

RAPID REPORT

LRRC8A protein is indispensable for swelling-activated and ATP-induced release of excitatory amino acids in rat astrocytes

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Key points

- Swelling-activated release of amino acids in the CNS is thought to be mediated by an unidentified volume-regulated anion channel.
- Two recent studies discovered that LRRC8 family members form a volume-regulated anion channel in non-neural cells.
- In this work we established a critical contribution of the *LRRC8A* gene product to swelling-activated glutamate and taurine release from primary rat astrocytes.
- We also found that LRRC8A is indispensable for glutamate and taurine release from non-swollen astrocytes when they are stimulated with ATP.
- These findings suggest that LRRC8A may play a role in physiological release of gliotransmitters, and mediate pathological glutamate release in the CNS disorders associated with cellular swelling.

Abstract In mammals, cellular swelling activates release of small organic osmolytes, including the excitatory amino acids (EAA) glutamate and aspartate, via a ubiquitously expressed volume-regulated chloride/anion channel (VRAC). Pharmacological evidence suggests that VRAC plays plural physiological and pathological roles, including excitotoxic release of glutamate in stroke. However, the molecular identity of this pathway was unknown. Two recent studies discovered that *LRRC8* gene family members encode heteromeric VRAC composed of LRRC8A plus LRRC8B-E, which mediate swelling-activated Cl⁻ currents and taurine release in human non-neural cells (Z. Qiu *et al. Cell* 157: 447, 2014; F.K. Voss *et al. Science* 344: 634, 2014). Here, we tested the contribution of LRRC8A to the EAA release in brain glia. We detected and quantified expression levels of *LRRC8A-E* in primary rat astrocytes with quantitative RT-PCR and then downregulated LRRC8A with gene-specific siRNAs. In astrocytes exposed to hypo-osmotic media, LRRC8A knockdown dramatically reduced swelling-activated release of the EAA tracer D-[³H]aspartate. In parallel HPLC assays, LRRC8A siRNA prevented hypo-osmotic media-induced loss of the endogenous intracellular L-glutamate and taurine. Furthermore, downregulation of LRRC8A completely ablated the ATP-stimulated release of D-[³H]aspartate and [¹⁴C]taurine from non-swollen astrocytes. Overall, these data indicate that LRRC8A is an indispensable component of a permeability pathway that mediates both swelling-activated and agonist-induced amino acid release in brain glial cells.

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Abbreviations CNS, central nervous system; DCPIB, 4-[(2-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1*H*-inden-5-yl)oxy]butanoic acid; EAA, excitatory amino acids; LRRC8, leucine-rich repeat containing protein 8; VRAC, volume-regulated anion channel.

Introduction

Animal cells endure constant changes in their volume due to alterations in osmolarity of the extracellular and intracellular milieu, or changes in the transmembrane transport of osmotically active molecules (osmolytes). The majority of cell types, including those in the CNS, homeostatically regulate their volume by releasing or accumulating osmolytes via plasmalemmal channels and transporters (reviewed in Lang *et al.* 1998; Hoffmann *et al.* 2009). In response to swelling, cell volume is typically adjusted with a net loss of K⁺ ions via K⁺ channels and simultaneous release of negatively charged molecules through the ubiquitous volume-regulated anion channel (VRAC) (Lang *et al.* 1998; Hoffmann *et al.* 2009). In addition to conducting Cl⁻ and other inorganic anions, VRAC is permeable to small organic molecules, including the excitatory amino acids (EAA), glutamate and aspartate, but also taurine, inositol and others (Kimmelberg *et al.* 1990; Banderali & Roy, 1992; Manolopoulos *et al.* 1997).

VRAC, also known as volume-sensitive organic osmolyte–anion channel or volume-sensitive outwardly rectifying Cl⁻ channel can be readily detected in virtually every vertebrate cell type (reviewed in Strange *et al.* 1996; Nilius *et al.* 1997; Okada, 1997). Yet, the molecular identity of this channel remained an enigma for nearly three decades. Recently, two studies have identified the leucine-rich repeat containing protein 8A (LRRC8A) as an essential component of VRAC, and four other members of the same family (LRRC8B–E) as complementary VRAC subunits in non-neural cells (Qiu *et al.* 2014; Voss *et al.* 2014).

