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Upregulation of Excitatory Amino Acid Transporters by Coexpression of Janus Kinase 3

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Abstract Janus kinase 3 (JAK3) contributes to cytokine receptor signaling, confers cell survival and stimulates cell proliferation. The gain of function mutation $JAK3^{A572V}$ is found in acute megakaryoplastic leukemia. Replacement of ATP coordinating lysine by alanine yields inactive $JAK3^{K855A}$. Most recent observations revealed the capacity of JAK3 to regulate ion transport. This study thus explored whether JAK3 regulates glutamate transporters EAAT1-4, carriers accomplishing transport of glutamate and aspartate in a variety of cells including intestinal cells, renal cells, glial cells, and neurons. To this end, EAAT1, 2, 3, or 4 were expressed in *Xenopus* oocytes with or without additional expression of mouse wild-type JAK3, constitutively active $JAK3^{A568V}$ or inactive $JAK3^{K851A}$, and electrogenic glutamate transport was determined by dual electrode voltage clamp. Moreover, Ussing chamber was employed to determine electrogenic glutamate transport in intestine from mice lacking functional JAK3 ($jak3^{-/-}$) and from corresponding wild-type mice ($jak3^{+/+}$). As a result, in EAAT1, 2, 3, or 4 expressing oocytes, but not in oocytes injected with water, addition of glutamate to extracellular bath generated an inward current (I_g), which was significantly increased following coexpression of JAK3. I_g in oocytes expressing EAAT3 was further increased by $JAK3^{A568V}$ but not by $JAK3^{K851A}$. I_g in EAAT3 + JAK3 expressing oocytes was significantly decreased by JAK3 inhibitor WHI-P154 (22 μM). Kinetic analysis revealed that JAK3 increased maximal I_g and significantly reduced the glutamate concentration required for half maximal I_g (K_m). Intestinal electrogenic glutamate transport was significantly lower in $jak3^{-/-}$ than in $jak3^{+/+}$ mice. In conclusion, JAK3 is a powerful regulator of excitatory amino acid transporter isoforms.

Keywords Intestine · EAAT · Oocytes · Mice · JAK3

Introduction

The Janus kinase 3 (JAK3) participates in the signaling of cytokine receptors (Cornejo et al. 2009; Ghoreschi et al. 2009; Imada and Leonard 2000; O'Shea et al. 2002; Shuai and Liu 2003). As shown in lymphocytes and tumor cells JAK3 confers cell survival and cell proliferation (de Totero et al. 2008; Fainstein et al. 2008; Nakayama et al. 2009). Along those lines, the gain of function mutation $JAK3^{A572V}$ (Haan et al. 2011) may lead to acute megakaryoplastic leukemia (Malinge et al. 2008; Walters et al. 2006). Conversely, JAK3 inhibitors stimulate apoptosis of neoplastic cells (Kim et al. 2010; Uckun et al. 2007). On the other hand, JAK3 may facilitate apoptosis (Yamaoka et al. 2005). JAK3 is involved in the cellular response to hypoxia and ischemia–reperfusion (Ananthakrishnan et al. 2005; Nagel et al. 2012; Wang et al. 2008). The kinase is inactivated by replacement of the ATP coordinating lysine by alanine in the catalytic subunit ($JAK3^{K855A}$) (Haan et al. 2011).

The pleiotropic effects of JAK3 include regulation of transport across the cell membrane (Umbach et al. 2013; Warsi et al. 2013). This study explored whether JAK3 influences the glutamate transporters EAAT1-4, carriers mediating intestinal and renal transport of acidic amino acids (Castagna et al. 1997) as well as clearance of excitatory amino acids from synaptic clefts (Fainstein et al.

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2008; Kim et al. 2010; Nakayama et al. 2009; Uckun et al. 2007; Yamaoka et al. 2005). Defective glutamate transporters participates in the pathophysiology of several neuronal disorders including ischemic stroke injury, epilepsy, Alzheimer's disease, Huntington's disease, and amyotrophic lateral sclerosis (Kim et al. 2010).

Materials and Methods

Constructs

Constructs encoding wild-type EAAT1, EAAT2, EAAT3, and EAAT4 (Hosseinzadeh et al. 2011), wild-type murine JAK3 (Imagenes, Berlin, Germany), gain of function mutant $JAK3^{A568V}$ (Haan et al. 2011) and inactive $JAK3^{K851A}$ mutant (Haan et al. 2011) were used for generation of cRNA as described previously (Alesutan et al. 2012; Hosseinzadeh et al. 2013).

