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Original Paper

Up-Regulation of Excitatory Amino Acid Transporters EAAT3 and EAAT4 by Lithium Sensitive Glycogen Synthase Kinase GSK3ß

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Key Words
Neuroexcitability • Lithium • Glutamate transporter • SLC1A1 • SLC1A6

Abstract
Background: Cellular uptake of glutamate by the excitatory amino-acid transporters (EAATs) decreases excitation and thus participates in the regulation of neuroexcitability. Kinases impacting on neuronal function include Lithium-sensitive glycogen synthase kinase GSK3ß. The present study thus explored whether the activities of EAAT3 and/or EAAT4 isoforms are sensitive to GSK3ß. ***Methods:*** cRNA encoding wild type EAAT3 (SLC1A1) or EAAT4 (SLC1A6) was injected into *Xenopus* oocytes without or with additional injection of cRNA encoding wild type GSK3ß or the inactive mutant ^{K85A}GSK3ß. Dual electrode voltage clamp was performed in order to determine glutamate-induced current (I_{EAAT}). ***Results:*** Appreciable I_{EAAT} was observed in EAAT3 or EAAT4 expressing but not in water injected oocytes. I_{EAAT} was significantly increased by coexpression of GSK3ß but not by coexpression of ^{K85A}GSK3ß. Coexpression of GSK3ß increased significantly the maximal I_{EAAT} in EAAT3 or EAAT4 expressing oocytes, without significantly modifying apparent affinity of the carriers. Lithium (1 mM) exposure for 24 hours decreased I_{EAAT} in EAAT3 and GSK3ß expressing oocytes to values similar to I_{EAAT} in oocytes expressing EAAT3 alone. Lithium did not significantly modify I_{EAAT} in oocytes expressing EAAT3 without GSK3ß. ***Conclusions:*** Lithium-sensitive GSK3ß is a powerful regulator of excitatory amino acid transporters EAAT3 and EAAT4.

Introduction

Glutamate transporters clear glutamate from synaptic clefts and thus counteract excitotoxicity [1-5]. The EAAT3 isoform is expressed in neurons [6-13], retinal ganglion cells [14], and glial cells [12, 15-17]. EAAT4 is expressed in cerebellar Purkinje cells and clears glutamate from the synapses connecting the climbing fibers with the Purkinje cells [10, 18].

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Dysregulation of EAAT3 has been implicated in schizophrenia [19-25], epilepsy [26-30] and hepatic encephalopathy [31], dysregulation of EAAT4 has been associated with schizophrenia [19, 20].

Regulators of neuronal excitability include the glycogen synthase kinase GSK3ß [32], a serine/threonine kinase inhibited by the antidepressant Lithium [33]. GSK3ß is phosphorylated and down-regulated by protein kinase B (PKB/Akt) [34].

The present study explored whether GSK3ß influences the activity of the glutamate transporters EAAT3 (SLC1A1) or EAAT4 (SLC1A6). To this end, the carriers were expressed in *Xenopus* oocytes without and with additional expression of wild type GSK3ß or inactive ^{K85A}GSK3ß and electrogenic glutamate transport quantified by dual electrode voltage clamp.

Materials and Methods

Constructs

For generation of cRNA [35, 36], constructs were used encoding wild-type human EAAT3 [37], EAAT4 [37], wild type GSK3ß and inactive mutant ^{K85A}GSK3ß [38]. The constructs were generated by site-directed mutagenesis (QuikChange II XL Site-Directed Mutagenesis Kit; Stratagene, Heidelberg, Germany) according to the manufacturer's instructions [39]. The mutants were sequenced to verify the presence of the desired mutation and used for generation of cRNA as described previously [40-42].

