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Cytoskeleton-related trafficking of the EAAC1 glutamate transporter after activation of the $G_{q/11}$-coupled neurotensin receptor NTS1

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Abstract The possible modulation of the glutamate transporter EAAC1 by a class A G protein-coupled receptor was studied in transfected C6 glioma cells stably expressing the high-affinity neurotensin receptor NTS1. Brief exposure (5 min) to neurotensin increased Na^+-dependent D-[^{3}H]aspartate uptake by about 70%. The effect of neurotensin was found to result from an increase in cell surface expression of EAAC1 and accordingly, cytochalasin D and colchicine were shown to block the effect of neurotensin on aspartate uptake, suggesting that the cytoskeleton participates in this regulation. Neither protein kinase C nor phosphatidylinositol 3-kinase activities, two intracellular signaling pathways known to modulate EAAC1, was required for EAAC1-mediated aspartate transport regulation by neurotensin. Together, these results provide evidence for an acute regulation of EAAC1 trafficking after activation of a G protein-coupled receptor.

Key words: Trafficking; Glioma; G protein-coupled receptor; Phospholipase C; EAAC1

1. Introduction

Glutamate is the major mediator of excitatory signals in the mammalian brain [1]. Within glutamatergic synapses, a variety of membrane proteins participate in the regulation of the intensity and length of postsynaptic excitation. Diverse cell surface glutamate receptors play determining roles in ensuring signal transmission whereas specific glutamate transporters contribute to reduce the glutamate concentration in the synaptic cleft. Although many studies have been focused on the activity of glutamate receptors, modulation of transporter function has recently received much more attention as it is now clearly established that glutamate uptake is required to prevent glutamate-mediated excitotoxic injuries [2–4].

To date, five high-affinity Na^+-dependent glutamate transporters have been characterized: the glial GLT-1 and GLAST, the neuronal EAAC1 and EAAT4, and the retinal transporter EAAT5 [5]. Many reports have shown that transporters for diverse neurotransmitters are subject to regulation, supporting evidence for the possible cross-talk between these transporters and other proteins in the same synaptic element. Accordingly, the activity and cell surface expression of glutamate transporters can be acutely altered by manipulating intracellular signaling pathways, including protein kinases, nitric oxide synthesis, and arachidonic acid production [6–8]. In C6 glioma cells, protein kinase C (PKC) activation was shown to increase the activity and cell surface expression of EAAC1, whereas phosphatidylinositol 3-kinase (PI3-K) inhibition leads to a decrease in these parameters [6,9].

In order to evaluate the possible involvement of G protein-coupled receptors (GPCR) in the modulation of EAAC1 activity, we have used a model of transfected C6 glioma cells expressing the high-affinity neurotensin (NT) receptor (NTS1 receptor) as a prototypical member of the class A GPCR family known to preferentially activate $G_{q/11}$-type G proteins, leading to the activation of phospholipase C (PLC) and subsequent mobilization of intracellular Ca^{2+} and activation of PKC. In this model, brief exposure to NT was found to dramatically increase cell surface expression of EAAC1 and glutamate uptake through a PKC-independent mechanism. This study is the first report on the regulation of EAAC1 trafficking and activity expression after activation of a GPCR.

2. Materials and methods

2.1. Materials

All radioisotopes were purchased from Amersham Pharmacia Biotech (Roosendaal, The Netherlands). Sulfo-NHS-biotin and immunopure immobilized monomeric streptavidin were from Pierce (Perbio Science, Erembodegem-Aalst, Belgium). L-Aspartate, cytochalasin D, colchicine, wortmannin, D-sphingosine, phorbol 12-myristate 13-acetate (PMA) and phenylarsine oxide (PAO) were from Sigma (Bornem, Belgium). H89, KN-62, PD98059, Ro-31-8220 were obtained from Calbiochem (Merck Eurolab, Leuven, Belgium).

2.2. Cell culture

Rat C6 glioma cells expressing the NTS1 receptor were obtained by transfection (calcium phosphate co-precipitation method) using the rat NTS1 cDNA sequence inserted in the expression vector $pcDNA_3$. Transfected C6 cells were selected in the presence of 600 μg/ml Geneticin (G418). The clone that displayed the highest [^{3}H]NT binding (B_{max} and K_D values of 1.39 ± 0.63 pmol/mg protein and 0.26 ± 0.03 nM, respectively) was expanded for the study presented here. Cells were routinely grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 20 μg/ml proline, 100 U/ml penicillin and 100 μg/ml streptomycin. All cultures were maintained at 37°C in a water-saturated atmosphere containing 5% CO_2.

