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Down-Regulation of Excitatory Amino Acid Transporters EAAT1 and EAAT2 by the Kinases SPAK and OSR1

Abeer Abousaab[1] · Jamshed Warsi[1] · Bernat Elvira[1] · Ioana Alesutan[1] · Zohreh Hoseinzadeh[1] · Florian Lang[1]

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Abstract SPAK (SPS1-related proline/alanine-rich kinase) and OSR1 (oxidative stress-responsive kinase 1) are cell volume-sensitive kinases regulated by WNK (with-no-K[Lys]) kinases. SPAK/OSR1 regulate several channels and carriers. SPAK/OSR1 sensitive functions include neuronal excitability. Orchestration of neuronal excitation involves the excitatory glutamate transporters EAAT1 and EAAT2. Sensitivity of those carriers to SPAK/OSR1 has never been shown. The present study thus explored whether SPAK and/or OSR1 contribute to the regulation of EAAT1 and/or EAAT2. To this end, cRNA encoding EAAT1 or EAAT2 was injected into *Xenopus* oocytes without or with additional injection of cRNA encoding wild-type SPAK or wild-type OSR1, constitutively active ^{T233E}SPAK, WNK insensitive ^{T233A}SPAK, catalytically inactive ^{D212A}SPAK, constitutively active ^{T185E}OSR1, WNK insensitive ^{T185A}OSR1 or catalytically inactive ^{D164A}OSR1. The glutamate (2 mM)-induced inward current (I_{Glu}) was taken as a measure of glutamate transport. As a result, I_{Glu} was observed in EAAT1- and in EAAT2-expressing oocytes but not in water-injected oocytes, and was significantly decreased by coexpression of SPAK and OSR1. As shown for EAAT2, SPAK, and OSR1 decreased significantly the maximal transport rate but significantly enhanced the affinity of the carrier. The effect of wild-type SPAK/OSR1 on EAAT1 and EAAT2 was mimicked by ^{T233E}SPAK and ^{T185E}OSR1, but not by ^{T233A}SPAK, ^{D212A}SPAK, ^{T185A}OSR1, or ^{D164A}OSR1. Coexpression of either SPAK or OSR1 decreased the EAAT2 protein abundance in the cell membrane of EAAT2-expressing oocytes. In conclusion, SPAK and OSR1 are powerful negative regulators of the excitatory glutamate transporters EAAT1 and EAAT2.

Keywords Neuronal excitation · Glutamate · Oxidative stress-responsive kinase 1 · SPS1-related proline/alanine-rich kinase · WNK

Introduction

SPAK (SPS1-related proline/alanine-rich kinase) (Castaneda-Bueno and Gamba 2010; Rafiqi et al. 2010; Yang et al. 2010) and OSR1 (oxidative stress-responsive kinase 1) (Lin et al. 2011; Villa et al. 2008) are cell volume-sensitive kinases contributing to the regulation of epithelial ion transport and blood pressure (Gagnon and Delpire 2012). SPAK and OSR1 are phosphorylated by WNK (with-no-K[Lys]) kinases (Glover et al. 2011; O'Reilly et al. 2003; Rafiqi et al. 2010; Vitari et al. 2005, 2006), which are similarly involved in the regulation of ion transport and blood pressure (Achard et al. 2001; Capasso et al. 2005; Flatman 2008; Furgeson and Linas 2010; Glover et al. 2011; Kahle et al. 2010; O'Reilly et al. 2003; Uchida 2010; Wilson et al. 2001). SPAK and OSR1 are effective by stimulating NaCl (NCC) and Na^+, K^+, $2Cl^-$ (NKCC) cotransporters (Delpire and Gagnon 2006; 2008; Gagnon and Delpire 2010, 2012; Gimenez 2006; Glover and O'Shaughnessy 2011; Huang et al. 2008; Kahle et al. 2010; Lin et al. 2011; Mercier-Zuber and O'Shaughnessy 2011; Richardson and Alessi 2008; Richardson et al. 2011; Villa et al. 2008; Vitari et al. 2005, 2006), as well as a number of ion channels (Falin et al. 2009, 2011; Miyazaki and Strange 2012; Park et al. 2010). WNK-SPAK/OSR1

✉ Florian Lang
florian.lang@uni-tuebingen.de

[1] Department of Physiology, University of Tübingen, Gmelinstr. 5, 72076 Tübingen, Germany

kinase pathway-dependent functions include neuronal excitability (Alessi et al. 2014; Yang et al. 2013). By activation of NKCC-dependent Cl^- uptake and inhibition of KCC-dependent Cl^- exit, SPAK/OSR1 increase intracellular Cl^- concentration, thus decreasing the Cl^- gradient across the cell membrane (Alessi et al. 2014; Yang et al. 2013). A decreased Cl^- gradient jeopardizes the ability of the inhibitory neurotransmitter GABA to hyperpolarize neurons by activation of Cl^- channels and thus enhances neuronal excitability (Ben-Ari et al. 2012).

Neuroexcitation is further influenced by clearance of the excitatory transmitters glutamate and aspartate from synaptic clefts, a function of excitatory amino acid transporters (Beart and O'Shea 2007; Estrada Sanchez et al. 2008; Foran and Trotti 2009; Markowitz et al. 2007; Sheldon and Robinson 2007) including the excitatory amino acid transporter isoforms EAAT1 and EAAT2 (Amara and Fontana 2002; Beart and O'Shea 2007; Lehre and Danbolt 1998). EAAT1 (Berger and Hediger 1998; Cholet et al. 2002; Fukaya et al. 1999; Kimmich et al. 2001; Sandhu et al. 2002; Suarez et al. 2000; Suzuki et al. 2001; Ullensvang et al. 1997; Utsumi et al. 2001) and EAAT2 (Milton et al. 1997) are expressed in astrocytes. In addition, EAAT1 is expressed in oligodendrocytes (Domercq and Matute 1999; Domercq et al. 1999), neurons (Gaillet et al. 2001; Plachez et al. 2000; Rothstein et al. 1994), retina (Barnett and Pow 2000; Derouiche and Rauen 1995), taste buds (Lawton et al. 2000), cochlea (Furness and Lehre 1997; Li et al. 1994), vestibular organ (Takumi et al. 1997), circumventricular organ (Berger and Hediger 1998), adrenal and pineal glands (Lee et al. 2001; Redecker and Pabst 2000), as well as bone cells (Gray et al. 2001; Huggett et al. 2000; Mason et al. 1997; Nomura and Takano-Yamamoto 2000).

