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Regulation of the glutamate transporter EAAT3 by mammalian target of rapamycin mTOR

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ABSTRACT

The serine/threonine kinase mammalian target of rapamycin (mTOR) is stimulated by insulin, growth factors and nutrients and confers survival of several cell types. The kinase has previously been shown to stimulate amino acid uptake. In neurons, the cellular uptake of glutamate by the excitatory amino-acid transporters (EAATs) decreases excitation and thus confers protection against excitotoxicity. In epithelia, EAAT3 accomplishes transepithelial glutamate and aspartate transport. The present study explored, whether mTOR regulates EAAT3 (SLC1A1). To this end, cRNA encoding EAAT3 was injected into *Xenopus* oocytes with or without cRNA encoding mTOR and the glutamate induced current (I_{glu}), a measure of glutamate transport, determined by dual electrode voltage clamp. Moreover, EAAT3 protein abundance was determined utilizing chemiluminescence. As a result, I_{glu} was observed in *Xenopus* oocytes expressing EAAT3 but not in water injected oocytes. Coexpression of mTOR significantly increased I_{glu}, an effect reversed by rapamycin (100 nM). mTOR coexpression increased EAAT3 protein abundance in the cell membrane. The decay of I_{glu} following inhibition of carrier insertion with brefeldin A in oocytes coexpressing EAAT3 with mTOR was similar in the presence and absence of rapamycin (100 nM). In conclusion, mTOR is a novel powerful regulator of EAAT3 and may thus contribute to protection against neuroexcitotoxicity.

1. Introduction

The serine/threonine kinase mammalian target of rapamycin (mTOR) is stimulated by growth factors [1], insulin [2], leptin [3] and nutrients [4]. mTOR is inhibited by hypoxia [5]. The kinase participates in the regulation of cell proliferation, cell growth and cell survival [1,4,6]. Effects of the kinase include up-regulation of cellular amino-acid uptake [7].

According to previous studies inhibition of mTOR causes cell shrinkage [8] as well as impaired energy metabolism, lactate production and reactive oxygen species formation [9]. mTOR contributes to the pathophysiology of several disorders such as malignancy, cardiac hypertrophy, diabetes and obesity [4]. Along those lines, mTOR is considered a pharmacological target in the treatment of cancer [10] and diabetes [2]. On the other hand, dysregulation of mTOR signaling is observed in several neurodegenerative disorders [3].

Survival of neurons is challenged by neuroexcitotoxicity and may be fostered by glutamate clearance from synaptic clefts, a function of Na^+ coupled excitatory amino acid transporters (EAATs) [11–15]. Defective glutamate transporters may result in neurodegenerative disorders including amyotrophic lateral sclerosis, epilepsy, Huntington's disease, Alzheimer's disease and ischemic stroke injury [13]. The EAAT3 isoform (SLC1A1; also known as EAAC1) further accomplishes neuronal cysteine uptake [16,17] and transepithelial transport in kidney and intestine [18]. Loss of function SLC1A1 mutations cause human dicarboxylic aminoaciduria and in some patients mental retardation [18].

Little is known, however, about the influence of mTOR on glutamate transporters. The present study thus explored the influence of mTOR on the glutamate transporter EAAT3.

2. Materials and methods

2.1. Constructs

Constructs encoding wild-type human EAAT3 [19,20] and wild-type human mTOR [21] have been used for generation of cRNA as described previously [22].

2.2. Voltage clamp in Xenopus oocytes

For determination of electrogenic transport, *Xenopus laevis* oocytes were prepared as previously described [23]. Fifteen nano grams of cRNA encoding wild-type mTOR was injected one day

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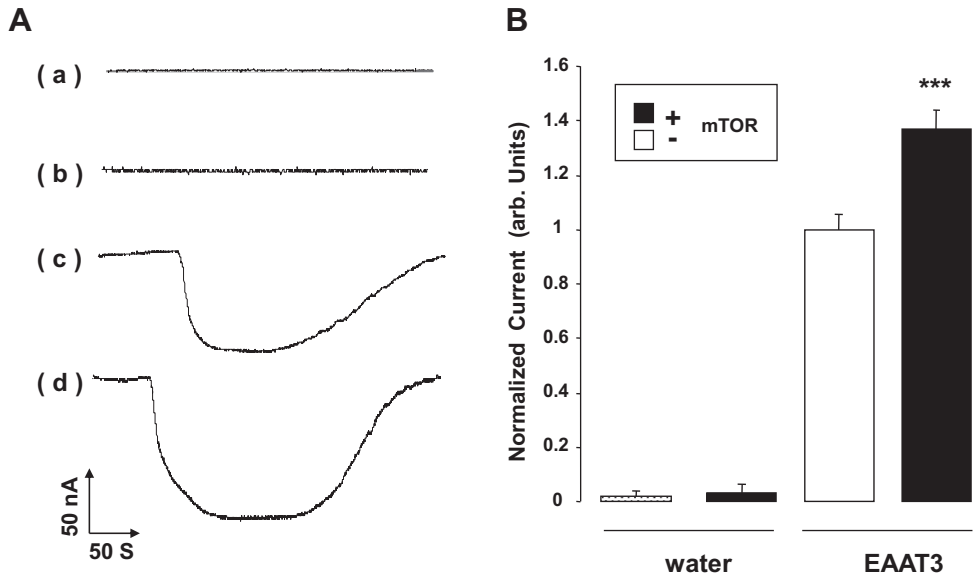

