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Transcriptional Regulation of Glutamate Transporters: From Extracellular Signals to Transcription Factors

Zila Martinez-Lozada, **Alain M. Guillem**, and **Michael B. Robinson**

Departments of Pediatrics and Systems Pharmacology and Experimental Therapeutics, Children's Hospital of Philadelphia Research Institute, University of Pennsylvania, PA 19104

Abstract

Glutamate is the predominant excitatory neurotransmitter in the mammalian CNS. It mediates essentially all rapid excitatory signaling. Dysfunction of glutamatergic signaling contributes to developmental, neurologic, and psychiatric disease. Extracellular glutamate is cleared by a family of five Na^+-dependent glutamate transporters. Two of these transporters (GLAST and GLT-1) are relatively selectively expressed in astrocytes. Other of these transporters (EAAC1) is expressed by neurons throughout the nervous system. Expression of the last two members of this family (EAAT4 and EAAT5) is almost exclusively restricted to specific populations of neurons in cerebellum and retina, respectively. In this review, we will discuss our current understanding of the mechanisms that control transcriptional regulation of the different members of this family. Over the last two decades our understanding of the mechanisms that regulate expression of GLT-1 and GLAST has advanced considerably; several specific transcription factors, cis-elements, and epigenetic mechanisms have been identified. For the other members of the family, little or nothing is known about the mechanisms that control their transcription. It is assumed that by defining the mechanisms involved, we will advance our understanding of the events that result in cell specific expression of these transporters and perhaps begin to define the mechanisms by which neurologic diseases are changing the biology of the cells that express these transporters. This approach might provide a pathway for developing new therapies for a wide-range of essentially untreatable and devastating diseases that kill neurons by an excitotoxic mechanism.

Keywords

Glu uptake; astrocytes; EAAT; GLT-1; GLAST; EAAC1; transcriptional regulation

1. Introduction

L-Glutamate (Glu) is the major excitatory neurotransmitter in the mammalian CNS and activates both ligand-gated ion channels and G-protein coupled receptors (Fagg, Mena & Cotman, 1983, Nakanishi, 1992, Nakanishi, 1994, Robinson & Coyle, 1987). Even before the receptors were cloned (Hollman & Heinemann, 1994), there was strong evidence that

Address Correspondence to: Michael B. Robinson, Department of Pediatrics, 502N, Abramson Research Building, 3615 Civic Center Boulevard, Philadelphia, PA 19104-4318, Tel. 215-590-2205; Fax. 215-590-3779, Robinson@mail.med.upenn.edu.

Conflict of interest statement

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excessive activation of Glu receptors contributes to neuronal loss in a variety of neurological insults. This was based on the following observations: 1) Exogenous (and non-transported) receptor agonists are toxic to neurons *in vitro* and *in vivo*, and the potencies closely correlate with that observed for receptor activation (Schwarcz & Coyle, 1977, Schwarcz, Scholz & Coyle, 1978). 2) The patterns of damage caused by these agonists roughly paralleled those observed in humans with various disorders (Beal, Kowall, Ellison, Mazurek, Swartz & Martin, 1986, Schwarcz, Bennett & Coyle, 1977, Schwarcz, Whetsell & Mangano, 1983, Spencer, Nunn, Hugon, Ludolph, Ross, Roy et al, 1987). 3) Acute insults including stroke-like insults or traumatic brain injury were associated with increases in extracellular concentrations of Glu (Benveniste, Drejer, Schousboe & Diemer, 1984, Faden, Demediuk, Panter & Vink, 1989, Rossi, Oshima & Attwell, 2000, Rothman, 1984). 4) And finally, Glu receptor antagonists attenuate the damage caused by some of the acute insults (Gill, Foster & Woodruff, 1987). In the 1990's, it became clear that Glu-mediated excitotoxicity involved an apoptotic-necrotic continuum (Cheung, Pascoe, Giardina, John & Beart, 1998). This process of excitotoxicity has been implicated in virtually every neurologic disorder (for reviews, see Choi, 1992, Coyle & Puttfarcken, 1993, Faden, Demediuk, Panter & Vink, 1989, Fontana, 2015, Greene & Greenamyre, 1996, McDonald & Johnston, 1990). In spite of this strong set of complementary observations that were reproduced in several different laboratories, drug companies have not been successful in targeting Glu receptors in spite of the billions of dollars that were spent (for discussions, see Nicoletti, Bruno, Ngomba, Gradini & Battaglia, 2015, Wieronska, Zorn, Doller & Pilc, 2015). It appears that the side-effects caused by blocking the N-methyl-D-aspartate subtype of Glu receptor (psychotic symptoms, cell death) and possibly the fact that Glu receptor activation is required for essentially all human actions may have limited the utility of this strategy (Olney, 1994, Olney, Labruyere, Wang, Wozniak, Price & Sesma, 1991).

If, in fact, excessive activation of Glu receptors contributes to neurodegeneration observed after acute insults and/or in chronic neurodegenerative diseases, then it becomes important to understand the mechanisms that control extracellular concentrations of potentially toxic amino acids, including Glu and aspartate. To date, there is still no evidence of extracellular metabolism of either amino acid (for reviews, see Danbolt, 1994, Schousboe, 1981). Instead extracellular Glu concentrations are maintained below those required to chronically activate Glu receptors (Herman & Jahr, 2007) by a family of Na^+-dependent Glu transporters. This transport process was first identified and characterized in the early 1970s (Balcar & Johnston, 1972, Beart, 1976, Logan & Snyder, 1971). Then in the early 1990s, a family of five transporters that mediate sodium-dependent Glu uptake was cloned. The first three Glu transporters that were identified and cloned were named glutamate/aspartate transporter (GLAST), glutamate transporter 1 (GLT-1), and excitatory amino acid carrier 1 (EAAC1) (Kanai & Hediger, 1992, Pines, Danbolt, Bjørås, Zhang, Bendahan, Eide et al, 1992, Storck, Schulte, Hofmann & Stoffel, 1992). Shortly thereafter, the human homologues of these transporters were cloned and called excitatory amino acid transporters (EAAT1-3 respectively) (Arriza, Fairman, Wadiche, Murdoch, Kavanaugh & Amara, 1994). Two additional members of the family were also cloned; there were called EAAT4 and EAAT5 (Arriza, Eliasof, Kavanaugh & Amara, 1997, Fairman, Vandenberg, Arriza, Kavanaugh & Amara, 1995). The names of the genes that code for these transporters is different; they are

called SLC1A3, 2, 1, 6, & 7, respectively with capital letters for the human homologs and lower case letters for the rodent homologs. These transporters co-transport 3 molecules of Na^+ and 1 H^+ with one molecule of Glu; the countertransport 1 K^+ completes the cycle. This stoichiometry allows these transporters to generate up to a 1-million-fold concentration gradient across the membrane (Levy, Warr & Attwell, 1998, Owe, Marcaggi & Attwell, 2006, Wadiche, Arriza, Amara & Kavanaugh, 1995, Zerangue & Kavanaugh, 1996). Several reviews have discussed the pharmacology, localization, and biophysical properties of these transporters (Anderson & Swanson, 2000, Beart & O'Shea R, 2006, Danbolt, 2001, Gegelashvili & Schousboe, 1997, Kanner, 2006, Robinson, 1999, Robinson & Dowd, 1997, Ryan & Vandenberg, 2005, Seal & Amara, 1999, Shigeri, Seal & Shimamoto, 2004, Sims & Robinson, 1999, Tanaka, 2000, Trotti, Danbolt & Volterra, 1998, Vandenberg & Ryan, 2013). Therefore, in this review we will focus on their transcriptional regulation.

2. Differential localization of glutamate transporters

If one assumes that transcriptional mechanisms are the strongest driver of endogenous expression, it is important to first understand the expression patterns of the transporter subtypes. All five subtypes of the Glu transporters are enriched in different brain regions and different cell types. GLAST and GLT-1 are mainly expressed in astrocytes, while the other three subtypes are enriched in neurons (Chaudhry, Lehre, Campagne, Ottersen, Danbolt & Storm-Mathisen, 1995, Lehre, Levy, Ottersen, Storm-Mathisen & Danbolt, 1995, Pines et al, 1992, Regan, Huang, Kim, Dykes-Hoberg, Jin, Watkins et al, 2007, Rothstein, Martin, Levey, Dykes-Hoberg, Jin, Wu et al, 1994). GLAST, GLT-1 and EAAC1 are also found in oligodendroglia (DeSilva, Kabakov, Goldhoff, Volpe & Rosenberg, 2009, Domerq, Sánchez-Gómez, Areso & Matute, 1999, Martinez-Lozada, Waggener, Kim, Zou, Knapp, Hayashi et al, 2014, Pitt, Nagelmeier, Wilson & Raine, 2003), GLT-1 has also been observed in activated microglia (Lopez-Redondo, Nakajima, Honda & Kohsaka, 2000). GLT-1 is also expressed by neurons, but at much lower levels than those observed in astrocytes (for recent discussion, see Furness, Dehnes, Akhtar, Rossi, Hamann, Grutle et al, 2008, Petr, Sun, Frederick, Zhou, Dhamne, Hameed et al, 2015). GLAST is enriched in Bergmann glial cells of the cerebellum (Rothstein et al, 1994, Ruiz & Ortega, 1995), in Müller glial cells of the retina (Bringmann, Pannicke, Biedermann, Francke, Iandiev, Grosche et al, 2009), and in astrocytes in the olfactory bulb (Utsumi, Ohno, Onchi, Sato & Tohyama, 2001). GLT-1 is enriched in astrocytes in the cerebral cortex, hippocampus, lateral septum, striatum and spinal cord (Regan et al, 2007, Rothstein et al, 1994, Torp, Danbolt, Babaie, Bjoras, Seeberg, Storm-Mathisen et al, 1994). EAAC1 is observed in neurons in the forebrain, diencephalon, hindbrain, dorsal root ganglia and spinal cord (Bar-Peled, Ben-Hur, Biegon, Groner, Dewhurts, Furata et al, 1997, Furuta, Martin, Lin, Dykes-Hoberg & Rothstein, 1997). EAAT4 is almost exclusively expressed in Purkinje cells of the cerebellum (Dehnes, Chaudhry, Ullensvang, Lehre, Storm-Mathisen & Danbolt, 1998, Yamada, Watanabe, Shibata, Tanaka, Wada & Inoue, 1996). EAAT5 is almost exclusively expressed by photoreceptors and bipolar cells in the retina (Arriza et al, 1997, Pow & Barnett, 2000). Expression of these transporters is also differentially controlled during development. GLAST is found at relatively high levels early in development, while GLT-1 levels increase

dramatically during development (Furuta, Rothstein & Martin, 1997). This suggests that GLT-1 may be a marker of astrocyte maturation.

Although many think of these transporters as molecules that clear the 'neurotransmitter' Glu, several studies show that virtually all cells express Na^+-dependent Glu transporters. GLAST is expressed in heart, muscle, placenta, lung and liver (Gegelashvili & Schousboe, 1998). GLT-1 is expressed in pancreas and liver, but the levels are 100-fold or more higher in brain (Berger & Hediger, 2006). EAAC1 is expressed in intestine, kidney, heart, lung, placenta and liver (Nakayama, Kawakami, Tanaka & Nakamura, 1996). Low levels of EAAT4 mRNA are found in placenta, and EAAT5 is expressed in liver, kidney, intestine, heart, lung and skeletal muscle (Gegelashvili et al, 1998, Lee, Anderson, Stevens, Beasley, Barnett & Pow, 2013). These differential expression patterns strongly suggest that each of these transporters is under specific transcriptional regulation.

