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Regulation of the Glutamate Transporters by JAK2

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Key Words

Astrocytes • Neuroexcitability • Neurotransmission • Excitatory amino acid transporter • Leptin • Erythropoietin

Abstract

The Janus-activated kinase-2 JAK2 is involved in the signaling of leptin and erythropoietin receptors and mediates neuroprotective effects of the hormones. In theory, JAK2 could be effective through modulation of the glutamate transporters, carriers accounting for the clearance of glutamate released during neurotransmission. The present study thus elucidated the effect of JAK2 on the glutamate transporters EAAT1, EAAT2, EAAT3 and EAAT4. To this end, cRNA encoding the carriers was injected into *Xenopus* oocytes with or without cRNA encoding JAK2 and glutamate transport was estimated from glutamate induced current (I_{glu}). I_{glu} was observed in *Xenopus* oocytes expressing EAAT1 or EAAT2 or EAAT3 or EAAT4, but not in water injected oocytes. Coexpression of JAK2 resulted in an increase of I_{glu} by 83% (EAAT1), 67% (EAAT2), 42% (EAAT3) and 126% (EAAT4). As shown for EAAT4 expressing *Xenopus* oocytes, the effect of JAK2 was mimicked by gain of function mutation ^{V617F}JAK2 but not by the inactive mutant ^{K882E}JAK2. Incubation with JAK2 inhibitor AG490 (40 μM) resulted in a gradual decrease of I_{glu} by 53%, 79% and 92% within 3, 6 and 24 hours. Confocal microscopy and chemiluminescence analysis revealed that JAK2 coexpression increased EAAT4 protein abundance in the cell membrane. Disruption of transcription did not appreciably modify the up-regulation of I_{glu} in EAAT4 expressing oocytes. The decay of I_{glu} following inhibition of carrier insertion with brefeldin A was similar in oocytes expressing EAAT4 + JAK2 and oocytes expressing EAAT4 alone, indicating that JAK2 did not appreciably affect carrier retrieval from the membrane. In conclusion, JAK2 is a novel powerful regulator of glutamate transporters and thus participates in the protection against excitotoxicity.

Introduction

Janus-activated kinase-2 JAK2 is involved in the signaling of the leptin receptor [1]. In the brain leptin influences hypothalamic neurons and thus modifies appetite and mechanisms governing energy expenditure

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[1]. Leptin is effective through stimulation of the leptin receptor LEPRb, leading to activation of JAK2-dependent and -independent pathways. Leptin has further been shown to exert anticonvulsant activity, an effect considered to involve JAK2 and to result in part from modification of glutamate receptors [2, 3]. Excitotoxicity is further counteracted by erythropoietin, a hormone again signaling through JAK2 and interfering with glutamate signaling [4-6].

Excitotoxicity may be modified by the efficiency of glutamate clearance from synaptic clefts, which is a function of glutamate transporters [7-11]. Deranged function of the glutamate transporters has been implicated in the pathophysiology of several neurodegenerative disorders such as amyotrophic lateral sclerosis, epilepsy, Huntington's disease, Alzheimer's disease and ischemic stroke injury [9].

Little is known, however, about an influence of JAK2 on glutamate transporters. In the placenta, Leptin stimulates the system A amino acid transporter, an effect presumably involving JAK2 [12]. The present study thus explored, whether JAK2 influences the excitatory amino acid transporters EAAT1-4.

Materials and Methods

Constructs

Constructs encoding wild type human EAAT1 [13], EAAT2 [14, 15], EAAT3 [16, 17] and EAAT4 [18, 19] have been described previously. The JAK2 construct was generated from template human JAK2 cDNA provided by Imagenes (Berlin, Germany). Further, an inactive ^{K882E}JAK2 mutant [20] and the active ^{V617F}JAK2 mutant [21] were generated by site-directed mutagenesis (QuikChange II XL Site-Directed Mutagenesis Kit; Stratagene, Heidelberg, Germany) according to the manufacturer's instructions [22]. The following primers were used:

^{V617F}JAK2: 5'-AGC ATT TGG TTT TAA ATT ATG GAG TAT GT**T** TCT GTG GAG ACG AGA-3';

^{V617F}JAK2: 5'-TCT CGT CTC CAC AGA **A**AC ATA CTC CAT AAT TTA AAA CCA AAT GCT-3';

^{K882E}JAK2: 5'-GGG AGG TGG TCG CTG TA**G** AAA AGC TTC AGC ATA GT-3';

and ^{K882E}JAK2: 5'-ACT ATG CTG AAG CTT TT**C** TAC AGC GAC CAC CTC CC-3'.

