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ORIGINAL ARTICLE

Chronic treatment with anti-bipolar drugs suppresses glutamate release from astroglial cultures

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Abstract Astroglial cells are fundamental elements of most neurological diseases, including bipolar disorders in which astrocytes show morphological and functional deficiency. Here we report the suppression of astroglial glutamate release by chronic treatment with three anti-bipolar drugs, lithium salt (Li^+), carbamazepine (CBZ) and valproic acid (VPA). Release of glutamate was triggered by transient exposure of astrocytes to ATP (which activated purinoceptors) and 45 mM K^+ (which depolarised cell membrane to ~−30 mV). In both types of stimulation glutamate release was regulated by Ca^{2+} entry through plasmalemmal channels and by Ca^{2+} release from the endoplasmic reticulum (ER) intracellular stores. Exposure of astroglial cultures to Li^+, CBZ and VPA for 2 weeks led to a significant (more than 2 times) inhibition of glutamate release, which may alleviate the hyperactivity of the glutamatergic transmission in the brain of patients with bipolar disorders and thus contribute the underlying mechanism of drug action.

Keywords Astroglia · Neuropsychiatric diseases · Mood disorders · Bipolar disorder · Glutamate · Lithium · Carbamazepine · Valproic acid

Introduction

Astroglia, being responsible for homeostasis and defence of the central nervous system (CNS) contribute to the pathological progression and resolution of all neurological disorders (Giaume et al. 2007; Burda and Sofroniew 2014; Verkhratsky et al. 2012; Parpura et al. 2012). Astroglial dysfunction, pathological remodelling and atrophy have been observed in disorders manifested by disrupted higher cognitive functions including neurodegenerative and psychiatric diseases (Rajkowska and Stockmeier 2013; Verkhratsky et al. 2013, 2014a). Conceptually, reduced astroglial synaptic coverage and compromised ability of astrocytes to regulate homeostasis of major CNS transmitters (such as, for example glutamate, GABA and adenosine) may represent a mechanism for deregulated neurotransmission and aberrant synaptic connectivity which are key elements of pathological development of neuropsychiatric disorders (Bernstein et al. 2014; Sanacora and Banasr 2013).

Decrease in the number of astrocytes has been documented in post mortem tissues from subjects diagnosed with various forms of mood disorders including major depression and bipolar disorder (Czeh et al. 2013; Rajkowska 2014; Ongur et al. 1998). Mood disorders, both in humans and in animal models, are associated with

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a decreased expression of glial fibrilalry acidic protein (GFAP) and reduced densities of GFAP-positive astrocytes (Czeh et al. 2013; Miguel-Hidalgo et al. 2010). The GFAP belongs to the class of intermediate filament proteins; together with vimentin GFAP is the major component of astroglial cytoskeleton (Pekny and Pekna 2004). Increased expression of GFAP is universally acknowledged as a hallmark of reactive astrogliosis (Pekny and Pekna 2014), this latter being a complex evolutionary conserved defensive programme activated in astroglia in response to insults to CNS. Inhibition of astroglial reactivity, which can, in particular, be achieved by genetic deletion of GFAP and vimentin, generally exacerbates the evolution of neuropathology (Pekny et al. 2014; Sofroniew 2009). Suppressed astrogliosis, therefore could be one of the pathological steps in progression of mood disorders.

Astroglial cells in the CNS produce and secrete numerous neuroactive agents that include classical neurotransmitters (glutamate, ATP, or GABA), neuromodulators (D-serine, taurine or kynurenic acid), energy substrates (lactate), trophic factors (brain-derived neurotrophic factor, glia-derived neurotrophic factor or tumour-necrosis factor α), neuroactive peptide (atrial natriuretic peptide, secretogranin) and various molecules associated with brain plasticity such as, for example, trombospondins that regulate synaptogenesis in developing and regenerating CNS. Astroglial secretion, which represents a bona fide volume transmission, is fundamental for complex signalling in neuronal-glial networks (Parpura et al. 1994; Martineau et al. 2014; Parpura and Zorec 2010). Release of neuroactive substances from astrocytes is mediated by several molecular pathways that include diffusion through plasmalemmal channels, translocation by membrane transporters and exocytotic vesicular release. Astroglial exocytosis is regulated by cytosolic Ca^{2+} signals that originate from (1) Ca^{2+} entry through voltage- or ligand-gated channels, through transient receptor potential (TRP) channels or though Na^+/Ca^{2+} exchanger operating in the reverse mode; (2) Ca^{2+} release from intracellular endoplasmic reticulum (ER) store mediated by inositol-1,4,5-trisphosphate receptors ($InsP_3Rs$) or Ca^{2+}-gated Ca^{2+} channels generally known as ryanodine receptors (RyR) (Parpura and Grubisic 2011; Verkhratsky et al. 2014b). The $InsP_3$-induced Ca^{2+} release is controlled by metabotropic receptors, of which P2Y purinoceptors are abundantly expressed in astrocytes (Verkhratsky et al. 2009).