3. Why study transcriptional regulation of glutamate transporters?

Glu is a neurotransmitter, a source of energy through oxidation (Dienel & McKenna, 2014), a building block for the anti-oxidant glutathione (Brosnan & Brosnan, 2013, Had-Aissouni, 2012), the only precursor for the major inhibitory neurotransmitter (γ-aminobutyric acid-GABA), and is a building block for proteins. During development, Glutamatergic signaling participates in proliferation, migration, and differentiation (for reviews, see Jansson & Akerman, 2014, Lujan, Shigemoto & Lopez-Bendito, 2005, Nguyen, Rigo, Rocher, Belachew, Malgrange, Rogister et al, 2001). It also controls synapse formation and the shape of dendritic spines. Thus, it is critical to control extracellular Glu spatially and temporally during development and in the adult nervous system. In the adult nervous system, Glu transporters are found at such high levels (particularly GLT-1) that they function as buffers of the amount of Glu that is available for activation of receptors (Tong & Jahr, 1994) and/or they shape the excitatory post-synaptic currents at other synapses (for reviews, see Conti & Weinberg, 1999, Huang & Bergles, 2004, Marcaggi & Attwell, 2004, Otis, Brasnjo, Dzubay & Pratap, 2004, Tzingounis & Wadiche, 2007).

Decreased levels of Glu transporter proteins and/or mRNAs have been observed in animal models of stroke, head trauma, amyotrophic lateral sclerosis (ALS), Alzheimer's disease, epilepsy, and others. In many cases, similar changes have been observed in post-mortem specimens from patients with these diseases (for reviews, see Dunlop, 2006, Fontana, 2015, Kim, Lee, Kegelman, Su, Das, Dash et al, 2011 Sattler & Rothstein, 2006, Sheldon & Robinson, 2007, Yi & Hazell, 2006). In fact, a loss of GLT-1 that is consistently observed in both animal models of ALS (for review, see Rattray & Bendotti, 2006) and humans with ALS, prompted Rothstein and his colleagues to screen for compounds that increase GLT-1 expression. They identified the antibiotic ceftriaxone and showed that it delayed the onset of motor symptoms and death in a mouse model of ALS (Rothstein, Patel, Regan, Haenggeli, Huang, Bergles et al, 2005). Although ceftriaxone did not show a therapeutic benefit in a phase 3 clinical trial (Cudkowicz, Titus, Kearney, Yu, Sherman, Schoenfeld et al, 2014), several groups have shown therapeutic benefits of ceftriaxone in animal models of a wide-range of neurologic and psychiatric conditions (Amin, Hajhashemi, Abnous & Hosseinzadeh, 2014, Cui, Cui, Gao, Sun, Wang, Wang et al, 2014, Fontana, 2015, Hsu,

Hung, Chang, Liao, Ho & Ho, 2015, Inui, Alessandri, Heimann, Nishimura, Frauenknecht, Sommer et al, 2013, Soni, Reddy & Kumar, 2014). Glu transporters are regulated by a variety of mechanisms, including transcription, mRNA maturation and stabilization, post-translational modifications, trafficking to and from the plasma membrane (for reviews see Robinson, 2002, Robinson, 2006), and diffusion in the membrane (Benediktsson, Marrs, Tu, Worley, Rothstein, Bergles et al, 2012, Murphy-Royal, Dupuis, Varela, Panatier, Pinson, Baufreton et al, 2015, Shin, Nguyen, Pow, Knight, Buljan, Bennett et al, 2009).

In this review, we will focus on the mechanisms that control transcription of the transporters. This has mostly been approached by examining the effects of agents that activate cell surface receptors or by direct modulation of intracellular signals. In some cases, the effects of these agents have been linked to specific *cis* elements in the transporter genes and transcription factors that bind to these elements. There is some evidence to suggest that these regulatory events are influenced by epigenetic mechanisms (DNA methylation or histone acetylation), but this is still a relatively underexplored area.

One assumes that, as the field develops an understanding of the mechanisms that control transcription of these transporters, we will develop a better understanding of the specific signals and transcription factors that define populations of cells and/or subpopulations of cells in the brain. In some cases this information is starting to be used to define the mechanisms that contribute to decreases in transporter expression that accompany diverse neurologic insults. In spite of setbacks, it still seems appealing to consider the mechanisms that regulate Glu transporters as potential drug targets. It is also possible that developing an understanding of the transcriptional events that lead to altered expression of Glu transporters may lead to a broader mechanistic understanding of the pathogenesis of diverse neurologic and psychiatric diseases.

For the purposes of this review, we decided to simplify our discussion and include many studies in which steady state protein and/or mRNA levels change in response to an external stimulus. However there are examples of regulation of mRNA stability (Zelenaia, Gochenauer & Robinson, 1999), translation (Tian, Lai, Guo, Lin, Butchbach, Chang et al, 2007), or protein degradation (Wu, Xia, Lin, Cao, Chen, Liu et al, 2013). Therefore it seems possible that our simplification will end up being incorrect at least in some cases.

3.1 Transcriptional Regulation of SLC1A3/GLAST/EAAT1

The mouse GLAST gene was mapped to chromosome 15A2 (Hagiwara, Tanaka, Takai, Maeno-Hikichi, Mukainaka & Wada, 1996). Although the human gene was originally mapped to 5p13 (Takai, Yamada, Kawakami, Tanaka & Nakamura, 1995), one year latter it was re-mapped to chromosome 5p11–12 (Stoffel, Sasse, Duker, Muller, Hofmann, Fink et al, 1996). With the complete sequencing of both genomes, these locations have been verified. As the cellular, developmental, and regional expression patterns are shared between mouse/rodent and human (Bar-Peled et al, 1997, Furuta et al, 1997, Regan et al, 2007, Schmitt, Asan, Puschel & Kugler, 1997), it seems that core promoter elements are likely to be conserved through evolution. The fact that transgenic mice that utilize the entire human SLC1A3 gene to control discosoma red fluorescent protein (dsRFP) display complete overlap of GLAST with dsRFP further supports the notion that similar elements control

GLAST expression in mice and humans (Regan et al, 2007). Therefore, evolutionarily conserved regions of the promoter are likely to provide insights into the *cis* elements that may be involved in transcriptional control; these are presented in Figure 1.

Almost 20 years ago, the structures of both the mouse and human GLAST promoters were defined (Hagiwara et al, 1996, Stoffel et al, 1996). As might be expected, the proximal 2 kb of the promoter are highly conserved and all studies to date have focused on this region. Neither the mouse nor the human promoters contain a TATA box in this region but they both contain a GC box and the human gene also contains an E box. As is observed with several housekeeping genes, the transcription factors stimulating proteins 1 and 3 (Sp1, Sp3) bind to the GC box and upstream stimulating factor (USF1) binds to the E box in electrophoretic mobility assays (EMSA) (Kim, Choi, Chao & Volsky, 2003) (see figure 2A). Mutation of either the GC or E box dramatically reduces promoter reporter expression in human fetal astrocytes, suggesting that binding to both sites is required for transcription.

While working from the gene sequence has yielded some information about the *cis* and *trans* factors involved in controlling GLAST expression, an alternative strategy has been to identify agents that regulate GLAST protein and/or mRNA levels when applied to cells (generally astrocytes). Using this strategy, it has been possible to identify extracellular stimuli that either increase or decrease GLAST expression/transcription. We will first discuss the pathways that have been implicated in transcriptional activation (Figure 2A) and then the pathways that have been implicated in transcriptional repression (Figure 2B).

Astrocytes in culture have been an important model system to study transcriptional regulation of GLAST and in this system neurons increase astrocytic expression of GLAST (Schlag, Vondrasek, Munir, Kalandadze, Zelenaia, Rothstein et al, 1998, Swanson, Liu, Miller, Rothstein, Farrell, Stein et al, 1997). This effect is at least in part caused by secreted molecules (Gegelashvili, Danbolt & Schousboe, 1997, Schlag et al, 1998, Swanson et al, 1997). In earlier studies, it had been shown that dibutyryl-cyclic AMP (dbcAMP) increases glutamate uptake in astrocytes (Hertz, Bock & Schousboe, 1978) and three groups essentially simultaneously demonstrated that dbcAMP increases the levels of GLAST mRNA and protein in primary cultures of astrocytes from forebrain or retina (Eng, Lee & Lal, 1997, Schluter, Figiel, Rozyczka & Engele, Swanson et al, 1997). The effect of dbcAMP is blocked by protein-kinase A (PKA) inhibitors (Schlag et al, 1998). Shortly thereafter, Figuel and colleagues demonstrated that pituitary adenylate cyclase-activating polypeptide (PACAP) mimics the effects of neurons and that PACAP-directed antibodies block the effects of neuron-conditioned media (Figiel & Engele, 2000). Furthermore, inhibitors of a PACAP receptor (PAC1R) or PKA antagonists also blocked the effects of neuron-conditioned media. Together these studies are consistent with the notion that neurons use a PACAP, cAMP, PKA-dependent pathway to increase GLAST expression (see Figure 2A). Although these data are consistent with the activation of the transcription factor cAMP-response element binding protein (CREB), the transcription factor(s) or the *cis* element(s) of the promoter responsible for this effect have not been identified.

Steroids are neuroprotective in animal models of both acute insults and chronic neurodegenerative diseases (Baudry, Bi & Aguirre, 2013, Scott, Zhang, Wang, Vadlamudi &

Brann, 2012). Estrogen or tamoxifen increase GLAST mRNA and protein (Karki, Webb, Zerguine, Choi, Son & Lee, 2014, Lee, Sidoryk, Jiang, Yin & Aschner, 2009, Pawlak, Brito, Kuppers & Beyer, 2005). These effects are dependent upon activation of both the G protein-coupled receptor 30 (GPR30) and the nuclear receptors ERα and ERß (Karki et al, 2014, Lee, Sidoryk-Wegrzynowicz, Wang, Webb, Son, Lee et al, 2012), but there is some evidence that ERα may be more important for this effect (Sato, Matsuki, Ohno & Nakazawa, 2003). The effects of estrogen on GLAST are indirect and are mediated by estrogen-dependent induction of transforming growth factor – α (TGFα) (Dhandapani, Wade, Mahesh & Brann, 2005, Karki et al, 2014, Lee et al, 2009). Steroids or tamoxifen are thought to attenuate manganese-dependent neurotoxicity by a mechanism that depends on up-regulation of GLAST (Karki, Smith, Johnson & Lee, 2014, Lee et al, 2009).

In addition to TGFα, several other growth and neurotrophic factors increase expression of GLAST. Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1) and glial cell line-derived neurotrophic factor (GDNF) also increase GLAST mRNA and protein levels (Bonde, Sarup, Schousboe, Gegelashvili, Noraberg & Zimmer, 2003, Suzuki, Ikegaya, Matsuura, Kanai, Endou & Matsuki, 2001, Zelenaia, Schlag, Gochenauer, Ganel, Song, Beesley et al, 2000). Growth factors induce GLAST expression through Ras/Mitogen-extracellular signal regulated kinase (Ras/MEK), phosphatidylinositol-4,5-bisphosphate 3 kinase/Akt (PI3K/Akt) and PKA pathways (Dhandapani et al, 2005, Figiel, Maucher, Rozyczka, Bayatti & Engele, 2003, Lee et al, 2009) with a consequent activation of nuclear factor kappa B (NFκB)(Figiel et al, 2003, Karki et al, 2014) (see Figure 2A). Although inhibitors of NFκB signaling block these effects and exogenous expression of the active subunits of NFκB (p50 and p65) mimic these effects, a direct interaction between NFκB and the GLAST promoter has not been described (Figiel et al, 2003, Karki et al, 2014, Lin, You, Wei & Gean, 2014).