Upregulation of EAAT2 activity has been shown to confer neuroprotection (Rothstein et al. 2005), and impaired expression or activity of EAAT2 is followed by neuroexcitotoxicity (Gibb et al. 2007; Rothstein et al. 1996; Tanaka et al. 1997; Vorwerk et al. 2000).

The present study explored whether EAAT1 and/or EAAT2 are sensitive to SPAK and/or OSR1. To this end, EAAT1 or EAAT2 was expressed in *Xenopus* oocytes without or with additional expression of the kinases. The glutamate-induced current was determined by dual-electrode voltage clamp and taken as a measure of glutamate transport.

Materials and Methods

Ethical Statement

All experiments conform with the 'European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes' (Council of Europe No 123, Strasbourg 1985) and were conducted according to the German law for the welfare of animals, and the surgical procedures on the adult *Xenopus laevis* frogs were reviewed and approved by the respective government authority of the state Baden-Württemberg (Regierungspräsidium) prior to the start of the study (Anzeige für Organentnahme nach §36).

Constructs

Constructs encoding human wild-type EAAT1 (Boehmer et al. 2003), human wild-type EAAT2 (Boehmer et al. 2006; Gehring et al. 2009; Shigeri et al. 2001), human wild-type SPAK, constitutively active ^{T233E}SPAK, WNK insensitive ^{T233A}SPAK, catalytically inactive ^{D212A}SPAK (Vitari et al. 2005), human wild-type OSR1, constitutively active ^{T185E}OSR1, WNK insensitive ^{T185A}OSR1, and catalytically inactive ^{D164A}OSR1 (Pathare et al. 2012) were used for generation of cRNA as described previously (Almilaji et al. 2013a, 2014b). The constructs were a kind gift from Dario Alessi (University of Dundee).

Voltage Clamp in Xenopus Oocytes

Xenopus oocytes were prepared as previously described (Hosseinzadeh et al. 2013a; Pakladok et al. 2013). 10 ng cRNA encoding EAAT1 or EAAT2 and 10 ng of cRNA encoding wild-type, constitutively active or inactive kinase were injected 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), and pH7.4 (Almilaji et al. 2013b, 2014a). The voltage clamp experiments were performed at room temperature 3 days after the first injection (Almilaji et al. 2014b; Fezai et al. 2014). Glutamate-induced currents were taken as a measure of glutamate transport (Dermaku-Sopjani et al. 2013; Elvira et al. 2014). 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 analysis (Axon Instruments) (Hosseinzadeh et al. 2013b, 2014; Munoz et al. 2013). 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 (Warsi et al. 2014a, b, c). For kinetic analysis, the glutamate-induced current (I_{Glu}) was plotted against the respective glutamate concentration (s) and maximal current (I_{max}) as well as concentration required for halfmaximal

current (k_m) calculated using the equation $I_{Glu} = I_{max} \cdot s/(k_m + s)$.

Detection of EAAT2 Cell Surface Expression by Chemiluminescence

For detection of EAAT2 cell surface expression, the oocytes were first incubated with primary polyclonal rabbit anti-EAAT2 antibody (extracellular) (1:500, Alomone Labs) and subsequently with secondary, HRP-conjugated anti-rabbit IgG antibody (1:1000, Cell Signaling). 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 aluminometer (Walter Wallac 2 plate reader, Perkin Juegesheim, Germany) by integrating the signal over 1 s. Results display normalized relative light units.

Statistical Analysis

Data are provided as mean ± SEM, n represents the number of oocytes investigated. As different batches of oocytes may yield different results, comparisons were always made within a given oocyte batch. All voltage clamp 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 (Tukey or Kruskal–Wallis test) or *t* test, as appropriate. Results with $p < 0.05$ were considered statistically significant.

Results

The present study explored the effect of SPAK (SPS1-related proline/alanine-rich kinase) and/or OSR1 (oxidative stress-responsive kinase 1) on electrogenic glutamate transport by the excitatory amino acid transporters EAAT1 and EAAT2. To this end, cRNA encoding EAAT1 or EAAT2 was injected into *Xenopus laevis* oocytes with or without additional injection of cRNA encoding wild-type or mutant kinases.

The glutamate-induced inward current (I_{Glu}) in dual-electrode voltage clamp experiments was taken as a measure of electrogenic glutamate transport. As shown in Fig. 1, I_{Glu} was negligible in water-injected oocytes indicating that the oocytes did not express appreciable endogenous electrogenic glutamate transport. In contrast, addition of glutamate (2 mM) to the extracellular fluid bathing EAAT1-expressing *Xenopus laevis* oocytes was followed by a sizable I_{Glu}. The additional coexpression of wild-type SPAK was followed by a significant decrease of I_{Glu}.

Similar observations were made in EAAT2-expressing oocytes (Fig. 2). Again, I_{Glu} was negligible in water-injected oocytes but large in EAAT2-expressing *Xenopus laevis* oocytes. Additional coexpression of wild-type SPAK was followed by a significant decrease of I_{Glu} in EAAT2-expressing oocytes.

Additional experiments explored whether SPAK coexpression modifies the maximal I_{Glu} and/or the affinity of EAAT2. To this end, EAAT2 was expressed in *Xenopus laevis* oocytes without or with additional expression of SPAK and subsequently exposed to glutamate concentrations ranging from 10 µM to 5 mM. As illustrated in Fig. 3, I_{Glu} was a function of the extracellular glutamate concentration. Maximal I_{Glu} was significantly ($p < 0.05$) lower in *Xenopus laevis* oocytes expressing EAAT2 together with SPAK (27.6 ± 0.5 nA, $n = 7$) than in *Xenopus laevis* oocytes expressing EAAT2 alone (67.3 ± 2.3 nA, $n = 6$). The concentration required for half maximal I_{Glu} (apparent K_m) was significantly lower in *Xenopus laevis* oocytes expressing EAAT2 together with SPAK (89 ± 26 µM, $n = 7$) than in *Xenopus laevis* oocytes expressing EAAT2 alone (365 ± 51 µM, $n = 6$).