Underlined bases indicate mutation sites. The mutants were sequenced to verify the presence of the desired mutation. The mutants were used for generation of cRNA as described previously [23].

Voltage clamp in Xenopus oocytes

For determination of electrogenic transport, *Xenopus laevis* oocytes were prepared as previously described [24]. Ten ng of wild type JAK2 cRNA were injected on the first day and 10 ng EAAT1-4 cRNA on the same day after preparation of the oocytes. All experiments were performed at room temperature 3-4 days after injections. Two-electrode voltage-clamp recordings were performed at a holding potential of -60 mV. 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 9.0 software package for data acquisition and analysis (Axon Instruments, USA) [25]. 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 [26]. Fifty mg/l gentamycin and, where indicated, AG490 (40 µM), actinomycin D (10 µM) or brefeldin A (5 µM) were added to the solution. Glutamate was added to the solutions at the indicated concentrations. 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.

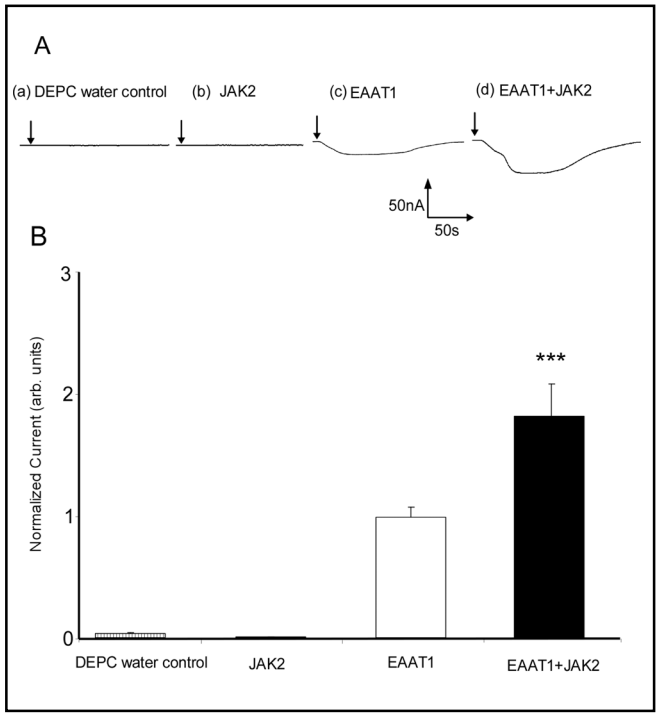
Immunohistochemistry and confocal microscopy

To determine EAAT4 cell surface expression by immunohistochemistry and chemiluminescence, defolliculated oocytes were first injected with 10 ng cRNA encoding either wild type JAK2 or JAK2-mutant (^{V617F}JAK2 or ^{K882E}JAK2) or with water and at the same day with 10 ng cRNA encoding EAAT4-HA which contains an HA epitope inserted extracellularly. After 3-4 days of injection, occytes were fixed with 4% paraformaldehyde for at least 12 h, oocytes were cryoprotected in 30% sucrose, frozen in mounting medium, and placed on a cryostat. Sections were collected at a thickness of 8 µm on coated slides and stored at -20°C. For immunostainings, sections were dehydrated at room temperature, fixated in acetone/methanol (1:1) for 15 min at room temperature, washed in PBS and pre-incubated for 1 h in 5% bovine serum albumin in PBS. The sections were incubated with primary rat anti-HA antibody for detection of EAAT4, (diluted 1:100, clone 3F10, Roche, Germany) for overnight in a moist chamber at 4°C. After washing with PBS a secondary antibody goat anti-rat FITC was used (diluted 1:1000, Cell Signaling Technology, MA, USA). The sections were mounted in prolong-gold antifad (Invitrogen). Oocytes were analyzed by a fluorescence laser scanning microscope (LSM 510, Carl Zeiss MicroImaging GmbH, Germany) with A-Plan 20x/0.48 Ph2. Brightness and contrast settings were kept constant during imaging of all oocytes in each injection series. Due to autofluorescence of the oocyte yolk, unspecific immunofluorescence was observed inside the oocyte.