Besides its fundamental role in cell volume regulation, VRAC is thought to be involved in numerous other physiological processes, including proliferation, apoptosis, cell migration and release of biologically active molecules (Lang *et al.* 1998; Okada *et al.* 2001; Hoffmann *et al.* 2009). In the CNS, VRAC activity is probably important for systemic osmosensing and for intercellular signalling via release of physiologically active compounds (Deleuze *et al.* 1998; Mongin & Kimmelberg, 2002). Moreover, this channel is thought to play a major pathological role in brain disorders that are associated with cellular swelling (reviewed in Mongin & Kimmelberg, 2005a). In stroke, hyponatraemia and epilepsy, VRAC is hypothesized to mediate release of EAA from swollen astrocytes and in this way contribute to the excitotoxic damage to neurons. In rodent models of stroke, the VRAC blockers tamoxifen and 4-[(2-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1*H*-inden-5-yl)oxy]butanoic acid (DCPIB), reduce ischaemic brain damage by as much as 80% (Kimmelberg *et al.* 2000; Zhang *et al.* 2008).

Although pharmacological evidence for VRAC involvement in stroke pathogenesis is thought to be

fairly conclusive, recent studies established that VRAC blockers also interfere with other pathologically relevant glutamate transporters (Ye *et al.* 2009; Bowens *et al.* 2013). Furthermore, several previous studies suggested that swelling-activated release of organic molecules, including EAA, may be mediated by a separate pathway (Lambert & Hoffmann, 1994; Mongin *et al.* 1999; Stutzin *et al.* 1999). The discovery that VRAC is comprised of LRRC8 gene family members in non-CNS cells opened up an opportunity to test whether the same proteins are also responsible for EAA release in the CNS.

Methods

Ethics statement

All animal procedures in this study were approved by the Institutional Animal Care and Use Committee of the Albany Medical College, and conformed to the NIH Guidelines for Care and Use of Laboratory Animals.

Astrocyte culture

Primary astrocytes were prepared from cortices of neonatal Sprague–Dawley rats as recently described (Hyzinski-García *et al.* 2011). Briefly, postnatal day 0–1 pups were killed by rapid decapitation. Aseptically isolated cortical tissue was dissociated with recombinant protease TrypLE. The resulting cell suspension was plated at low density into T75 flasks and grown to confluency in minimal essential medium containing 10% heat inactivated horse serum and antibiotics (all from Life Technologies/Invitrogen, Carlsbad, CA, USA). The purity of astrocyte cultures was ≥98% as routinely checked with the anti-GFAP antibody (St. Louis, MO, USA, Sigma-Aldrich).

Gene expression analysis

Expression levels of the LRRC8 family members were measured by quantitative RT-PCR. mRNA specimens were isolated from cells grown on 60 mm dishes using RNAqueous-4PCR kit (Life Technologies/Ambion, Austin, TX, USA) and converted to cDNA with iScript cDNA synthesis kit (BioRad, Hercules, CA, USA). The expression levels were determined by quantitative PCR using a CFX96 Real Time PCR setup (BioRad) and quantitative primers from Qiagen (Valencia, CA, USA), which are listed in Table 1. The expression levels were normalized within each sample to the transcripts of three housekeeping genes.

siRNA transfections

Gene expression knockdowns were performed using commercially available gene-specific siRNA constructs

Table 1. List of qPCR primers and siRNA constructs

Target gene	qPCR primer cat. no.	siRNAs name/cat. no. (manufacturer)
LRRC8A	QT01575483	siLRRC8A_1/SI01725339 (Q)
	QT00390978	siLRRC8A_3/SI01725353 (Q)
		siLRRC8A_4/SI01725360 (Q)
		siLRRC8A_mix/J090547-02-0005 (GE)
LRRC8B	QT00434805	N/A
LRRC8C	QT01583897	N/A
LRRC8D	QT00370111	N/A
LRRC8E	QT01591352	N/A
GAPDH	QT00199633	N/A
RPL13a	QT00178675	N/A
RPS20	QT00431333	N/A