Fig. 1. Effect of mTOR on electrogenic glutamate transport in EAAT3 expressing *Xenopus laevis* oocytes. (A) Original tracings of glutamate (500 μM)-induced current (I_g) in *Xenopus* oocytes injected with water (a), with mTOR cRNA alone (b) or injected with EAAT3 cRNA without (c) or with (d) mTOR cRNA. (B) Arithmetic means ± SEM (7–31) of glutamate (500 μM) induced currents (I_{glu}) in oocytes injected with water (dotted bar), injected with EAAT3 cRNA alone (white bar) or injected with mTOR cRNA (black bars) either alone (left black bar) or together with EAAT3 cRNA (right black bar). ***$p < 0.001$ indicates statistically significant difference from the oocytes expressing EAAT3 alone.

after preparation of oocytes and 5 ng cRNA encoding EAAT3 was injected one day after mTOR injection. The oocytes were maintained at 17 °C in ND96 solution containing 88.5 mM NaCl, 2 mM KCl, 1.8 mM $MgC1_2$, 0.1 mM $CaC1_2$, 5 mM HEPES, pH was adjusted to 7.5 by addition of NaOH. Tretracycline (Sigma, 0.11 mM), Ciprofloxacin (Sigma, 4 μM), Gentamycin (Refobacin© – 0.2 mM) and Theophyllin (Euphylong©, 0.5 mM) as well as sodium pyruvate (Sigma, 5 mM) were added to the solution. All experiments were performed at room temperature 6 days after mTOR injection. Two-electrode voltage-clamp recordings were obtained at a holding potential of −60 mV [24]. The data were filtered at 10 Hz, and recorded with a GeneClamp 500 amplifier, a DigiData 1300 A/D–D/A converter and the pClamp 9.02 software package for data acquisition and analysis (Axon Instruments, Union City, CA, USA) [25,26]. The control solution (superfusate/ND96) contained 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES, pH 7.4. Rapamycin (100 nM) or brefeldin A (5 μM) were added to the solutions where indicated. Glutamate was added to the solutions at a concentration of 500 μM. The final solutions were titrated to pH 7.4 using NaOH. The flow rate of the superfusion was 20 ml/min and a complete exchange of the bath solution was reached within about 10 s [27].

2.3. Detection of EAAT3 cell surface expression by chemiluminescence

For detection of EAAT3 cell surface expression the oocytes were first incubated with 0.5 μg/mL primary mouse anti-glutamate transporter EAAC1 antibody (Invitrogen, USA) and subsequently with secondary, HRP-conjugated, sheep anti-mouse IgG antibody (1:2500, GE Healthcare, UK). Individual oocytes were placed in 96 well plates with 20 μl of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA) and chemiluminescence of single oocytes was quantified in a luminometer (Walter Wallac 2 plate reader, Perkin Elmer, Juegesheim, Germany) by integrating the signal over a period of 1 s. Results display normalized relative light units. Integrity of the measured oocytes was assessed by visual control after the measurement to avoid unspecific light signals from the cytosol [28].

