22 Sodium-Calcium Exchanger Modulates the L-Glutamate Ca_i^{2+} Signalling in Type-1 Cerebellar Astrocytes

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Abstract

We have previously demonstrated that rat type-1 cerebellar astrocytes express a very active Na^+/Ca^{2+} exchanger which accounts for most of the total plasma membrane Ca^{2+} fluxes and for the clearance of Ca_i^{2+} induced by physiological agonist. In this chapter, we have explored the mechanism by which the reverse Na^+/Ca^{2+} exchange is involved in agonist-induced Ca^{2+} signalling in rat cerebellar astrocytes. Laser-scanning confocal microscopy experiments using immunofluorescence labelling of Na^+/Ca^{2+} exchanger and RyRs demonstrated that they are highly co-localized. The most important finding presented in this chapter is that L-glutamate activates the reverse mode of the Na^+/Ca^{2+} exchange by inducing a Na^+ entry through the electrogenic Na^+-glutamate co-transporter and not through the ionophoric L-glutamate receptors as confirmed by pharmacological experiments with specific blockers of ionophoric L-glutamate receptors, electrogenic glutamate transporters and the Na/Ca exchange.

Keywords

Na^+/Ca^{2+} exchange • CICR • Glutamate • Glutamate transporter

22.1 Introduction

The Na^+/Ca^{2+} exchanger a plasma membrane counter-transport system plays a critical role in the control of intracellular calcium. In its forward mode (Ca^{2+} efflux), the exchanger has an important physiological role for the rapid extrusion of large amounts of Ca^{2+} from the cell. However, the physiological role of the exchanger, working in its reverse mode (Ca^{2+} entry), is still

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controversial (Blaustein and Lederer 1999). In principle, there are two non-exclusive possibilities for the involvement of the reverse exchange in Ca_i^{2+} signalling in type-1 cerebellar astrocytes: (1) Ca_i^{2+} entering by the reverse exchanger directly triggers calcium-dependent processes and/or (2) Ca_i^{2+} entering through the exchanger serves as messenger for a Ca_i^{2+} signal amplification through a Ca_i^{2+}-induced-Ca_i^{2+}-release (CICR) mechanism. In favour of the first possibility are the reports that L-glutamate (L-Glu) through activation of kainate receptor channels leads to the influx of Na^+ ions which activates the reverse Na^+/Ca^{2+} exchange, thus leading to $[Ca^{2+}]_i$ increase (Goldman et al. 1994; Takuma et al. 1996). A similar mechanism has been proposed to explain the glutamate-induced homocysteic acid release from cortical astrocytes (Benz et al. 2004). On the other hand, the existence and functional significance of CICR coupled to ryanodine receptors (RyRs) is well documented in astrocytes (Verkhratsky and Kettenmann 1994). Nevertheless, the existence and functional relevance of RyRs in type-1 cerebellar astrocytes, if any, have not been demonstrated.

The experiments reported here examine the role of Ca^{2+} entry through reverse Na^+/Ca^{2+} exchange as a mechanism for inducing amplification of Ca_i^{2+} signals that occur during conditions of agonist activation. Using microspectrofluorometric measurements, pharmacological tools, immunofluorescence labelling and laser-scanning confocal microscopy (LSCM) analyses, we present for the first time evidences that in rat type-1 cerebellar astrocytes, (1) Ca^{2+} entry during operation of reverse Na^+/Ca^{2+} markedly increases $[Ca^{2+}]_i$ by a CICR mechanism, followed by the opening of store-operated Ca^{2+} channels (SOCC); (2) immunofluorescence labelling of both Na^+/Ca^{2+} exchanger and RyRs using confocal microscopy demonstrates that they are highly co-localized; and (3) unexpectedly, physiological agonist concentrations of L-Glu increase $[Ca^{2+}]_i$ through activation of the reverse exchange as a result of Na^+ entry through the electrogenic glutamate transporters.

