J Membrane Biol (2012) 245:157–163
DOI 10.1007/s00232-012-9424-8

# Downregulation of the Creatine Transporter SLC6A8 by JAK2

**Manzar Shojaiefard · Zohreh Hosseinzadeh · Shefalee K. Bhavsar · Florian Lang**

Received: 4 November 2011 / Accepted: 16 February 2012 / Published online: 11 March 2012


**Abstract** Janus-activated kinase-2 (JAK2) participates in the regulation of the $Na^+$-coupled glucose transporter SGLT1 and the $Na^+$-coupled amino acid transporter SLC6A19. Concentrative cellular creatine uptake is similarly accomplished by $Na^+$-coupled transport. The carrier involved is SLC6A8 (CreaT). The present study thus explored whether JAK2 regulates the activity of SLC6A8. To this end, cRNA encoding SLC6A8 was injected into *Xenopus* oocytes with or without cRNA encoding wild-type JAK2, constitutively active $^{V617F}$JAK2 or inactive $^{K882E}$JAK2. Electrogenic creatine transport was determined in those oocytes by dual-electrode voltage-clamp experiments. In oocytes injected with cRNA encoding SLC6A8 but not in oocytes injected with water or with cRNA encoding JAK2 alone, addition of 1 mM creatine to the extracellular bath generated an inward current ($I_{crea}$). In SLC6A8 expressing oocytes $I_{crea}$ was significantly decreased by coexpression of JAK2 or $^{V617F}$JAK2 but not by coexpression of $^{K882E}$JAK2. According to kinetic analysis, coexpression of JAK2 decreased the maximal transport rate without significantly modifying the affinity of the carrier. In oocytes expressing SLC6A8 and $^{V617F}$JAK2 $I_{crea}$ was gradually increased by the JAK2 inhibitor AG490 (40 μM). In SLC6A8 and JAK2 coexpressing oocytes the decline of $I_{crea}$ following disruption of carrier insertion with brefeldin A (5 μM) was similar in the absence and presence of JAK2. In conclusion, JAK2 is a novel regulator of the creatine transporter SLC6A8, which downregulates the carrier, presumably by interference with carrier protein insertion into the cell membrane.

**Keywords** Creatine uptake · Energy depletion · Brefeldin · AG490 · Erythropoietin · Leptin

## Introduction

Janus-activated kinase-2 (JAK2) participates in the signaling of several hormones, such as leptin (Morris and Rui 2009), growth hormone (Brooks and Waters 2010), erythropoietin (Spivak 2010), thrombopoietin (Spivak 2010), granulocyte colony-stimulating factor (Spivak 2010) and further cytokines (Lopez et al. 2010; Spivak 2010). JAK2 is activated by oxidative stress, ischemia, and hypertonicity (Kurdi and Booz 2009; Garnovskaya et al. 2003; Gatsios et al. 1998). Enhanced JAK2 activity may foster the development of malignancy, and JAK2 inhibitors are considered potential drugs in the treatment of myeloproliferative disorders (Baskin et al. 2010; Ho et al. 2010; Oh and Gotlib 2010; Pardanani et al. 2011; Santos and Verstovsek 2011; Tefferi et al. 2009; Tefferi 2010). The gain-of-function mutation $^{V617F}$JAK2 is found in, and presumably contributes to, the development of myeloproliferative disease (Mahfouz et al. 2011). The pleiotropic effects of JAK2 include transport regulation (Gong et al. 1998; Yokota et al. 1998). Recently, JAK2 has been shown

M. Shojaiefard and Z. Hosseinzadeh contributed equally to this work and share the position of first author.

M. Shojaiefard · Z. Hosseinzadeh · S. K. Bhavsar · F. Lang (✉)
Department of Physiology I, University of Tübingen, Gmelinstr. 5, 72076 Tübingen, Germany
e-mail: florian.lang@uni-tuebingen.de

M. Shojaiefard
Department of Physiology, Fasa University of Medical Science, Fasa, Iran

Z. Hosseinzadeh
Department of Science, Shahid Chamran University, Ahwaz, Iran

to upregulate $Na^+$-coupled glucose transport (Hosseinzadeh et al. 2011) and the $Na^+$-coupled amino acid transporter SLC6A19 (Bhavsar et al. 2011).