Voltage Clamp in *Xenopus laevis* Oocytes

Xenopus oocytes were prepared as previously described (Henrion et al. 2012; Mia et al. 2012). Where not indicated otherwise, 10 ng cRNA encoding EAAT1, EAAT2, EAAT3, or EAAT4 were injected on the first day and 10 ng cRNA encoding JAK3 (wild type), $JAK3^{A568V}$ or $JAK3^{K851A}$ were injected on the second day or the same day after preparation of the oocytes (Pakladok et al. 2013; Pathare et al. 2012a). The oocytes were maintained at 17 °C in ND96 solution containing (in mM): 88.5 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 2.5 NaOH, 5 HEPES, 5 sodium pyruvate ($C_3H_3NaO_3$), pH 7.4, supplemented with gentamycin (100 mg/l), tetracycline (50 mg/l), ciprofloxacin (1.6 mg/l), and theophylline (90 mg/l). The voltage clamp experiments were performed at room temperature 3 days after injection. Two-electrode voltage clamp recordings (Almilaji et al. 2013a; Hosseinzadeh et al. 2012a) were performed at a holding potential of −60 mV. The data were filtered at 10 Hz and recorded with a Digidata A/D–D/A converter (1322A Axon Instruments) and Clampex 9.2 software for data acquisition and Clampfit 9.2 software for analysis (Axon Instruments) (Bogatikov et al. 2012; Hosseinzadeh et al. 2012b). 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. Glutamate was added to the solutions at a concentration of 2 mM, unless otherwise stated. Where indicated the JAK3 inhibitor WHI-P154 (22 μM) was added for 24 h prior to the experiments. 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 (Almilaji et al. 2013b; Dermaku-Sopjani et al. 2013).

Ussing Chamber Experiments

All animal experiments were conducted according to the German law for the welfare of animals and according to the guidelines of the American Physiological Society and were approved by local authorities (Regierungspräsidium Tübingen). Experiments have been performed using intestinal segments from 16-week-old female gene targeted mice lacking functional JAK3 ($jak3^{-/-}$) and from corresponding wild-type mice ($jak3^{+/+}$) (Pathare et al. 2012b). The mice were fed a control diet (1314, Altromin, Heidenau, Germany) and had free access to tap drinking water.

For analysis of electrogenic intestinal glutamate transport, jejunal segments were mounted into a custom made mini-Ussing chamber with an opening of 0.00769 cm^2. Under control conditions, the serosal and luminal perfusate contained (in mM): 115 NaCl, 2 KCl, 1 $MgCl_2$, 0.4 KH_2PO_4, 1.25 $CaCl_2$, 1.6 K_2HPO_4, 5 sodium pyruvate, 25 $NaHCO_3$ (pH 7.4, NaOH). Where indicated, glutamate (20 mM) was added to the luminal perfusate at the expense of mannitol (20 mM) (all substances were from Sigma, Schnelldorf, Germany, or from Roth, Karlsruhe, Germany).

In all Ussing chamber experiments, the transepithelial potential difference (V_t) was determined continuously and the transepithelial resistance (R_t) was estimated from the voltage deflections (ΔV_t) elicited by imposing test currents (I_t). The resulting R_t was calculated according to Ohm's law (Hosseinzadeh et al. 2013).

Statistical Analysis

Data are provided as mean ± SEM, n represents the number of oocytes or intestinal segments investigated. All voltage clamp 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 or t test, as appropriate. Results with $P < 0.05$ were considered statistically significant.