In our previous experiments we found that chronic treatment of mice with classical anti-bipolar drugs lithium (Li^+) salts, valproic acid (VPA) and carbamazepine (CBZ) affects astroglial Ca^{2+} dynamics by remodelling Ca^{2+} signalling toolkit. This remodelling included significant down-regulation of canonical TRP channel TRPC1 with a consequent suppression of store-operated Ca^{2+} entry (Yan et al. 2013).

In the present study we studied the astroglia-targeted action of anti-bipolar drugs further, by investigating their effects on glutamate release from astrocytes.

Methods

Cell cultures

Primary cultures of astrocytes were prepared from the neopallia of the cerebral hemispheres of newborn CD-1 mice as previously described and grown in Dulbecco's Minimum Essential Medium (DMEM) with 7.5 mM glucose (Hertz et al. 1998; Hertz 2012). After 2 weeks in vitro, 0.25 mM dibutyryl cyclic AMP (dBcAMP) was included in the medium. These dBcAMP-supported cultures are highly enriched in astrocytes (>95 % purity of glial fibrillary protein-(GFAP-) and glutamine synthetase-expressing astrocytes). Addition of dBcAMP leads to a morphological and functional differentiation as evidenced by the extension of cell processes and increases in several metabolic and functional activities characteristic of astrocytes in situ.

Drug treatment

After 3 weeks in vitro, culture medium (DMEM with 7.5 mM glucose and 0.25 mM dBcAMP), was supplemented with either (1) lithium carbonate at a concentration of 0.125, 0.25 or 0.5 mM, (2) CBZ at concentrations of 25 or 50 μM, or (3) VPA at a concentration of 0.1 or 1 mM. Astrocytes were exposed to these treatments for 2 weeks. For controls, astroglial cells were kept for 2 weeks in normal culture medium.

Superfusion

After removal of culture medium, the cultures were incubated for 30 min in PBS (137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 2 mM KH_2PO_4; 1 mM $CaCl_2$; 0.5 mM $MgCl_2$) containing 7.5 mM glucose at 37 °C to allow equilibration. The cells were then transfered to PBS, in which KH_2PO_4 was 5 mM and subsequently stimulated by 200 μM ATP or 45 mM K^+ added to this PBS (a change between PBS and PBS + ATP or high-K^+ was made at 5 min and the washout at 10 min); the rate of superfusion was 500 μl/min. The superfusates were collected every minute. The peak release of glutamate by stimulation was calculated as the amount of glutamate released in response the stimulation (fraction 6, the highest level of glutamate release) minus the amount of glutamate release under resting conditions just before the stimulation (fraction 5). At the end of experiment, the cells were dissolved in 1 ml of 1 M NaOH for protein determination by Lowry method

(Lowry et al. 1951). One ml of 80 % ethanol was added to the culture to extract intracellular glutamate.

HPLC

Content of glutamate was measured as described previously (Peng et al. 1991). HPLC was performed with a Waters HPLC system with a mode of Waters 2475 fluorescence detector and a Waters C18 (4.6 × 150 mm) column (Agilent Technologies, Palo Alto, CA, USA), using a 0.1 M potassium acetate (pH 5.9)/methanol gradient. Superfusates without further treatment, or cell extracts after free drying and dissolving in the borate buffer, were precolumn derivatized with OPA. The solvent flow was 1.0 ml per min and the initial methanol concentration was 10 %. This was increased to 25 % over 10 min, to 47 % at 12 min and to 100 % at 14 min. Thereafter it was returned to 10 % at 16 min to equilibrate for 5 min before the next injection.