Additional experiments aimed to shed light on the activity of SPAK mutants. To this end, EAAT1 was expressed with or without additional expression of the respective SPAK mutants. As shown in Fig. 4, the effect of wild-type SPAK was mimicked by the constitutively active ^{T233E}SPAK but neither by the WNK insensitive inactive ^{T233A}SPAK nor by the catalytically inactive ^{D212A}SPAK.

Similar observations were made in EAAT2-expressing oocytes. As illustrated in Fig. 5, the effect of wild-type SPAK was mimicked by the constitutively active ^{T233E}SPAK but not by WNK insensitive inactive ^{T233A}SPAK or by catalytically inactive ^{D212A}SPAK.

The down-regulation of EAATs activity by SPAK may, at least in part, has resulted from an influence of the kinase on the carrier protein surface abundance in the cell membrane. Chemiluminescence was thus employed to quantify carrier protein abundance in the cell membrane. As shown in Fig. 6, coexpression of wild-type SPAK was followed by a significant decrease of EAAT2 carrier protein surface abundance.

Similar experiments were performed to elucidate the effect of OSR1 on EAAT1 and EAAT2 activity. As shown in Fig. 7, the coexpression of wild-type OSR1 was followed by a significant decrease of I_{Glu} in EAAT1-expressing *Xenopus* oocytes. As illustrated in Fig. 8, the coexpression of wild-type OSR1 was similarly followed by a significant decrease of I_{Glu} in EAAT2-expressing *Xenopus* oocytes.

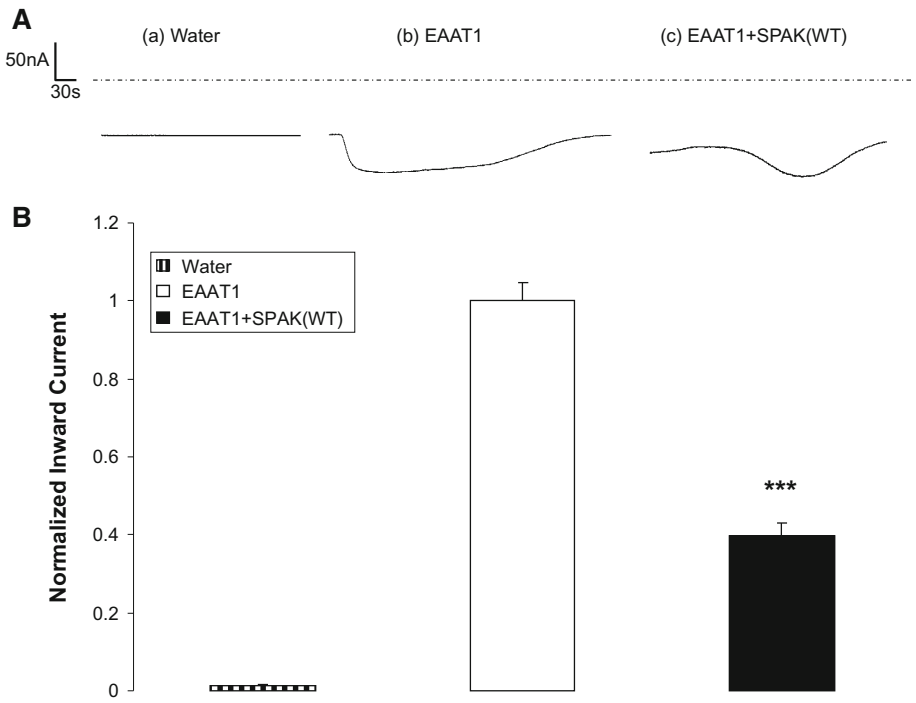

Fig. 1 Coexpression of SPAK decreases electrogenic glutamate transport in EAAT1-expressing *Xenopus laevis* oocytes. **a** Representative original tracings showing glutamate (2 mM)-induced current (I_{glu}) in *Xenopus laevis* oocytes injected with water (*a*) or expressing EAAT1 without (*b*), or with (*c*) additional coexpression of wild-type SPAK. **b** Arithmetic mean ± SEM ($n = 10$–14) of I_{glu} in *Xenopus laevis* oocytes injected with water (*striated bar*), or expressing EAAT1 without (*white bar*) or with (*black bar*) wild-type SPAK. ***($p < 0.001$) indicates statistically significant difference from the absence of SPAK

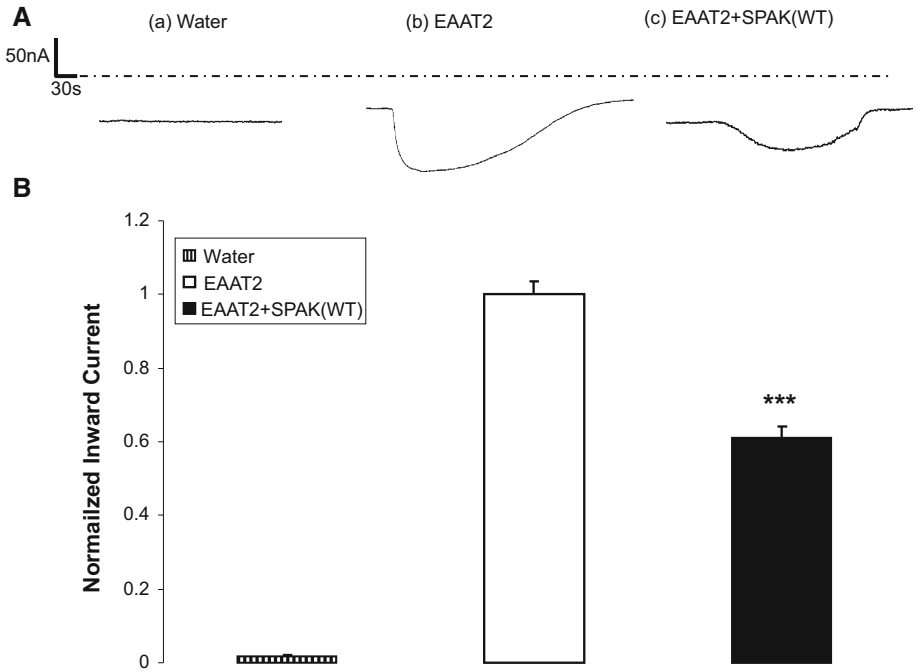

Fig. 2 Coexpression of SPAK decreases electrogenic glutamate transport in EAAT2-expressing *Xenopus laevis* oocytes. **a** Representative original tracings showing I_{glu} in *Xenopus laevis* oocytes injected with water (*a*) or expressing EAAT2 without (*b*), or with (*c*) additional coexpression of wild-type SPAK. **b** Arithmetic mean ± SEM ($n = 14$–20) of I_{glu} in *Xenopus laevis* oocytes injected with water (*striated bar*), or expressing EAAT2 without (*white bar*) or with (*black bar*) wild-type SPAK. ***($p < 0.001$) indicates statistically significant difference from the absence of SPAK