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Abbreviations: EAAC1, neuronal glutamate transporter; NT, neurotensin; NTS1, high-affinity neurotensin receptor; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; PI3-K, phosphatidylinositol 3-kinase; C6, rat glioma cells; C6-NTS1, stably transfected C6 cells expressing the rat NTS1 receptor; PAO, phenylarsine oxide

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2.3. Measurement of Na^+-dependent transport activity

For uptake assays, cells were grown on 24-well plates. At 80% confluence, plates were placed at the surface of a 37°C water bath, rinsed twice with 1 ml of preheated buffer (25 mM HEPES pH 7.4, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 6 mM glucose) supplemented with either 140 mM NaCl or 140 mM choline chloride and then treated with drugs or vehicle. As in the majority of studies concerning glutamate transporters, aspartate, a non-metabolizable transported analog of glutamate, was used to evaluate the uptake activity [5,10]. Except for saturation studies, D-[^{3}H]aspartate was used at a final concentration of 30 nM. For saturation studies, D-[^{3}H]aspartate (30 nM) was diluted with unlabeled L-aspartate to achieve final aspartate concentrations of 1–200 μM. Inhibitors were added 15 min before the addition of NT. The uptake was stopped after 6 min with three rinses of ice-cold choline buffer (in these experimental conditions, uptake was found to be linear up to 20 min). The cells were solubilized with 0.5 ml of 1 N NaOH. 200 μl of the radioactive lysate was counted in a scintillation counter. After experiments, protein assays were achieved in order to ensure that any drugs affect the protein content in the wells [11]. The specific Na^+-dependent uptake was estimated by subtracting the values obtained with cells incubated in the choline chloride-containing buffer.

2.4. Cellular trafficking of glutamate EAAC1 transporter

Biotinylation experiments were performed as previously described [6] with minor modifications. C6 glioma cells were grown in six-well plates to at least 80% confluence. After rinsing with phosphate-buffered saline (PBS) containing 0.1 mM Ca^{2+} and 0.1 mM Mg^{2+}, cells were incubated with 0.8 ml of sulfo-NHS-biotin (1.5 mg/ml in PBS–Ca^{2+}/Mg^{2+}) for 30 min at 4°C with mild shaking. Thereafter, cells were rinsed twice with cold PBS–Ca^{2+}/Mg^{2+} containing 100 mM glycine and incubated with the same solution for 45 min at 4°C to quench the unbound biotin reagent. Cell lysis was performed with radioimmunoprecipitation assay (RIPA) buffer (100 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) supplemented with protease inhibitors (250 μM phenylmethylsulfonyl fluoride and protease inhibitor cocktail from Sigma) buffer for 1 h at 4°C with brisk shaking. After centrifugation at 16 000 × *g* at 4°C for 20 min, 150 μl supernatant was incubated with an equal volume of streptavidin bead suspension for 1 h at room temperature. After centrifugation (15 min at 16 000 × *g* at 4°C), the supernatant was collected for analysis of the non-biotinylated intracellular fraction while the pelleted biotinylated cell surface proteins were washed four times with 0.8 ml of RIPA buffer and resuspended in 50 μl of Laemmli buffer containing 50 mM dithiothreitol. After 15 min centrifugation at 16 000 × *g*, supernatants were stored at −20°C until analyzed.

2.5. Immunoblot analyses

Samples were thawed and boiled for 5 min. Cell extracts were analyzed by 10% SDS–PAGE according to Laemmli [12]. After electroblotting to nitrocellulose membranes, proteins were probed with affinity-purified anti-EAAC1 (0.5 μg/ml) (ADI, USA) and subsequently, the antigen–antibody complex was detected with a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:3000) (Sigma). The immunoreactive proteins were detected with enhanced chemiluminescence reagents. Quantitative densitometry analysis of the autoradiograms was performed using a digital image analysis system (MCID 4; Imaging Research, St. Catharines, ON, Canada).