Detection of EAAT4 cell surface expression by chemiluminescence

The oocytes were incubated with 0.5 µg/mL primary rat monoclonal anti-HA antibody (clone 3 F10, Roche, Mannheim, Germany) and subsequently with secondary, HRP-conjugated goat anti-rat IgG (H&L) antibody (1:1000, Cell Signaling Technology, MA, USA). 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

Fig. 1. Coexpression of JAK2 increased electrogenic glutamate transport in EAAT1 expressing *Xenopus laevis* oocytes. A. Representative original tracings of glutamate (2 mM)-induced currents (I_{glu}) in *Xenopus* oocytes injected with water (a), injected with JAK2 alone (b), or expressing EAAT1 without (c) or with (d) additional co-expression of JAK2. B. Arithmetic means ± SEM of glutamate (2 mM) induced normalized currents (I_{glu}) in oocytes injected with water (perpendicularly striped bar, n = 23), injected with JAK2 alone (horizontally striped bar, n = 5), expressing EAAT1 without (white bar, n = 23) or with (black bar, n = 20) additional coexpression of JAK2. ***p < 0.001 indicates statistically significant difference from the absence of JAK2.



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 [27].

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. Data were tested for significance using ANOVA, and results with $P < 0.05$ were considered statistically significant.

Results

Electrogenic glutamate transport was minimal in non-injected or water injected *Xenopus laevis* oocytes (Fig. 1). In oocytes expressing EAAT1, however, glutamate (2 mM) induced an inward current (I_{glu}) reflecting electrogenic entry of Na^+ and glutamate. I_{glu} was significantly increased by additional injection of cRNA encoding Janus-activated kinase-2 JAK2 (Fig. 1). The injection of JAK2 alone was not followed by the appearance of glutamate induced currents (Fig. 1), ruling out the theoretical possibility that the observed increase of I_{glu} in EAAT1 expressing oocytes following additional coexpression of JAK2 was due to up-regulation of an endogenous electrogenic glutamate carrier. Thus, JAK2 enhanced EAAT1 activity.

The glutamate transporter EAAT2 similarly mediated electrogenic glutamate transport (Fig. 2). In oocytes expressing EAAT2, glutamate (2 mM) induced an inward current (I_{glu}), which was again significantly enhanced by coexpression of JAK2.

