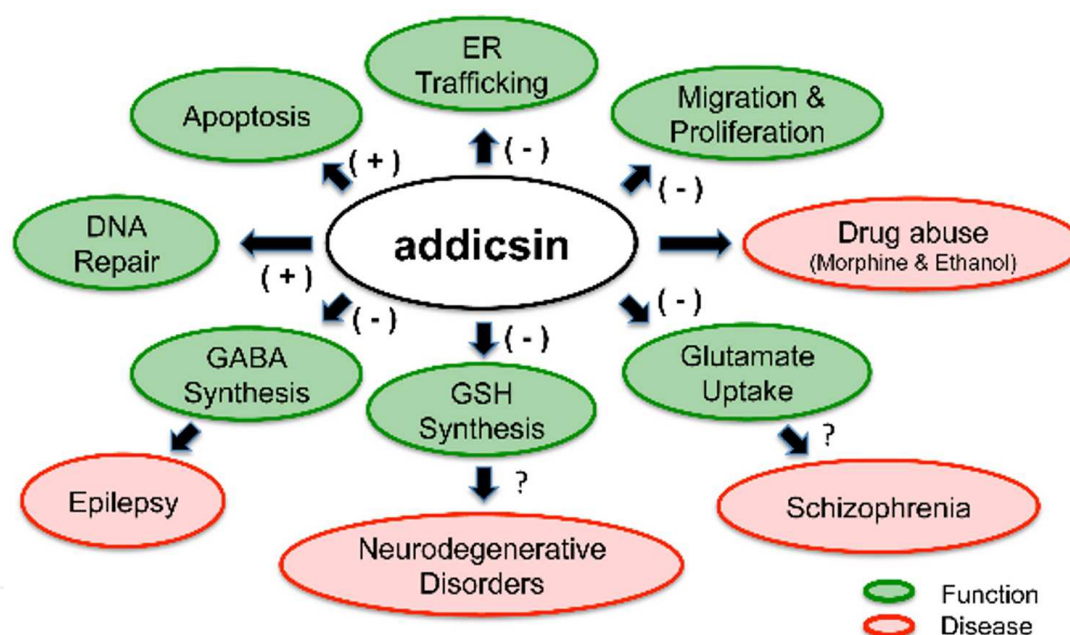


Fig. 2. A scheme of the proposed physiological functions of addicisin

Second, *in vivo* functional studies are still needed to clarify the physiological and pathological functions of addicisin. Accumulating evidence suggests that addicisin participates in various physiological and pathological processes *in vivo*, but the molecular mechanisms controlling the selective interaction of addicisin with multiple targets, including receptors and transporters, are unknown. Furthermore, many reports demonstrate that the physiological and pathological roles of addicisin are observed when expression of addicisin is increased by various stresses, including oxidative and chemical stress. Thus, the production of animal models that overexpressed addicisin in a tissue- or region-specific manner may be useful to analyze addicisin functions in various tissues, including the brain. At present, no studies have been undertaken in tissues outside the brain, although addicisin is ubiquitously expressed in kidney, heart, and liver (Butchbach et al., 2002; Ikemoto et al., 2002).

We believe that studies using transgenic or conditional knockin/knockout animal models will lead to novel insights into addictin function. Of particular interest is whether dysfunctional addictin expression or function can lead to neurodegenerative diseases through dysregulation of EAAC1 or other proteins. Finally, we hope that studies on addictin will continue to advance our understanding of the role of addictin in the pathogenesis of diseases, such as drug abuse, and lead to the development of curative therapies.

5. Conclusion

In this chapter, we argued that Arl6ip1 is a novel addictin-interacting protein that indirectly promotes PKC-dependent, EAAC1-mediated glutamate uptake by inhibiting the interaction of addictin with EAAC1 at the plasma membrane. Based on these findings, we proposed the regulatory model of EAAC1-mediated glutamate uptake illustrated in Fig. 3. In this model, EAAC1-mediated glutamate uptake activity can be negatively and positively regulated by PKC activity depending on dynamic modulation by addictin complexes. Thus, the cellular dynamics of addictin is a key element regulating EAAC1-mediated glutamate uptake. The study of addictin is still in its infancy, but future findings on the physiological and pathophysiological functions of addictin could greatly clarify the role of EAAC1 (and other proteins regulated by addictin) in health and disease.