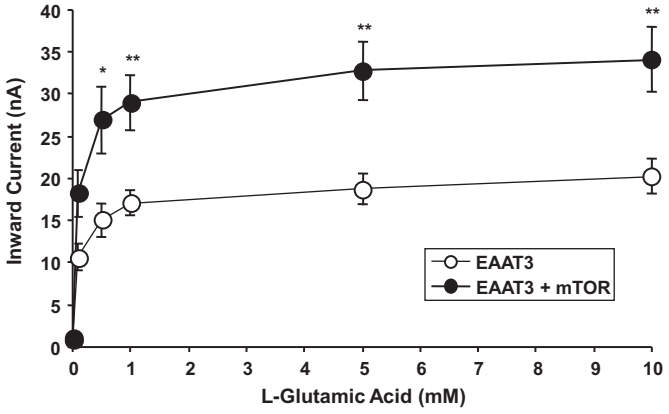

Fig. 2. Effect of mTOR on EAAT3 transport kinetics. Arithmetic means ± SEM ($n = 13 – 14$) of glutamate induced current (I_g) as a function of glutamate concentration in *Xenopus* oocytes expressing EAAT3 without (open circles) or with (closed circles) mTOR. *($p < 0.05$), **$p < 0.01$ indicates statistically significant difference from the oocytes expressing EAAT3 alone.

2.4. Statistical analysis

Data are provided as means ± SEM, n represents the number of oocytes investigated. All experiments were repeated with at least three batches of oocytes; in all repetitions qualitatively similar data were obtained. Data were tested for significance using ANOVA, and results with $p < 0.05$ were considered statistically significant.

3. Results

To explore, whether mammalian target of rapamycin mTOR modifies the activity of the excitatory amino acid transporter EAAT3, experiments were performed in *Xenopus* oocytes expressing EAAT3 without and with additional coexpression of mTOR. Glutamate (500 μM) induced inward current (I_{glu}) was taken as a measure of glutamate transport. In EAAT3 expressing oocytes but not