22.2 Role of the Sodium-Calcium Exchanger in the Control of L-Glutamate Ca_i^{2+} Signalling in Type-1 Cerebellar Astrocytes

Figure 22.1a shows a run in which a cell was exposed to a long 70-s pulse to a 0NaCa solution, producing a larger increase in intracellular Ca^{2+}. Readmission of external Na causes the Ca_i^{2+} to drop to a sloping plateau which was cut short by superfusing the cell with a Na^+-containing Ca^{2+}-free medium (Na_0Ca) lowering the Ca_i^{2+} to nearly resting values. This experiment suggests that in this preparation, substantial Ca^{2+} entry through the reverse exchange may activate the release of Ca^{2+} from intracellular Ca^{2+} stores, and the opening of the SOCC (store-operated calcium channels). The protocol of Fig. 22.1b was designed to disable the forward Na^+/Ca^{2+} exchange with a 50-s pulse of 0NaCa (Ca^{2+} entry mode, horizontal slash) and then rapidly enable the forward exchange (Ca^{2+} extrusion mode) for about 40 s by rapidly superfusing with the test Na_0Ca medium (vertical arrows). This protocol was repeated during nine consecutive pulses. The results of Fig. 22.1b indicate that in the absence of external Ca^{2+}, the forward mode of the exchange lowers the Ca_i^{2+} faster and to a greater extent than in its presence. More importantly, they also show that the peak of the Ca_i^{2+}-dependent fluo-3 signal induced by the reverse exchange decreases progressively after each period of activation of the forward exchange mode. The fact that this decrease is due to depletion of ryanodine-sensitive Ca_i^{2+} stores is confirmed by the absence of Ca_i^{2+} release by the ryanodine receptor agonist 4-C*m*C (end of experiment).

The pharmacological experiments of Fig. 22.1 indicate that ryanodine receptors are somehow involved in the amplification of the Ca^{2+} signal during reverse operation of exchanger in this glial cell type. Therefore, the next step was focused on the immunolocalization of this transporter in the plasma membrane of these cells as well as on the spatial relationship between the plasmalemmal Na^+/Ca^{2+} exchanger and the