The creatine transporter SLC6A8 (CreaT) belongs to a superfamily of $Na^+$, $Cl^-$-coupled transporters for neurotransmitters (e.g., dopamine, GABA, serotonin and norepinephrine), amino acids (e.g., glycine) (Christie 2007; Dodd and Christie 2001; Sora et al. 1994) and the organic osmolytes betaine (Takenaka et al. 1995) and taurine (Uchida et al. 1992).

SLC6A8 is expressed in a wide variety of tissues, such as skeletal muscle, kidney, small intestine, heart, brain, and retina (Braissant and Henry 2008; Guimbal and Kilimann 1993; Mak et al. 2009; Mellott et al. 2007). Genetic defects affecting SLC6A8 result in mental retardation with seizures (Alcaide et al. 2010, 2011; Ardon et al. 2010; Battini et al. 2007, 2011; Braissant et al. 2010, 2011; Hahn et al. 2002; Jensen et al. 2011; Longo et al. 2011; Mancardi et al. 2007; Mercimek-Mahmutoglu et al. 2010; Puusepp et al. 2009; Rosenberg et al. 2007; Salomons et al. 2003; Skelton et al. 2011; Stockler et al. 2007; van de Kamp et al. 2011). Decreasing SLC6A8 protein abundance has been observed in the failing heart (Neubauer et al. 1999), and SLC6A8 deficiency may compromise cardiac function (Anselm et al. 2008). SLC6A8 activity is regulated by AMP-activated kinase AMPK (Li et al. 2010), cyclosporin A (Tran et al. 2000), mammalian target of rapamycin (mTOR) (Shojaiefard et al. 2006), serum and glucocorticoid inducible kinase isoforms (Shojaiefard et al. 2005) and PIKfyve (Strutz-Seebohm et al. 2007). SLC6A8 has been suggested to be phosphorylated by Src (Wang et al. 2002). SLC6A8 is further regulated by extracellular and cytosolic creatine levels (Brault et al. 2003; Loike et al. 1988). Expression of the creatine transporter is increased by growth hormone (Omerovic et al. 2003).

The present study explored whether SLC6A8 is regulated by JAK2. SLC6A8 was expressed in *Xenopus* oocytes with or without wild-type JAK2, constitutively active $^{V617F}$JAK2 or inactive $^{K882E}$JAK2; and the electrogenic creatine transport was determined utilizing dual-electrode voltage-clamp.

## Materials and Methods

### Constructs

Constructs were used encoding wild-type bovine SLC6A8 (CreaT) (Dodd and Christie 2001) and wild-type human JAK2 (Imagenes, Berlin, Germany). Further, an inactive $^{K882E}$JAK2 mutant (Feng et al. 1997) and the $^{V617F}$JAK2 mutant (Mahfouz et al. 2011) were generated by site-directed mutagenesis (QuikChange II XL Site-Directed Mutagenesis Kit; Stratagene, Heidelberg, Germany) according to the manufacturer's instructions (Mohamed et al. 2010). The following primers were used: $^{V617F}$JAK2, 5′-AGCATTTGGTTTTAAATTATGGAGTATGTTTCTG TGGAGACGAGA-3′; $^{V617F}$JAK2, 5′-TCTCGTCTCCAC AGAAACATACTCCATAATTTAAAACCAAATGCT-3′; $^{K882E}$JAK2, 5′-GGGAGGTGGTCGCTGTAGAAAAGCT TCAGCATAGT-3′; and $^{K882E}$JAK2, 5′-ACTATGCTGAA GCTTTTCTACAGCGACCACCTCCC-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 (Gehring et al. 2009).