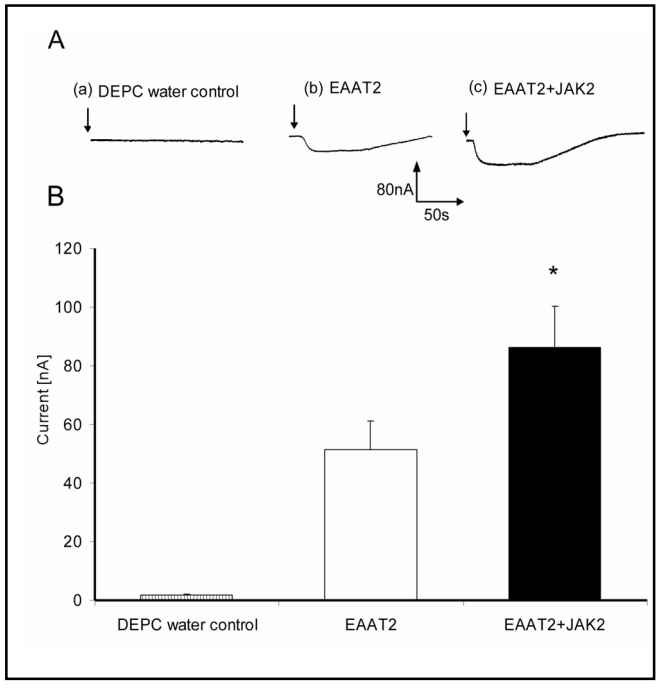


Fig. 2. JAK2 coexpression increased electrogenic glutamate transport in EAAT2 expressing *Xenopus laevis* oocytes. A. Representative original tracings of glutamate (2 mM)-induced currents (I_{glu}) in *Xenopus* oocytes injected with water (a), expressing EAAT2 without (b) or with (c) additional co-expression of JAK2. B. Arithmetic means ± SEM of glutamate (2 mM) induced currents (I_{glu}) in oocytes injected with water (striped bar, n = 19), expressing EAAT2 without (white bar, n = 18) or with (black bar, n = 19) additional coexpression of JAK2. *p < 0.05 indicates statistically significant difference to currents in oocytes expressing EAAT2 alone.

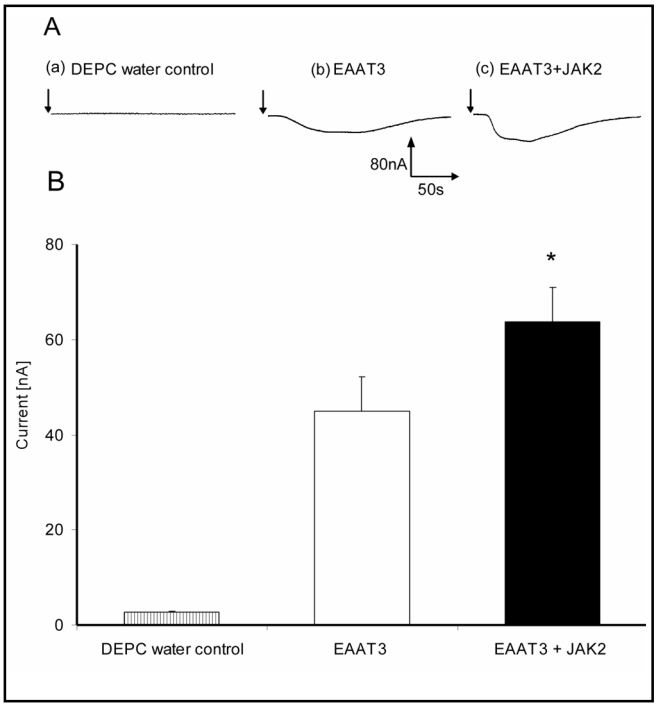


Fig. 3. Coexpression of JAK2 increased electrogenic glutamate transport in EAAT3 expressing *Xenopus laevis* oocytes. A. Representative original tracings of glutamate (2 mM)-induced currents (I_{glu}) in *Xenopus* oocytes injected with water (a), expressing EAAT3 without (b) or with (c) additional co-expression of JAK2. B. Arithmetic means ± SEM of glutamate (2 mM) induced normalized currents (I_{glu}) in oocytes injected with water (striped bar, n = 14), expressing EAAT3 without (white bar, n = 15) or with (black bar, n = 14) additional coexpression of JAK2. *p < 0.05 indicates statistically significant difference from the absence of JAK2.