2.6. Statistical analysis

Statistical differences were determined by Student's *t*-test for two-group comparison. For multiple comparisons, data were analyzed by one-way ANOVA followed by the Newman–Keuls test.

3. Results

3.1. Effect of NT on D-[^{3}H] aspartate uptake activity

Exposure of C6-NTS1 cells to 100 nM NT for 5 min markedly increased the specific Na^+-dependent L-[^{3}H]glutamate (30 nM) uptake by 82% as compared to non-treated cells (not shown). Similarly, the same treatment caused a 95% increase in the specific uptake of D-[^{3}H]aspartate (30 nM). Further determination of the effect of NT was obtained by non-linear analysis of the specific uptake of isotopic dilutions of D-[^{3}H]aspartate/L-aspartate revealing that the 5 min NT (100 nM) treatment increased the uptake capacity by 67% (V_{max} values of 174 ± 21 and 292 ± 40 pmol/mg protein/min for control and NT-treated cells, respectively; $n = 6$, $P < 0.001$, paired Student's *t*-test) with no modification of the affinity for the substrate (K_m values of 7.86 ± 0.65 and 7.34 ± 0.56 μM, respectively) (Fig. 1A). The effect of NT on aspartate uptake

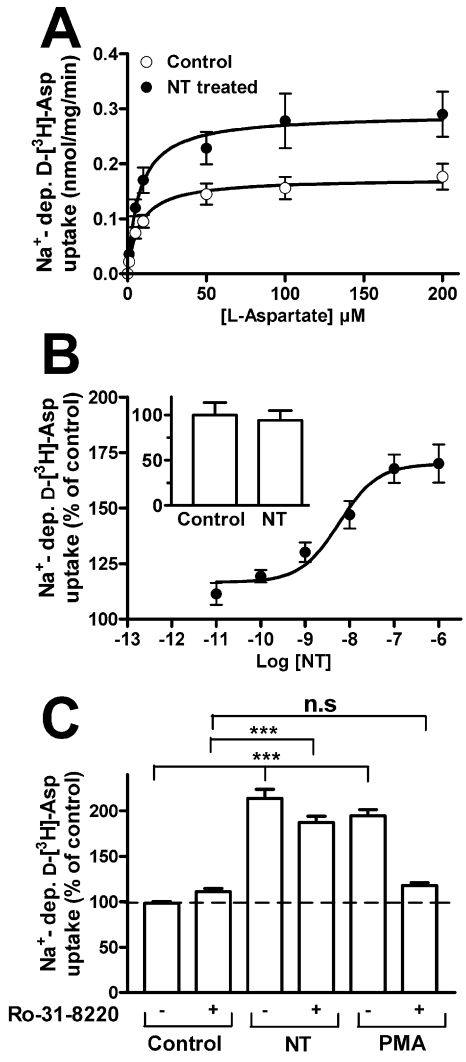

Fig. 1. Effect of NT on Na^+-dependent D-[^{3}H]aspartate uptake in C6-NTS1 cells. A: Saturation curve for aspartate uptake measured in C6-NTS1 cells treated for 5 min with NT (closed symbols) or vehicle (open symbols). Data shown correspond to means ± S.E.M. from six experiments performed in triplicate. B: Effect of pretreating C6-NTS1 cells with increasing concentrations of NT for 5 min on aspartate uptake. Inset shows the effect of NT on aspartate uptake in non-transfected C6 cells. Data are expressed as percent of control and correspond to means ± S.E.M. of four independent experiments performed in triplicate. C: Effect of the PKC inhibitor Ro-31-8220 on NT- and PMA-stimulated aspartate uptake in C6-NTS1 cells. Data shown are means ± S.E.M. of at least four independent experiments performed in triplicate. ***$P < 0.001$, significant difference versus corresponding control, n.s. denotes no significant difference (one-way ANOVA followed by Newman–Keuls test).