Abbreviation: N/A, not applicable. All primers for qPCR were from Qiagen. siRNA constructs were from either Qiagen (Q) or GE Health Care/Dharmacon (GE).

listed in Table 1. Cells grown to ~50–70% confluency were transfected with one or several siRNAs and Lipofectamine[®] RNAiMAX reagent as previously optimized for primary astrocytes (Bowens *et al.* 2013). As a negative control we used Alexa-488-labelled Allstars scrambled siRNA from Qiagen. After 4–8 h of incubation with siRNA/Lipofectamine complexes, transfection media were replaced with fresh culture media. This transfection technique produces negligible cell toxicity. Changes in the mRNA expression levels were quantified at 48 h post-transfection, and all functional assays were performed at 72 h post-transfection. Based on the Alexa-488 fluorescence analysis in negative controls, the efficacy of transfections routinely reached 95%.

Amino acid release assays

Glutamate transport was traced using a non-metabolizable glutamate analogue, D-[³H]aspartate, and [¹⁴C]taurine, as previously described (Hyzinski-Garcia *et al.* 2011; Bowens *et al.* 2013). Cells grown on either 18 × 18 mm coverslips or in 12-well plates were preloaded in serum-containing medium with radiolabelled amino acids overnight. For the majority of assays, we used a Lucite perfusion chamber, in which cells were superfused with Hepes-buffered media at a rate of 1.2 ml min⁻¹. Basal iso-osmotic medium contained (in mM): 135 NaCl, 3.8 KCl, 1.2 MgSO₄, 1.3 CaCl₂, 1.2, KH₂PO₄, 10 D-glucose, 10 Hepes (pH 7.4, osmolarity ≈ 290 mosmol l⁻¹). To activate the VRAC pathway, cells were exposed to hypo-osmotic medium, in which [NaCl] was reduced by 50 mM, while all other components remained the same (osmolarity ≈ 200 mosmol l⁻¹). One minute superfusate fractions were collected and analysed for the presence of released D-[³H]aspartate and [¹⁴C]taurine in a

liquid scintillation counter TriCarb 2900TR (Perkin Elmer, Waltham, MA, USA). To increase the throughput, some of the experiments were done in a multiwell plate format, in which media in individual wells were changed once every 10 min and the integral D-[³H]aspartate release was compared between basal iso-osmotic conditions and during exposure to hypo-osmotic medium.

HPLC analysis of intracellular amino acid content

Endogenous amino acid contents were determined using a reverse-phase HPLC as described in detail elsewhere (Hyzinski-Garcia *et al.* 2011). Briefly, cells were lysed in medium containing 5 mM Hepes and 1 mM EDTA (pH 7.4), and amino acid levels were quantified after derivatization with *o*-phthalaldehyde and 2-mercaptoethanol using an Agilent 1200 HPLC setup (Agilent Technologies, Santa Clara, CA, USA) and Eclipse XBD-C18 column. Calculated concentrations were further normalized to protein content in each individual well.

Statistical analysis

All data are presented as means ± S.E.M. Statistical significance of differences between experimental groups was determined using one-way or two-way ANOVA, as appropriate, and *post hoc* corrections for multiple comparisons. When several experimental groups were normalized to controls, we used Student's one-sample *t* test with Bonferroni correction for multiple comparisons. Origin 8.1 (OriginLab, Northampton, MA, USA) and Prism 5.0 (GraphPad Software, San Diego, CA, USA) were used in all statistical analyses.

Results

LRRC8 family expression levels in primary rat astrocytes

We first quantified mRNA expression levels for all five members of the LRRC8 family. The expression was normalized within each sample to three housekeeping genes, GAPDH, RPL13a and RPS20, with highly reproducible results regardless of using one or several genes for normalization. As shown in Fig. 1, mRNA levels for LRRC8A, which forms the indispensable VRAC subunit in human non-CNS cells (Qiu *et al.* 2014), were ~0.2% of the levels of the highly abundant glycolytic enzyme GAPDH. Three other LRRC8 species, LRRC8B, LRRC8C and LRRC8D showed approximately 2-fold higher abundance as compared to LRRC8A, while LRRC8E levels were ~10-fold lower. There was very little variation in the mRNA expression levels between three independently tested astrocyte cultures.