Results

The pleiotropic effects of JAK3 include regulation of ion transport. This study explored whether JAK3 influences the function of the excitatory amino acid transporter isoforms EAAT1-4. To this end, cRNA encoding EAAT1, EAAT2, EAAT3, or EAAT4 was injected into *Xenopus laevis* oocytes with or without additional injection of cRNA encoding wild-type JAK3. Electrogenic glutamate transport was estimated from the glutamate-induced inward current (I_g) utilizing dual electrode voltage clamp (TEVC). As illustrated in Fig. 1, addition of 2 mM glutamate to the extracellular bath did not generate an appreciable I_g in

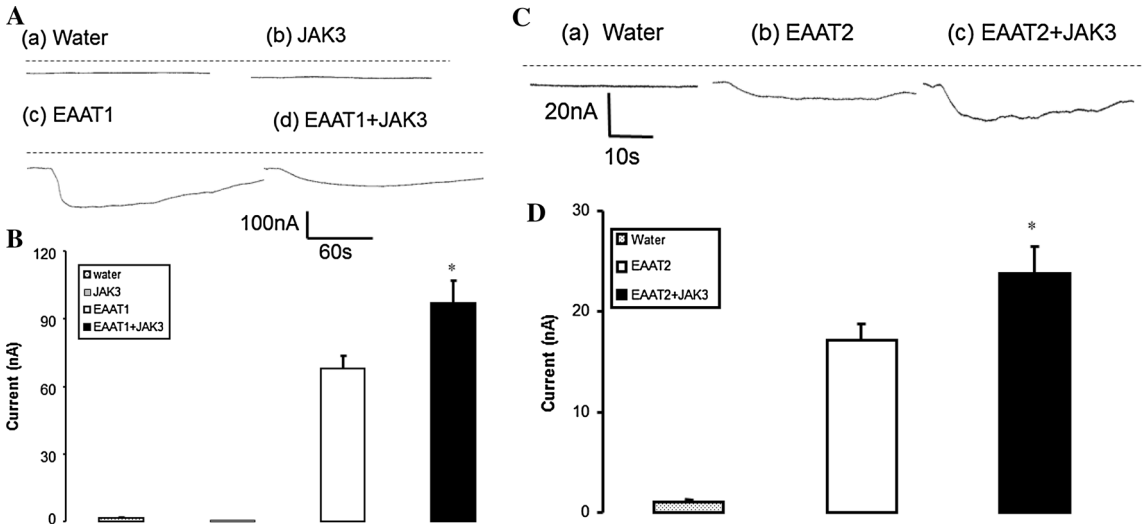

Fig. 1 Effect of wild-type JAK3 on electrogenic glutamate transport in EAAT1 or EAAT2-expressing *Xenopus laevis* oocytes. **A** Representative original tracings showing glutamate (2 mM)-induced current in *Xenopus* oocytes injected with water (*a*), expressing JAK3 alone (*b*), expressing EAAT1 alone (*c*), or expressing EAAT1 with additional coexpression of wild-type JAK3 (*d*). **B** Arithmetic mean ± SEM ($n = 10$–27) of glutamate (2 mM)-induced current in *Xenopus* oocytes injected with water (*dotted bar*) or expressing JAK3 alone (*gray bar*) or expressing EAAT1 alone (*white bar*), or expressing EAAT1 together with wild-type JAK3 (*black bar*). **C** Representative original tracings showing glutamate (2 mM)-induced current in *Xenopus* oocytes injected with water (*a*), expressing EAAT2 alone (*b*), or expressing EAAT2 with additional coexpression of wild-type JAK3 (*c*). **D** Arithmetic mean ± SEM ($n = 18$) of glutamate (2 mM)-induced current in *Xenopus* oocytes injected with water (*dotted bar*) or expressing EAAT2 alone (*white bar*), or expressing EAAT2 together with wild-type JAK3 (*black bar*). *Asterisk* indicates statistically significant ($P < 0.05$) difference from *Xenopus* oocytes expressing EAAT1 or EAAT2 alone