### Voltage Clamp in *Xenopus* Oocytes

*Xenopus* oocytes were prepared as previously described (Bohmer et al. 2010). We injected SLC6A8 cRNA (15 ng) on the 1st day and wild-type JAK2 cRNA (10 ng) on the 2nd day or the same day after preparation of oocytes. Oocytes were maintained at 17°C in ND96 solution containing (in mM) 96 NaCl, 4 KCl, 1.8 $MgCl_2$, 0.1 $CaCl_2$, 5 HEPES (pH 7.4) (Rexhepaj et al. 2010). Gentamycin (100 mg/l), theophylline (90 mg/l), tetracyclin (50 mg/l), ciprofloxacin (1.6 mg/l) and, where indicated, the JAK2 inhibitor AG490 (40 μM), actinomycin D (10 μM) or brefeldin A (5 μM) were added to the respective solutions. Voltage-clamp experiments were performed at room temperature 4 days after injection. 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 Digidata A/D–D/A converter and Clampex V.9 software for data acquisition and analysis (Axon Instruments, Foster City, CA). The control superfusate (ND96) contained (in mM) 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$ and 5 HEPES (pH 7.4). Creatine was added to the solutions at a concentration of 1 mM, unless otherwise stated. The flow rate of the superfusion was approximately 20 ml/min, and a complete exchange of the bath solution was reached within about 10 s.

### Statistical Analysis

Data are provided as means ± SEM, and $n$ represents the number of oocytes investigated. All experiments were repeated with at least two to 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

To explore whether JAK2 influences SLC6A8, the carrier was expressed with or without additional expression of the

kinase and SLC6A8-mediated transport was estimated from the creatine (1 mM)—induced inward current ($I_{crea}$). Addition of 1 mM creatine to the extracellular fluid did not induce an appreciable $I_{crea}$ in non-injected or water-injected *Xenopus* oocytes. Thus, *Xenopus* oocytes do not express appreciable endogenous electrogenic creatine transport (Fig. 1a). Moreover, $I_{crea}$ was negligible in *Xenopus* oocytes expressing wild-type JAK2 alone (Fig. 2b). A sizable $I_{crea}$ was, however, observed in *Xenopus* oocytes injected with cRNA encoding SLC6A8 (CreaT). The coexpression of wild-type JAK2 was followed by a significant decrease of $I_{crea}$ in *Xenopus* oocytes expressing SLC6A8 (Fig. 1b).

Additional experiments were performed to elucidate whether JAK2 was effective by modifying the maximal transport rate or the affinity of the carrier. As revealed by kinetic analysis of the creatine-induced currents in SLC6A8-expressing *Xenopus* oocytes (Fig. 1c), the maximal current approached 21.7 ± 1.1 nA ($n = 3$) in *Xenopus* oocytes expressing SLC6A8 alone and 10.3 ± 0.6 nA ($n = 3$) in *Xenopus* oocytes expressing SLC6A8 together with $^{V617F}$JAK2. Thus, the maximal transport rate was significantly lower in *Xenopus* oocytes expressing SLC6A8 together with $^{V617F}$JAK2 than in *Xenopus* oocytes expressing SLC6A8 alone. The creatine concentration required for half-maximal current ($K_m$) approached 167.4 ± 33.6 μM ($n = 3$) in *Xenopus* oocytes expressing SLC6A8 alone and 101.6 ± 25.2 μM ($n = 3$) in *Xenopus* oocytes expressing SLC6A8 together with $^{V617F}$JAK2. The $K_m$ was not significantly different between *Xenopus* oocytes expressing SLC6A8 together with $^{V617F}$JAK2 and those expressing SLC6A8 alone. Accordingly, coexpression of JAK2 decreased SLC6A8 activity at least in part by reducing the maximal current.