The effects of growth factors on GLAST expression also seem to depend on activation of the Janus kinase/signal transducer activator of transcription (JAK/STAT) pathway. Using an *in vivo* model, Raymond and colleagues demonstrated that fibroblast growth factor-2 (FGF2) blocks the damage caused by hypoxia (Raymond, Li, Mangin, Huntsman & Gallo, 2011). They show that hypoxia causes decreases in GLAST, phospho-JAK, and phospho-STAT. They also show that these effects are blocked by FGF2 and that the effects of FGF2 are blocked by an inhibitor of JAK/STAT signaling. Prolonged stress also causes a decrease in GLAST protein and an increase in Glu in cerebrospinal fluid (Feng, Guo, Liu, Wang, Wang, Gao et al, 2015). These effects were also strongly linked to inhibition of JAK/STAT signaling. At present, it is not clear if STAT directly interacts with the GLAST promoter, but these studies strongly suggest that the JAK/STAT pathway contributes to maintenance of GLAST levels *in vivo*. Together these studies also suggest that several different signaling pathways may function downstream of growth factors to regulate transcription of GLAST; it will be important to learn if these pathways converge or function in parallel/independently.

As these transporters play an important role in regulating a potential toxin (Glu itself), it should not be surprising that Glu receptors are also linked to transcriptional regulation of GLAST. In fact, some of the subtypes of Glu receptors are linked to increases in GLAST, while others decrease GLAST. A selective group II metabotropic Glu receptor (mGluR)

agonist increases GLAST mRNA and protein (Aronica, Gorter, Ljlst-Keizsers, Rozemuller, Yankaya, Leenstra et al, 2003, Gegelashvili, Dehnes, Danbolt & Schousboe, 2000). Using pharmacological strategies, this effect was linked to the ERK/PI3K/NFkB signaling pathway (Lin et al, 2014)(Figure 2A). The *in vivo* relevance of this effect is supported by the observation that mice deleted of one of the members of the group II receptors, mGluR3, have lower levels of GLAST protein (Lyon, Kew, Corti, Harrison & Burnet, 2008). This suggests that tonic activation of mGluR3 maintains GLAST expression *in vivo*.

In contrast to the effects of mGluR3 activation, agonists of group I mGluRs decrease GLAST expression (Aronica et al, 2003, Gegelashvili et al, 2000). This effect has only been studied using a group I mGluRs selective agonist and an antagonist. Therefore more studies are needed to identify the downstream signaling pathways and transcription factors involved.

The ionotropic Glu receptors (iGluRs) also regulate GLAST expression in cerebellar Bergmann glia. Lopez-Bayghen and Ortega demonstrated that Glu and the Glu receptor agonist, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), decrease GLAST mRNA and protein levels (Lopez-Bayghen, Espinoza-Rojo & Ortega, 2003). This variant of Glu receptor is Ca^{2+} permeable, and the consequent increase in intracellular Ca^{2+} is known to activate protein kinase C (PKC) (Burnashev, Khodorova, Jonas, Helm, Wisden, Monyer et al, 1992). Inhibition of PKC blocks the effect of Glu and exogenous expression of constitutively active PKCα mimics the effects of Glu, strongly suggesting that activation of PKC is necessary and sufficient for this effect (Lopez-Bayghen et al, 2003, Lopez-Bayghen & Ortega, 2004). They showed that exogenous expression of c-jun or c-fos, subunits of the transcription factor activator protein-1 (AP1), decrease GLAST levels. They also demonstrated that Glu, AMPA, or a PKC activator increase AP-1 binding to a sequence in the proximal GLAST promoter using EMSAs (Lopez-Bayghen et al, 2004). Together these studies demonstrate that Glu down-regulates GLAST expression through AMPA receptors that are coupled to PKC and AP-1 (see figure 2B). In a subsequent study, this same group showed that Glu and AMPA also increases the interaction of the transcription factor Ying Yang 1 (YY1) with the GLAST promoter, and overexpression of this transcription factor decreases GLAST expression (Rosas, Vargas, Lopez-Bayghen & Ortega, 2007). These studies suggest that the effects of AMPA may depend on both AP-1 and YY1. Using the same model, Poblete-Naredo and her colleagues demonstrated that insulin increases YY1 binding to the GLAST promoter by EMSA and decreases GLAST expression (Poblete-Naredo, Angulo, Hernandez-Kelly, Lopez-Bayghen, Aguilera & Ortega, 2009).

The cytokine, tumor necrosis factor α (TNFα), decreases GLAST protein levels (Korn, Magnus & Jung, 2005). However astrocytes grown in media containing dbcAMP are resistant to the effects of TNFα (Tilleux & Hermans, 2008). As dbcAMP simulates some aspects of astrocyte maturation, these results suggest that regulation of GLAST transcription may vary at different stages of astrocyte maturation. This effect of TNFα does not generalize to all molecules that decrease GLAST expression as endothelin 1 (ET1) decreases GLAST protein levels even in the presence of dbcAMP, PACAP, EGF or TGFα (Rozyczka, Figiel & Engele, 2004). Although a role for ET1-dependent regulation of GLAST has not been defined *in vivo*, increases in ET1 are correlated with decreases in GLAST levels observed in Alzheimer's disease (Luo & Grammas, 2010).

Environmental toxins also decrease GLAST expression. For example, chronic manganese exposure has been associated with a Parkinsonian-like disease (Kwakye, Paoliello, Mukhopadhyay, Bowman & Aschner, 2015). In astrocytes in culture, Mn causes a decrease in GLAST expression that is associated with an increase in TNFα. The decrease is blocked by an inhibitor of TNFα synthesis or a receptor antagonist (Lee et al, 2009). Arsenic exposure has also been associated with neurological dysfunction (e.g. impaired learning and memory, mood disorders and diminished IQ) (for a review see Tyler & Allan, 2014). Arsenite decreases GLAST expression, transport activity, and increases the binding of the transcription factors Nrf2 and AP-1 to the GLAST promoter (Castro-Coronel, Del Razo, Huerta, Hernandez-Lopez, Ortega & Lopez-Bayghen, 2011).

In summary, several different extrinsic signals regulate GLAST protein and mRNA. It is assumed that these effects are dependent upon increased transcription, but in most cases this has not been formally demonstrated. Several different signaling pathways have been implicated in this regulation (see Figure 2), but it is not clear if these pathways function independently. There is also a need to carefully define the specific transcription factors involved and the *cis* promoter elements required. At least two different sets of evidence suggest that different populations of astrocytes employ different mechanisms to control expression of GLAST (Gegelashvili, Civenni, Racagni, Danbolt, Schousboe & Schousboe, 1996, Schluter et al, 2002). Therefore it is likely that as different subpopulations of astrocytes are molecularly characterized (for discussion see Matyash & Kettenmann, 2010, Rusnakova, Honsa, Dzamba, Stahlberg, Kubista & Anderova, 2013, Schitine, Nogaroli, Costa & Hedin-Pereira, 2015, Walz, 2000, Zhang & Barres, 2010), it will be possible to link differential control of GLAST expression to these subtypes.

3.2 Transcriptional Regulation of SLC1A2/GLT-1/EAAT2

The human GLT1 gene (SLC1A2) was mapped to chromosome 11 bands p13-p12 (Li & Francke, 1995). The mouse GLT-1 gene (Slc1a2) was mapped to the middle region of chromosome 2 (Kirschner, Copeland, Gilbert, Jenkins & Amara, 1994). As is observed with GLAST, the gene contains 10 exons that range from 127 bp to 251 bp and there is no TATA box in the proximal promoter. There is a GC box with five Sp1 binding sites (Su, Leszczyniecka, Kang, Sarkar, Chao, Volsky et al, 2003), but it is not known if these sites are required for GLT-1 expression. As is true for GLAST, when a bacterial artificial chromosome containing the human SLCA2 gene is used to control expression of enhanced green fluorescent protein (eGFP), expression of reporter closely correlates with GLT-1 expression in transgenic mice (Regan et al, 2007). This suggests that transcriptional control is similar between the two species, and it suggests that studies of evolutionarily conserved domains in the promoter region may be informative.

While GLAST and GLT-1 are both enriched in astrocytes, GLT-1 expression uniquely correlates with synaptogenesis (Furuta et al, 1997), suggesting that GLT-1 is a marker of astrocyte maturation. When astrocytes are maintained in culture they assume a polygonal (fibroblast-like) shape. Almost forty years ago dbcAMP was demonstrated to induce a dramatic change in astrocyte morphology to a more stellate shape that is somewhat similar to that observed with mature astrocytes *in vivo* (Moonen, Heinen & Goessens, 1976). Two

groups essentially simultaneously realized that rat astrocytes in culture express little or no GLT-1 protein, but co-culturing astrocytes with neurons induces expression of GLT-1 in astrocytes (Schlag et al, 1998, Swanson et al, 1997). In fact, several subsequent studies have documented low levels of GLT-1 in mouse astrocyte cultures, but neurons also increase GLT-1 transcription in this system (Apricó, Beart, Crawford & O'Shea, 2004, Ghosh, Lane, Krizman, Sattler, Rothstein & Robinson, 2015, O'Shea, Lau, Farso, Diwakarla, Zagami, Svendsen et al, 2006). The effect of neurons is at least in part dependent upon soluble factors but it may also depend on contact (Drejer, Meier & Schousboe, 1983, Gegelashvili et al, 1997, Gegelashvili et al, 2000, Yang, Gozen, Watkins, Lorenzini, Lepore, Gao et al, 2009, Zelenaia et al, 2000). dbcAMP mimics this effect of neurons (Eng et al, 1997, Schlag et al, 1998, Swanson et al, 1997). Interestingly, inducing stellation using inhibitors of Rho kinase inhibitors also increases GLAST and GLT-1 protein, although the effect on GLT-1 is much larger (Lau, O'Shea, Broberg, Bischof & Beart, 2011).

Figiel and colleagues tested PACAP as a potential mediator of the effect of neurons because it was known that neurons release PACAP and that PACAP activates adenylate cyclase (Figiel et al, 2000). They showed that anti-PACAP directed antibodies or a PACAP receptor (PAC_1 receptor) antagonist block the effects of neuron-conditioned media (Figiel et al, 2000). Using pharmacological approaches, they also demonstrated that blocking either PKA or PKC attenuate the effects of PACAP, but a PKC inhibitor had a bigger effect. Inhibitors of NFκB also block PACAP-dependent induction of GLT-1 (Figiel et al, 2003). Expression of dominant-negative inhibitors of NFκB in astrocytes blocks neuron-dependent induction of GLT-1 (or eGFP under the control of a bacterial artificial chromosome GLT-1 promoter) (Ghosh, Yang, Rothstein & Robinson, 2011). Exogenous expression of either of two different NFκB subunits, p65 or p50, induce expression of GLT-1 and both subunits interact with the GLT-1 promoter *in vivo* as demonstrated with ChIP. This interaction was not observed in a tissue that does not express GLT-1 protein. While it seems logical that activation of cAMP and PKA would signal through CREB there is no evidence that CREB is activated by PACAP in astrocytes, however, neuron-conditioned media or cAMP increase CREB phosphorylation (activation) in astrocytes (Gegelashvili et al, 2000, Schluter et al, 2002). The cAMP/PKA/CREB pathway has also been linked to expression of GLT-1 *in vivo*. Using chronic unpredictable stress to create an animal model of depression, Liu and colleagues observe decreases in cAMP, the catalytic subunit of PKA, phospho-CREB and GLT-1 levels (Liu, 2015). An inhibitor of phosphodiesterase type 4, prevents all of these changes, strongly implicating this pathway in the regulation of GLT-1 *in vivo*. They also find that inhibition of phosphodiesterase partially corrects the 'depression phenotype' suggesting that down-regulation of GLT-1 may contribute to the pathology of this disease. Together these studies suggest that the cAMP/PKA/CREB signaling pathway contributes to GLT-1 regulation *in vitro* and *in vivo*, but there is also dependence of NFκB signaling (see Figure 3A). It is not known if these two transcription factors function together or independently.