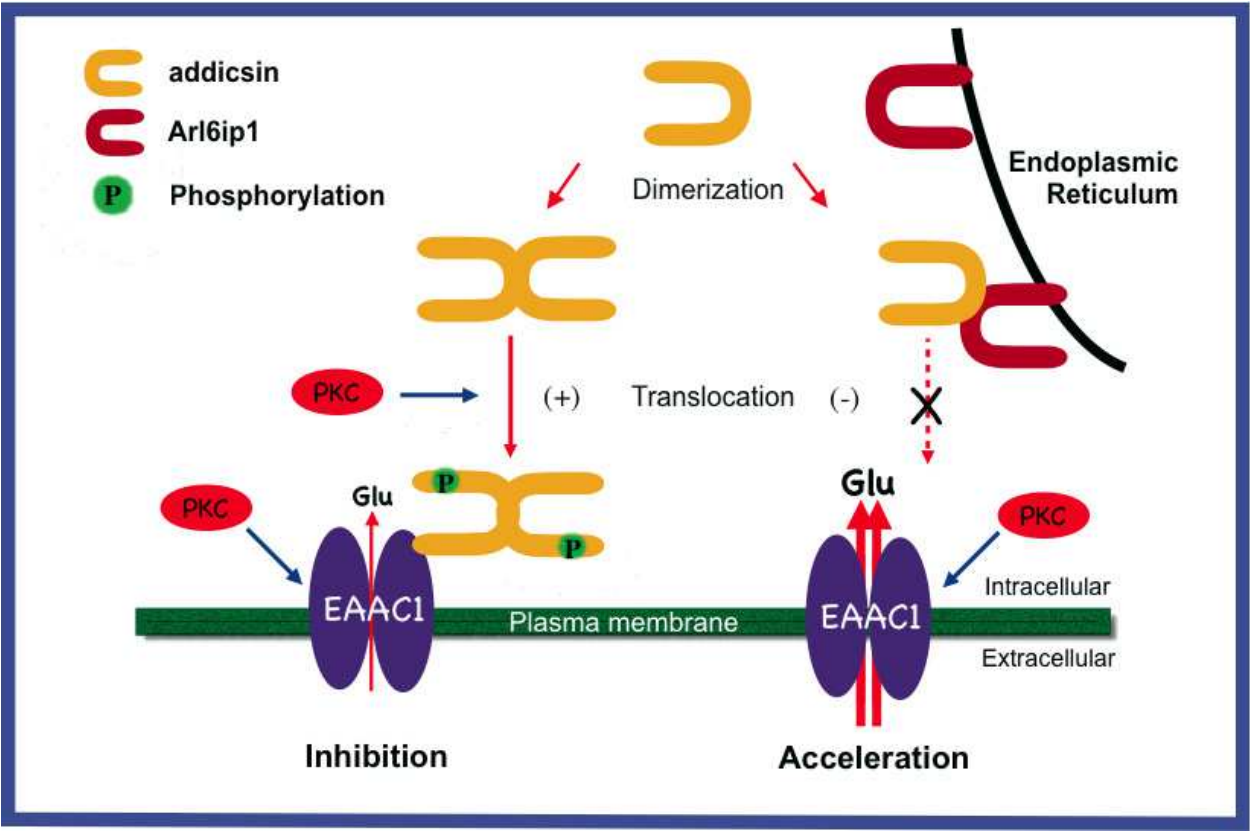


Fig. 3. A regulatory model of EAAC1-mediated glutamate uptake in C6BU-1 cells

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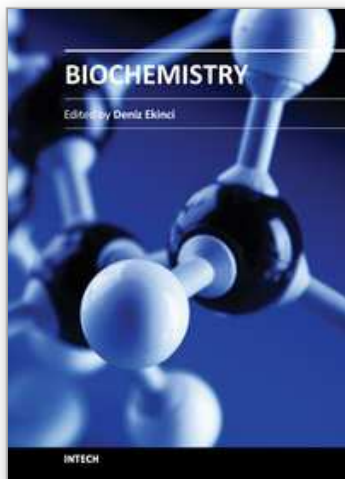
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## **Biochemistry**

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Over the recent years, biochemistry has become responsible for explaining living processes such that many scientists in the life sciences from agronomy to medicine are engaged in biochemical research. This book contains an overview focusing on the research area of proteins, enzymes, cellular mechanisms and chemical compounds used in relevant approaches. The book deals with basic issues and some of the recent developments in biochemistry. Particular emphasis is devoted to both theoretical and experimental aspect of modern biochemistry. The primary target audience for the book includes students, researchers, biologists, chemists, chemical engineers and professionals who are interested in biochemistry, molecular biology and associated areas. The book is written by international scientists with expertise in protein biochemistry, enzymology, molecular biology and genetics many of which are active in biochemical and biomedical research. We hope that the book will enhance the knowledge of scientists in the complexities of some biochemical approaches; it will stimulate both professionals and students to dedicate part of their future research in understanding relevant mechanisms and applications of biochemistry.

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Phone: +86-21-62489820  
Fax: +86-21-62489821

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