Statistics

Differences between multiple groups were evaluated by one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) multiple comparison test for unequal replications. The level of significance was set at $p < 0.05$.

Materials

Most chemicals, including CBZ, VPA, nifedipine, PPADs (Benzene-2,4-Disulfonic Acid), ATP (Adenosine 5′-triphosphate), SKF96365 1-[2-(4-Methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl-1H-imidazole hydrochloride) and dibutyryl cyclic AMP (dBcAMP) were purchased from Sigma (St.Louis, MO, USA). Lithium carbonate (Li_2CO_3) was obtained from Shanghai Hengxin Chemical Reagent (Shanghai, China). Ryanodine was purchased from Calbiochem (La Jolla, CA, USA).

Results

ATP-induced glutamate release

The background release of glutamate in the presence of physiological (5.4 mM) K^+ concentration amounts to 0.2–0.5 nmol per mg/protein per min; exposure to 200 μM ATP however transiently increased glutamate content to 0.9–1.5 nmol per mg protein per min (Fig. 1). The peak of the release stimulated by ATP was on average, 1.13 ± 0.08 nmol per mg protein per min (from Fig. 1a–d; $n = 20$). The ATP-induced release of glutamate was suppressed (to 0.60 ± 0.06 nmol/mg/min, $n = 4$, $p < 0.01$; Fig. 1a) by 10 μM PPADS, a broad inhibitor of P2 receptors. Similarly, ATP-stimulated glutamate release was inhibited by removal of extracellular Ca^{2+} (nominally Ca^{2+}-free solution with $[Ca^{2+}] \sim 100$ μM to 0.78 ± 0.08 nmol/mg/min, $n = 4$, $p < 0.01$; Fig. 1b). Exposure of astrocytes to broad spectrum inhibitor of store-operated Ca^{2+} entry (SOCE) and of TRPC1 channels SKF-96365 (7.5 μM), potently suppressed stimulated glutamate release (to 0.25 nmol/mg/min ± 0.03 nmol/mg/min, $n = 4$, $p < 0.01$, Fig. 1c) and also reduced resting release of glutamate (to 0.12 ± 0.01 nmol/mg/min, $p < 0.01$; Fig. 1c) indicating the role for SOCE in background release of neurotransmitter.

Chronic treatment with each of three anti-bipolar drugs caused a significant decrease of ATP-stimulated glutamate release (Fig. 1d–f). Chronic exposure to Li^+ significantly decreased ATP-induced glutamate release from astrocytes (Fig. 1d). In cells treated with 0.25 mM Li^+, the amount of glutamate release by ATP decreased to 64 % of the control (peak release was 0.97 ± 0.06 nmol/mg/min, $n = 4$, $p < 0.05$); at 0.5 and at 1 mM Li^+ suppression of glutamate release was almost complete (ATP-induced release at 0.5 mM was 0.73 ± 0.08 nmol/mg/min, $n = 4$, $p < 0.01$ and at 1 mM 0.61 ± 0.06 nmol/mg/min, $n = 4$, $p < 0.01$, respectively; Fig. 1d). Chronic treatment with CBZ at 25 μM for 2 weeks decreased glutamate release to 64 % of control value (ATP-induced release at 25 μM CBZ was 0.97 ± 0.12 nmol/mg/min, $n = 4$, $p < 0.05$; Fig. 1e), and at 50 μM it decreased to 58 % of control value (0.88 ± 0.19 nmol/mg/min, $n = 4$, $p < 0.01$; Fig. 1e). Chronic treatment with VPA had similar inhibitory effect on glutamate release (Fig. 1f). At 0.1 mM of VPA, ATP-induced glutamate release was decreased to 66 % of control (stimulated release was 0.99 ± 0.09 nmol/mg/min, $n = 4$, $p < 0.01$; Fig. 1f), and at 1 mM it further decreased to 51 % of control value (0.77 ± 0.05 nmol/mg/min, $n = 4$, $p < 0.01$; Fig. 1f).