As illustrated in Fig. 9, the maximal glutamate-induced current was again significantly ($p < 0.05$) lower in *Xenopus laevis* oocytes expressing EAAT2 together with OSR1 (26.2 ± 2.1 nA, $n = 6$) than in *Xenopus laevis* oocytes expressing EAAT2 alone (75.0 ± 6.1 nA, $n = 6$). Again, the concentration required for half maximal I_{Glu} (apparent K_m) was significantly lower in *Xenopus laevis* oocytes expressing EAAT2 together with OSR1 (82 ± 17 μM, $n = 6$) than in *Xenopus laevis* oocytes expressing EAAT2 alone (220 ± 29 μM, $n = 6$).

Coexpression of EAAT1 with constitutively active ^{T185E}OSR1, but not with WNK insensitive inactive ^{T185A}OSR1 or catalytically inactive ^{D164A}OSR1 significantly downregulated I_{Glu} (Fig. 10). Similarly, coexpression of EAAT2 with constitutively active ^{T185E}OSR1, but not with WNK insensitive inactive ^{T185A}OSR1 or

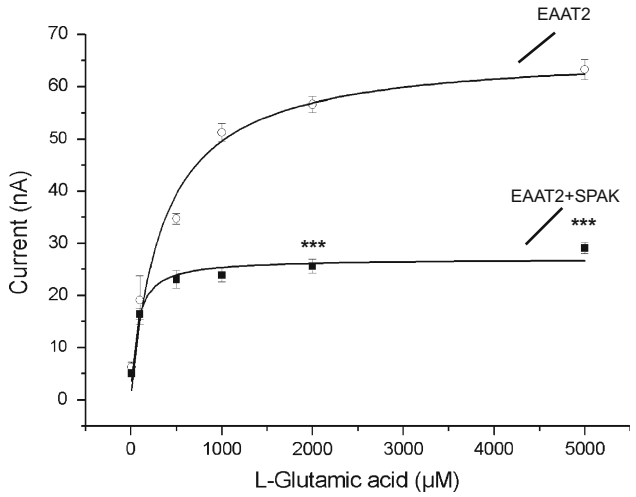

Fig. 3 Coexpression of SPAK decreases maximal electrogenic glutamate transport in EAAT2-expressing *Xenopus laevis* oocytes. Arithmetic mean ± SEM ($n = 7$) of I_{glu} as a function of glutamate concentration in *Xenopus laevis* oocytes expressing EAAT2 without (*white circles*), or with (*black squares*) additional coexpression of wild-type SPAK. ***($p < 0.001$) indicates statistically significant difference from oocytes expressing EAAT2 alone

catalytically inactive ^{D164A}OSR1 significantly downregulated I_{Glu} (Fig. 11).

Again, chemiluminescence was employed to quantify carrier protein abundance in the cell membrane. As shown in Fig. 12, coexpression of wild-type OSR1 was followed by a significant decrease of EAAT2 carrier protein surface abundance.

Discussion

The present study reveals that SPAK and OSR1 are powerful negative regulators of the excitatory amino acid transporters EAAT1 and EAAT2. Coexpression of either SPAK or OSR1 decreases the glutamate-induced inward current (I_{Glu}) in both, EAAT1- and EAAT2-expressing oocytes. As shown for EAAT2, SPAK, and OSR1 decrease the maximal transport rate. The kinases simultaneously enhance the affinity of EAAT2, an effect preventing a decline of current at very low substrate concentrations.