As illustrated in Fig. 2, the effect of JAK2 was mimicked by the gain-of-function mutant $^{V617F}$JAK2 but not by the inactive mutant $^{K882E}$JAK2. The effect of $^{V617F}$JAK2 tended to be higher than the effect of wild-type JAK2, though it did not reach statistical significance. Possibly, wild-type JAK2 is not fully activated in *Xenopus* oocytes.

Treatment of *Xenopus* oocytes expressing both SLC6A8 and $^{V617F}$JAK2 with the JAK2 inhibitor AG490 (40 μM) was followed by a gradual increase of $I_{crea}$ (Fig. 3). The effect of the inhibitor on $I_{crea}$ reached statistical significance within 24 h of preincubation with AG490. At 6 h incubation, however, the inhibitor did not appreciably affect $I_{crea}$.

The decrease of $I_{crea}$ in SLC6A8-expressing *Xenopus* oocytes by coexpression of $^{V617F}$JAK2 could have resulted from accelerated clearance of carrier protein from the cell membrane. To test this possibility, SLC6A8-expressing *Xenopus* oocytes were treated with 5 μM brefeldin A, a substance that blocks the insertion of new carrier protein into the cell membrane. As shown in Fig. 4a, the creatine-induced current of SLC6A8 and $^{V617F}$JAK2-expressing *Xenopus*