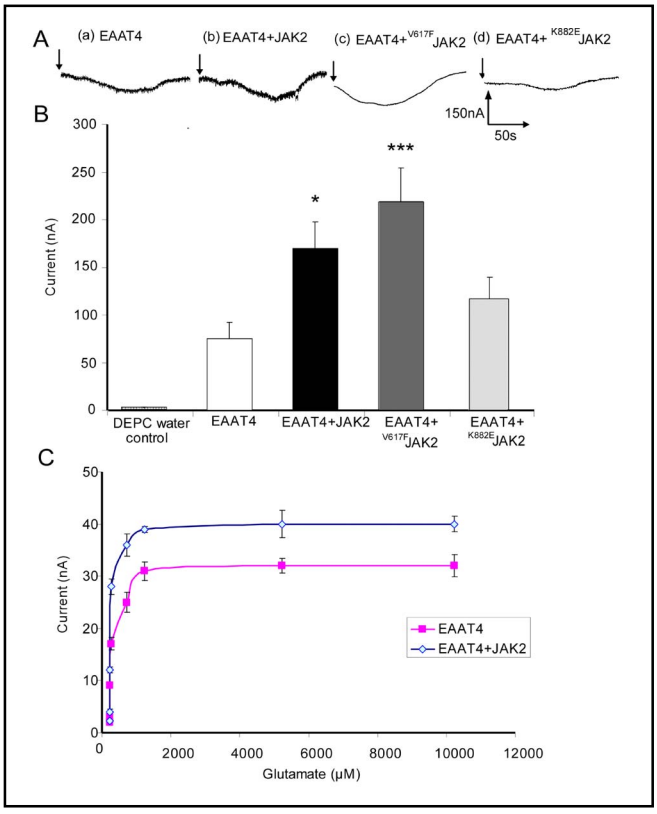


Fig. 4. JAK2 coexpression increased electrogenic glutamate transport in EAAT4 expressing *Xenopus laevis* oocytes. A. Representative original tracings of glutamate (2 mM)-induced currents (I_{glu}) in *Xenopus* oocytes expressing EAAT4 without (a) or with (b) additional co-expression of JAK2 or of (c) ^{V617F}JAK2 or (d) ^{K882E}JAK2. B. Arithmetic means ± SEM of glutamate (2 mM) induced currents (I_{glu}) in oocytes injected with water (striped bar, n = 15), or expressing EAAT4 without (white bar, n = 16) or with (black bar, n = 16) additional coexpression of JAK2 or ^{V617F}JAK2 (dark grey bar, n = 15) or ^{K882E}JAK2 (light grey bar, n = 16) *p<0.05, ***p < 0.001 indicates statistically significant difference to current in oocytes expressing EAAT4 alone. C. Arithmetic means ± SEM (n = 3) of glutamate induced currents (I_{glu}) as a function of glutamate concentration in *Xenopus laevis* oocytes expressing EAAT4 without or with JAK2. The values are significantly (p<0.05) different between the presence and absence of JAK2 at all concentrations tested except at 0.1 and 1 µM.

Electrogenic glutamate transport was further observed in oocytes expressing EAAT3 (Fig. 3). In those oocytes glutamate (2 mM) induced an inward current (I_{glu}), which was again increased by coexpression of JAK2.

Glutamate further induced an inward current (I_{glu}) in oocytes expressing EAAT4 (Fig. 4). I_{glu} was in EAAT4 expressing *Xenopus laevis* oocytes again significantly enhanced by coexpression of JAK2. The effect of JAK2 was mimicked by the gain of function mutation ^{V617F}JAK2 but not by the inactive mutant ^{K882E}JAK2 (Fig. 4 A, B). The effect of ^{V617F}JAK2 tended to be higher than the effect of wild type JAK2, an effect, however, not reaching statistical significance. Possibly, wild type JAK2 is not fully activated in *Xenopus* oocytes. Kinetic analysis of the glutamate-induced currents in EAAT4-expressing *Xenopus* oocytes (Fig. 4C) yielded a maximal current of 31.8 ± 1.3 nA (n = 3). Coexpression of JAK2 significantly enhanced the maximal current to 39.6 ± 0.8 nA (n = 3). Calculation of the glucose concentration required for halfmaximal current (K_M) yielded values of 35.6 ± 0.1 µM (n = 3) in the absence and of 22.5 ± 2.9 µM (n = 3) in the presence of JAK2, values significantly different