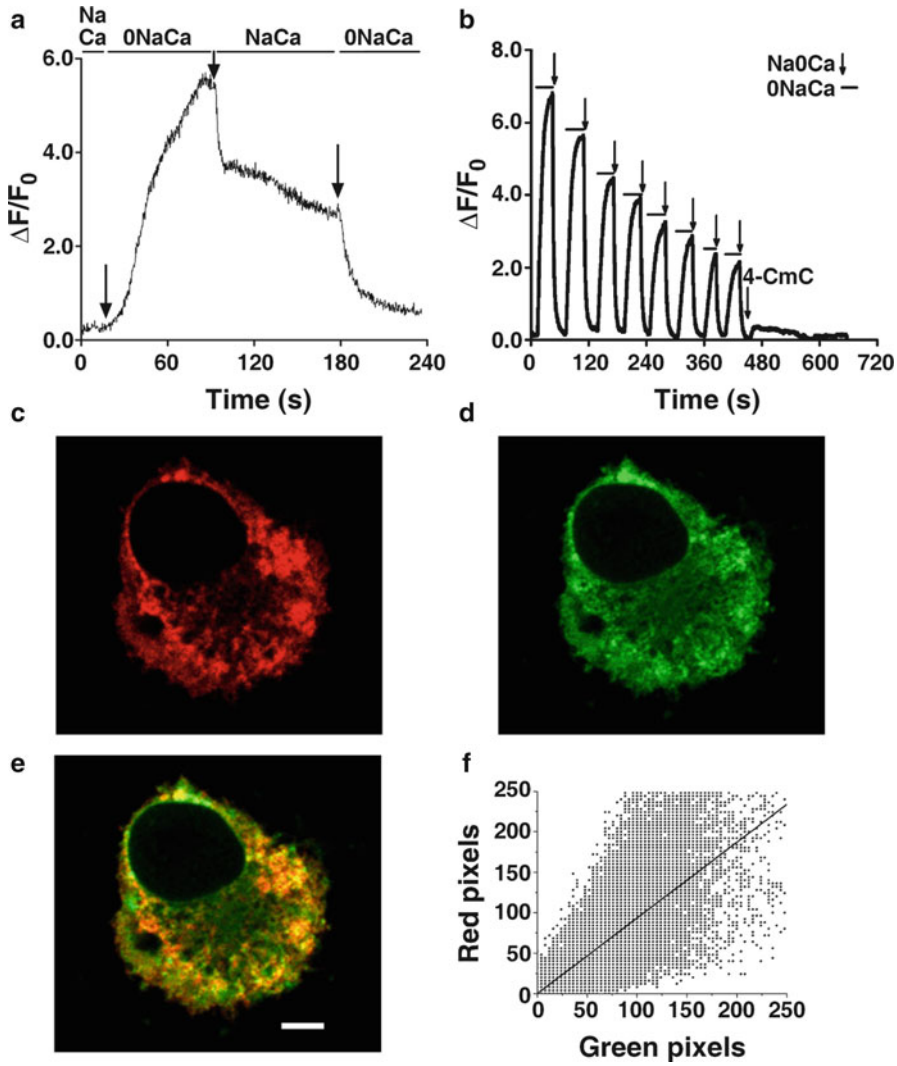

Fig. 22.1 (**a**) Effect of a 0NaCa pulse induces a large Ca_i^{2+} signal, which, upon re-exposure to the normal medium, (NaCa) is partially reversed reaching a sloping plateau value. Additions of a solution containing Na^+ but no Ca^{2+} bring the signal to background levels. (**b**) The effect of nine consecutive reverse (0NaCa) and forward (Na_0Ca) short pulses causes a progressive decrease in the Ca_i^{2+} signal until it reaches a constant small Ca^{2+} value. At the end of experiment, the ryanodine agonist 4-C*m*C fails to release Ca^{2+} from ryanodine-sensitive Ca^{2+} stores, thus indicating that activation of the reverse exchange empty the calcium accumulated in the endoplasmic reticulum. (**C**) and (**D**) show the immunofluorescent labelling of Na^+/Ca^{2+} exchanger (in *red*) with affinity-purified antibodies raised against the cardiac sarcolemmal Na^+/Ca^{2+} exchanger Alexa Fluor 546-Na/Ca and ryanodine receptors (in *green*) with Bodipy-FL-ryanodine, respectively. (**e**) and (**f**) show the merge of the images obtained with the two different labels (*orange* colour corresponding to regions of overlap) and the mathematical analysis of co-localization (Pearson's correlation of 0.89) (*white bar* indicate 10 μm)

underlying endoplasmic reticulum (ER), in particular the ryanodine receptors. For this, cells were incubated first with a purified canine cardiac Na^+/Ca^{2+} exchanger mouse monoclonal antibody and second with a secondary labelled goat anti-mouse Igm antibody Na^+/Ca^{2+} exchange Alexa Fluor 546. The immunofluorescence of the Na^+/Ca^{2+} exchanger (red colour) in a representative cell is presented in Fig. 22.1c. In all cells studied ($n=6$), the labelling was punctual suggesting a