Voltage clamp in Xenopus oocytes

Xenopus oocytes were prepared as previously described [43-45]. 10 ng of wild type GSK3ß or ^{K85A}GSK3ß cRNA were injected on the first day and 10 ng EAAT3 or EAAT4 cRNA on the same day after preparation of the oocytes. The oocytes were maintained at 17°C in ND96-A solution containing (in mM): 88.5 NaCl, 2 KCl, 1 $MgC1_2$, 1.8 $CaC1_2$, 2.5 NaOH, 5 HEPES, 5 sodium pyruvate ($C_3H_3NaO_3$), Gentamycin (100 mg/l), Tetracycline (50 mg/l), Ciprofloxacin (1.6 mg/l), Theophiline (90 mg/l). The pH was titrated to 7.4 using NaOH. Where indicated, Lithium (1 mM) was added. The voltage clamp experiments [46-48] were performed at room temperature 3 days after injection. Two-electrode voltage-clamp recordings were performed at a holding potential of -70 mV. The data were filtered at 10 Hz and recorded with a Digidata A/D-D/A converter and Clampex V.9 software for data acquisition and analysis (Axon Instruments)[49-51]. The control superfusate ND96 contained (in mM) 93.5 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 2.5 NaOH and 5 HEPES (pH 7.4). The flow rate of the superfusion was approx. 20 ml/min, and a complete exchange of the bath solution was reached within about 10 s. Glutamate was added to the solutions at the indicated concentrations. The flow rate of the superfusion was approx. 20 ml/min, and a complete exchange of the bath solution was reached within about 10 s [52-54].

Statistical analysis

Data are provided as means ± SEM, n represents the number of oocytes investigated. All experiments were repeated with at least 3 batches of oocytes; in all repetitions qualitatively similar data were obtained. As expression of injected cRNA may vary from batch to batch of oocytes, comparisons were always made within the same oocyte batch [55, 56]. Data were tested for significance using ANOVA or t-test, as appropriate. Results with $p < 0.05$ were considered statistically significant.

Results

The present study explored whether the glycogen synthase kinase GSK3ß modifies the function of the glutamate transporters EAAT3 and EAAT4. To this end, cRNA encoding EAAT3 or EAAT4 was injected into *Xenopus laevis* oocytes with or without additional injection of cRNA encoding GSK3ß. Glutamate-induced current determined by dual electrode voltage clamp was taken as measure of transport.

As illustrated in Fig. 1, addition of glutamate to the superfusate did not elicit an appreciable current in water-injected oocytes. Accordingly, the oocytes did not express

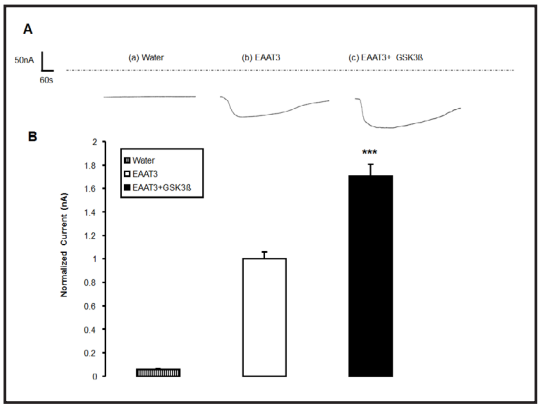


Fig. 1. Effect of wild-type GSK3ß on electrogenic glutamate transport in EAAT3 expressing *Xenopus laevis* oocytes. A: Representative original tracings showing glutamate (1 mM) - induced current (I_{EAAT}) in *Xenopus laevis* oocytes injected with water (a) or expressing EAAT3 without (b) or with (c) additional co-expression of wild-type GSK3ß. B: Arithmetic means ± SEM (n = 19-23) of I_{EAAT} in *Xenopus laevis* oocytes injected with water (striated bar), or expressing EAAT3 without (white bar) or with wild-type GSK3ß (black bar). *** (p<0.001) indicates statistically significant difference from the absence of GSK3ß.

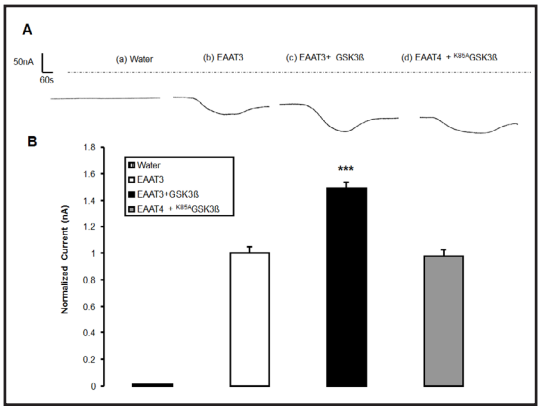


Fig. 2. Effect of inactive mutant ^{K85A}GSK3ß on electrogenic glutamate transport in EAAT3 expressing *Xenopus laevis* oocytes. A: Representative original tracings showing glutamate (1 mM) - induced current (I_{EAAT}) in *Xenopus laevis* oocytes injected with water (a), expressing EAAT3 alone (b) or expressing EAAT3 with additional co-expression of wild-type GSK3ß (c), or catalytically inactive ^{K85A}GSK3ß (d). B: Arithmetic means ± SEM (n = 13-15) of I_{EAAT} in *Xenopus laevis* oocytes injected with water (striated bar) or expressing EAAT3 without (white bar) or with wild-type GSK3ß (black bar), or catalytically inactive ^{K85A}GSK3ß (grey bar) *** (p<0.001) indicates statistically significant difference from oocytes expressing EAAT3 alone.