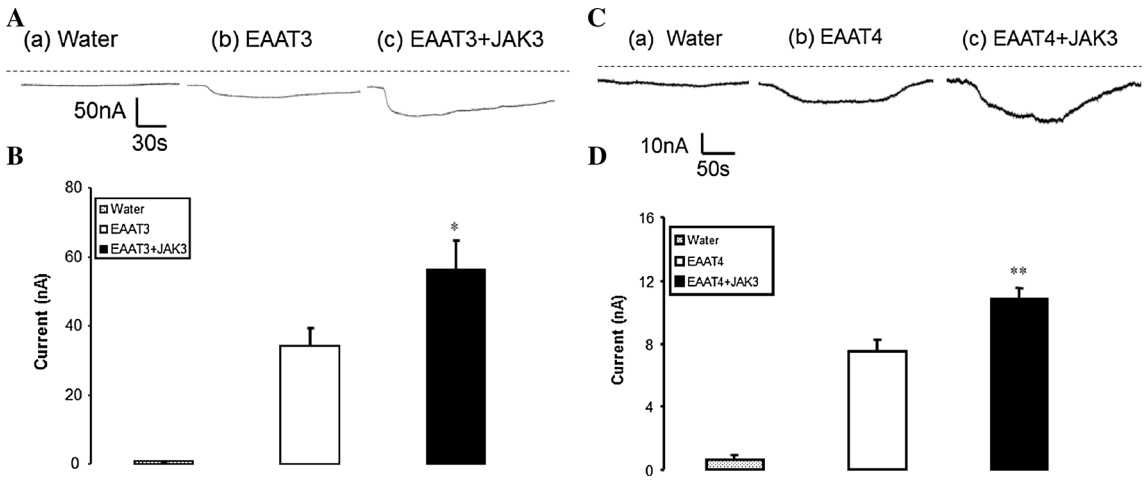

Fig. 2 Effect of wild-type JAK3 on electrogenic glutamate transport in EAAT3 or EAAT4-expressing *Xenopus laevis* oocytes. **A** Representative original tracings showing glutamate (2 mM)-induced current in *Xenopus* oocytes injected with water (*a*), expressing EAAT3 alone (*b*), or expressing EAAT3 with additional coexpression of wild-type JAK3 (*c*). **B** Arithmetic mean ± SEM ($n = 24$–25) of glutamate (2 mM)-induced current in *Xenopus* oocytes injected with water (*dotted bar*) or expressing EAAT3 alone (*white bar*), or expressing EAAT3 together with wild-type JAK3 (*black bar*). **C** Representative original tracings showing glutamate (2 mM)-induced current in *Xenopus* oocytes injected with water (*a*), expressing EAAT4 alone (*b*), or expressing EAAT4 with additional coexpression of wild-type JAK3 (*c*). **D** Arithmetic mean ± SEM ($n = 10$) of glutamate (2 mM)-induced current in *Xenopus* oocytes injected with water (*dotted bar*) or expressing EAAT4 alone (*white bar*), or expressing EAAT4 together with wild-type JAK3 (*black bar*). $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate statistically significant difference from *Xenopus* oocytes expressing EAAT3 and EAAT4 alone, respectively

water-injected *Xenopus* oocytes or oocytes injected with JAK3 alone, indicating that *Xenopus* oocytes do not express appreciable electrogenic glutamate transport. In contrast, addition of 2 mM glutamate to the bath generated a large I_g in *Xenopus* oocytes expressing EAAT1 (Fig. 1A, B), EAAT2 (Fig. 1C, D), EAAT3 (Fig. 2A, B), or EAAT4

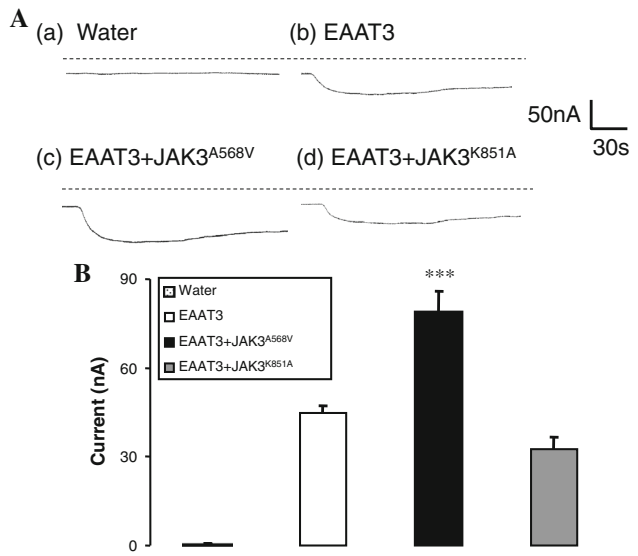

Fig. 3 Effect of wild-type JAK3, active mutant JAK3^{A568V}, or catalytically inactive coexpression on electrogenic glutamate transport in EAAT3-expressing *Xenopus laevis* oocytes. **A** Representative original tracings showing glutamate (2 mM)-induced current in *Xenopus* oocytes injected with water (*a*), expressing EAAT3 alone (*b*), or expressing EAAT3 with additional coexpression of constitutively active JAK3^{A568V} (*c*), or catalytically inactive JAK3^{K851A} (*d*). **B** Arithmetic mean ± SEM (n = 12–16) of glutamate (2 mM)-induced current in *Xenopus* oocytes injected with water (*dotted bar*) or expressing EAAT3 alone (*white bar*), or expressing EAAT3 together with constitutively active JAK3^{A568V} (*black bar*), or catalytically inactive JAK3^{K851A} (*gray bar*). *** indicates statistically significant ($P < 0.001$) difference from *Xenopus* oocytes expressing EAAT3 alone