As is observed with GLAST, growth factors also increase GLT-1 expression. EGF or TGFα increase GLT-1 mRNA, protein levels, and GLT-1-mediated uptake in cultured cortical astrocytes. These effects are blocked by inhibitors of receptor tyrosine kinase, PI3K, or NFκB (Zelenaia et al, 2000). In a later study, we demonstrated that expression of a dominant-negative variant of Akt kinase blocks the effect of EGF and expression of a

constitutively active form of Akt mimics the effect of EGF (Li, Toan, Zelenaia, Watson, Wolfe, Rothstein et al, 2006). These studies are consistent with a growth factor receptor/PI3K/Akt/NFκB pathway regulating transcription of GLT-1 (see Figure 3A). The effects of EGF and TGFα on GLT-1 expression have been replicated by others (Figiel et al, 2003). We initially found that platelet-derived growth factor (PDGF) increases GLT-1 protein levels in astrocyte-enriched cultures, but these effects were associated with an increase in the number of $A2B5^+$ oligodendroglial precursor cells that express GLT-1 (Zelenaia et al, 2000). We observed no effect of PDGF on GLT-1 expression in astrocyte cultures devoid of these cells (Zelenaia et al, 2000), but others have reported that PDGF increases GLT-1 protein levels (Figiel et al, 2003). There are also differences in the effects of GDNF and brain-derived neurotrophic factor (BDNF). While neither GDNF nor BDNF have an effect on GLT-1 in one study (Figiel et al, 2003), other groups have shown that GDNF (Bonde et al, 2003) or BDNF (Rodriguez-Kern, Gegelashvili, Schousboe, Zhang, Sung & Gegelashvili, 2003) increase expression of GLT-1. The effects of BDNF are blocked by pharmacological inhibition of the ERK/NFκB signaling pathway (Rodriguez-Kern et al, 2003). In addition, the work from Lau and colleagues shows that in stellated astrocytes GLT-1 and BDNF are co-regulated (Lau, Kovacevic, Tingleff, Forsythe, Cate, Merlo et al, 2014, Lau, Perreau, Chen, Cate, Merlo, Cheung et al, 2012). These differences may reflect differential regulation of GLT-1 in different populations of astrocytes. It is also possible that the presence of neurons changes the response of astrocytes to these stimuli. Cortical astrocytes were used in our studies and those of Rodriguez-Kern, while Figiel and colleagues used forebrain astrocytes. Bonde and colleagues used organotypic slice cultures (Bonde et al, 2003, Figiel et al, 2003, Rodriguez-Kern et al, 2003, Zelenaia et al, 2000). It is not clear if all of the effects of PDGF are related to increased proliferation of the $A2B5^+$ cells or if PDGF also has a direct effect on GLT-1 expression in astrocytes.

Estrogen also increases GLT-1 expression (Pawlak et al, 2005). The effects of estrogen are mediated through both nuclear receptors (ERα, ERß) and G-protein coupled receptor, GPR30. Activation of estrogen receptors increases binding of the transcription factors CREB and NFκB to GLT-1 promoter in EMSA and chromatin immunoprecipitation (ChIP) assays (Karki, Webb, Smith, Lee, Son, Aschner et al, 2013, Karki et al, 2014). Mutation of the putative binding site for CREB (−308), or mutation of all three NFκB binding sites (−251, −272 and −583) in the GLT-1 promoter block estrogen- or dbcAMP-dependent activation of promoter reporter constructs (Lee et al, 2012). The effects of estrogen are, at least in part dependent on estrogen-dependent up-regulation of TGFα that in turn serves as an autocrine factor to regulate GLT-1 expression by the MEK/ERK and PI3K/Akt signaling pathways (Karki et al, 2014, Lee et al, 2012). Other selective estrogen receptor modulators (tamoxifen and raloxifene) also increase TGFα mRNA and protein levels (Karki et al, 2013). Exogenous/over-expression of CREB, p65, or p50 activate the TGFα and the GLT-1 promoters in promoter-reporter assays. Together these studies suggest that estrogen regulates GLT-1 expression through two mechanisms. First, it up-regulates expression of TGFα which in turn activates PI3K/Akt/NFκB and MEK/ERK/NFκB signaling pathways. It also appears that estrogen activates GLT-1 expression through GPR30/cAMP/PKA/CREB signaling pathway (see Figure 3A). Several neuroprotective roles had been attributed to estrogen (for discussions see Karki et al, 2014, Simpkins, Singh, Brock & Etgen, 2012), the previous

results suggest that some of these neuroprotective roles may be associated with the induction of GLT-1 expression.

Glucocorticoids also increase GLT-1 mRNA and protein levels (Autry, Grillo, Piroli, Rothstein, McEwen & Reagan, 2006, Zschocke, Bayatti, Clement, Witan, Figiel, Engele et al, 2005). The effect of the synthetic glucocorticoid, dexamethasone is blocked by antagonists of either the glucocorticoid or the mineralocorticoid receptors (GR and MR, respectively)(Figure 3A). The downstream signals or transcription factors involved in the regulation have not been identified, but it is thought that chronic stress results in glucocorticoid-dependent up-regulation of GLT-1 (Autry et al, 2006, Reagan, Rosell, Wood, Spedding, Munoz, Rothstein et al, 2004).

Both ATP (Frizzo, Frizzo, Amadio, Rodrigues, Perry, Bernardi et al, 2007) and adenosine (Wu, Lee, Kim, Johng, Rohrback, Kang et al, 2011) increase GLT-1 expression. Pharmacological approaches demonstrated that P2Y (ATP receptors) and A1 (adenosine receptors) mediate these effects. An inhibitor of ERK signaling blocks the effects of ATP. As described above, the effects of ERK activation are blocked by inhibitors of NFκB. This suggests that the effects of ATP depend upon NFκB, but this has not been examined.

As an alternate approach to identifying substances that activate cell surface receptors or manipulate intracellular signaling molecules, we and others have identified evolutionarily conserved domains with the 5' non-coding region of Slc1a2 and used this information to identify cis-elements or transcription factors that control transcription. As indicated above, this strategy has been validated with bacterial artificial chromosome mice. For example, we identified sequences within the proximal promoter region (which is highly conserved, see Figure 1) that are required for neuron-dependent expression of reporter in astrocytes (Yang et al, 2009). Through sequential deletion and site-directed mutagenesis a region (−688 to −679) that contains *cis*-elements essential for GLT-1 promoter activity was identified in the proximal promoter. Using this sequence as 'bait', mass spectrometry was used to identify kappa B-motif binding phosphoprotein (KBBP) as a transcription factor that binds to this region. Knock down of KBBP was shown to reduce GLT-1 or reporter activity in mice engineered to express eGFP under the control of the complete human GLT-1 gene. Decreased expression of KBBP correlates with the loss of eGFP observed in ricin-induced lesions or in an animal model of ALS.

Allritz and colleagues examined basal promoter activity upon transduction of rat or fetal human astrocytes (Allritz, Bette, Figiel & Engele, 2010). They found that deletions of nucleotides -216 through -502 in human promoter or -399 through -557 of the rat promoter sequence dramatically reduce reporter activity (Allritz et al, 2010). It is somewhat unclear how to interpret these results because cultured rat astrocytes, unlike cultured mice astrocytes, don't normally express much GLT-1. Therefore, these elements may or may not be important for the increase in GLT-1 that is observed upon astrocyte maturation.

While the proximal 2.5kb promoter of GLT-1 gene is highly conserved and has been well characterized, there are several additional evolutionary conserved domains distal to this region out to ~12.5kb from the translation start site (Figure 1) (Ghosh et al, 2015). Analyses

of promoter reporter mice generated by Rothstein and his colleagues have revealed that the proximal 7.9 kb of the promoter is not sufficient to direct astrocytic expression of reporter protein, reporter is observed mostly in neurons (Rothstein unpublished observations; for discussion, see Ghosh et al, 2015). When 8.3 kb of promoter is used to control expression, reporter protein is essentially exclusively found in astrocytes, but not all astrocytes express the reporter (Yang, Vidensky, Jin, Jie, Lorenzini, Frankl et al, 2011). These studies have three implications. First, they suggest that the region between 7.9 and 8.3 kb is required to direct astrocytic expression. In fact, we recently showed that Pax6 interacts with this region *in vitro* (EMSA) and *in vivo* (ChIP). ShRNA directed knockdown of Pax6 attenuates neuron-dependent induction of GLT-1 and exogenous expression of Pax6 increases GLT-1 expression (Ghosh et al, 2015). Secondly, these data suggest that different subtypes of astrocytes engage different mechanisms to control expression of GLT-1 *in vivo*. A similar conclusion has been drawn from *in vitro* analyses (Drejer et al, 1983, Gegelashvili et al, 1996, Schluter et al, 2002). Finally, these studies suggest that the evolutionarily conserved domains that are distal to 8.3 kb are important for expression of GLT-1 in a subtype of astrocytes. This has not been explored.

Rothstein and colleagues used a screen of 1,040 FDA-approved drugs, to identify β-lactam antibiotics that increase GLT-1 levels. To understand the mechanism, they used reporter promoter assays *in-vitro* and *in-vivo*, and found that this promoter was activated by the β-lactam antibiotics ceftriaxone and amoxicillin (Rothstein, Patel, Regan, Haenggeli, Huang, Bergles et al, 2005). They also demonstrated that ceftriaxone induces neuroprotection in mouse models of oxygen glucose deprivation, threo-hydroxyaspartate-induced motor neuron loss, and in a mouse model of ALS with the gene of superoxide dismutase 1 mutated (Rothstein et al, 2005). Since this time over 100 papers have been published most of them demonstrate a neuroprotective role of ceftriaxone and other β-lactam antibiotics (for review see Fontana, 2015, Soni et al, 2014). However the molecular mechanism responsible the effect are not well understood. Lee and colleagues demonstrated that NFκB inhibitors block ceftriaxone-dependent GLT-1 expression (Lee, Su, Emdad, Gupta, Sarkar, Borjabad et al, 2008). They showed that ceftriaxone increases the binding of NFκB to GLT-1 promoter using EMSA. They demonstrated that mutation of GLT-1 promoter at −272 blocks the effect of ceftriaxone but is also reduces basal activity. Finally, they demonstrated that ceftriaxone induces translocation of p65 to the nucleus and the degradation of IkBa (Lee et al, 2008). These results strongly suggest that NFκB is responsible for the effect of ceftriaxone, however it is still not known how ceftriaxone activates NFκB.

Besides ceftriaxone, several other drugs that increase GLT-1 expression are also neuroprotective. A similar screening assay was used to identify harmine, a β -carboline alkaloid, which increases GLT-1 protein and mRNA levels (Li, Sattler, Yang, Nunes, Ayukawa, Akhtar et al, 2011). Riluzole, an anti-convulsant agent, also increases GLT-1 protein and Glu uptake (Azbill, Mu & Springer, 2000, Carbone, Duty & Rattray, 2012). Together these data suggest that increasing the expression of GLT-1 may be neuroprotective, but there is evidence that over-expression of GLT-1 can also exacerbate the damage observed with certain acute insults (Li, Hala, Seetharam, Poulsen, Wright & Lepore, 2015, Poulsen, Schousboe, Sarup, White & Schousboe, 2006).