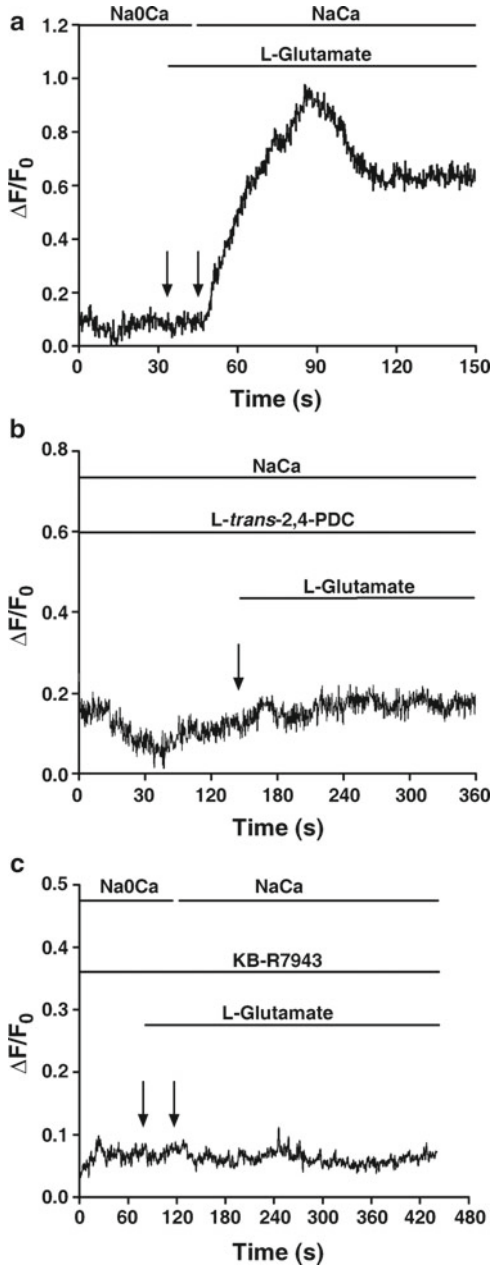

Fig. 22.2 *The effect of low (<30 μM)* L-*Glu on the* $Ca^{2+}{}_i$*-dependent fluo-3 signal in the presence and absence of extracellular* Ca^{2+}. (**a**) This astrocyte was perfused from the beginning with a medium containing no external Ca^{2+} (Na_0Ca). Notice that no effect of L-Glu is observed under this condition. Addition of 2 mM external Ca^{2+} rapidly induces a biphasic response. (**b**) The effect of L-*trans*-2,4-PDC (100 μM), a blocker of the electrogenic glutamate transporter, completely eliminates the L-Glu-induced increase in Ca_i^{2+}.(**c**) The effect of the Na^+/Ca^{2+} exchange inhibitor the KB-R7943 (10 μM) over the L-Glu-induced Ca_i^{2+} rise even at 1 mM [L-Glu]

cluster of Na^+/Ca^{2+} exchange molecules. Labelling of the exchanger was more intense at cell edges suggesting that the exchanger is distributing in an organized manner in the astrocyte plasmalemma. Figure 22.1d shows the localization of ryanodine receptors (green colour) in the same cell using Bodipy-FL-ryanodine, a specific ryanodine receptor marker (Hua et al. 2004). Figure 22.1e shows the co-localization (orange colour) of the Na^+/Ca^{2+} exchanger and the ryanodine receptors. Figure 22.1 shows that from co-localization analysis, the observed overlap was found to be highly significant with a Pearson`s correlation of about 0.89. This indicates that the Na^+/Ca^{2+} exchanger is indeed co-localized with some of the ER, in particular with the ryanodine receptors.

Figure 22.2a shows that in the absence of external Ca, 30-μM L-Glu does not modify the calcium fluorescence signal. Following addition of external Ca^{2+} in the continuous presence of L-Glu, a substantial increase in the $[Ca^{2+}]_i$ was observed, thus indicating that the fluo-3 signal induced by L-Glu is mediated by Ca^{2+} entering from the extracellular medium. On the other hand, Fig. 22.2b shows that the specific blocker of the electrogenic Na^+-glutamate co-transporter, L-*trans*-2,4,-PDC, completely eliminates the L-Glu induced increase in Ca_i^{2+}. Figure 22.2c shows that in presence of a potent inhibitor of the Na^+/Ca^{2+} exchanger, KB-R7943 (Iwamoto et al. 1998) and a preincubation (60 s) with 10 μM of the inhibitor completely block the L-Glu effect.

22.3 Conclusion

The present work demonstrates that in type-1 cerebellar astrocytes in culture, the Ca^{2+} signal generated by Ca^{2+} entry through the reverse Na^+/Ca^{2+} exchange is greatly amplified by a Ca_i^{2+}-induced Ca^{2+} release mechanism which involves ryanodine receptors and ryanodine-sensitive Ca^{2+} stores. While the presence of RyRs has been demonstrated in this preparation, their physiological significance was not clear (Langley and Pearce 1994; Simpson et al. 1998; Matyash et al. 2002; Golovina and Blaustein 2000; Beck et al.

2004; Aley et al. 2006). Caffeine may induce Ca^{2+} release from RyRs-operated Ca^{2+} stores in different neurons (Uneyama et al. 1993; Usachev et al. 1993; Kano et al. 1995; Llano et al. 2000) and glia preparations, (Verkhratsky and Shmilgol 1996; Beck et al. 2004).

In addition, and most importantly, for the first time, we provide evidence that the intracellular Ca^{2+} signal induced by physiological concentrations of the excitatory amino acid L-glutamate is the result of Na^+ entry through the electrogenic glutamate transporter that activates the reverse Na^+/Ca^{2+} exchange and leads to Ca^{2+} entry, with a concomitant increase in $[Ca^{2+}]_i$. The finding of a functional co-expression of Na^+/Ca^{2+} exchangers with ryanodine receptors strongly supports the idea that the original Ca^{2+} signal due to Ca_i^{2+} entry through the exchanger is largely amplified by a CICR process.