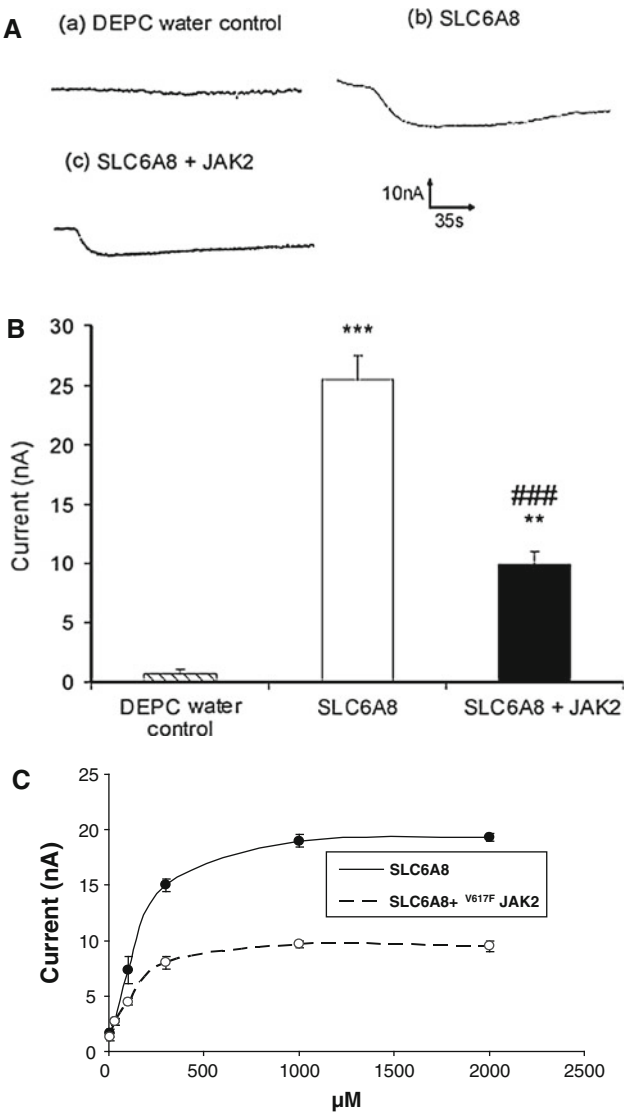


**Fig. 1** Coexpression of JAK2 inhibits electrogenic creatine transport in SLC6A8-expressing *Xenopus* oocytes. **a** Representative original tracings showing creatine (1 mM)—induced current ($I_{crea}$) in *Xenopus* oocytes injected with water (*a*), expressing SLC6A8 without (*b*) or with (*c*) additional coexpression of wild-type JAK2. **b** Arithmetic means ± SEM ($n = 16$–33) of $I_{crea}$ in *Xenopus* oocytes injected with water and expressing JAK2 alone (DEPC water control) or SLC6A8 without (SLC6A8) or with (SLC6A8 + JAK2) additional coexpression of wild-type JAK2. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. absence of SLC6A8; $^{\#\#\#}P < 0.001$ vs. absence of JAK2. **c** Arithmetic means ± SEM ($n = 3$) of $I_{crea}$ as a function of creatine concentration in *Xenopus* oocytes expressing SLC6A8 without (*closed circles, solid line*) or with (*open circles, dashed line*) additional coexpression of wild-type JAK2

oocytes declined at similarly rapid rates in the presence of brefeldin A. The observation suggests that $^{V617F}$JAK2 decreases SLC6A8 activity by a mechanism other than accelerating carrier clearance from the cell membrane.

Inhibition of transcription by incubation (1–3 days) with actinomycin D (10 μM) did not significantly modify the

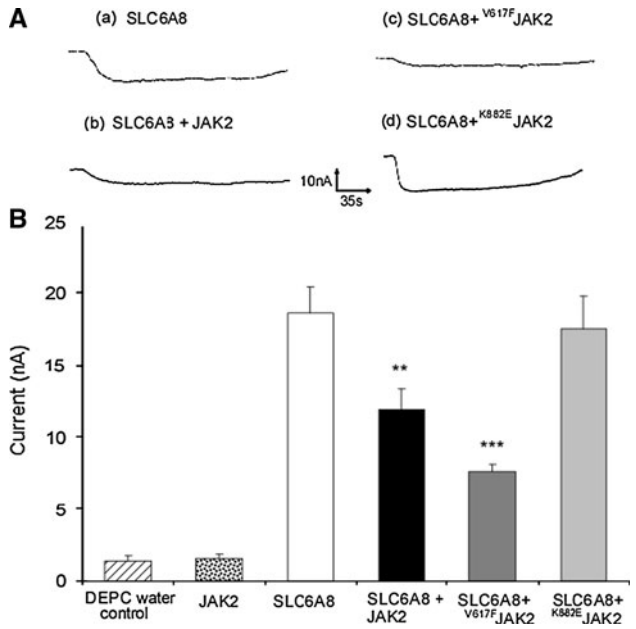


**Fig. 2** Effect of JAK2 is mimicked by $^{V617F}$JAK2 but not by the inactive mutant $^{K882E}$JAK2. **a** Representative original tracings showing creatine (1 mM)—induced current ($I_{crea}$) in *Xenopus* oocytes injected with SLC6A8 alone (*a*) or coexpressing SLC6A8 with JAK2 (*b*), $^{V617F}$JAK2 (*d*) or SLC6A8 with the inactive mutant $^{K882E}$JAK2 (*c*). **b** Arithmetic means ± SEM ($n = 13$–23) of $I_{crea}$ in *Xenopus* oocytes expressing SLC6A8 without (SLC6A8) or with wild-type JAK2 or SLC6A8 with constitutively active $^{V617F}$JAK2 (SLC6A8 + $^{V617F}$JAK2) or SLC6A8 with the inactive mutant $^{K882E}$JAK2 (SLC6A8 + $^{K882E}$JAK2). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. expression of SLC6A8 alone

current induced by SLC6A8 either in the presence or in the absence of JAK2 (Fig. 4b).