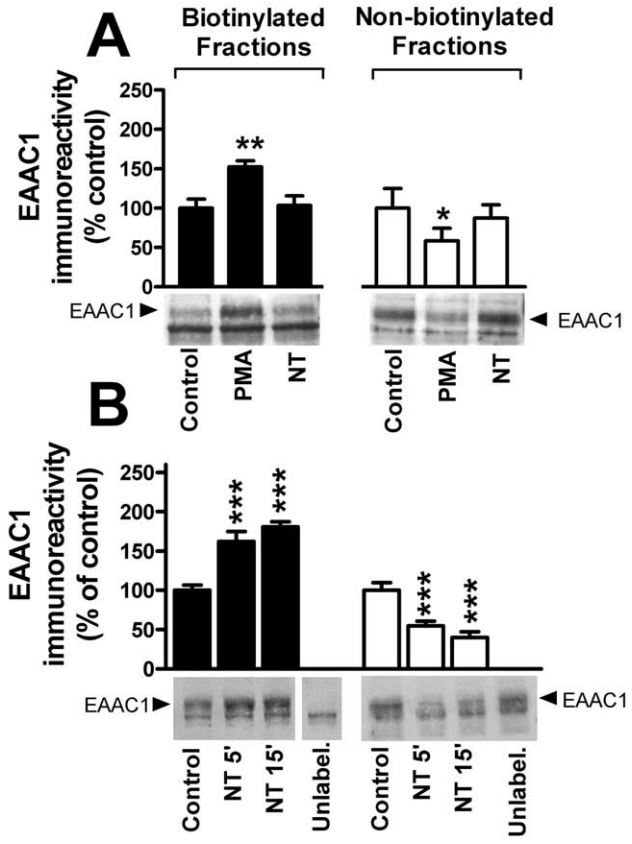

Fig. 2. Modulation of cell surface expression of EAAC1 by NT. Biotinylation of membrane proteins was performed to distinguish cell surface (biotinylated protein fractions) and intracellular proteins (non-biotinylated protein fractions) as described in Section 2. A: Non-transfected C6 cells. B: C6-NTS1 transfected cells. Typical autoradiograms are shown in the lower panels (arrows indicate the band corresponding to the EAAC1 transporter) whereas upper panels show the quantitation by gray-scale densitometry of the EAAC1 immunoreactivity (means ± S.E.M. from three different experiments). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ as compared to control (one-way ANOVA followed by Newman–Keuls test).

was concentration-dependent (Fig. 1B). The maximal effect was obtained when cells were incubated in the presence of 100 nM NT and the pEC_{50} value for NT was 8.27 ± 0.08, in the same range as the potency of NT at mediating the functional activation of NTS1 receptor (NT was shown to stimulate phosphoinositide hydrolysis with a pEC_{50} value of 9.05 ± 0.07). As expected, the effect of NT was not observed when tested on non-transfected C6 cells (Fig. 1B, inset), further confirming the involvement of the NTS1 receptor.

3.2. Role of intracellular signaling cascades in the stimulation of EAAC1-mediated aspartate uptake by NT

Activation of PKC by phorbol ester was previously shown to modulate EAAC1 activity in C6 glioma cells. Accordingly, exposure of C6-NTS1 cells to PMA resulted in a robust increase in specific aspartate uptake (97% increase as compared to control, $P < 0.001$) that was completely prevented when cells were preincubated with the specific PKC inhibitor Ro-31-8220 (0.5 μM) (Fig. 1C). In contrast, this inhibitor (used at the same concentration) failed to block the NT-mediated increase in aspartate uptake. Similar results were obtained with D-sphingosine, another specific PKC inhibitor (Table 1). Based on two recent studies indicating the possible involvement of PI3-K in the regulation of EAAC1 in C6 cells [6,9], we also evaluated the possible implication of this enzyme in the modulation of aspartate uptake by NT. As previously reported, inhibition of this enzyme with wortmannin (100 nM) was found to decrease the basal activity of EAAC1 to 37% of control (Table 1). However, when wortmannin-pretreated cells were exposed to NT, the neuropeptide was found to significantly increase the residual aspartate uptake (137% increase as compared to control wortmannin-pretreated cells, $P < 0.001$). These results indicate that PKC and PI3-K activities are not involved in the rapid effect of NT on aspartate uptake in C6-NTS1 cells.

Although NTS1 receptor is known to preferentially mediate intracellular responses through $G_{q/11}$-type G proteins, multiple studies have demonstrated functional coupling with $G_{i/o}$- and G_s-type G proteins [13,14], leading to additional signaling cascades, including modulation of adenylyl cyclase and induction of mitogen-activated protein kinases (MAPK). As shown in Table 1, neither the PKA inhibitor H89, nor the MAPK inhibitor PD98059 significantly prevented the effect of NT. Similarly, KN-62, an inhibitor of calcium calmodulin-dependent protein kinase II, and L-NAME, a nitric oxide synthase inhibitor, were unable to block the NT-stimulated aspartate uptake in C6-NTS1 cells.