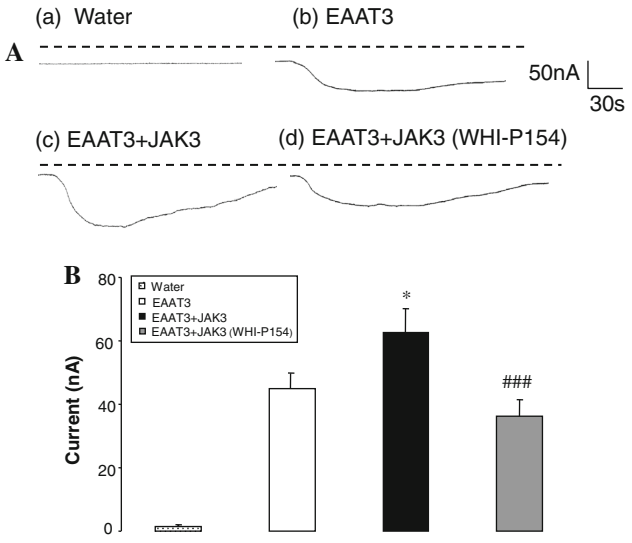

Fig. 4 Effect of JAK3 inhibitor WHI-P154 on electrogenic glutamate transport in EAAT3 and JAK3-expressing *Xenopus laevis* oocytes. **A** Representative original tracings showing glutamate (2 mM)-induced current in *Xenopus* oocytes injected with water (*a*), expressing EAAT3 alone (*b*), or expressing EAAT3 with additional coexpression of wild-type JAK3 either in the absence (*c*) or presence (d) of JAK3 inhibitor WHI-P154 (22 μM). **B** Arithmetic mean ± SEM (n = 10–20) of glutamate (2 mM)-induced current in *Xenopus* oocytes injected with water (*dotted bar*) or expressing EAAT3 alone (*white bar*), or expressing EAAT3 together with wild-type JAK3 either in the absence (*black bar*) or presence (*gray bar*) of JAK3 inhibitor WHI-P154 (22 μM). *Asterisk* indicates statistically significant ($P < 0.05$) difference from *Xenopus* oocytes expressing EAAT3 alone, ### statistically significant ($P < 0.001$) difference from the absence of WHI-P154

(Fig. 2C, D). The additional expression of JAK3 was followed by a significant increase of I_g in EAAT1, EAAT2, EAAT3, or EAAT4 expressing *Xenopus* oocytes (Figs. 1, 2).

As illustrated in Fig. 3, the stimulating effect of wild-type JAK3 on EAAT3 was mimicked by the active mutant JAK3^{A568V} but not by the inactive mutant JAK3^{K851A}. Thus, kinase activity was required for the stimulation of glutamate transport by JAK3. As illustrated in Fig. 4, I_g in EAAT3 + JAK3 expressing oocytes was significantly decreased by JAK3 inhibitor WHI-P154 (22 μM, 24 h).

Additional experiments explored whether JAK3 influences glutamate transport by modifying maximal transport rate or affinity of the EAAT3 carrier. To this end, *Xenopus* oocytes expressing EAAT3 alone or together with JAK3 were exposed to glutamate concentrations ranging from 10 μM to 10 mM (Fig. 5). Kinetic analysis of the glutamate-induced currents yielded a maximal current of 39.4 ± 2.5 nA (n = 8–10) in *Xenopus* oocytes expressing EAAT3 alone. Coexpression of JAK3 significantly ($P < 0.05$) increased the maximal current to 58.2 ± 8.7 nA (n = 8–10). The glutamate concentration required for the half maximal current (K_m) was significantly ($P < 0.05$)