significant endogenous electrogenic glutamate transport. In EAAT3 expressing oocytes, however, the addition of glutamate to the superfusate was followed by appearance of an inward current. The additional co-expression of wild-type GSK3ß was followed by a significant increase of glutamate-induced current in EAAT3 expressing *Xenopus laevis* oocytes.

In contrast to wild-type GSK3ß, the inactive ^{K85A}GSK3ß mutant did not significantly modify the glutamate induced-current in EAAT3 expressing *Xenopus* oocytes (Fig. 2).

In order to test whether GSK3ß co-expression modifies the maximal glutamate-induced current or the affinity of the carrier, the current induced by glutamate concentrations ranging from 1 µM to 5 mM was determined in *Xenopus laevis* oocytes expressing EAAT3 without or with additional expression of wild-type GSK3ß. As illustrated in Fig. 3, the glutamate induced-current was a function of the extracellular glutamate concentration. Kinetic analysis revealed that the maximal glutamate induced current was significantly (p<0.001) lower in *Xenopus laevis* oocytes expressing EAAT alone (61.92 ± 4.17 nA, n = 10) than in *Xenopus laevis* oocytes expressing EAAT3 together with wild-type GSK3ß (86.03 ± 3.41 nA, n = 10). The concentration required for half-maximal glutamate induced current was not significantly different between *Xenopus* oocytes expressing EAAT3 alone (652.04 ± 61.96 µM, n = 10) and *Xenopus* oocytes expressing EAAT3 together with wild-type GSK3ß (562.36 ± 80.88 µM, n = 10).

As GSK3ß could be inhibited by Lithium, additional experiments were performed in *Xenopus* oocytes expressing both EAAT3 and wild-type GSK3ß with or without prior exposure to 1 mM Lithium. As illustrated in Fig. 4, a 24 hours treatment with Lithium significantly decreased the glutamate-induced current in *Xenopus* oocytes expressing both EAAT3 and wild-type GSK3ß. In *Xenopus* oocytes expressing EAAT3 alone, however, a 24 hours treatment with Lithium did not significantly modify the glutamate-induced current

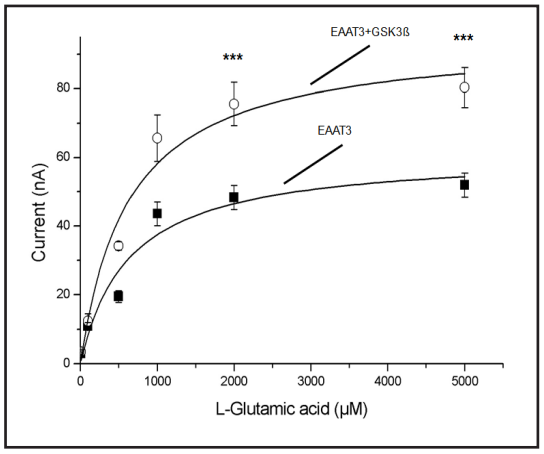


Fig. 3. Electrogenic glutamate transport in EAAT3-expressing *Xenopus laevis* oocytes as a function of glutamate concentration without and with presence of wild-type GSK3ß. Arithmetic means ± SEM (n = 10) of I_{EAAT} as a function of glutamate concentration in *Xenopus laevis* oocytes expressing EAAT3 without (black squares), or with (white circles) additional co-expression of wild-type GSK3ß. *** (p<0.001) indicates statistically significant difference from oocytes expressing EAAT3 alone.