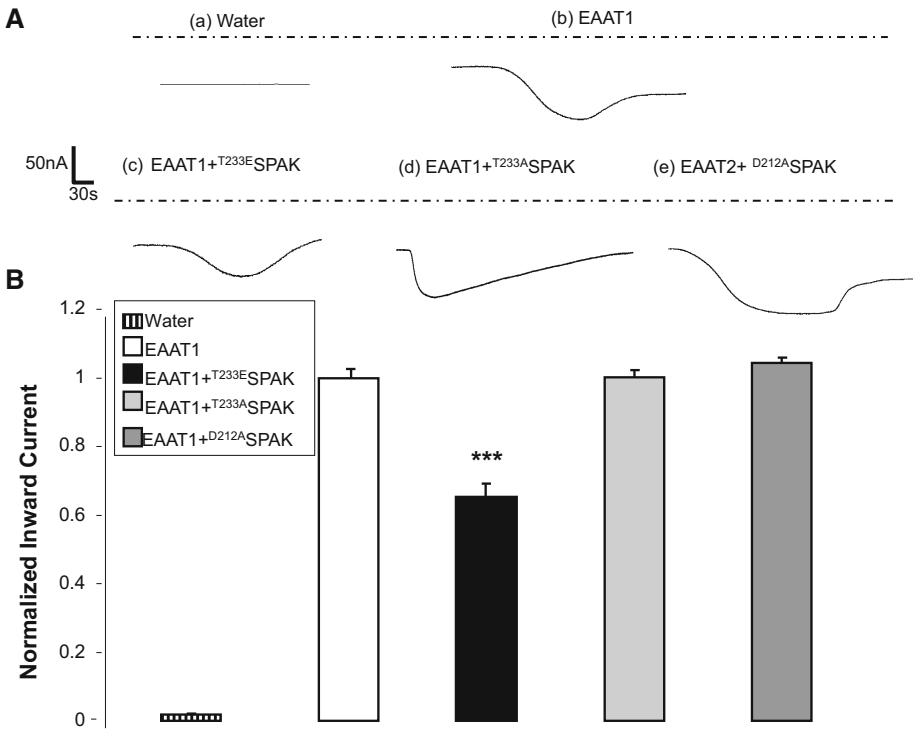

Fig. 4 Constitutively active ^{T233E}SPAK but not inactive mutants ^{T233A}SPAK or ^{D212A}SPAK decrease electrogenic glutamate transport in EAAT1-expressing *Xenopus laevis* oocytes. **a** Representative original tracings showing I_{glu} in *Xenopus laevis* oocytes injected with water (*a*), expressing EAAT1 alone (*b*) or with additional coexpression of constitutively active ^{T233E}SPAK (*c*) WNK1 insensitive ^{T233A}SPAK (*d*), or catalytically inactive ^{D212A}SPAK (*e*). **b** Arithmetic mean ± SEM ($n = 10$–15) of I_{glu} in *Xenopus laevis* oocytes injected with water (*striated bar*), or expressing EAAT1 without (*white bar*) or with constitutively active ^{T233E}SPAK (*black bar*), WNK insensitive ^{T233A}SPAK (*light grey bar*), or catalytically inactive ^{D212A}SPAK (*dark grey bar*). ***($p < 0.001$) indicates statistically significant difference from oocytes expressing EAAT2 alone

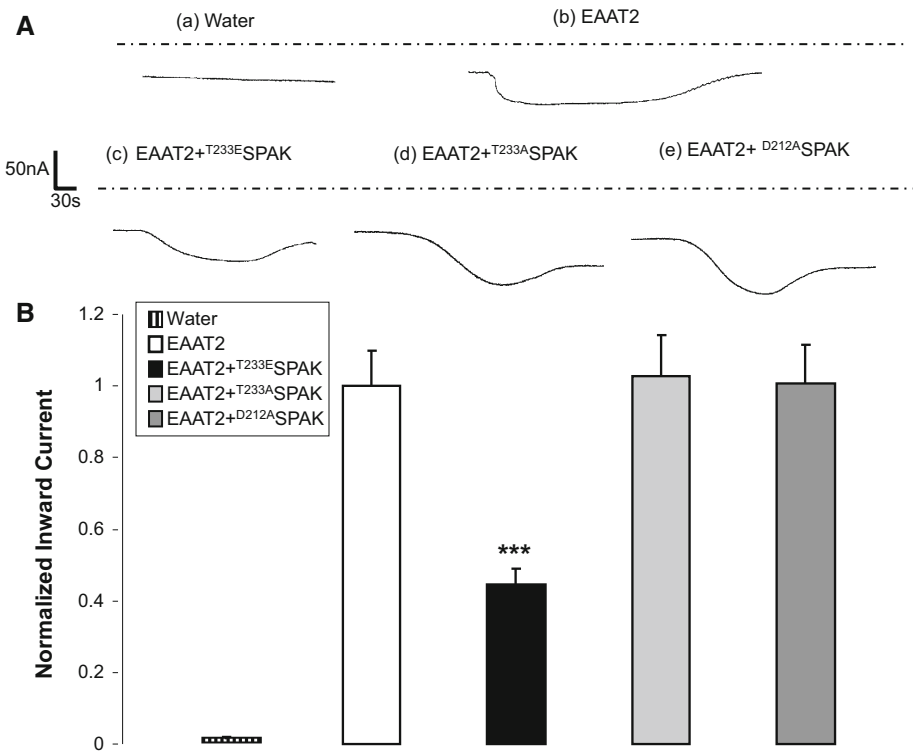

Fig. 5 Constitutively active ^{T233E}SPAK but not inactive mutants ^{T233A}SPAK or ^{D212A}SPAK decrease electrogenic glutamate transport in EAAT2-expressing *Xenopus laevis* oocytes. **a** Representative original tracings showing I_{glu} in *Xenopus laevis* oocytes injected with water (*a*), expressing EAAT2 alone (*b*) or with additional coexpression of constitutively active ^{T233E}SPAK (*c*) WNK1 insensitive ^{T233A}SPAK (*d*), or catalytically inactive ^{D212A}SPAK (*e*). **b** Arithmetic mean ± SEM ($n = 10$–15) of I_{glu} in *Xenopus laevis* oocytes injected with water (*striated bar*), or expressing EAAT2 without (*white bar*) or with constitutively active ^{T233E}SPAK (*black bar*), WNK insensitive ^{T233A}SPAK (*light grey bar*), or catalytically inactive ^{D212A}SPAK (*dark grey bar*). ***($p < 0.001$) indicates statistically significant difference from oocytes expressing EAAT2 alone

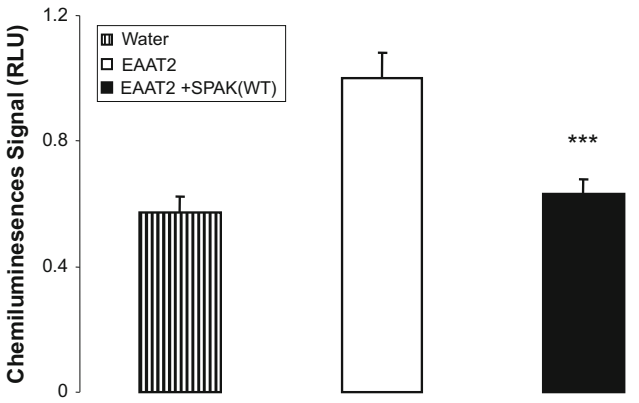

Fig. 6 Coexpression of SPAK decreases the EAAT2 protein abundance within the plasma membrane of *Xenopus laevis* oocytes. Arithmetic mean ± SEM ($n = 53$–59) of the normalized EAAT2 protein abundance assessed by chemiluminescence in oocytes injected with water (*striated bar*), or expressing EAAT2 alone (*white bar*), or together with wild-type SPAK (*black bar*). ***($p < 0.001$) indicates statistically significant difference from *Xenopus* oocytes expressing EAAT2 alone

The effects of wild-type SPAK on EAAT1 and EAAT2 are mimicked by constitutively active ^{T233E}SPAK but not by WNK insensitive ^{T233A}SPAK or catalytically inactive ^{D212A}SPAK. Similarly, the effects of wild-type OSR1 on EAAT1 and EAAT2 are mimicked by constitutively active ^{T185E}OSR1, but not by WNK insensitive ^{T185A}OSR1 or catalytically inactive ^{D164A}OSR1. The effects of SPAK or OSR1 on EAAT1 and EAAT2 thus require phosphorylation of SPAK and OSR1 at the respective WNK kinase phosphorylation site as well as an intact catalytic site pointing to the involvement of SPAK- and OSR1-dependent phosphorylation. The observations do not necessarily mean, however, that SPAK and OSR1 are effective by directly phosphorylating the EAAT1 and EAAT2 carrier proteins. Instead, the kinases might modify the function of other EAAT1 and EAAT2 regulating signalling molecules.