Previous studies have shown that the Na^+/Ca^{2+} exchanger working in its reverse mode can induce Ca^{2+} entry in cultured astrocytes (Goldman et al. 1994; Takuma et al. 1994; Blaustein and Lederer 1999). Moreover, Ca^{2+} influx via the exchanger may be responsible for $[Ca^{2+}]_i$ increases under certain pathological conditions (Kin-Lee et al. 1992; Matsuda et al. 1996). Type-1 cerebellar astrocytes express a highly active Na^+/Ca^{2+}exchanger responsible for the balance of the plasma membrane Ca^{2+} fluxes under resting physiological conditions (Rojas et al. 2004). In different preparations, there is evidence of an intimate association between the Na^+/Ca^{2+} exchanger and internal Ca^{2+} stores (Juhaszova et al. 1996). Such association is well established in smooth muscle cells where the exchanger is in close proximity to the sarcoplasmic reticulum (SR) so that Ca^{2+} release from the SR through RyRs is closely coupled to its extrusion by the exchanger (Nazer and van Breemen 1998). Furthermore, in neurons, there is evidence for a functional (Hurtado et al. 2002) and spatial association of the exchanger with the intracellular Ca^{2+} stores (Juhaszova et al. 1996). Micci and Cristensen (1998) working in catfish retinal neurons have studied the interaction between the exchanger and caffeine-sensitive Ca^{2+} stores showing that reverse operation of the Na^+/Ca^{2+} exchanger refills Ca^{2+}-depleted ER. For the case of astrocytes, however, the relationship between the Na^+/Ca^{2+} exchanger and the RyRs is unknown.

One of the aims of the present work was to investigate whether the magnitude of the increase in $[Ca^{2+}]_i$ observed when the operation of the Na^+/Ca^{2+} exchanger was reversed was due solely to Ca^{2+} entry or whether this entry could trigger further Ca^{2+} release from RyRs-operated intracellular Ca^{2+} stores. During long (>60 s) Na^+ gradient reversal pulses, the increase in $[Ca^{2+}]_i$ is much larger and leads to depletion of RyRs-operated intracellular Ca^{2+} store, indicating the presence of a CICR mechanism. Furthermore, depletion of intracellular Ca^{2+} stores causes the activation of SOCC, as confirmed by the extracellular Ca^{2+} dependency (Fig. 22.1a) and sensitivity to 2-APB (Lo et al. 2002; Rojas et al. 2007) of a late, residual component of the Ca^{2+} signal. The presence of ryanodine receptors in type-1 cerebellar astrocytes has been confirmed using conventional Ca^{2+} imaging confocal microscopy and immunocytochemistry techniques. The close proximity of the Na^+/Ca^{2+} exchanger to the ER membranes, where the RyRs are localized, allows the former to rapidly extrude Ca^{2+} ions released from the ER before their recapture by the ER Ca^{2+}-ATPase. This leads to depletion of the ER Ca^{2+} stores as demonstrated by the consecutive reverse-forward pulse experiments. The fact that no release of Ca^{2+} is observed at the end of the run in the presence of the ryanodine agonist 4-C*m*C or a combination (Fig. 22.1b) demonstrates that the exchanger is capable of depleting the ER.

An important discovery in glial cell research is that $[Ca^{2+}]_i$ increase may trigger glutamate release from astrocytes which then mediates Ca_i^{2+} increases in nearby neurons, thus indicating a crosstalk between neurons and astrocytes (Parpura et al. 1994; Jeftinija et al. 1997; Pasti et al. 1995; Calegari et al. 1999; Araque et al. 2000; Fellin and Carmignoto 2004). Benz et al. (2004) have demonstrated the importance of the Na^+/Ca^{2+} exchanger in the glutamate response in cortical astrocytes from mice. Their experiments show that 500-μM L-Glu induces a Ca_i^{2+}-dependent release of homocysteic acid from astrocytes through activation of glutamate receptors, leading to an