## Discussion

The present study uncovers a novel mechanism in the regulation of the creatine transporter SLC6A8 (CreaT). JAK2 decreased the electrogenic transport of creatine in SLC6A8-expressing *Xenopus* oocytes. The effect was mimicked by the gain-of-function mutant $^{V617F}$JAK2 but not by the inactive $^{K882E}$JAK2, indicating that kinase activity was required for this effect. Coexpression of $^{V617F}$JAK2 did not significantly affect the affinity for creatine but rather decreased the maximal transport rate. According to the experiments in the presence of brefeldin A, JAK2 did not accelerate carrier retrieval from the cell membrane. Thus, JAK2 was presumably effective through inhibition of the carrier or interference with transporter protein insertion into the cell membrane. However, as brefeldin A is expected to affect the trafficking of a wide variety of molecules, which could at least in theory participate in the regulation of SLC6A8, the interpretation of experiments in the presence of brefeldin A may involve some

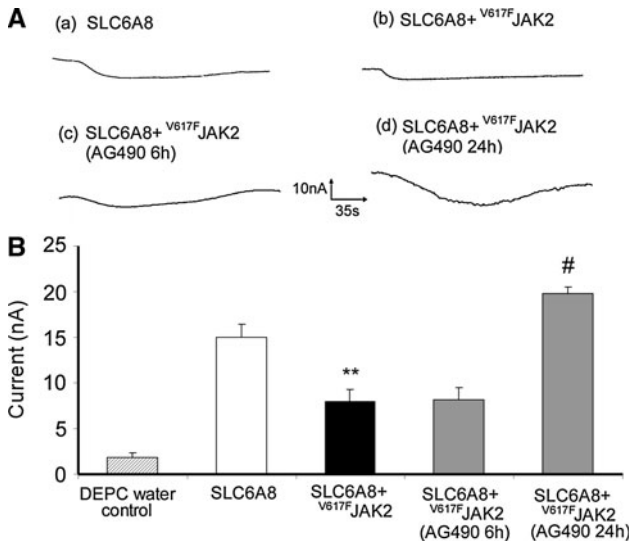


**Fig. 3** Effect of $^{V617F}$JAK2 is reversed by the JAK2 inhibitor AG490. **a** Representative original tracings showing creatine (1 mM)—induced current ($I_{crea}$) in *Xenopus* oocytes injected with SLC6A8 (*a*) or SLC6A8 + $^{V617F}$JAK2 without (*b*) or with 6 h (*c*) or 24 h (*d*) pretreatment with AG490 (40 μM). **b** Arithmetic means ± SEM ($n = 4$–5) of $I_{crea}$ in *Xenopus* oocytes expressing SLC6A8 with constitutively active $^{V617F}$JAK2 in the absence of inhibitor (0 h) or following pretreatment with AG490 (40 μM) for the indicated time periods. $^{**}P < 0.001$ vs. expression of SLC6A8 alone, $^{\#}P < 0.001$ vs. absence of AG490 (SLC6A8 + $^{V617F}$JAK2)

uncertainty. The inefficacy of the inhibitor following short incubation may result from delayed access of the inhibitor to the kinase or may be an indirect effect of the kinase on carrier activity. For instance, the slow effect of the inhibitor could result from regulation of protein abundance rather than direct inactivation of the transporter. The insensitivity of the JAK2 effect to the presence of actinomycin suggests that the observed effect of JAK2 does not depend on transcription.

Given the close relation of SLC6A8 to the osmolyte transporters BGT and TAUT (Nash et al. 1994), the downregulation of SLC6A8 by JAK2 appears somewhat surprising. JAK2 activity is stimulated by hypertonicity (Garnovskaya et al. 2003; Gatsios et al. 1998) and the kinase enhances the activity of the cell volume regulatory $Na^+/H^+$ exchanger (NHE) (Coaxum et al. 2009). Osmotic cell shrinkage upregulates the activity of osmolyte transporters (Handler and Kwon 1996; Hoffmann and Pedersen 2006; Pasantes-Morales and Cruz-Rangel 2010), a regulation opposite to the presently observed JAK2-dependent downregulation of SLC6A8. It must be kept in mind that the osmolyte carriers are regulated by gene expression (Handler and Kwon 1996) and by cell volume–sensitive signaling involving the mTOR (Shojaiefard et al. 2006), serum and glucocorticoid inducible kinase (SGK1) (Shojaiefard et al. 2005) and phosphatidylinositol-3-phosphate-5-kinase (PIKfyve) (Strutz-Seebohm et al. 2007). Those mechanisms could upregulate SLC6A8 during cell