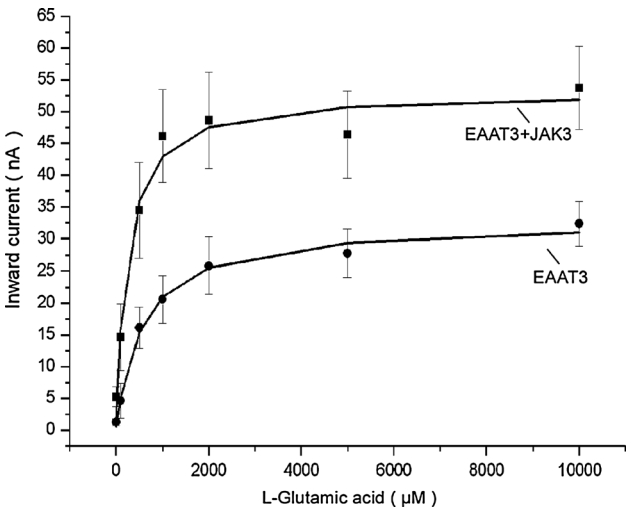

Fig. 5 Effect of wild-type JAK3 coexpression on the kinetics of electrogenic glutamate transport in EAAT3-expressing *Xenopus laevis* oocytes. Arithmetic mean ± SEM (n = 8–10) of glutamate-induced current as a function of glutamate concentration in *Xenopus* oocytes expressing EAAT3 alone (*black circles*) or together with wild-type JAK3 (*black squares*)

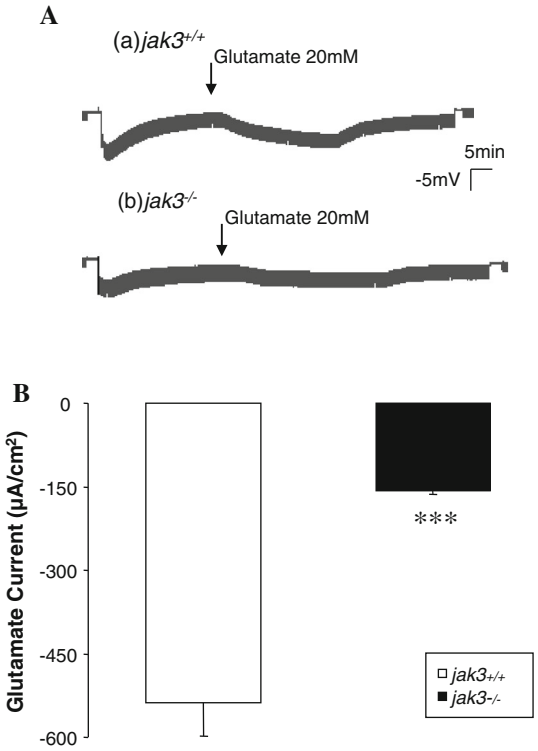

Fig. 6 Glutamate-sensitive transepithelial current in jejunum from $jak3^{+/+}$ and $jak3^{-/-}$ mice. **A** Representative original tracings of the transepithelial jejunal potential difference in wild-type mice ($jak3^{+/+}$) (*a*) and in gene targeted mice lacking functional Jak3 ($jak3^{-/-}$) (*b*). *Arrows* highlight the addition of glutamate (20 mM). **B** Arithmetic mean ± SEM ($n = 4$) of the glutamate-induced equivalent short-circuit current in jejunum from $jak3^{+/+}$ (*white bar*) and $jak3^{-/-}$ mice (*black bar*). *** indicates statistically significant ($P < 0.001$) difference from wild-type mice ($jak3^{+/+}$)

higher (623 ± 139 μM) in oocytes expressing EAAT3 alone than in oocytes expressing both, EAAT3 and JAK3 (234 ± 61 μM). Thus, coexpression of JAK3 may increase EAAT3 activity by enhancing the maximal current and by reducing the glutamate concentration required for the half maximal current.

In order to test whether the stimulation of EAAT3 by JAK3 plays a role in vivo, glutamate-induced current was measured in jejunal tissue utilizing Ussing chamber experiments. Glutamate-induced current was determined in intestine from mice lacking functional JAK3 ($jak3^{-/-}$) and from corresponding wild-type mice ($jak3^{+/+}$). As illustrated in Fig. 6, the glutamate-induced current was significantly higher in intestine from $jak3^{+/+}$ mice than in intestine from $jak3^{-/-}$ mice.