LRRC8A siRNA knockdown suppresses hypo-osmotic medium-induced amino acid release and preserves intracellular amino acid content

We next knocked down astrocytic LRRC8A using several siRNA constructs. As seen in Fig. 2A, the most effective downregulation ($\geq 80\%$) was seen in cells treated with either the LRRC8A siRNA mix (siLRRC8A_mix; GE Health Care/Dharmacon, Lafayette, CO, USA), or the siRNA construct siLRRC8A_4. The subsequent screening in a multiwell format established that siLRRC8A_4 and siLRRC8A_mix reduced swelling-activated D-[³H]aspartate release by 65% ($P < 0.001$) and 73% ($P < 0.001$), respectively (Fig. 2B). Consistent with the mRNA expression data, siLRRC8A_1 and siLRRC8A_3 also caused reductions in the amino acid efflux, but with an intermediate efficacy (Fig. 2B). We then looked at the kinetics of EAA release in the siLRRC8A_mix-treated cells. As seen in Fig. 3A, the LRRC8A protein knockdown decreased the maximum rate of D-[³H]aspartate efflux under hypo-osmotic condition by 78% ($P < 0.001$) as compared to negative control siRNA, and the integral 10 min release values by 68% ($P = 0.002$). To assure that hypo-osmotic medium stimulated EAA release was due to changes in medium osmolarity (cell swelling) rather than reduction in the extracellular [NaCl], we performed additional controls in which NaCl was replaced with equiosmolar mannitol. In these latter experiments no changes in D-[³H]aspartate release were found as compared to iso-osmotic baseline (data not shown, $n = 4$, $P < 0.001$ hypo-osmotic vs. low [NaCl] medium). Interestingly, in cells treated with siLRRC8A_mix we additionally found significant

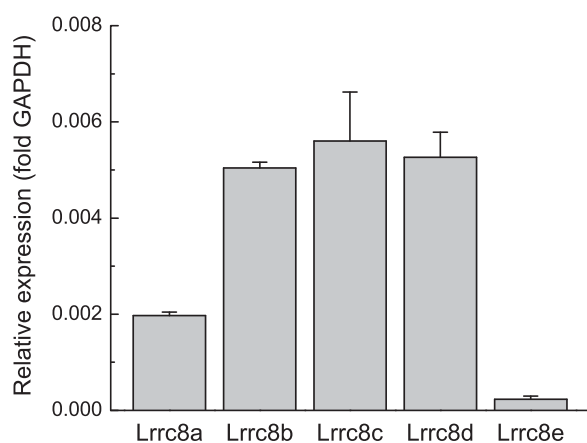


Figure 1. mRNA expression levels for five members of the LRRC8 protein family in primary rat astrocytes

mRNA expression levels for LRRC8A-E were detected using quantitative RT-PCR and normalized to GAPDH levels within the same samples. Similar normalization was performed for RPL13a and RPS20 (not shown). Data are the means \pm S.E.M. for three independently prepared astrocytic cultures.

decrease in the basal EAA release rates ($P = 0.03$; Fig. 3A), suggesting that VRAC is at least partially responsible for amino acid 'leak' from non-swollen cells.