Not surprisingly, GLT-1 transcription is also regulated in other subtypes of glia. In some cases the regulation appears to parallel that observed in astrocytes. For example, neuron conditioned medium increases Glu uptake and GLT-1 protein in primary cultures of microglia (Nakajima, Yamamoto, Kohsaka & Kurihara, 2008). In other cases, the regulation is opposite to that observed in astrocytes at least *in vitro*. While TNFα decreases GLT-1 expression in most experiments using astrocytes (Boycott, Wilkinson, Boyle, Pearson & Peers, 2008, Sitcheran, Gupta, Fisher & Baldwin, 2005, Su et al, 2003), it increases expression of GLT-1 in activated microglia (Persson, Brantefjord, Hansson & Ronnback, 2005). In oligodendroglia, TNFα decreases GLT-1 expression (Pitt et al, 2003). In patients, with multiple sclerosis, the levels of GLT-1 in oligodendrocytes are decreased in areas of active lesions (Pitt et al, 2003). The fact that TNFα levels are increased in these same lesions suggests that TNFα may contribute to the loss of GLT-1 in these patients.

Some of the transcription factor(s) that underlie suppression of GLT-1 expression in astrocytes have been identified. Although NFκB binding to the promoter contributes to activation (see above), Sitcheran and colleagues used both EMSA and DNA-based affinity purification to demonstrate that TNFα increases NFκB binding to GLT-1 promoter (Sitcheran et al, 2005). These results support a bidirectional regulation of GLT-1 by NFκB. When it is activated by EGF/TGFα (or presumably neurons), it increases GLT-1 expression, however when activated by TNFα, it decreases GLT-1 expression. Sitcheran and colleagues found that TNFα increases the binding of N-myc to the GLT-1 promoter. This transcription factor also contributes to GLT-1 repression, as its overexpression decreases basal and NFκB-induced activation of GLT-1 (Sitcheran et al, 2005). Together these studies suggest that the interaction of NFκB with other transcription factors may regulate the direction of the effect of NFκB. The TNFα-dependent repression of GLT-1 has been associated with the decrease in GLT-1 expression observed after hypoxia (Boycott et al, 2008). The interaction of N-myc with the promoter is high at post-natal day 0 and decreases as GLT-1 expression increases during development (Gupta & Prasad, 2014). Thus, it is possible that repression of the GLT-1 promoter may contribute to the low expression observed early in development.

In addition to NFκB and N-myc, TNFα also increases the binding of the transcription factor YY1 to the GLT-1 promoter. Exogenous expression of YY1 decreases GLT-1 promoter activity. Furthermore, mutation of the 'putative' YY1 binding site in the GLT-1 promoter or expression of siRNA directed against YY1 increases GLT-1 promoter activity, suggesting that YY1 represses basal GLT-1 expression (Karki, Webb, Smith, Johnson, Lee, Son et al, 2014). Interestingly NFκB is itself a regulator of YY1 expression as exogenous expression of p65 activates the promoter of YY1 (Karki et al, 2014)(Figure 3B). Exogenous expression of p65 increases GLT-1 promoter activity, however when p65 is expressed with YY1 there is a decrease in GLT-1 promoter activity (Karki et al, 2014). Thus as is observed with N-myc, binding of YY1 to the GLT-1 promoter switches the effect of NFκB from activation to suppression.

TNFα also increases the expression of the chemokine, macrophage inflammatory protein-2γ (MIP2γ) in astrocytes (Fang, Han, Hong, Tan & Tian, 2012). Exogenous expression of MIP2γ decreases GLT-1 mRNA and protein, localization of GLT-1 in raft domains and Glu uptake. In fact, inhibition of signaling pathways that normally activate the GLT-1 promotor

(e.g. NFκB, PI3K, PKA, MEK/ERK), block MIP2γ-dependent suppression of the promoter (Fang et al, 2012)(see Figure 3). This suggests that the ability of TNFα to switch activation to repression may extend to other signals.

Other extracellular stimuli also seem to switch signals that normally result in promoter activation to signals that suppress the GLT-1 promoter. For example, endothelins decrease GLT-1 protein levels and this effect is blocked by an inhibitor of PKA (Rozyczka et al, 2004). Dopamine also decreases GLT-1 protein and mRNA in astrocytes isolated from striatum (Brito, Rozanski, Beyer & Kuppers, 2009). Using pharmacological tools the authors demonstrate that D1 receptors mediate this effect. Although the downstream signaling components responsible for GLT-1 repression have not been identified, D1 receptors are normally coupled to increased cAMP and might be expected to activate the PKA signaling pathway (Figure 3B).

Several signals suppress GLT-1 expression. For example, retinoic acid or a specific retinoid × receptor (RXR) agonist decrease GLT-1 levels (Chan, Her, Liaw, Chen & Tzeng, 2012). The authors demonstrate that retinoic acid increases the binding of RXR to the retinoic acid response element (RARE) at −632 to −612 of GLT-1 promoter using EMSA (Chan et al, 2012). Aronica and colleagues demonstrated that DHPG, a specific agonist of group 1 mGluRs, decreases GLT-1 protein levels (Aronica et al, 2003). Antagonists of mGluR1 block the loss of GLT-1 observed after transient global ischemia, suggesting the mGluR1 activation may stimulate the loss of GLT-1 observed with these insults (Chen, Hsu-Chou, Lu, Chiang, Huang, Wang et al, 2005).

Amyloid-β (Aβ) peptides, the major component of amyloid plaques observed in Alzheimer's disease, decrease Glu uptake and GLT-1 protein levels. Aβ increases the phosphorylation/activation of ERK, JNK and p38 MAPK. p38 MAPK is activated by oxidative stress, accordingly trolox, an antioxidant, blocks the Aβ-dependent decrease in Glu uptake (Matos, Augusto, Oliveira & Agostinho, 2008). Human cytomegalovirus infection can result in birth defects that affect primarily the CNS. Infection of astrocytes with this virus decreases expression of GLT-1, GLAST and glutamine synthetase. Except for the fact that inhibition of PKC blocks these effects, nothing is known about the mechanisms involved (Zhang, Li, Wang, Qian, Song & Hu, 2014). Down-regulation of GLT-1 also has been implicated in human immunodeficiency virus (HIV)-associated dementia (Wang, Pekarskaya, Bencheikh, Chao, Gelbard, Ghorpade et al, 2003). An HIV-inducible gene, astrocyte-elevated gene (AEG), decreases GLT-1 promoter activity. The phosphatase and tensin homolog (PTEN), a negative regulator of PI3K/Akt signaling, mimics the effect of AEG (Kang, Su, Sarkar, Emdad, Volsky & Fisher, 2005).

From these analyses, it has become clear that many different signals can increase transcription of GLT-1 and under certain circumstances these signals can switch from induction to suppression. Many of these signals have been implicated in the loss of GLT-1 that is observed in various neurologic insults. Although no unifying concepts have emerged, it seems likely that these studies will provide mechanistic insights into the pathogenesis of various disease processes. Several groups are also focused on therapeutically targeting GLT-1 expression (for recent review, see Takahashi, Foster & Lin, 2015).

3.3 Transcriptional Regulation of SLC1A1/EAAC1/EAAT3

The gene that encodes human EAAC1 (SLC1A1) was localized to chromosome 9 band p24 using fluorescence *in situ* hybridization (Smith, Weremowicz, Kanai, Stelzner, Morton & Hediger, 1994). The mouse homolog is located in chromosome 19 at the centromere (http://www.ncbi.nlm.nih.gov/gene). In comparison to GLAST and GLT-1, there is much less evolutionarily conserved sequence in the 5' non-coding region for the EAAC1 gene (Figure 1). To date, no bacterial artificial chromosome EAAC1 reporter mice have been generated. Therefore, it is possible that the elements required for *in vivo* expression are relatively small. It is also possible that the control of EAAC1 expression is different in mice and humans. In fact, there is one example of differential expression (see below).

Several studies have suggested that EAAC1 may be more important for the synthesis of the anti-oxidant glutathione than for the clearance of neurotransmitter pools of Glu by importing cysteine (and possibly Glu) (Aoyama & Nakaki, 2013). For example, in mice deleted of EAAC1 there is a delayed neuronal degeneration that is associated with decreased glutathione; this damage is blocked by N-acetylcysteine (Aoyama, Suh, Hamby, Liu, Chan, Chen et al, 2006, Berman, Chan, Brennan, Reyes, Adler, Suh et al, 2011). Consistent with this general role of EAAC1, evolutionarily conserved antioxidant responsive elements (ARE) are found in the promoter (Escartin, Won, Malgorn, Auregan, Berman, Chen et al, 2011). One of the transcription factors that bind to these elements is nuclear factor (erythroid-derived 2)-like 2 (Nrf2). In fact, activators of Nrf2 or exogenous expression of Nrf2 increase EAAC1 expression in C6 glioma cells that endogenously express EAAC1 and not the other transporters (Escartin et al, 2011). They also demonstrated that Nrf2 binds to the ARE sequence in the EAAC1 promoter *in vivo* (Figure 4). Selective expression of Nrf2 in neurons *in vivo* increases both EAAC1 and glutathione levels (Escartin et al, 2011).

Ma and colleagues identified a binding site for the regulatory factor X1 (RFX1) in human EAAC1 promoter. Using C6 and SH-SY5Y cell lines, the authors demonstrated that transfection of RFX1 increases both EAAC1 protein levels and activates a promoter reporter. In addition knockdown of RFX1 decreases EAAC1 expression in cultured rat cortical neurons (Ma, Zheng & Zuo, 2006).

Bianchi and colleagues demonstrated that all trans retinoic acid (ATRA) treatment increases EAAC1 mRNA and protein levels in C6 glioma (Bianchi, Gazzola, Tognazzi & Bussolati, 2008)(Figure 4). An agonist for the retinoic acid receptor β (RARβ) or exogenous expression of this receptor mimics the effect of ATRA. It appears that this effect is dependent on synthesis of an intermediary protein as a protein synthesis inhibitor blocks the ATRA-dependent increase in mRNA. RARβ expression increases after ATRA treatment, suggesting that RARβ may be the intermediate of ATRA-dependent EAAC1 increase. Accordingly they identified two putative-binding sites for RARβ (at −191 and −2696) in EAAC1 rat promoter (Bianchi, Gazzola, Cagnin, Kagechika & Bussolati, 2009). This site is not evolutionarily conserved; therefore it is not clear if these effects will extend to humans.

As mentioned above, neurons regulate expression of the astrocytic transporters. Although essentially nothing is known about the mechanism, there is a reciprocal interaction; astrocyte-conditioned media increases expression EAAC1 (Canolle, Masmejean, Melon,

Nieoullon, Pisano & Lortet, 2004). It is also interesting to note that the circadian rhythm changes EAAC1 expression in a region-dependent fashion (Cagampang, Rattray, Powell, Chong, Campbell & Coen, 1996). The signaling pathway (s), transcription factor(s) or *cis*-elements responsible of this regulation have not been identified. Finally, EAAC1 levels are decreased in mice deleted of mGluR2, suggesting that mGluR may regulate basal expression of EAAC1 (Lyon et al, 2008).

3.4 Transcriptional Regulation of SLC1A6/EAAT4 and SLC1A7/EAAT5

The human EAAT4 gene (SLC1A6) localizes to chromosome 19 band 13.12. The mouse gene (Slc1a6) is mapped in chromosome 10 in the centromeric region (http://www.ncbi.nlm.nih.gov/gene). Gincel and colleagues generated promoter reporter mice using a bacterial artificial chromosome containing the human EAAT4 gene plus 107kb of upstream sequence and 54kb downstream of the last exon (Gincel, Regan, Jin, Watkins, Bergles & Rothstein, 2007). The expression of reporter protein correlates with EAAT4 protein, suggesting that there is evolutionary conservation of transcriptional regulation. Essentially nothing is known about the transcriptional regulation of EAAT4, except that subjecting rats to chronic restraint stress lowers EAAT4 protein levels (Zink, Vollmayr, Gebicke-Haerter & Henn, 2010).