High K^+-induced glutamate release

Exposure of astrocytes to 45 mM K^+ triggered transient release of glutamate, which, on average, peaked at 0.64 ± 0.05 nmol/mg/min ($n = 13$). Removal of the extracellular Ca^{2+} reduced K^+-stimulated glutamate release to 0.51 ± 0.03 nmol/mg/min, $n = 4$, $p < 0.01$; (Fig. 2a). The high K^+-induced release of glutamate was suppressed by 50 μM ryanodine to 0.36 ± 0.02 nmol/mg/min, $n = 4$, $p < 0.05$; (Fig. 2b). Similarly, nifedipine, an inhibitor of voltage-gated L-type Ca^{2+} channels, at 100 nM decreased high K^+-induced glutamate release to 0.30 ± 0.06 nmol/mg/min, $n = 4$, $p < 0.01$ (Fig. 2c).

Chronic treatment with 1 mM Li^+ significantly suppressed high K^+-induced glutamate release from astrocytes

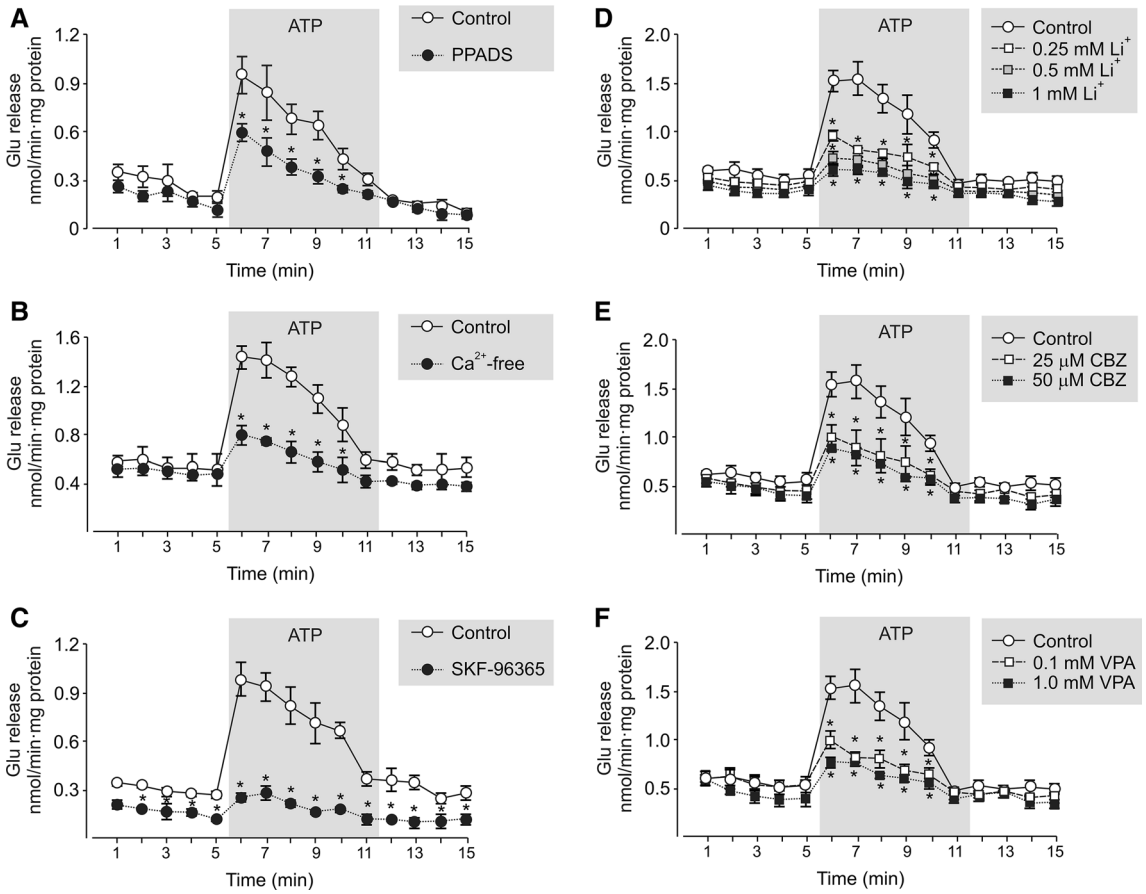

Fig. 1 Chronic treatment with anti-bipolar drugs suppresses ATP-induced glutamate release from astrocytes. **a** ATP-induced glutamate release from astrocytes is mediated by P2 purinoceptors. The dynamics of glutamate release in response to ATP (200 μM) application in control conditions and in the presence of broad P2 antagonist PPADS (10 μM) are presented. **b** Traces showing glutamate release in response to ATP (200 μM) application in control and in Ca^{2+}-free extracellular solution indicate the role for plasmalemmal Ca^{2+} entry as well as the importance of intracellular Ca^{2+} release. **c** The ATP-induced glutamate release from astroglia is sensitive to SOCE/TRP inhibitor SKF-96365 (7.5 μM) thus highlighting contribution of store-operated Ca^{2+} entry mechanism in regulation of glutamate secretion. **d** The ATP-induced glutamate release in control and in cells chronically treated with 0.25, 0.5 and 1 mM Li^+. **e** The ATP-induced glutamate release in control and in cells