To validate and extend these results using an independent approach, we also measured the effect of RNAi on intracellular content of endogenous amino acids using an HPLC analysis. For brevity, we present the data for two major cytosolic osmolytes, L-glutamate and taurine, only. In untransfected cells, exposure to hypo-osmotic medium reduced the intracellular taurine content by 50% (Fig. 3B; $P = 0.015$), and very similar 46% reduction

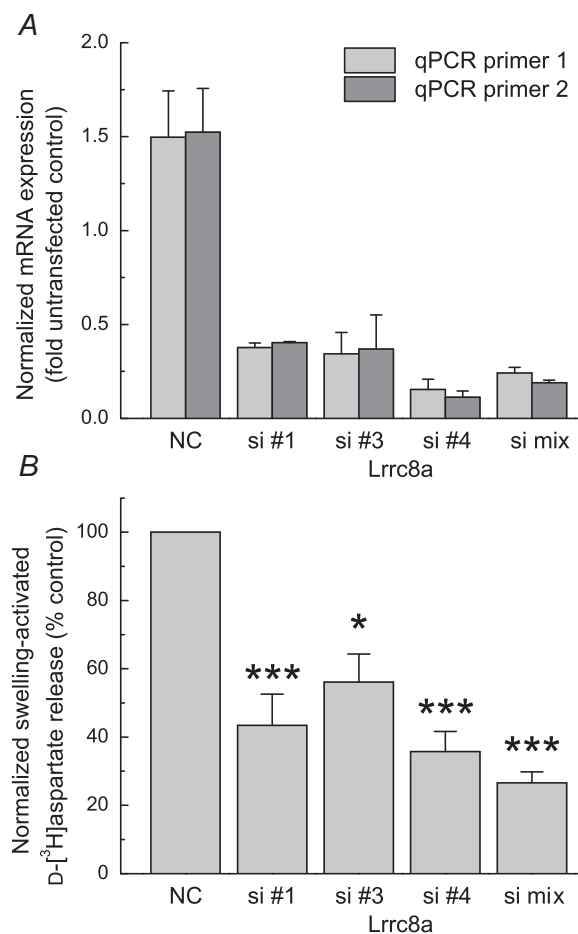


Figure 2. Effect of LRRC8A knockdown on astrocytic mRNA expression levels and swelling-activated glutamate (D-[³H]aspartate) release

A, quantification of the LRRC8A mRNA expression levels in cells treated with three individual LRRC8A siRNAs (Qiagen), or the LRRC8A siRNA mix (GE Health Care/Dharmacon). Data are the means \pm S.E.M. of six independent qRT-PCR assays in two independently prepared astrocyte cultures. Expression levels were normalized to mRNA levels in non-transfected cells in the same experiment. B, multiwell assays of the swelling-activated D-[³H]aspartate release from cells treated with LRRC8A siRNAs or the siRNA mix. Data are the means \pm S.E.M. of six independent measurements in two astrocyte cultures. * $P < 0.05$, *** $P < 0.001$ vs. NC siRNA-treated cells (Student's one population t test after Bonferroni correction). NC, negative control.

was found in cells transfected with the negative control siRNA (Fig. 3B; $P = 0.002$). These results resemble previously published findings (Hydzinski-Garcia *et al.* 2011 and references therein). LRRC8A knockdown largely prevented this effect: only a small 12% reduction in taurine content was found in swollen cells ($P = 0.035$; Fig. 3B). In a very similar fashion, the LRRC8A knockdown prevented loss of the endogenous L-glutamate from the

hypo-osmotically swollen cells (Fig. 3B). It is important to note that decreases in the intracellular amino acid content are determined exclusively by changes in medium osmolarity (cellular swelling). In our previous study (Hydzinski-Garcia *et al.* 2011) iso-osmotic replacement of NaCl with mannitol produced no effect on endogenous taurine and L-glutamate levels.