The human EAAT5 gene (SLC1A7) localizes to chromosome 1 band 32.3, and the mouse gene (Slc1a7) is mapped in the centromere of chromosome 4 (http://www.ncbi.nlm.nih.gov/gene). Nothing is known about the events that control transcriptional regulation of EAAT5.

3.5 Epigenetic Regulation

As is true for transcriptional regulation, most studies of epigenetic regulation have focused on just two transporters, GLAST and GLT-1. There is evidence that methylation contributes to the different expression patterns and localization during brain development (Danbolt, 2001, Freeman, 2010, Furuta et al, 1997, Perisic, Holsboer, Rein & Zschocke, 2012).

DNA methylation is mediated by a family of DNA methyltransferases (DNMT), these enzymes transfer a methyl group from S-adenosyl-L-methionine to the carbon 5 of cytosine. Normally this methylation occurs in CpG islands which are defined by repeats of the nucleotides cytosine and guanine that occur 10–20 times more frequently than would be expected to occur by chance (e.g. 1 in 16). Generally it is thought that hyper-methylation reduces transcription and hypo-methylation increases transcription (Robertson & Wolffe, 2000); methylation may preclude binding of transcription factors (Perisic et al, 2012).

The GLT-1 promoter has several CpG islands (−1473 to −1146, −685 to −491, −247 to −20, etc) that are methylated (Yang, Gozen, Vidensky, Robinson & Rothstein, 2010, Zschocke, Allritz, Engele & Rein, 2007), and as expected there are several evolutionarily conserved putative transcription factor binding sites in these regions (Su et al, 2003). There are a couple of studies to suggest that demethylation of the GLT-1 promoter is required for transcriptional activation. First, co-culturing neurons with astrocytes reduces methylation of the GLT-1 promoter and differential methylation is associated with different gel shifts by EMSA (Yang et al, 2010). Second, differential methylation of the GLT-1 promoter also contributes to the region-specific effects of glucocorticoids; in forebrain, where the promoter

is hypo-methylated, they up-regulate GLT-1 expression and in brainstem/cerebellum, where the region is hyper-methylated, they have no effect (Zschocke et al, 2005)(Figure 3).

Several diseases that result in lower transporter expression are associated with changes in methylation. For example, hypermethylation of the GLT-1 promoter is observed in brain tumors. It has been suggested that this contributes to the decreased expression of GLT-1 observed with some of these tumors and that this decrease allows for excitotoxic expansion of the tumor (Groot, Liu, Fuller & Yung, 2005). Abnormal control of methylation underlies the basis of Rett syndrome, a neurodevelopmental disorder caused by mutations in the DNMT methyl-CpG-binding protein 2 (MeCP2), that results in dysregulation of both GLAST and GLT-1 (Amir, Van den Veyver, Wan, Tran, Francke & Zoghbi, 1999, Dunn & MacLeod, 2001, Guy, Hendrich, Holmes, Martin & Bird, 2001, Okabe, Takahashi, Mitsumasu, Kosai, Tanaka & Matsuishi, 2012). In patients who have died of ALS, there is evidence that hypermethylation of the GLT-1 promoter correlates with the decrease of GLT-1 expression (Yang et al, 2010).

Histone modifications also play an important role in epigenetic regulation. Two enzymes carry out histone modifications: histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs acetylate the ε-amino group of lysine residues in histones, and HDACs remove this acetyl group (for review see Kuo & Allis, 1998).

HDAC inhibitors like trichostatin A and valproic acid (VPA) increase the expression of EAATs. For example, VPA increases the levels of GLAST, GLT-1 and EAAC1 mRNA and protein in astrocytes and oligodendrocytes (Bianchi, Franchi-Gazzola, Reia, Allegri, Uggeri, Chiu et al, 2012, Hassel, Iversen, Gjerstad & Tauboll, 2001, Rosas et al, 2007). The fact that only 1 to 7% of all genes are thought to be regulated by HDACs (Butler & Bates, 2006) and that all the EAATs that had been studied to date are regulated by HDACs suggest that epigenetic regulation of this family of transporters may be important.

In addition to modification of histones, HATs and HDAC also acetylate and de-acetylate transcription factors. VPA decreases binding of the transcription factor YY1 to the GLAST promoter (Aguirre, Rosas, Lopez-Bayghen & Ortega, 2008) and increases GLAST mRNA and protein levels in cerebellum and hippocampus (Hassel et al, 2001, Rosas et al, 2007, Ueda & Willmore, 2000)(Figure 2A). There is also evidence that the HATs, p300 or p300/CBP associated factor, acetylate YY1, while HDACs de-acetylate YY1 (Yao, Yang & Seto, 2001). Although VPA is not a particularly selective drug, it is tempting to speculate that VPA regulates GLAST expression by modulating acetylation of YY1. This has not been directly tested.

VPA also regulates the expression of GLT-1, but the direction of the effect is region dependent. It increases expression in cortex and hippocampus and decreases expression in cerebellum (Perisic et al, 2012). There is evidence that this effect is influenced by methylation of the promoter; thus interactions between methylation and acetylation likely regulate GLT-1 expression.

4. Conclusions and summary

During the last 15 years, several groups examined the signals that regulate expression of the two astroglial Glu transporters. Together these studies identify a complex web of signals that either up- or down-regulate expression of these transporters. In some cases, these signals converge on seemingly common transcription factors. In other cases, the direction of an effect caused by one signal can be switched by the presence of a second signal. While many of the signals regulate both transporters, there are differences that may underlie the unique maturation-associated increases in GLT-1. Although it has not been the topic of extensive analysis, it seems likely that different populations of astrocytes engage different mechanisms to control expression of these transporters. Developing an understanding of the mechanisms involved may lead to new insights into the mechanisms that generate astrocyte heterogeneity. Epigenetic modifications seem likely to contribute to this differential control but this is still relatively unexplored. Virtually every neurologic disease is associated with altered expression of one or both of these transporters. Several recent studies have implicated specific pathways in the loss of transporters observed in various models of disease. While there is hope that this approach will lead to new therapies, it will certainly help define pathways that are dysregulated and thereby presumably lead to a better mechanistic understanding of the pathogenesis of disease. Remarkably, there have been relatively few analyses of the other three transporters. It is somewhat surprising that there have been so few analyses of EAAC1. This might be an ideal target given its known role in limiting oxidant-mediated damage, however the low evolutionary conservation in the promoter region may be hindering the identification of *cis* and *trans* elements involved in the regulation of transcription. This low conservation may also have implications for extensions to humans.

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I (Michael Robinson) would also like to thank Dr. Joseph Coyle for his mentorship and friendship. In early 1985, I accepted a post-doctoral position in his laboratory. Upon sharing this information with colleagues, I was immediately impressed by the fact that I repeatedly heard “What a great guy”. Of course, I also knew that I had been fortunate to land a position with an outstanding scientist. Joe and his group (including one of the editors of this special volume) were among the first investigators to demonstrate that excessive activation of Glu receptors is toxic to neurons; they also defined many of the mechanisms involved in this cell loss. Joe had also been involved in pioneering analyses of the monoamine transporters, the targets of many psychoactive molecules. I joined Joe’s laboratory to study the acidic dipeptide N-acetylaspartylglutamate (a topic of two chapters in this volume). During this time, many lasting friendships were formed. When a faculty position came along, I decided to establish a laboratory focused on analyses of Glu transporters because it was becoming clear that ‘excitotoxicity’ was likely involved in virtually all neurologic diseases. By this time, it had also become clear that Glu transporters were the only mechanism to clear extracellular Glu. Joe, our kids, and a group of colleagues have been enjoying an annual ‘retreat’ for just about 20 years doing the same thing that he and I did our Dads. I couldn’t have made a better choice for my post-doctoral position. I am grateful for his friendship and his unassuming inspirational style.

Abbreviations

Aβ	Amyloid beta
AEG	Astrocyte elevated gene-1
ALS	Amyotrophic Lateral Sclerosis

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP-1	Activating protein-1
ARE	Antioxidant response element
ATRA	All trans retinoic acid
BDNF	Brain derived neurotrophic factor
bFGF	Basic fibroblast growth factor
ChIP	Chromatin immunoprecipitation
CREB	cAMP response element binding protein
dbcAMP	dibutyryl-cyclic AMP
DNMT	DNA methyltransferase
dsRFP	Discosoma red fluorescent protein
EAAC1	excitatory amino acid carrier-1
EAATs	excitatory amino acid transporters
EGF	Epidermal growth factor
eGFP	enhanced green fluorescent protein
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular signal-regulated kinase
ET1	Endothelin 1
FGF	Fibroblast growth factor
GDNF	Glial cell line-derived neurotrophic factor
GLAST	Glutamate/aspartate transporter
GLT1	Glutamate transporter 1
Glu	L-Glutamate
GPR30	G protein-coupled receptor 30
HAT	Histone acetyltransferase enzyme
HDAC	Histone deacetylases
HIV	Human immunodeficiency virus
IGF-1	Insulin-like growth factor-1
iGluRs	Ionotropic glutamate receptors

JAK	Janus kinase
KBBP	kappa-B motif-binding phosphoprotein
LPS	lipopolysaccharide
MeCP2	Methyl CpG binding protein 2
mGluRs	Metabotropic glutamate receptors
NFκB	Nuclear factor kappa-B
Nrf2	Nuclear factor (erythroid-derived)-like 2
MEK	Mitogen-extracellular signal regulated kinase
PACAP	Pituitary adenylate cyclase-activating polypeptide
PDGF	Platelet-derived growth factor
PI3K	Phosphatidylinositol-4,5-bisphosphate 3 kinase
PKA	Protein kinase A
PKC	Protein kinase C
PTEN	Phosphatase and tensin homolog
RARβ	Retinoic acid receptor β
RARE	Retinoic acid response element
RFX1	Regulatory factor X 1
RXR	Receptor X retinoide
Sp1,Sp3	Stimulating protein 1 and 3
STAT	Signal Transducer and Activator of Transcription
TGFα	Transforming growth factor – α
TNFα	Tumor necrosis factor α
USF1	Upstream stimulating factor
VPA	Valproic acid
YY1	Ying Yang 1