According to chemiluminescence, both, SPAK and OSR1 decreased the EAAT2 protein abundance in the cell membrane, an observation explaining the decrease of maximal transport rate following coexpression of the

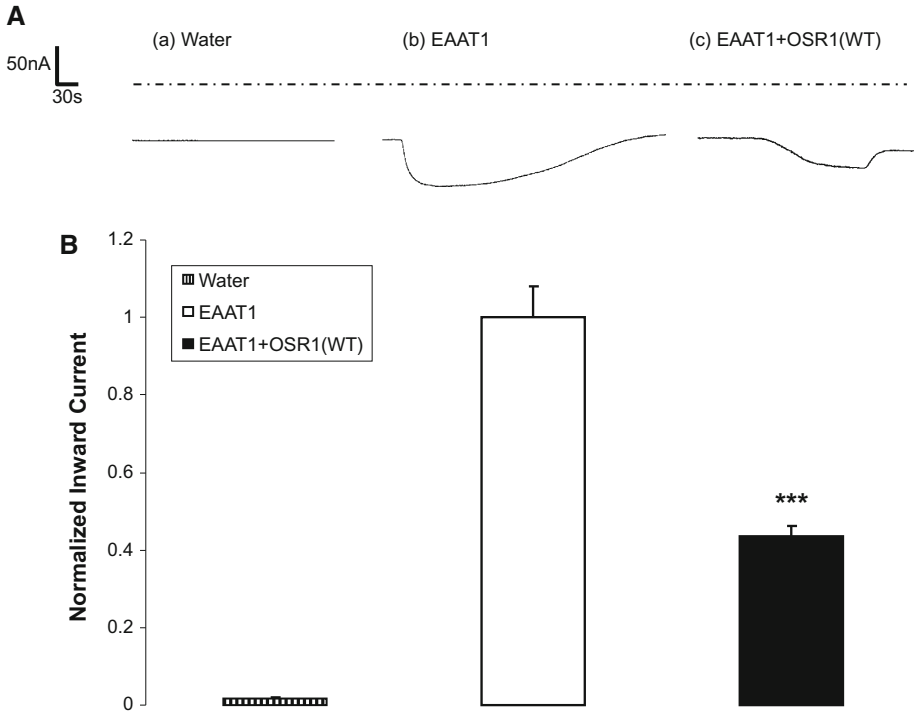

Fig. 7 Coexpression of OSR1 decreases electrogenic glutamate transport in EAAT1-expressing *Xenopus laevis* oocytes. **a** Representative original tracings showing I_{glu} in *Xenopus laevis* oocytes injected with water (*a*) or expressing EAAT1 without (*b*) or with (*c*) additional coexpression of wild-type OSR1. **b** Arithmetic mean ± SEM ($n = 14$–18) of I_{glu} in *Xenopus laevis* oocytes injected with water (*striated bar*), or expressing EAAT1 without (*white bar*) or with (*black bar*) wild-type OSR1. ***($p < 0.001$) indicates statistically significant difference from the absence of OSR1

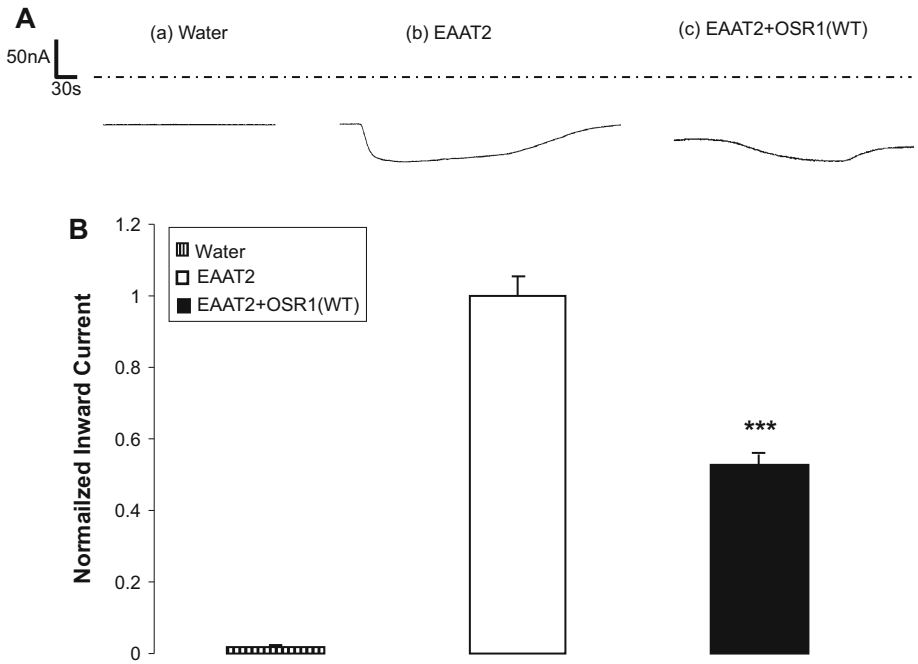

Fig. 8 Coexpression of OSR1 decreases electrogenic glutamate transport in EAAT2-expressing *Xenopus laevis* oocytes. **a** Representative original tracings showing I_{glu} in *Xenopus laevis* oocytes injected with water (*a*) or expressing EAAT2 without (*b*) or with (*c*) additional coexpression of wild-type OSR1. **b** Arithmetic mean ± SEM ($n = 14$–20) of I_{glu} in *Xenopus laevis* oocytes injected with water (*striated bar*), or expressing EAAT2 without (*white bar*) or with (*black bar*) wild-type OSR1. ***($p < 0.001$) indicates statistically significant difference from the absence of OSR1

carriers. The increase of apparent affinity may reflect an influence on carrier function.