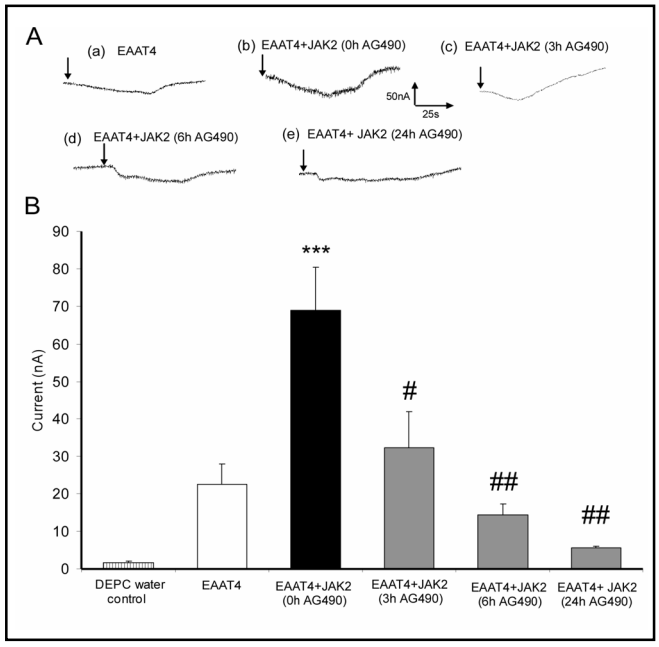


Fig. 5. Effect of JAK2 inhibitor AG490 on the activity of EAAT4 in oocytes coexpressing JAK2. A. Representative original tracings showing glutamate (2 mM)-induced currents (I_{glu}) in *Xenopus* oocytes injected with EAAT4 alone (a), or expressing EAAT4 together with JAK2 incubated in the absence (b) or presence of the JAK2 inhibitor AG490 (40 μM) for 3 hours (c), 6 hours (d) or 24 hours (e). B. Arithmetic means ± SEM of glutamate (2 mM) induced currents (I_{glu}) in oocytes injected with water (striped bar, n = 14), expressing EAAT4 without (white bar, n = 15) or with JAK2 (black and grey bars, n = 15) in the absence (black bar) or presence (light grey bars) of the JAK2 inhibitor AG490 (40 μM) for the indicated time periods. ***p < 0.001 indicates statistically significant difference to current in oocytes expressing EAAT4 alone, #p<0.01, ##p<0.001 from the absence of AG490 i. e. EAAT4+JAK2 (0h AG490).

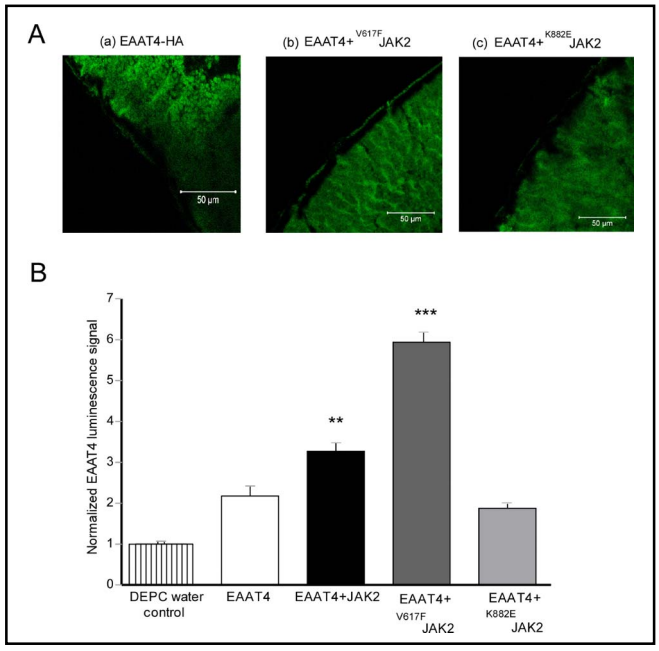


Fig. 6. Coexpression of JAK2 increased the EAAT4 abundance within the plasma membrane of oocytes. A. Confocal microscopy of *Xenopus* oocytes expressing EAAT4 alone (left) or with additional coexpression of gain of function mutation ^{V617F}JAK2 (middle) or of kinase dead mutant ^{K882E}JAK2 (right). Two different preparations of oocytes were analzyed. B. Chemiluminescence analysis of surface EAAT4 expression assessed by chemiluminescence in oocytes injected with water (striped bar, n = 40) or expressing EAAT4 alone (white bar, n = 42), together with wild type JAK2 (black bar, n = 36), with gain of function mutation ^{V617F}JAK2 (dark grey bar, n = 31) or with inactive ^{K882E}JAK2 (light grey bar, n = 20). Cell surface expression was normalized to the mean relative light units value obtained in oocytes injected with water. *indicates statistically significant (p<0.05) difference to *Xenopus* oocytes expressing EAAT4 alone.

(p <0.05). The observation suggested that coexpression of JAK2 enhanced EAAT4 activity by increasing both, the maximal current and the affinity of the carrier.