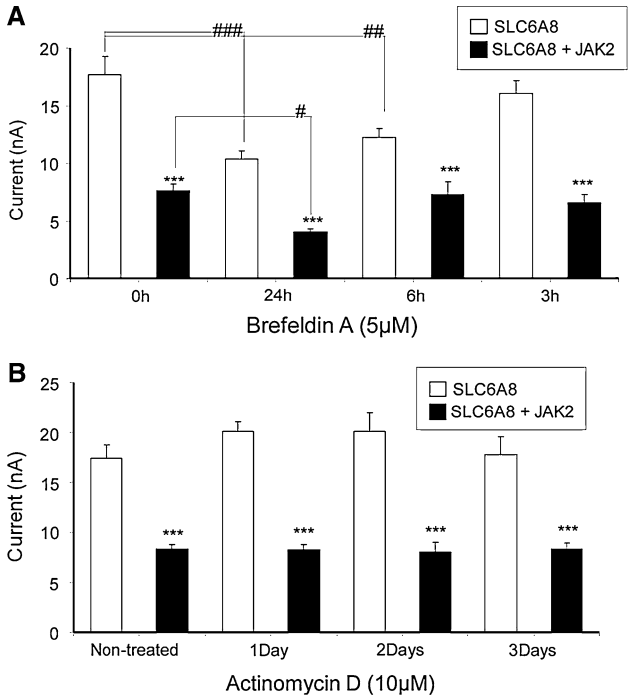


**Fig. 4** Effects of brefeldin A and actinomycin D on SLC6A8 and SLC6A8 + JAK2 expressing *Xenopus* oocytes. **a** Arithmetic means ± SEM ($n = 7$–16) of creatine (1 mM)—induced current ($I_{crea}$) in *Xenopus* oocytes injected with cRNA encoding SLC6A8 without (*white bars*) and with (*black bars*) JAK2 in the presence and absence of 5 μM brefeldin A for 0–24 h prior to measurement. $^{***}P < 0.001$ vs. absence of JAK2, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. absence of brefeldin A. **b** Arithmetic means ± SEM ($n = 8$–9) of $I_{crea}$ in *Xenopus* oocytes injected with cRNA encoding SLC6A8 without (*white bars*) and with (*black bars*) JAK2 in the presence and absence of 10 μM actinomycin D 1–3 days prior to measurement. $^{***}P < 0.001$ vs. absence of JAK2

shrinkage. The creatine transporter is further regulated by Src (Wang et al. 2002) and AMP-activated kinase AMPK (Li et al. 2010). Nevertheless, the present observations raise a question about the functional significance of JAK2-sensitive SLC6A8 downregulation. The decrease of carrier activity would decrease $Na^+$ entry, which may be protective during energy depletion or otherwise compromised $Na^+$ extrusion by the $Na^+/K^+$-ATPase. On the other hand, creatine may reversibly bind phosphate (Speer et al. 2004), and decreased abundance of cytosolic creatine may decrease the ability of the cell to replenish ATP upon energy depletion.

## Summary

This study presents evidence, for the first time, that JAK2 regulates the creatine transporter SLC6A8 (CreaT). It appears that JAK2 associates with the carrier and regulates its trafficking to the surface and, thus, cell membrane abundance.

**Acknowledgments** The authors acknowledge the meticulous preparation of the manuscript by Lejla Subasic and Sari Rübe and technical support by Elfriede Faber. This study was supported by the Deutsche Forschungsgemeinschaft (GRK 1302, SFB 773 B4/A1, La 315/13-3).

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