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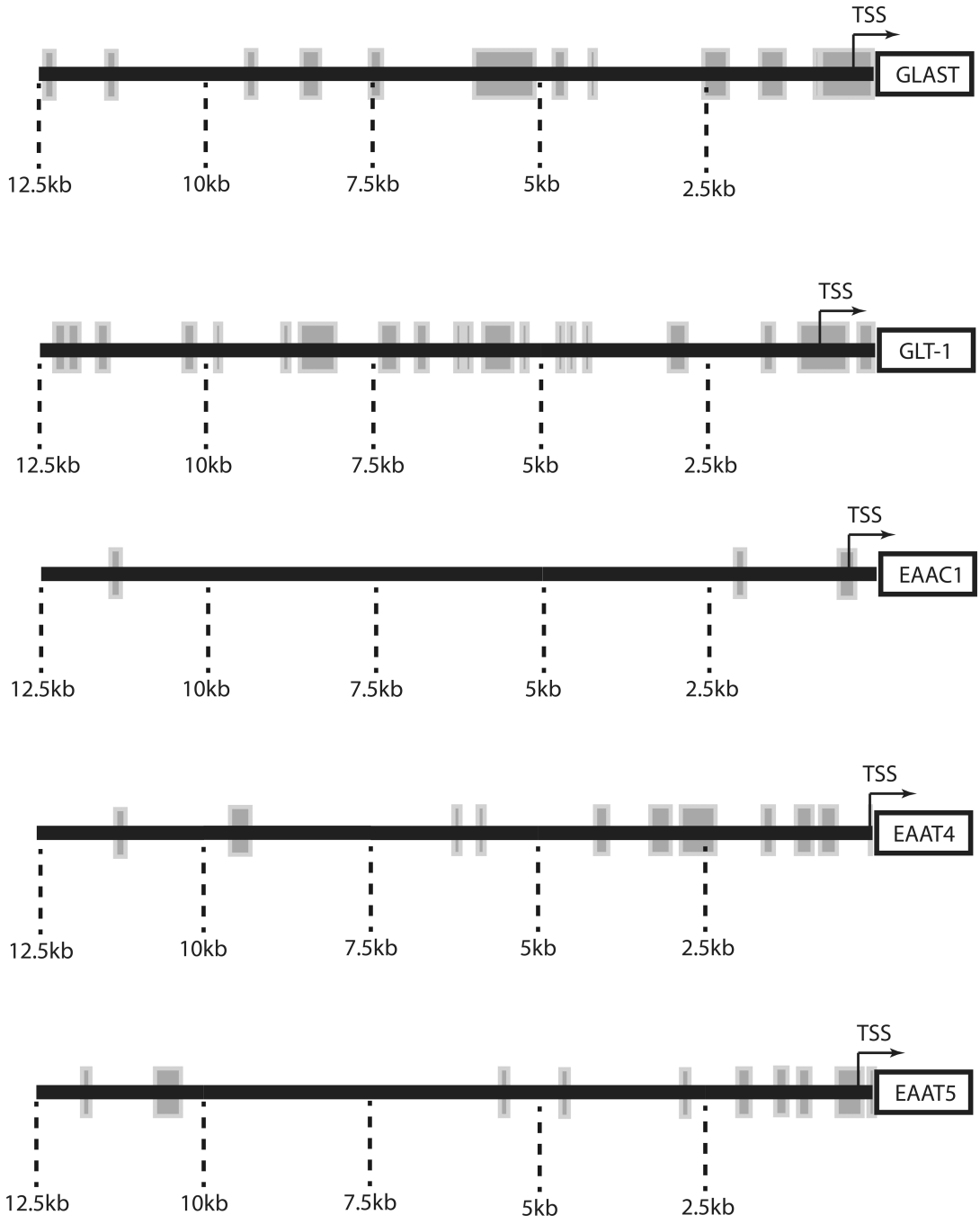
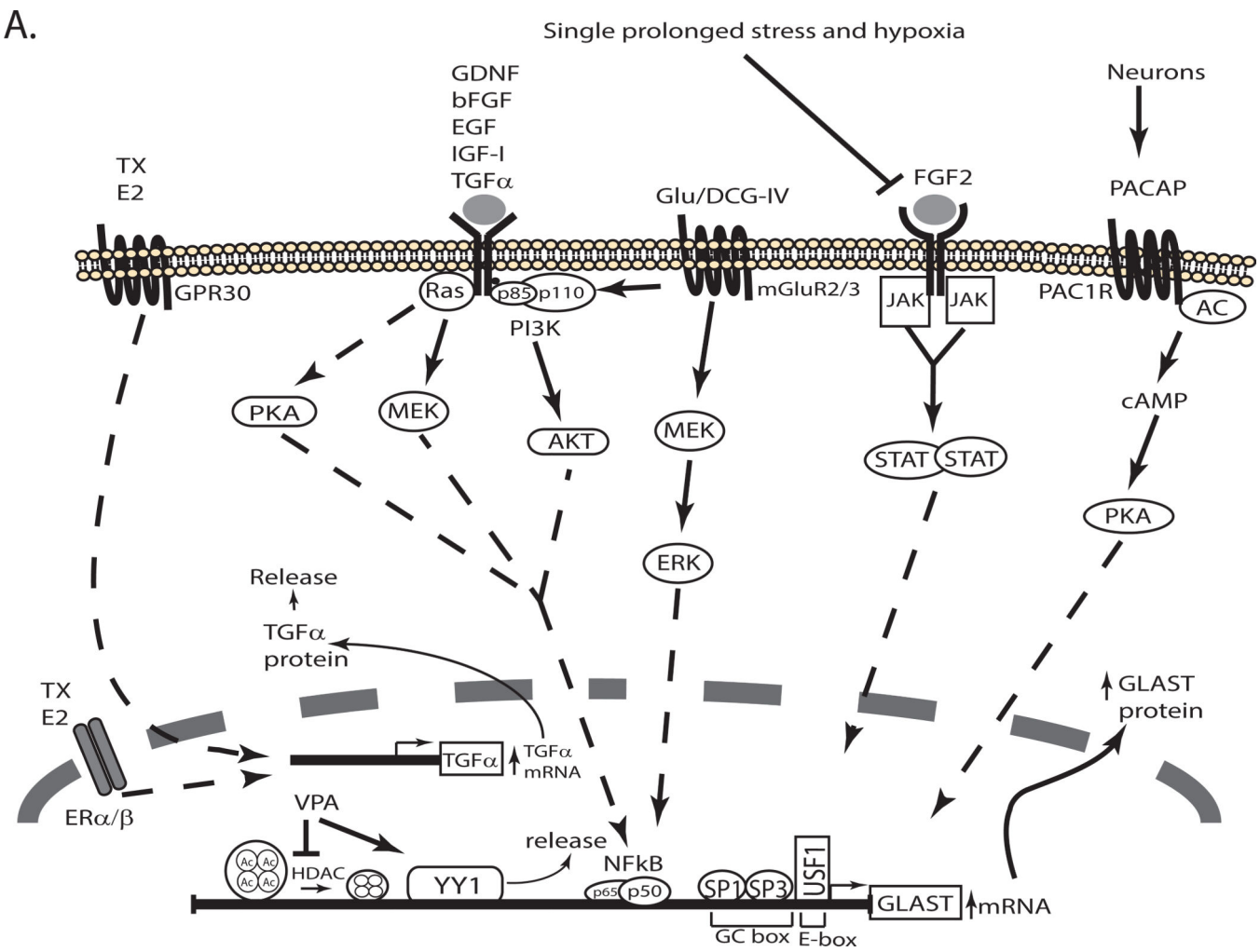


Figure 1. Schematic representation of evolutionary conserved domains in the promoter regions of Glu transporters

The mouse and human homologs of the genes encoding GLAST, GLT-1, EAAC1, EAAT4 or EAAT5 were aligned using an online resources (DCODE database; http://ecrbase.dcode.org). Rectangular boxes represent evolutionary conserved domains, defined as regions of ≥70% homology for at least 100 nucleotides. It is important to remember that enhancer elements can be outside the regions aligned and that some of the distal conserved regions may not be involved in transport regulation and may instead, regulate the neighboring gene.



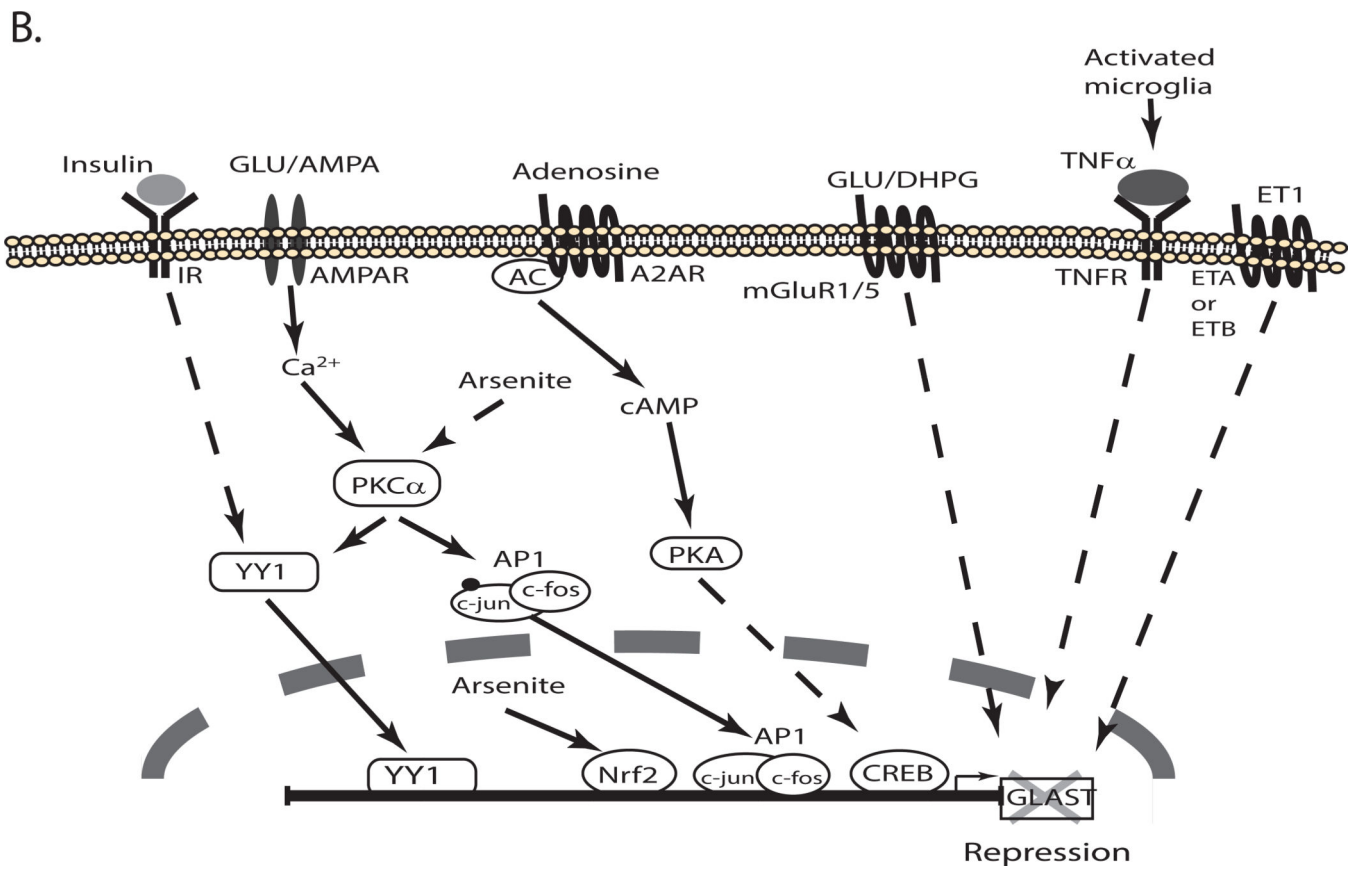
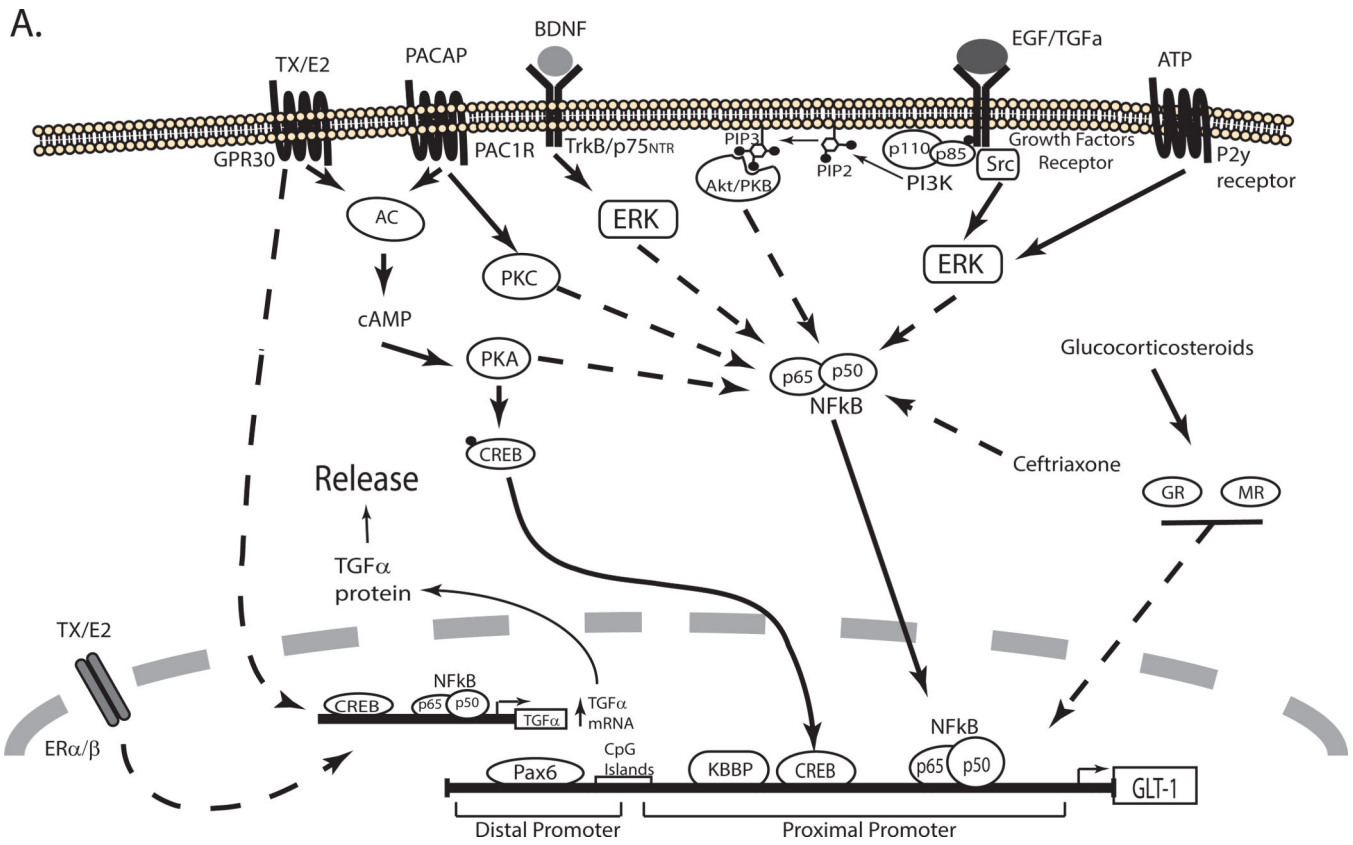