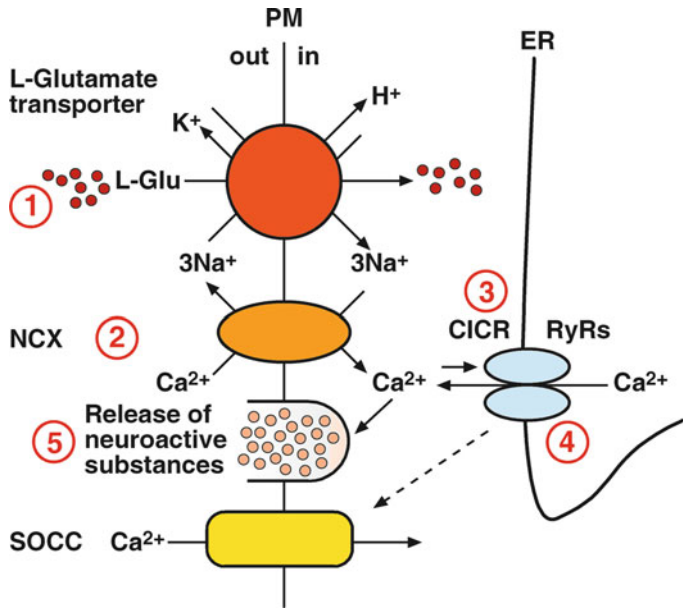

Fig. 22.3 *Role of Na^+/Ca^{2+} exchanger in glutamate-induced rise of intracellular Ca^{2+} in rat cerebellar type 1 astrocytes.* The events that lead to glutamate-induced rise in intracellular Ca^{2+} involve (*1*) Na^+ entry through the electrogenic glutamate co-transporter, (*2*) activation of the reverse Na^+/Ca^{2+} exchange (NCX) by the rise in intracellular Na^+ (Na^+ inward current through the glutamate transporter), (*3*) rise in the $[Ca^{2+}]_i$ near the RyRs to trigger a CICR from the ER, (*4*) activation by Ca^{2+} of RyRs followed by Ca^{2+} release from ryanodine channels leading to an amplification of the original Ca^{2+} entry through the exchanger and (*5*) opening of the store-operated calcium channels and release of neuroactive substrates

influx of Na^+ and to an increase in Ca^{2+} entry through the reverse Na^+/Ca^{2+} exchange (Benz et al. 2004). Previous electrophysiological studies in rat type-1 cerebellar astrocytes show that application of as low as 30-μM L-Glu produced large inward currents which remains inward going at potentials up to $^+$80 mV being the result of the presence of an electrogenic glutamate uptake carrier (Wyllie et al. 1991). In cells kept up to 4 days in culture, quisqualate, kainate and NMDA failed to produce any current indicating the absence at this early stage of glutamate ionotropic receptors in rat type-1 cerebellar astrocytes (Wyllie et al. 1991). These authors showed that even in older cultures, in which ionotropic glutamate receptors are well expressed, most of the L-Glu-induced inward current can be ascribed to the Na^+-glutamate co-transporter (Wyllie et al. 1991). Based on these findings, we considered the possibility that the electrogenic Na^+-glutamate transporter might be involved in the L-Glu-dependent $[Ca^{2+}]_i$ increase in type-1 cerebellar astrocytes through an increase in $[Na^+]_i$.

The major finding in the present work is that activation of the reverse Na^+/Ca^{2+} exchange by physiological [L-Glu] is not the consequence of Na^+ entry through ionotropic receptors as occurs in other astrocyte preparations (Benz et al. 2004) but the result of Na^+ entry through the electrogenic glutamate transporter (see the scheme of Fig. 22.3). An important role of the electrogenic glutamate transporter in the L-Glu-induced Ca_i^{2+} increase and its relationship with the reverse Na^+/Ca^{2+} exchange are supported by the demonstration that (1) no effect of L-Glu is observed in the absence of external Ca^{2+}, (2) inhibition of the ionotropic glutamate receptors does not impair the Ca_i^{2+} rise induced by L-Glu, (3) inhibition of the Na^+/Ca^{2+} exchanger completely blocks the L-Glu effect, (4) L-Glu effect is abolished by depletion of the ryanodine-sensitive intracellular stores (by 4-C*m*C) and (5) specific inhibition of

the electrogenic Na^+-glutamate co-transporter completely eliminates the L-Glu effect.

Considering that the transport current generated by the glutamate transporter is evoked by the inward movement of two positive charges per transported glutamate (1Glu:1H^+:3Na^+ entering vs. 1K^+ moving outward; Greever and Rauen 2005) and an average inward current of 800 pA/cm^2 for a 30-μM L-Glu (Wyllie et al. 1991), then for a hypothetical type-1 astrocyte resembling a rectangular triangle of 25 μm in the base and an approximate astrocyte volume of 1.2×10^{-6} μl, enough Na^+ will enter the astrocyte during L-Glu activation as to induce increases of the intracellular [Na^+] in tens of millimolar in less than 10 s, sufficient to greatly activate the reverse mode of the Na^+/Ca^{2+}.

Finally, an interesting recent finding is an acute up-regulation of the Na^+-glutamate transporter mediated by metabotropic glutamate receptors in rat cortical astrocytes, in which activation of mGluR5a induces a PKC-dependent up-regulation of GLT-1 activity (Vermeiren et al. 2005). Further experiments are necessary to link this cross regulation with our proposed model.

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