chronically treated with 25 and 50 μM CBZ. **f** The ATP-induced glutamate release in control and in cells chronically treated with 0.1 and 1 mM VPA. The values are averages ± S.E.M. from 5 samples for control group and 4 samples for PPADS group (**a**), values are averages ± S.E.M. from 4 samples for control, Ca^{2+}-free and SKF96365 groups (**b**, **c**), values are averages ± S.E.M. from 7 samples for control group and 4 samples for Li^+, CBZ and VPA groups (**d**–**f**). *Statistically significant ($P < 0.05$) difference from control group

(on average K^+-stimulated glutamate release peaked at 0.55 ± 0.04 nmol/mg/min, $n = 4$, $p < 0.01$; Fig. 2d). Similarly, glutamate release was inhibited in astrocytes chronically treated with 50 μM CBZ (the amplitude of stimulated release was 0.25 ± 0.04 nmol/mg/min, $n = 4$, $p < 0.01$; Fig. 2e) and with 1 mM VPA (peak value for glutamate release was 0.27 ± 0.04 nmol/mg/min, $n = 4$, $p < 0.01$; Fig. 2f).

Intracellular glutamate

Chronic treatment with Li^+, CBZ or VPA had no significant effect on intracellular content of glutamate in astroglia, as shown in Fig. 3.

Discussion

In the present paper we performed systematic study of glutamate release in astrocytes in response to stimulation of purinoceptors with ATP and in response to cell depolarisation with "high-extracellular K^+ voltage-clamp". We further investigated how chronic treatment with three major anti-bipolar drugs (Li^+, CBZ or VPA) impacts on the glutamate secretion from astroglia. First, we found that acute exposure to both ATP (at 200 μM) and high K^+ (45 mM, which depolarised cells to ~−30 mV) induced a release of glutamate from astrocytes. The average peak release was 1.13 ± 0.08 nmol per mg protein per min for ATP and 0.64 ± 0.05 nmol per mg protein per min for K^+