Discussion

This study discloses a novel regulator of the excitatory amino acid transporter isoforms EAAT1, 2, 3, and 4. All four carrier isoforms are stimulated by coexpression of JAK3. The effect of wild-type JAK3 on EAAT3 is mimicked by the active $JAK3^{A568V}$ but not by the inactive $JAK3^{K851A}$, indicating that JAK3 kinase activity is required for the stimulating effect of JAK3 on the glutamate transporters.

The effect of JAK3 on glutamate transport via EAAT3 was abrogated by the JAK3 inhibitor WHI-P154. Kinetic analysis revealed that JAK3 increased maximal I_g and significantly reduced the glutamate concentration required for half maximal I_g (K_m).

The in vivo functional significance of JAK3 sensitive glutamate transport is supported by the observation that intestinal electrogenic glutamate transport was significantly lower in intestinal segments isolated from gene targeted mice lacking functional JAK3 ($jak3^{-/-}$) than in intestinal segments isolated from $jak3^{+/+}$ mice. The difference between untreated $jak3^{-/-}$ mice and untreated $jak3^{+/+}$ mice reveals that JAK3 deficiency modifies intestinal transport under non-stimulated conditions. It cannot fully be ruled out, however, that the effect of JAK3 deficiency on intestinal glutamate transport is indirect. JAK3 is predominantly expressed in hematopoietic cells and participates in the signal transduction of the common gamma chain subfamily of cytokine receptors (Cornejo et al. 2009). JAK3 is expressed in macrophages (Johnston et al. 1996) and microglia (Liva et al. 1999), which both may express EAATs (Gras et al. 2012), At least in theory, JAK3 participates in the regulation of amino acid uptake into tumor cells expressing JAK3. Whether or not JAK3 influences glutamate transport in other tissues such as brain remains to be shown. JAK3 presumably contributes to interleukin 6 stimulated signaling in neurons (Orellana et al. 2005), which are known to express EAAT1 (Gaillet et al. 2001; Rothstein et al. 1994). EAAT1 mediates glutamate uptake into a wide variety of further cells (Hosseinzadeh et al. 2011) including glial cells (Amara and Fontana 2002; Beart and O'Shea 2007; Berger and Hediger 1998; Cholet et al. 2002; Domercq et al. 1999; Sandhu et al. 2002; Ullensvang et al. 1997), retina (Barnett and Pow 2000; Derouiche and Rauen 1995), cochlea (Furness and Lehre 1997; Li et al. 1994), vestibular organ (Takumi et al. 1997), taste buds (Lawton et al. 2000), adrenal and pineal glands (Lee et al. 2001; Redecker and Pabst 2000), and bone cells (Gray et al. 2001; Mason et al. 1997). EAAT2 is expressed in astrocytes (Gibb et al. 2007; Lehre and Danbolt 1998; Milton et al. 1997; Rothstein et al. 1996, 2005). EAAT3 is expressed in glial cells (Miralles et al. 2001; van Landeghem et al. 2007), blood–brain barrier (O'Kane et al. 1999), neurons (Amara and Fontana 2002; Collin et al. 2003; Furuta et al. 1997, 2005; Huang et al. 2004; Nieoullon et al. 2006; Schmitt et al. 2003; Shashidharan et al. 1997), retinal ganglion cells (Schniepp et al.

2004), blood platelets (Rainesalo et al. 2005; Zoia et al. 2004), heart (King et al. 2004), glomerular podocytes (Gloy et al. 2000), epididymis (Cooper et al. 2003), and placenta (Matthews et al. 1999; Noorlander et al. 2004). EAAT4 is specifically expressed in cerebellar Purkinje cells (Huang et al. 2004). At least in theory, glutamate uptake may be modified by JAK3 in some of those cells.

In conclusion, JAK3 is a powerful stimulator of all four excitatory amino acid transporters EAAT1-4. The kinase up-regulates the maximal transport rate and reduces the glutamate concentration required for half maximal EAAT3-mediated glutamate current. The in vivo significance of JAK3 sensitive glutamate transport is illustrated by the observation of reduced intestinal electrogenic glutamate transport in gene targeted mice lacking functional JAK3.

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