As shown for EAAT4 expressing *Xenopus* oocytes, JAK2 inhibitor AG490 (40 μM) decreased the glutamate induced current. Pre-incubation of the oocytes with the JAK2 inhibitor AG490 (40 μM) reversed the stimulating effect of JAK2 expression (Fig. 5A, B). The effect of the inhibitor was slow and reached statistical significance within 3 hours of pre-incubation with AG490.

The up-regulation of the glutamate transporters by JAK2 could have resulted in part from an increase of carrier protein abundance in the cell membrane. Immunohistochemistry and confocal microscopy together with chemiluminescence analysis was thus applied to test for altered carrier protein abundance at the cell surface. As illustrated in Fig. 6A and Fig. 6B, JAK2 indeed significantly increased the EAAT protein abundance in the cell membrane.

At least in theory, JAK2 could enhance EAAT4 protein abundance by influencing transcription of EAAT4 or a regulator thereof. To estimate the potential contribution of altered transcription, further experiments were performed with and without actinomycin D (10 μM), an inhibitor of transcription. As a result, actinomycin D failed to significantly modify the effect of JAK2 on EAAT4 (Fig. 7A).

The enhanced EAAT4 protein abundance in the cell membrane of JAK2 co-expressing oocytes could have resulted from accelerated insertion of new carriers into or delayed clearance of carriers from the cell membrane. To discriminate between those two possibilities the

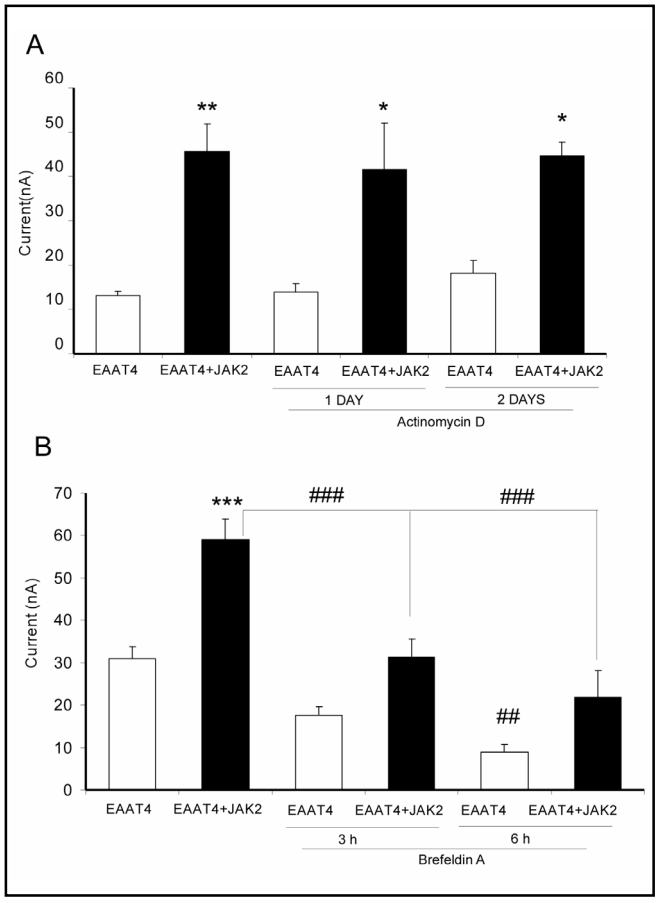


Fig. 7. Effect of actinomycin D and brefeldin A on EAAT4 activity in presence and absence of JAK2. A: Arithmetic means ± SEM (n = 4-8) of glutamate (2 mM)-induced currents (I_{glu}) in *Xenopus* oocytes injected with EAAT4 without (white bars) or with (black bars) JAK2 in the absence (left) and presence (right) of 10 µM actinomycin D 1-2 days prior to the measurement. B: Arithmetic means ± SEM (n = 15-19) of glutamate (2 mM)-induced current (I_{glu}) in *Xenopus* oocytes injected with EAAT4 without (white bars) and with (black bars) JAK2 in the absence (left) and presence (right) of 5 µM brefeldin for 0-6 hours prior to the measurement. ***indicates statistically significant ($p<0.001$) difference from the absence of JAK2. ##, ###indicates significant difference from the absence of brefeldin ($p<0.01$, $p<0.001$).