At least in theory, the down-regulation of EAAT1 and EAAT2 could delay the clearance of glutamate from the synaptic cleft and thus impede termination of excitation. Accordingly, down-regulation of excitatory glutamate transporters could contribute to the increase of neuroexcitability by the kinases. SPAK/OSR1 enhance neuroexcitation at least in part by activation of NKCC-dependent Cl^- uptake and inhibition of KCC-dependent Cl^- exit with subsequent increase of intracellular Cl^- concentration, decrease of Cl^- gradient across the cell membrane, and thus decreased ability of the inhibitory neurotransmitter GABA to hyperpolarize neurons by activation of Cl^- channels (Alessi et al. 2014; Ben-Ari et al. 2012; Yang et al. 2013). Whatever underlying mechanisms, deranged regulation of SPAK/OSR1 may contribute to

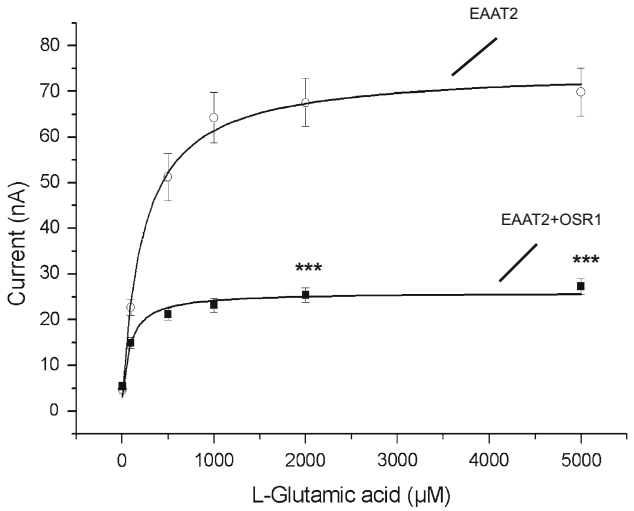

Fig. 9 Coexpression of OSR1 decreases maximal electrogenic glutamate transport in EAAT2-expressing *Xenopus laevis* oocytes. Arithmetic mean ± SEM ($n = 6$) of I_{glu} as a function of glutamate concentration in *Xenopus laevis* oocytes expressing EAAT2 without (*white circles*), or with (*black squares*) additional coexpression of wild-type OSR1.***($p < 0.001$) indicates statistically significant difference from oocytes expressing EAAT2 alone

several clinical conditions associated with neuronal hyperactivity, such as epilepsy, spasticity, neuropathic pain, schizophrenia, and autism (Alessi et al. 2014; Yang et al. 2013). As shown in mice, EAAT1 deficiency may lead to locomotor hyperactivity, abnormal behaviour with reduced preference for a novel social stimulus, reduced acoustic startle response, and impaired memory consolidation (Karlsson et al. 2008, 2009). Defective EAAT2 may contribute to several neurological disorders including Alzheimer disease (Li et al. 1997; Tian et al. 2007), schizophrenia (Lang et al. 2007), HIV-associated dementia (Rumbaugh et al. 2007), multiple sclerosis (Pampliega et al. 2008; Vercellino et al. 2007), leukomalacia (Desilva et al. 2008), epilepsy (Rakhade and Loeb 2008; Rakhade et al. 2007), brain trauma (van Landeghem et al. 2006), hypoxia and stroke (Boycott et al. 2007; Hurtado et al. 2008; Munch et al. 2008; Sheldon and Robinson 2007), reward dependence (Matsumoto et al. 2007), as well as amyotrophic lateral sclerosis (ALS) (Gibb et al. 2007; Rothstein et al. 1992, 1995).

By stimulating NaCl cotransporters and Na^+, K^+, $2Cl^-$ cotransporters and by inhibiting KCl cotransporters (Delpire and Gagnon 2006, 2008, 2010, 2012; Gimenez 2006;

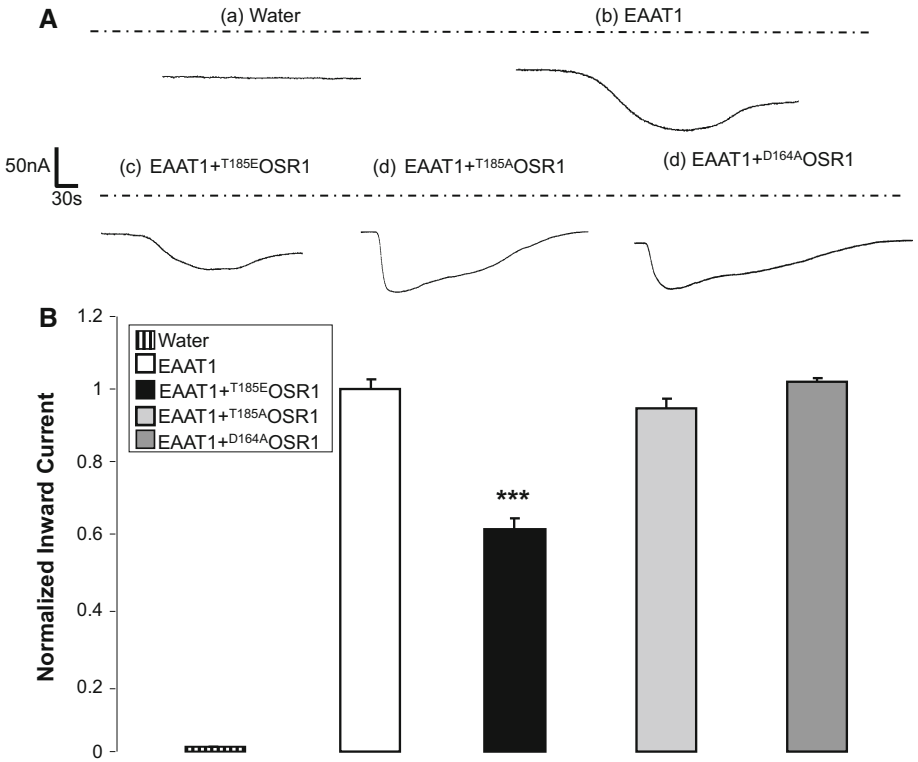

Fig. 10 Constitutively active ^{T185E}OSR1 but not inactive mutants ^{T185A}OSR1 or ^{D164A}OSR1 decrease electrogenic glutamate transport in EAAT1-expressing *Xenopus laevis* oocytes. **a** Representative original tracings showing I_{glu} in *Xenopus laevis* oocytes injected with water (*a*), expressing EAAT1 alone (*b*) or with additional coexpression of constitutively active ^{T185E}OSR1 (*c*) WNK1 insensitive ^{T185A}OSR1 (*d*), or catalytically inactive ^{D164A}OSR1 (*e*). **b** Arithmetic mean ± SEM ($n = 9$–18) of I_{glu} in *Xenopus laevis* oocytes injected with water (*striated bar*) expressing EAAT1 without (*white bar*) or with constitutively active ^{T185E}OSR1 (*black bar*), WNK insensitive ^{T185A}OSR1 (*light grey bar*), or catalytically inactive ^{D164A}OSR1 (*dark grey bar*). ***($p < 0.001$) indicates statistically significant difference from oocytes expressing EAAT1 alone