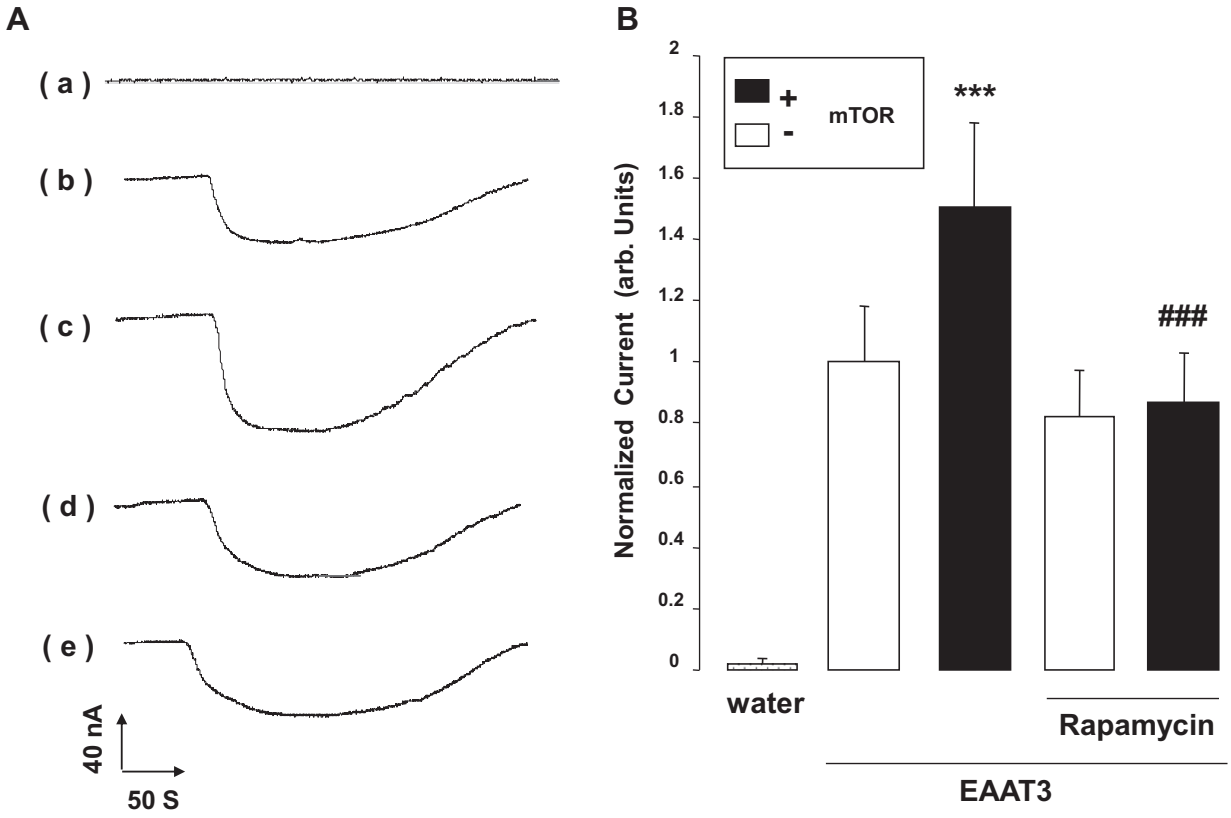

Fig. 3. Reversal of the mTOR effect by rapamycin. (A) Original tracings of glutamate (500 μM) induced current (I_g) in *Xenopus* oocytes injected with water (a) or with EAAT3 without (b, d) or with (c, e) additional co-expression of mTOR in the absence (a–c) and presence (d, e) of rapamycin (100 nM). (B) Arithmetic means ± SEM ($n = 5 - 22$) of glutamate (500 μM) induced currents (I_g) in oocytes injected with water (dotted bar) or injected with EAAT3 cRNA without (white bars) or with (black bars) additional co-expression of mTOR in the absence (left bars) or presence (right bars) of rapamycin (100 nM, 48 h). $^{***}p < 0.001$ indicates statistically significant difference from oocytes expressing EAAT3 alone. $^{###}p < 0.001$, indicates statistically significant difference from absence of rapamycin.

in water injected *Xenopus. laevis* oocytes glutamate exposure resulted in the generation of I_{glu} (Fig. 1), indicating that I_{glu} was due to EAAT3 activity and not due to an endogenous electrogenic transport system. I_{glu} was in EAAT3 expressing *Xenopus* oocytes significantly increased by additional injection of cRNA encoding mTOR (Fig. 1). The injection of mTOR alone did not result in an appreciable I_{glu} (Fig. 1), an observation ruling out the theoretical possibility that the observed increase of I_{glu} in EAAT3 expressing oocytes following additional coexpression of mTOR was due to up-regulation of an endogenous electrogenic glutamate transporter.

A kinetic analysis of glutamate induced current in EAAT3 expressing *Xenopus* oocytes (Fig 2) yielded a maximal I_{glu} of 18.6 ± 0.7 nA ($n = 14$). The glutamate concentration required for halfmaximal I_{glu} (K_M) was 90.3 ± 19.8 μM. The coexpression of mTOR did not significantly modify apparent K_M (92.2 ± 20.2 μM), but significantly increased the maximal I_{glu} to 32.5 ± 1.3 nA ($n = 13$).

In EAAT3 and mTOR expressing *Xenopus* oocytes, mTOR inhibitor rapamycin (100 nM) decreased I_{glu} (Fig. 3). The effect of the inhibitor was slow and reached statistical significance after 48 h of treatment with rapamycin (100 nM).

At least in theory, the increase of I_{glu} in EAAT3 expressing oocytes following coexpressing of mTOR could have resulted from an increase of carrier protein abundance in the cell membrane. Thus, chemiluminescence analysis was employed to test for altered carrier protein abundance within the cell surface. As illustrated in Fig. 4A, the coexpression of mTOR indeed significantly increased the EAAT3 protein abundance in the cell membrane. The enhanced EAAT3 protein abundance in the cell membrane of EAAT3 and mTOR coexpressing oocytes could have resulted from either, accelerated insertion of new carriers into the cell membrane, or delayed clearance of carriers from the cell membrane. To discriminate between those two possibilities *Xenopus* oocytes expressing both, EAAT3 and mTOR were treated with 5 μM brefeldine A for 24 h, which blocks the insertion of new carrier protein into the cell membrane. The experiment was performed in both the absence and presence of rapamycin (100 nM). As illustrated in Fig. 4B, I_{glu} declined in the presence of brefeldin A at a similar rate in the absence and presence of rapamycin, indicating that rapamycin did not affect retrieval of carrier protein from the cell membrane. This observation suggests that mTOR increases EAAT3 protein abundance by stimulating carrier insertion into the cell membrane rather than by inhibiting carrier clearance from the cell membrane.

4. Discussion

The present study reveals a novel function of the mammalian target of rapamycin mTOR, i.e. the upregulation of the excitatory amino acid transporter EAAT3. The effect of mTOR is at least in part due to an increase of carrier protein abundance in the cell membrane.