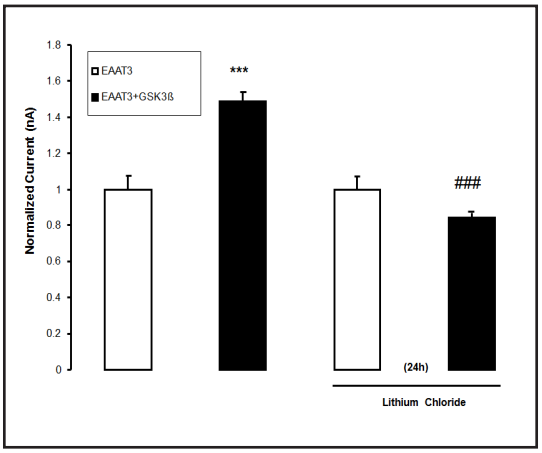
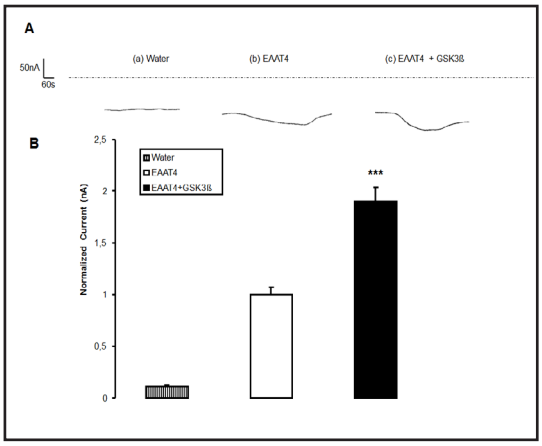


Fig. 4. Effect of Lithium on electrogenic glutamate transport in EAAT3 and GSK3ß expressing *Xenopus laevis* oocytes. Arithmetic means ± SEM (n = 17) of I_{EAAT} in *Xenopus* oocytes expressing EAAT3 alone (white bars), or expressing EAAT3 together with wild-type GSK3ß (black bars) without (left bars) or with (right bars) prior exposure to GSK3ß inhibitor Lithium (1 mM) for 24 hours. ***(p< 0.001) indicates statistically significant difference from *Xenopus* oocytes expressing EAAT3 alone, ### (p< 0.001) indicates statistically significant (p< 0.001) difference from the absence of Lithium.

Fig. 5. Effect of wild-type GSK3ß on electrogenic glutamate transport in EAAT4 expressing *Xenopus laevis* oocytes. A: Representative original tracings showing glutamate (1 mM) - induced current (I_{EAAT}) in *Xenopus laevis* oocytes injected with water (a) or expressing EAAT4 without (b) or with (c) additional co-expression of wild-type GSK3ß. B: Arithmetic means ± SEM (n = 23) of I_{EAAT} in *Xenopus laevis* oocytes injected with water (striated bar), or expressing EAAT4 without (white bar) or with wild-type GSK3ß (black bar). *** (p<0.001) indicates statistically significant difference from the absence of GSK3ß.



(Fig. 4). Thus, Lithium abrogated the stimulating effect of GSK3ß on EAAT3, but did not influence EAAT3 activity in the absence of GSK3ß.

Similar observations were made in oocytes expressing EAAT4. As shown in Fig. 5, addition of glutamate to the superfusate did again not elicit an appreciable current in water-injected oocytes. In EAAT4 expressing oocytes, however, the addition of glutamate to the superfusate was followed by appearance of an inward current. The additional co-expression of wild-type GSK3ß was followed by a significant increase of glutamate induced-current in EAAT4 expressing *Xenopus laevis* oocytes. In contrast to wild-type GSK3ß, the inactive ^{K85A}GSK3ß mutant did not significantly modify the glutamate-induced current in EAAT4 expressing *Xenopus* oocytes (Fig. 6).

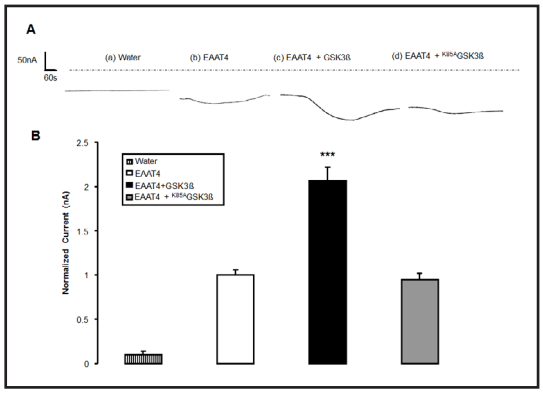


Fig. 6. Effect of inactive mutant ^{K85A}GSK3ß on electrogenic glutamate transport in EAAT4 expressing *Xenopus laevis* oocytes. A: Representative original tracings showing glutamate (1 mM) - induced current (I_{EAAT}) in *Xenopus laevis* oocytes injected with water (a), expressing EAAT4 alone (b) or expressing EAAT4 with additional co-expression of wild-type GSK3ß (c), or catalytically inactive ^{K85A}GSK3ß (d). B: Arithmetic means ± SEM (n = 14-16) of I_{EAAT} in *Xenopus laevis* oocytes injected with water (striated bar) or expressing EAAT4 without (white bar) or with wild-type GSK3ß (black bar), or catalytically inactive ^{K85A}GSK3ß (grey bar) *** (p<0.001) indicates statistically significant difference from oocytes expressing EAAT4 alone.