LRRC8A is critical for the agonist-stimulated excitatory amino acid release

There is a significant interest in the agonist-induced astrocytic glutamate release in non-swollen cells due to its proposed role in astrocyte-to-neuron communication (reviewed in Haydon & Carmignoto, 2006). Therefore, we also tested if the LRRC8A function is important for the ATP-stimulated amino acid release in cultured glia. To this end, we exposed astrocytes to $50 \mu\text{M}$ of ATP, and measured the release of the glutamate analogue D-[^3H]aspartate, and simultaneously the release of another major osmolyte [^{14}C]taurine. Numerous previous studies demonstrated that purinergic signalling transiently stimulates release of several small organic osmolytes, including glutamate, aspartate and taurine, in non-swollen glial cells and in cells subjected to physiological levels of swelling (see for example Mongin & Kimelberg, 2002; Cheema *et al.* 2005). In cells transfected with the negative control siRNA, ATP triggered a transient ~ 2.5 -fold increase in the release of D-[^3H]aspartate (Fig. 4A) and the same phenomenon was observed for the concomitant [^{14}C]taurine efflux (Fig. 4B). Previous studies established that the effect of ATP is mediated by activation of metabotropic P2Y₁ receptor with additional contribution of the UTP/UDP-sensitive P2Y_{2/4/6} (Mongin & Kimelberg, 2002). Importantly, transfection with the siLRRC8A_4 completely abolished the ATP-induced efflux of both amino acids ($P = 0.002$ and $P < 0.001$ for D-[^3H]aspartate and [^{14}C]taurine, respectively), and showed a trend for reduction in their basal release levels ($P = 0.09$ and $P = 0.02$ for D-[^3H]aspartate and [^{14}C]taurine, respectively).

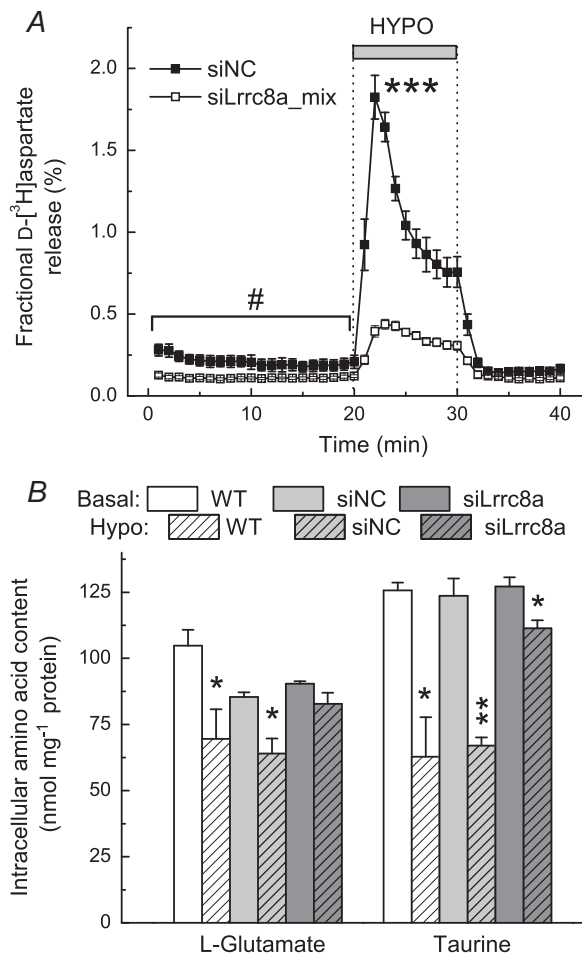


Figure 3. Effect of LRRC8A knockdown on kinetics of glutamate (D-[^3H]aspartate) release and the intracellular levels of L-glutamate and taurine in astrocyte exposed to hypo-osmotic medium

A, kinetics of preloaded D-[^3H]aspartate release from primary rat astrocytes exposed to 30% reduction in medium osmolarity measured 72 h after treatment with either siNC or siLRRC8A_mix. Data are the means \pm S.E.M. of five to six independent experiments performed in two different cell cultures. *** $P < 0.001$, maximal release rates in siNC vs. siLRRC8A_mix (Student's paired t test). # $P < 0.05$, basal release values in siNC vs. siLRRC8A_mix (two-way ANOVA). B, HPLC analysis of the endogenous L-glutamate and taurine levels in untransfected cells (WT) and cells treated with siNC, or the siLRRC8A_mix. Data are the means \pm S.E.M. for three different cell cultures. * $P < 0.05$; ** $P < 0.01$, hypo-osmotic vs. iso-osmotic conditions for the same treatment (Student's paired t test). hypo, hypo-osmotic; siNC, negative control siRNA; WT, wild-type.