EAAT3 is expressed in neurons [29–36], retinal ganglion cells [37] and glial cells [35,38–40]. Defective EAAT3 may lead to schizophrenia [41–47], epilepsy [48–52] and hepatic encephalopathy [53]. As mTOR up-regulates EAAT3, it may, at least in theory, counteract those disorders. Notably, mTOR has been implicated in the protection of neurons against apoptosis and rapamycin treatment has been reported to enhance neuronal degeneration [54–56]. On the other hand, rapamycin has been reported to counteract

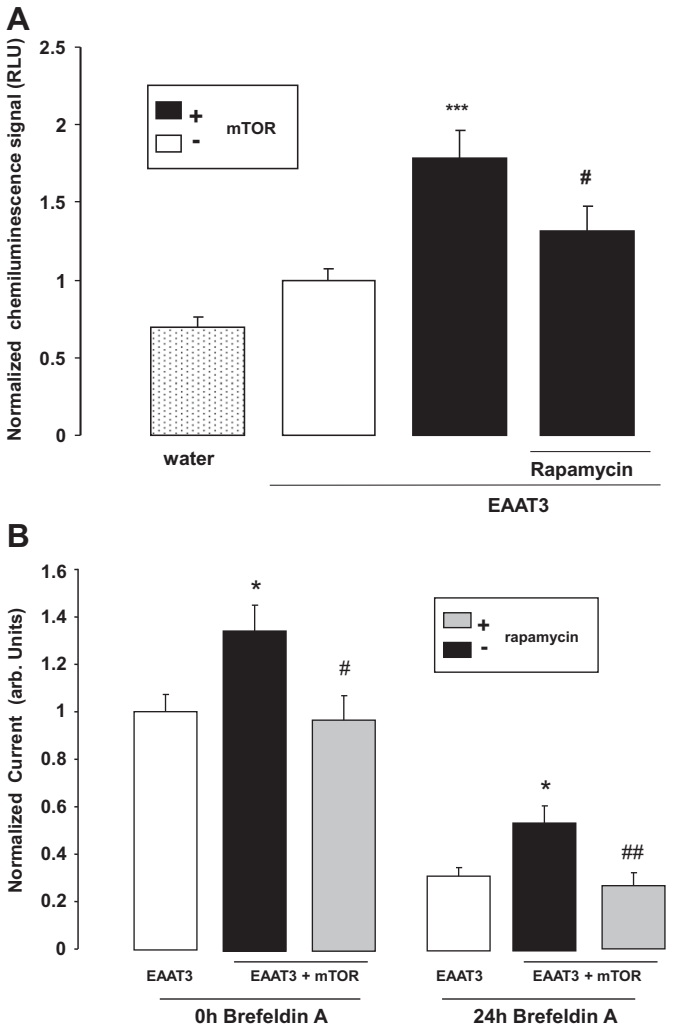

Fig. 4. Effect of mTOR on EAAT3 abundance within the plasma membrane of oocytes and decline of EAAT3 induced currents by brefeldine A. (A) EAAT3 cell surface expression assessed by chemiluminescence in *Xenopus* oocytes injected with water (dotted bar), with EAAT3 without (white bar) or with additional coexpression of mTOR (black bars) in the absence or presence of rapamycin (100 nM, 48 h, right bar), $^{***}p < 0.001$ indicates statistically significant difference from oocytes expressing EAAT3 alone, $^{\#}p < 0.05$, indicates statistically significant difference from oocytes coexpressing EAAT3 with mTOR in the absence of rapamycin. (B) Arithmetic means ± SEM ($n = 9 - 16$) of glutamate (500 μM)-induced current (I_g) in *Xenopus* oocytes injected with EAAT3 without (white bars) or with mTOR after incubation with (5 μM) brefeldin A for 24 h in the absence (black bars) or the presence of 100 nM rapamycin (grey bars). $^{*}p < 0.05$, indicates statistically significant difference from the oocytes expressing EAAT3 alone. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ indicates significant differences from the oocytes expressing EAAT3 with mTOR in the absence of rapamycin.

neuronal or sensory cell death [57–60]. Rapamycin may in part be effective by modulating autophagy [61]. Activation of mTOR inhibits and rapamycin stimulates autophagy [56,59,62]. The stimulation of autophagy reduces the cellular accumulation of protein aggregates and counteracts the progression of neurodenerative diseases [61]. Autophagy is inhibited by cell swelling, which could result from concentrative amino acid transport [63]. In view of the present observations the inhibitory effect of mTOR on autophagy could be at least in part be due to stimulation of EAAT3 and further amino acid transporters.

EAAT3 is further expressed in blood platelets [64,65], heart [66], renal podocytes [67], renal and intestinal epithelia [18], epididymis [68], placenta [69,70] and blood–brain barrier [71]. Whether transport of dicarboxylic amino acids in those tissues is indeed sensitive to mTOR activity, remains to be shown.

In conclusion, mTOR up-regulates the excitatory amino acid transporter EAAT3, an effect due to increase of carrier protein abundance in the cell membrane. The effect may modify cellular amino acid transport in neurons and a wide variety of nonexcitable cells.

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The authors declare that they have no conflict of interest to disclose.

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