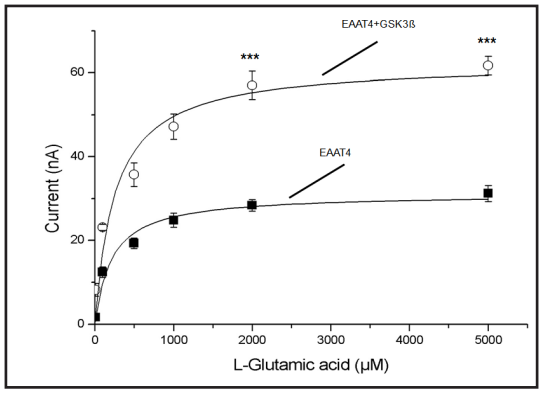


Fig. 7. Electrogenic glutamate transport in EAAT4-expressing *Xenopus laevis* oocytes as a function of glutamate concentration without and with presence of wild-type GSK3ß. Arithmetic means ± SEM (n = 8) of I_{EAAT} as a function of glutamate concentration in *Xenopus laevis* oocytes expressing EAAT4 without (black squares), or with (white circles) additional co-expression of wild-type GSK3ß. *** (p<0.001) indicates statistically significant difference from oocytes expressing EAAT4 alone.

Again, the current induced by glutamate concentrations ranging from 1 µM to 5 mM was determined in *Xenopus laevis* oocytes expressing EAAT4 without or with additional expression of wild-type GSK3ß. As illustrated in Fig. 7, the glutamate-induced current was a function of the extracellular glutamate concentration. Kinetic analysis revealed that the maximal glutamate-induced current was significantly (p<0.001) lower in *Xenopus laevis* oocytes expressing EAAT4 alone (31.11 ± 1.85 nA, n = 8) than in *Xenopus laevis* oocytes expressing EAAT4 together with wild-type GSK3ß (63.64 ± 2.80 nA, n = 7). The concentration required for half-maximal glutamate-induced current was not significantly different between *Xenopus* oocytes expressing EAAT4 alone (217.21 ± 26.08 µM, n = 8) and *Xenopus* oocytes expressing EAAT4 together with wild-type GSK3ß (298.39 ± 41.26 µM, n = 7).

Discussion

The present observations revealed that GSK3ß is a powerful regulator of the excitatory amino acid transporters EAAT3 and EAAT4. GSK3ß increased electrogenic glutamate transport in EAAT3 and EAAT4 expressing *Xenopus* oocytes. In contrast, the inactive mutant ^{K85A}GSK3ß failed to modify the glutamate induced current. Thus, kinase activity is apparently required for GSK3ß mediated regulation of EAAT3 and EAAT4 transport activity.

Coexpression of GSK3ß increased significantly the maximal glutamate-induced current, without significantly modifying affinity of the carriers. Possibly, GSK3ß increases the abundance of carrier protein in the plasma membrane.

At least in theory, dysregulation of excitatory amino acid transporters could contribute to the complex neuronal actions of the kinase. As were expected [33], the effect of GSK3ß on the glutamate induced current was abrogated by Lithium. Whether or not the effect on excitatory

amino acid transporters contributes to the pleotropic actions of this widely used antidepressant drug, cannot be answered by the present experiments.

Besides its potential impact on neuronal function, GSK3ß sensitive regulation of EAAT3 may modify a variety of further functions, as EAAT3 is expressed in a variety of cells and tissues, including blood platelets [57, 58], heart [59], renal podocytes [60], epididymis [61], placenta [62, 63] and blood-brain barrier [64]. In view of the present observations, glutamate uptake into the respective cells is expected to be sensitive to GSK3ß activity.

In conclusion, Lithium sensitive GSK3ß is a powerful stimulator of the excitatory amino acid transporters EAAT3 and EAAT4. Regulation of the carriers may thus contribute to the impact of neuronal and extracerebral functions of this ubiquitously expressed kinase.

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Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

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