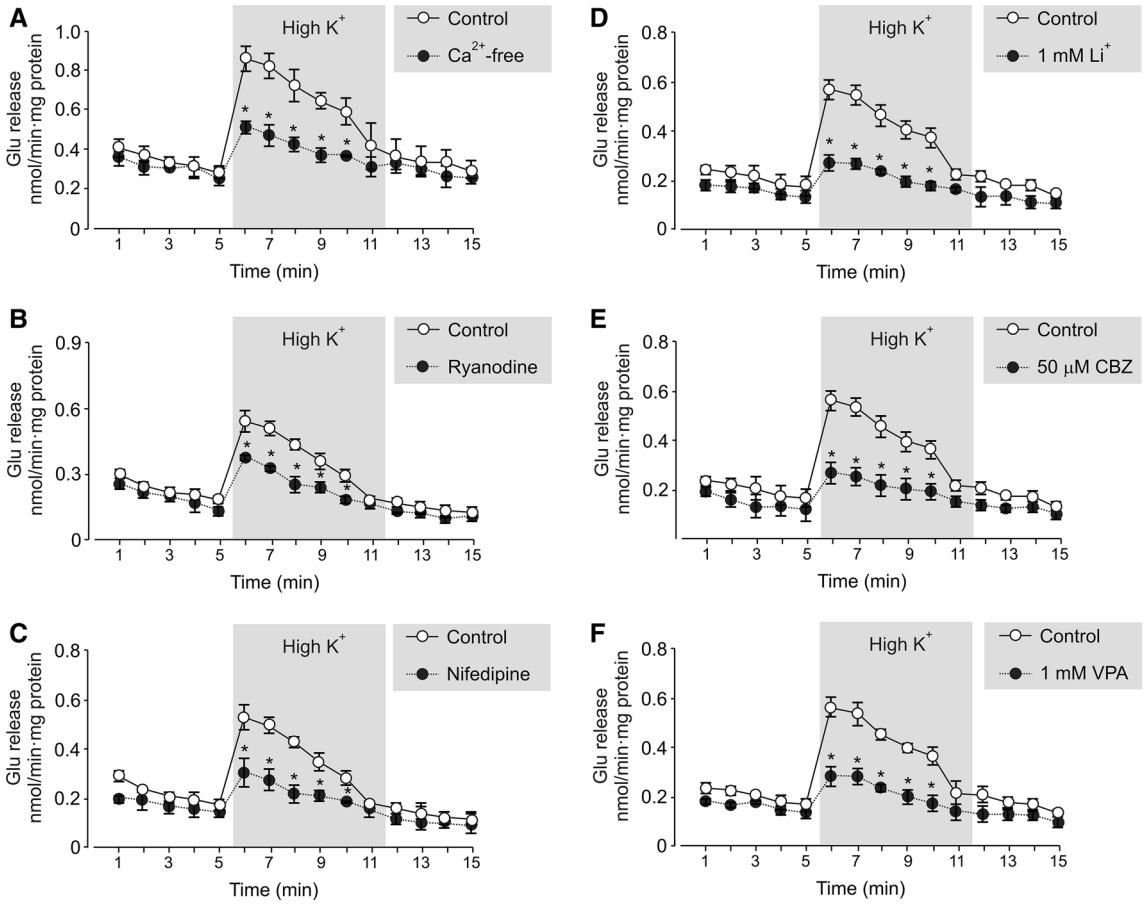

Fig. 2 Chronic treatment with anti-bipolar drugs suppresses depolarisation (high K^+)-induced glutamate release from astrocytes. Glutamate release from astrocytes requires the presence of extracellular Ca^{2+} and the activities of RyR and L-channels. **a** The depolarisation-induced glutamate release is partially suppressed in Ca^{2+}-free solution indicating the role for plasmalemmal Ca^{2+} entry. **b** The depolarisation-induced glutamate release is partially inhibited by 50 μM ryanodine solution marking the contribution of Ca^{2+}-induced Ca^{2+} release. **c** The depolarisation-induced glutamate release is partially suppressed by 100 nM nifedipine revealing the contribution of Ca^{2+} entry through voltage-gated Ca^{2+} channels. **d** The depolarisation (high K^+)-induced glutamate release in control and in cells chronically treated with 1 mM Li^+. **e** The depolarisation (high K^+)-induced glutamate release in control and in cells chronically treated with 50 μM CBZ. **f** The depolarisation (high K^+)-induced glutamate release in control and in cells chronically treated with 1 mM VPA. The values are averages ± S.E.M. from 4 samples for control and Ca^{2+}-free group (**a**), from 5 samples for control group and 4 samples for ryanodine and nifedipine groups (**b**, **c**), and averages ± S.E.M. from 5 samples for control group and 4 samples for lithium, CBZ and VPA groups (**d**–**f**). *Statistically significant ($P < 0.05$) difference from control group

depolarisation, which is in good agreement with previous reports (Jeremic et al. 2001). The ATP-induced glutamate release was partially inhibited by PPADS, indicating the involvement of P2 purinoceptors. Further, the ATP-evoked release of glutamate was partially suppressed by removal of extracellular Ca^{2+}, indicating possible role for P2X receptors, mediating Ca^{2+} influx as well as for P2Y receptors mediating Ca^{2+} release form the ER stores (Verkhratsky et al. 2009). In addition ATP-induced release of glutamate was sensitive to broad SOCE/TRP inhibitor SKF-96365 thus highlighting the contribution of the store-operated Ca^{2+} entry. These data are in full agreement with existing concept that considers multiple sources of Ca^{2+} that contribute to regulation of physiological astroglial secretion (Parpura and Grubisic 2011). Depolarisation-induced release of glutamate was similarly regulated by plasmalemmal Ca^{2+} entry through voltage-gated Ca^{2+} channels (hence partial sensitivity of the release to nifedipine), and by Ca^{2+}-induced Ca^{2+} release (which could be suppressed by ryanodine).