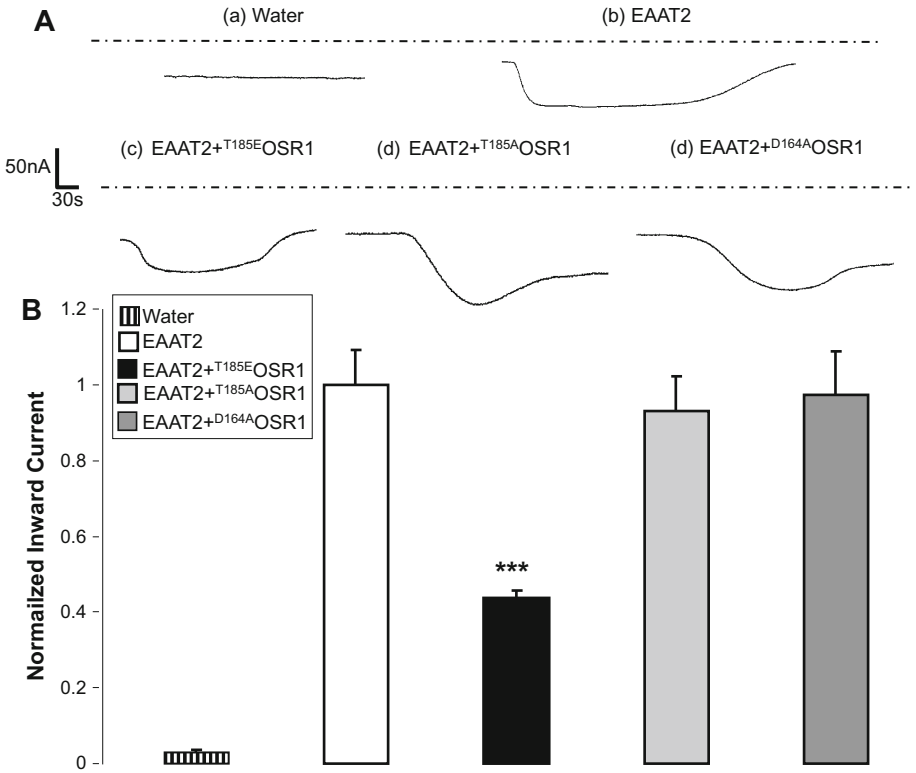

Fig. 11 Constitutively active ^{T185E}OSR1 but not inactive mutants ^{T185A}OSR1 or ^{D164A}OSR1 decrease electrogenic glutamate transport in EAAT2-expressing *Xenopus laevis* oocytes. **a** Representative original tracings showing I_{glu} in *Xenopus laevis* oocytes injected with water (*a*), expressing EAAT2 alone (*b*) or with additional coexpression of constitutively active ^{T185E}OSR1 (*c*) WNK1 insensitive ^{T185A}OSR1 (*d*), or catalytically inactive ^{D164A}OSR1 (*e*). **b** Arithmetic mean ± SEM ($n = 9$–18) of I_{glu} in *Xenopus laevis* oocytes injected with water (*striated bar*) expressing EAAT2 without (*white bar*) or with constitutively active ^{T185E}OSR1 (*black bar*), WNK insensitive ^{T185A}OSR1 (*light grey bar*), or catalytically inactive ^{D164A}OSR1 (*dark grey bar*). ***($p < 0.001$) indicates statistically significant difference from oocytes expressing EAAT2 alone

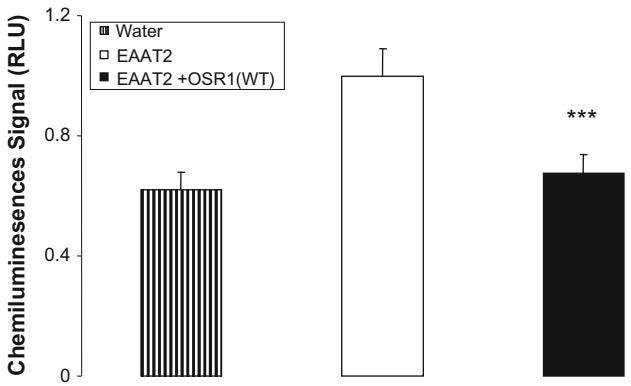

Fig. 12 Coexpression of OSR1 decreases the EAAT2 protein abundance within the plasma membrane of oocytes. Arithmetic mean ± SEM ($n = 53$–59) of the normalized EAAT2 protein abundance assessed by chemiluminescence in oocytes injected with water (*striated bar*), or expressing EAAT2 alone (*white bar*), or together with wild-type OSR1 (*black bar*). ***($p < 0.001$) indicates statistically significant difference from *Xenopus* oocytes expressing EAAT2 alone

Glover and O'Shaughnessy 2011; Huang et al. 2008; Kahle et al. 2010; Lin et al. 2011; Mercier-Zuber and O'Shaughnessy 2011; Richardson and Alessi 2008; Richardson et al. 2011; Villa et al. 2008, 2005, 2006), SPAK and OSR1 foster cellular KCl uptake and thus cell swelling (Hoffmann 2011; Hoffmann et al. 2009; Lang 2007). Inhibition of glutamate transport by SPAK/OSR1 is rather expected to decrease cell volume. Notably, inhibition of glutamate uptake with osmotically obliged water would be expected to rather increase cytosolic concentration of Cl^-, which inhibits WNK and thus SPAK/OSR1 activation (Alessi et al. 2014).

In conclusion, SPAK and OSR1 both down-regulate the activity of the glutamate transporters EAAT1 and EAAT2. The effect could impact on both neuronal excitability and cell volume regulation.

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Compliance with Ethical Standards

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

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