Discussion

The critical finding of this work is that LRRC8A is an essential component of VRAC in the CNS, and plays an indispensable role in swelling-activated and the agonist-induced EAA release from rat glial cells. This discovery has strong physiological and pathological implications for the CNS as outlined below.

The idea that VRAC plays an indispensable role in swelling-activated release of organic osmolytes in intact cells and *in situ* has a long history, but it has never been decisively tested because of unknown molecular identity of VRAC channel. This concept was developed based on

strong pharmacological similarity between VRAC currents recorded in electrophysiological experiments, radiotracer release of $^{36}\text{Cl}^-$ and $^{125}\text{I}^-$, and efflux of ^3H -labelled small organic osmolytes, including EAA, across several cell types (Jackson & Strange, 1993; Manolopoulos *et al.* 1997; Abdullaev *et al.* 2006; reviewed in Strange *et al.* 1996; Nilius *et al.* 1997). Additionally, several laboratories directly demonstrated electrophysiological VRAC currents carried by intracellular L-aspartate, L-glutamate or taurine (Banderali & Roy, 1992; Jackson & Strange, 1993; Manolopoulos *et al.* 1997). Nevertheless, the picture remained far from clear, because other studies identified substantial differences in either pharmacology or regulation of the

swelling-activated transmembrane transport of inorganic anions, D- ^3H aspartate, and ^3H taurine (Lambert & Hoffmann, 1994; Mongin *et al.* 1999; Stutzin *et al.* 1999). This led to the hypothesis that multiple swelling-activated pathways for inorganic anions and organic molecules exist in mammalian cells (reviewed in Hoffmann *et al.* 2009). Our study resolves this controversy and provides proof that, at least in primary glial cells, the LRRC8A protein is an indispensable component of the pathway for swelling-activated EAA release. This is consistent with the findings of two recent studies that uncovered the obligatory role for the LRRC8A for swelling-activated VRAC Cl^- currents and taurine release in non-CNS cells (Qiu *et al.* 2014; Voss *et al.* 2014).

In the CNS, VRAC is frequently discussed in the context of brain pathology because of the early discovery that swelling of primary astrocytes triggers substantial release of the excitotoxic glutamate via a VRAC-like pathway (Kimelberg *et al.* 1990). This latter finding resonated with numerous independent *in vivo* observations of astrocytic swelling in traumatic brain injury, stroke, hyponatraemia and other pathologies (reviewed in Mongin & Kimelberg, 2005a). Systemic application of the poorly selective VRAC blocker, tamoxifen or intracerebroventricular injection of the more selective inhibitor, DCPIB, dramatically reduces brain damage in rodent models of stroke (Kimelberg *et al.* 2000; Zhang *et al.* 2008). Furthermore, several groups found that structurally unrelated VRAC blockers, including DCPIB, strongly diminish pathological EAA release during cerebral ischaemia (Phillis *et al.* 1997; Feustel *et al.* 2004; Zhang *et al.* 2008). Even though the evidence for VRAC involvement in stroke appears to be quite conclusive, there are inherent problems associated with a strictly pharmacological analysis. VRAC blockers poorly discriminate between several transport pathways that have been suggested to contributing to glutamate release in stroke (Malarkey & Parpura, 2008; Ye *et al.* 2009; Bowens *et al.* 2013). Our current findings open an opportunity to use the RNAi-based tools and gene knockout animals for directly testing VRAC involvement in stroke and other neural disorders, and for further development of more refined pharmacological agents.

An additional point of interest is the potential contribution of VRAC to the physiological glutamate release in glia. In the past two decades, our views on physiological functions of astrocytes have undergone a dramatic transformation. These cells are capable of modulating activity of individual neurons and neuronal networks via release of glutamate and other physiologically active 'gliotransmitters' (see for example Parpura *et al.* 1994; Newman, 2003, and review of Haydon & Carmignoto, 2006). Such gliotransmitters are commonly assumed to be released via a Ca^{2+} -dependent vesicular mechanism (Haydon & Carmignoto, 2006) but alternative hypotheses have also been proposed, including contribution of VRAC