EAAT4-expressing *Xenopus* oocytes were treated with 5 µM Brefeldin A, which blocks the insertion of new carrier protein into the cell membrane. As shown in Fig. 7B, the glutamate induced current in the presence of Brefeldin A declined at a similar rate in oocytes expressing EAAT4 alone and in oocytes expressing EAAT4 together with JAK2. Twenty-four hours after Brefeldin A treatment EAAT4 activity was similarly low in oocytes expressing EAAT4 together with JAK2 as in oocytes expressing EAAT4 alone. This observation argues against a role of JAK2 in the carrier clearance from the cell membrane and suggests that JAK2 increases EAAT4 activity by stimulating carrier insertion into the cell membrane.

Discussion

The present observations unravel a novel regulator of glutamate transporters. The Janus-activated kinase-2 JAK2 up-regulates the activity of the four excitatory amino acid transporter isoforms EAAT1, EAAT2, EAAT3, EAAT4. The effect is at least partially due to an increase of carrier protein abundance in the cell membrane.

The effect of JAK2 could impact on the function of glutamatergic neurons and thus affect cerebral function. EAAT1 accomplishes glutamate uptake into glial cells [9]. Together with EAAT2 it is the most important carrier accounting for the clearance of glutamate released during neurotransmission [28]. The carrier is expressed mainly in astrocytes [29-32]. Expression has further been reported in oligodendrocytes [33], neurons [34, 35], retina [36, 37], taste buds [38], cochlea [39, 40], vestibular organ [41], circumventricular organ [29], adrenal and pineal glands [42, 43] as well as bone cells [44, 45].

EAAT2 is similarly expressed in astrocytes [46] and similarly contributes to glutamate reuptake from the synaptic cleft [47]. Upregulation of EAAT2 activity provides neuroprotection [48] and impaired expression or activity of EAAT2 leads to extracellular glutamate accumulation and neuroexcitotoxicity [49, 50].

EAAT3 is not only expressed in neurons [28, 51-57], retinal ganglion cells [58] and glial cells [59, 60] but is expressed in a wide variety of nonexcitable cells and non-neuronal tissues including blood platelets [61, 62], heart [63], renal podocytes [64], epididymis [65], placenta [66, 67] and blood-brain barrier [68].

EAAT4 is specifically expressed in cerebellar Purkinje cells and clears glutamate from the synapses connecting the climbing fibers with the Purkinje cells [54].

Deranged function of glutamate transporters affects mainly the function of the brain. Deranged EAAT2 function has been implicated in several neurological disorders including amyotrophic lateral sclerosis (ALS) [49, 69], Alzheimer disease [70, 71], schizophrenia [72], HIV associated dementia [73], multiple sclerosis [74, 75],

leukomalacia [76], epilepsy [77, 78], brain trauma [79], hypoxia and stroke [80, 81]. Moreover, gene variants in EAAT2 influence reward dependence [82]. Dysfunction of EAAT3 may result in dicarboxylic aminoaciduria, which can be associated with mental retardation [83] and has been implicated in obsessive-compulsive disorder [83], schizophrenia [72, 84, 85], epilepsy [78] and hepatic encephalopathy [86]. EAAT4 has been associated with schizophrenia [72, 84].

Beyond its putative role in glutamatergic transmission, JAK2 may affect glutamate transport in extracerebral tissues. JAK2 has previously been shown to participate in the regulation of cell proliferation [87, 88]. Accordingly, mutations in the gene encoding JAK2 underlie some myeloproliferative disorders [89] and JAK2 inhibitors are considered potential pharmacological candidates for the management of myelofibrosis [90, 91].

JAK2 dependent regulation of glutamate transporters may contribute to the cerebral effects of leptin, which protects against hypoxic neuronal injury [92] and excitotoxicity as well as seizures under a variety of conditions [2, 3, 93]. JAK2-dependent upregulation of glutamate transporters may further participate in the protective effect of erythropoeitin against excitotoxicity [6, 94].

In conclusion, JAK2 up-regulates the glutamate transporters EAAT1, EAAT2, EAAT3 and EAAT4, an effect presumably participating in the regulation of neuronal function and survival during ischemia and in the effect of neuroprotective hormones, such as leptin and erythropoietin.

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