Second, we found that chronic treatment with each of the three anti-bipolar drugs employed in this study substantially decreased astroglial release of glutamate in response to both ATP- and high K^+-stimulation. At the same time this treatment did not affect intracellular glutamate content in astrocytes, indicating that the release mechanisms were the primary targets. Decrease of astroglial glutamate release could possibly contribute to anti-bipolar effects of drugs since

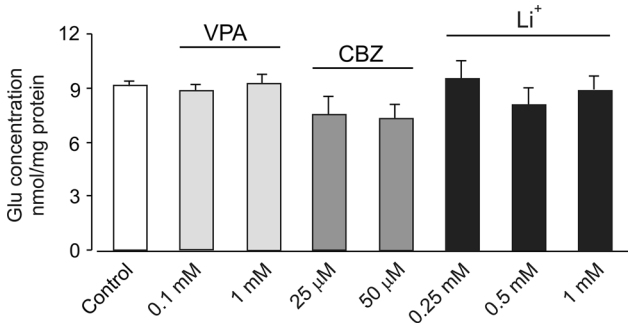

Fig. 3 Chronic treatment with lithium, CBZ or VPA does not affect intracellular glutamate content in astrocytes. The cells were treated with 0.25, 0.5 and 1 mM Li^+, 25 and 50 μM CBZ or 0.1 and 1 mM VPA for 2 weeks and compared with untreated control cultures. Content of glutamate in ethanol extracts was measured by HPLC. The values are averages ± S.E.M. from 7 samples for the control group and 4 samples for Li^+, CBZ and VPA groups

abnormal hyperactivity of the glutamatergic system has been documented in the brains of bipolar patients in vivo by magnetic resonance imaging (Ongur et al. 2008; Michael et al. 2003; Chen et al. 2010; Eastwood and Harrison 2010). There are also evidence that administration of riluzole, a drug affecting the astroglial glutamate uptake, modifies glutamate/glutamine metabolism in patients suffering from bipolar disorder (Brennan et al. 2010) and shows some therapeutic potential in this disease (Zarate and Manji 2008).

The action of anti-bipolar drugs on astroglia has a complex nature and affects multiple signalling pathways. We had reported previously that chronic exposure to Li^+, CBZ or VPA affects expression of multiple genes; for example it up-regulates expression of cytosolic phospholipase 2α while down-regulation expression of TRPC1 channels; this treatment also affects astroglial metabolism and causes intracellular alkalization (Yan et al. 2013; Li et al. 2007, 2009; Song et al. 2013). The decrease in expression of TRPC1 [in astroglia TRPC1 contributes substantially to SOCE (Golovina 2005; Reyes et al. 2013; Malarkey et al. 2008)] in particular may affect the Ca^{2+} content of the ER, and thus limit both Ca^{2+} release and plasmalemmal Ca^{2+} entry, which could be an important mechanism for suppression of astroglial glutamate secretion. Astroglial TRPC1 channels control several aspects of cytosolic ion signalling due to their polyionic permeability, as indeed in physiological conditions TRPC1 channels provide both Ca^{2+} and Na^+ influx (Verkhratsky et al. 2014b). Spatio-temporal fluctuations in Na^+ concentration regulate multiple molecular cascades responsible for astroglial homeostatic function, including for example, K^+ buffering, uptake and release of neurotransmitters and neuronal metabolic support (Kirischuk et al. 2012).

In conclusion, various classes of molecules used for pharmacological treatment of neurological disorders target astrocytes; although none of these molecules was originally designed as an astroglia-specific drug. Actions of these drugs on the nervous tissue may, conceivably, be mediated through astroglia (with subsequent neuronal consequences) and thus be rather different from contemporary concepts.

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Conflict of interest The authors declare no conflict of interest.

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