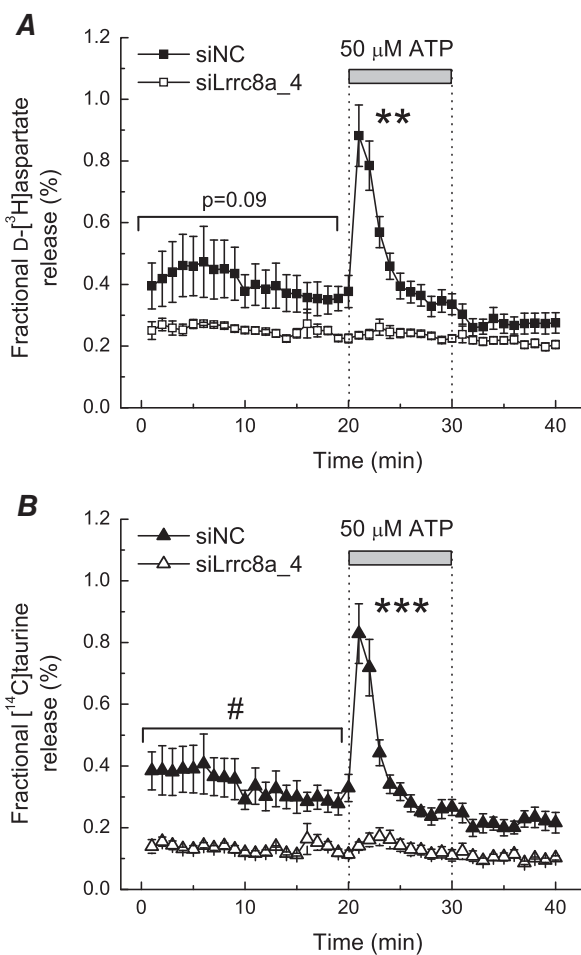


Figure 4. Effect of LRRC8A knockdown on the ATP induced of D- ^3H aspartate and ^{14}C taurine release from astrocytes

A, primary rat astrocytes were loaded with radiolabelled D-aspartate and taurine, and simultaneous D- ^3H aspartate (A) and ^{14}C taurine (B) efflux rates were measured before and after addition of 50 μM ATP in cells treated with either siNC, or the LRRC8A_4 siRNA (siLRRC8A_4). Data are the means \pm s.e.m. for five experiments performed in two different cell cultures. *** $P < 0.01$, siNC vs. siLRRC8A_4 under hypo-osmotic conditions; # $P < 0.05$, siNC vs. siLRRC8A_4 during the first 20 min of exposure to basal medium (two-way ANOVA). siNC, negative control siRNA.

(reviewed in Hamilton & Attwell, 2010). Although VRAC has no direct Ca^{2+} dependency, the activity of this channel is potently modulated by a variety of GPCR-coupled receptors and downstream signalling cascades involving Ca^{2+} -dependent protein kinases (see for example Mongin & Kimelberg, 2002, 2005b; and review of Fisher *et al.* 2008). Because of the limited selectivity of pharmacological tools and the unknown molecular identity, the contribution of VRAC to physiological gliotransmitter release has been difficult to corroborate or rule out, with the notable exception of the physiological release of taurine in the hypothalamus (Deleuze *et al.* 1998). In our study, the LRRC8A knockdown completely eliminated the ATP-induced release of preloaded D- ^3H aspartate and ^{14}C taurine, indicating the critical involvement of VRAC in the agonist-induced release of EAA and other cytosolic gliotransmitters. This finding opens a door for testing the relative contribution of the LRRC8A and other members of the same family to bidirectional astrocyte–neuron communication using an RNAi approach.

Based on the highly conserved nature of VRAC and strong similarity of VRAC currents among various cell types and *in situ*, it is possible that composition of astrocytic VRAC *in vivo* will be similar to that discovered in cultured cells. However, the definitive physiological and pathological contributions of the LRRC8A and other members of this new protein family will have to be further determined *in vivo*.

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Additional information

Competing interests

The authors declare no competing financial interests.

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