3.3. Effect of NT on cell surface expression of EAAC1

Previous studies revealed that both alteration in transporter activity and cell surface expression may contribute to the regulation of EAAC1 [6,9]. In order to study the effect of NT on the membrane trafficking of EAAC1, we biotinylated cell surface proteins using a membrane-impermeant biotin ester and

Table 1
Effects of signal transduction inhibitors on NT-stimulated D-[^{3}H]aspartate uptake

	Inhibitor	NT	Inhibitor+NT
D-Sphingosine (10 μM)	115 ± 6	183 ± 10	186 ± 11
L-NAME (1 μM)	122 ± 9	183 ± 10	176 ± 11
H89 (500 nM)	99 ± 3	179 ± 14	155 ± 6
KN-62 (1 μM)	85 ± 7	179 ± 14	156 ± 12
PD98059 (10 μM)	98 ± 3	141 ± 5	148 ± 7
Wortmannin (100 nM)	37 ± 1	212 ± 7	88 ± 7

C6-NTS1 cells were pretreated with either vehicle or inhibitor for 15 min before exposure to NT (100 nM). The uptake assay was performed 5 min later. In all cases, no statistical difference was observed between the groups preincubated with inhibitors plus NT and those preincubated with NT alone. The two groups were statistically different from the controls (vehicles) and the groups preincubated with inhibitor alone (one-way ANOVA followed by the Newman–Keuls test). Values are percentages of Na^+-dependent D-[^{3}H]aspartate uptake in control wells preincubated in the absence of NT or inhibitor and represent means ± S.E.M. of at least three independent experiments performed in triplicate.

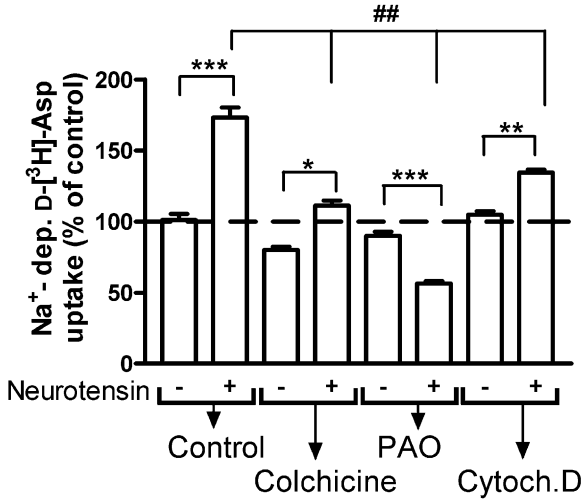

Fig. 3. Effect of colchicine (10 μM), PAO (10 μM) or cytochalasin D (30 μM) on basal and NT-stimulated aspartate uptake in C6-NTS1 cells. Cells were pretreated with the drugs for 15 min. Thereafter, NT (100 nM) was added and uptake assay was performed after 5 min. Results (percent of control) correspond to means ± S.E.M. of at least three independent experiments performed in triplicate. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ as compared to the corresponding control; $^{\#\#}P<0.01$ as compared to the NT-treated cells (one-way ANOVA followed by Newman–Keuls test).