Figure 2. Schematic representation of the signaling pathways that regulate transcription of GLAST/EAAT1/SLC1A3

Several signaling pathways activated in response to extracellular molecules regulate the expression of GLAST by activating transcription factors that interact with *cis* elements in the promoter. The pathways that increase or decrease GLAST transcription are depicted in different schematics (Panel A & B, respectively). For a detailed description see the text. TX: Tamoxifen, E2: Estrogen, Glu: Glu, AC: adenylate cyclase, ET1: endothelin-1, ETA, ETB: Endothelin receptors A and B.



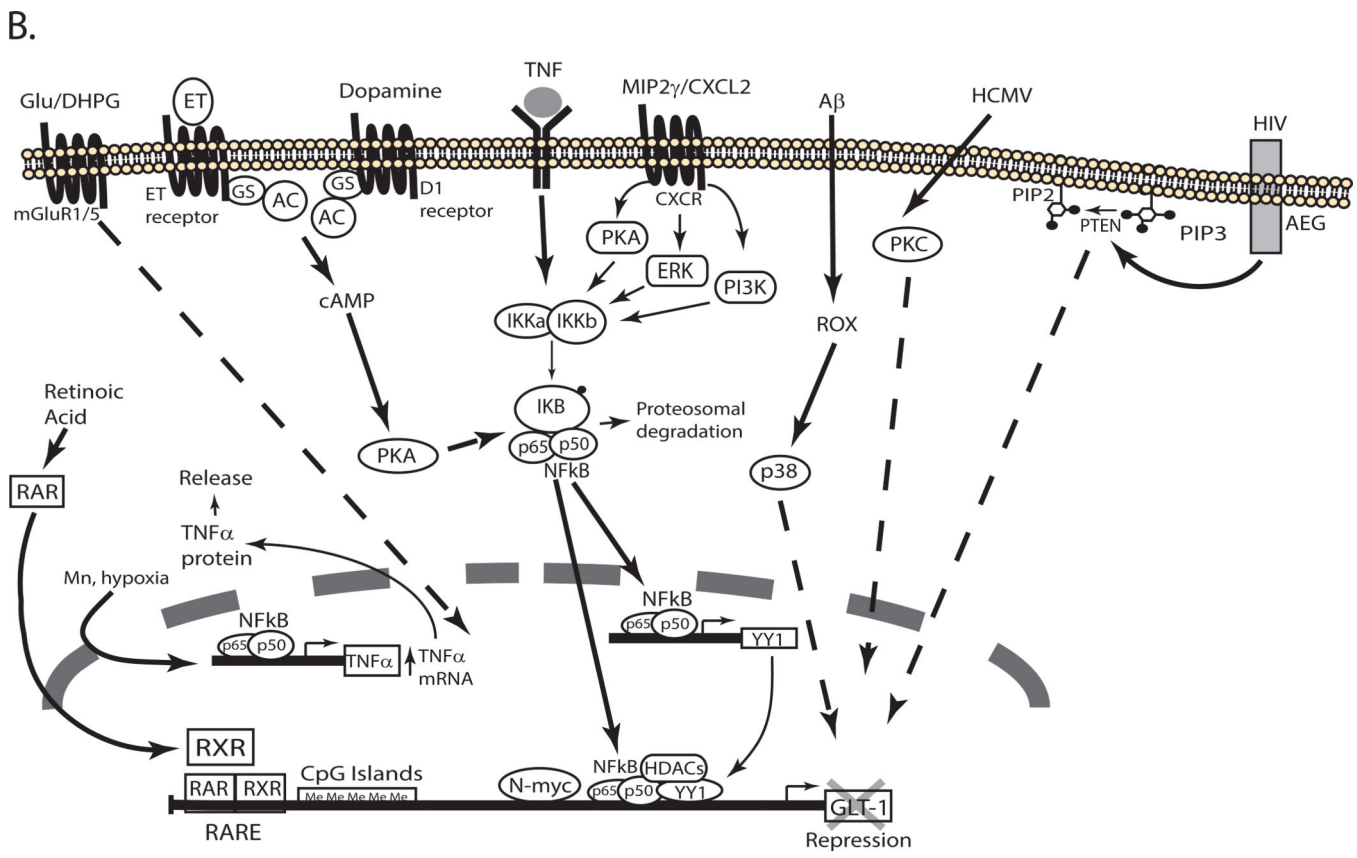


Figure 3. Schematic representation of the signaling pathways that regulate transcription of GLT-1/EAAT2/SLC1A2

Several signaling pathways activated in response to extracellular molecules regulate the expression of GLT-1 by activating transcription factors that interact with *cis* elements in the promoter. The pathways that increase or decrease GLT-1 transcription are depicted in different schematics (Panel A & B, respectively). For a detail description see the text. TX: tamoxifen, E2: estrogen, AC: adenylate cyclase, GR: glucocorticoid receptor, MR: mineralocorticoid receptor.

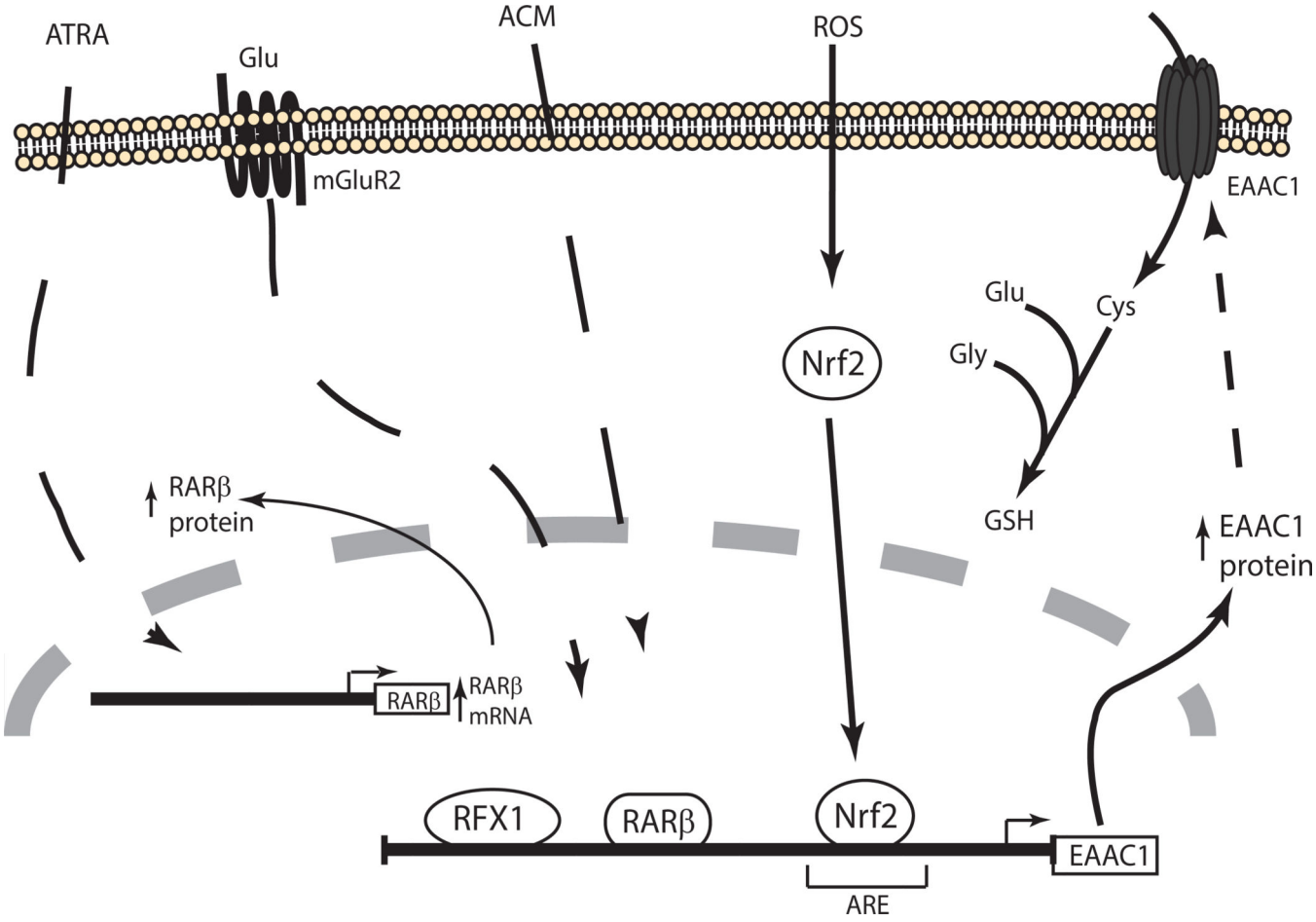


Figure 4. Schematic representation of the signaling pathways that regulate transcription of EAAC1/EAAT3/SLC1A1

Extracellular signals and transcription factors that have been associated with an increase in EAAC1 expression are shown. For a detail description see the text. ACM: astrocyte conditioned media, ROS: reactive oxygen species.