after recovery of biotinylated EAAC1 using streptavidin–agarose beads, the transporters expressed at the plasma membrane were detected by immunoblotting (see Fig. 2). The band corresponding to the biotinylated EAAC1 was identified with an apparent molecular weight of ~68 kDa. After removal of biotinylated material, EAAC1 immunoreactive proteins were detected in the supernatant, indicating the existence of intracellular EAAC1. This result is in good agreement with those demonstrating the cytoplasmic localization of EAAC1 in brain tissues [15,16]. Incubation of non-transfected C6 cells with PMA (500 nM, 30 min) caused an increase in the intensity of EAAC1 immunoreactivity in the biotinylated fraction (Fig. 2A) that correlated with a significant decrease in the corresponding non-biotinylated (intracellular) fraction (Fig. 2A). As expected, no change in the intensity of the biotinylated and intracellular EAAC1-positive fractions was detected when non-transfected C6 cells were challenged with NT (Fig. 2A). Similarly, incubation of C6-NTS1 cells with NT for 5 or 15 min resulted in a modification in the proportion of biotinylated and non-biotinylated EAAC1 levels, with the highest effect of NT observed after 15 min (about 80% increase as compared to control values) (Fig. 2B). Based on these observations, we investigated whether such redistribution of EAAC1 could explain the effect of NT on aspartate uptake, using inhibitors of cytoskeletal organization. As shown in Fig. 3, colchicine, a disrupting agent of microtubule formation, and cytochalasin D, an inhibitor of actin polymerization, were without effect on basal aspartate uptake but impaired the stimulatory effect of NT. Similarly, PAO, which blocks both externalization and internalization processes, dramatically inhibited the effect of NT on aspartate uptake. These results suggest that the increase in aspartate uptake induced by NT results from an alteration in the EAAC1 trafficking which requires an intact cytoskeleton.

4. Discussion

The activity and expression of many membrane transporters are known to be regulated through phosphorylation-dependent pathways [17–19]. In the case of the glutamate transporter EAAC1, recent studies have highlighted the possible implication of both PKC and PI3-K in the alteration of glutamate uptake by influencing its cell surface expression [6,9]. However, the physiological stimuli responsible for the activation of these kinases have not been identified and no data are available concerning the possible involvement of endogenous transmission systems acting via GPCRs in the regulation of EAAC1. In order to study such regulation, we established transfected C6 cells expressing the NTS1 receptor and examined whether its activation modulates the activity and/or cell surface expression of EAAC1. Exposure of C6-NTS1 cells to NT induces an increase in EAAC1-mediated D-[^{3}H]aspartate uptake within 5 min. Since the NTS1 receptor is preferentially coupled to PLC activation, most responses to NT are usually attributed to the intracellular Ca^{2+} mobilization [20] and PKC translocation [21,22]. However, in the present study, the PKC inhibitors Ro-31-8220 and D-sphingosine failed to prevent the effect of NT indicating that PKC is not the pathway required for the modulation of aspartate transport by NT. Similarly, the PI3-K inhibitor wortmannin was ineffective in blocking the effect of NT on the residual aspartate uptake.

The regulation of the uptake of many neurotransmitters involves the rapid translocation of intracellular pools of specific transporters [23–25]. Accordingly, the rapid modulation of EAAC1 activity is incompatible with the de novo synthesis of this transporter and our data suggest that the alteration of aspartate transport observed after NT treatment is the consequence of an increased density of cell surface EAAC1 transporters. Thus the effect of NT on aspartate uptake affects the maximal velocity of the transport but not the affinity for the substrate. Our biotinylation experiments indicate that the proportion of extracellularly exposed EAAC1 significantly increases after a brief exposure to NT, an effect that is compatible with the time required for changes related to trafficking of this transporter. Moreover, we demonstrate that the NT-induced aspartate transport was completely blocked by colchicine suggesting the participation of the microtubule network in the modulation of the cell distribution of EAAC1 by NT. The inhibitory effect of cytochalasin D, which impairs actin polymerization, promoting disruption of the cytoskeleton, indicates that the latter is also involved in the NT-induced translocation of EAAC1 to the cell surface.

Based on these observations and since none of the classical second messenger pathway inhibitors used were effective in preventing the effect of NT, one could suggest that cytoskeletal elements may participate in the interaction between EAAC1 and other intracellular regulatory proteins modulated by GPCRs. For instance, it has been demonstrated that tubulin directly interacts with GPCR kinases for which it constitutes a potential substrate [26,27]. Indeed, it has been clearly suggested that GPCRs or G proteins could directly interact with cytoskeletal elements [28,29] and modulate cytoskeletal dynamics and intracellular trafficking [28,30,31]. These functional interactions bring further complexity to the intracellular signals triggered by GPCR activation and could help to better understand the PKC- or PI3-K-independent mechanisms involved in the regulation of EAAC1 transporter activity by

these membrane receptors. Further studies are required to clarify such mechanisms that allow the rapid and reversible mobilization of EAAC1 transporter molecules in response to the activation of a $G_{q/11}$-coupled receptor and thereby participate in the control of the extracellular levels of glutamate and neuronal excitation

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