Ionotropic NMDA and P2X₁/₅ receptors mediate synaptically induced Ca²⁺ signalling in cortical astrocytes

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1. Introduction

Twenty years has passed since the discovery of propagating Ca²⁺ waves in astroglial cultures [1,2]. This seminal observation together with an in depth characterisation of multiple pathways triggering glial Ca²⁺ signalling led to a concept of glial Ca²⁺ excitability [3,4] that regarded local, global and propagating Ca²⁺ signals as a substrate for information processing in astroglial syncytia. It is generally acknowledged [5,6] that in physiological conditions glial Ca²⁺ signalling primarily results from the activation of metabotropic G-protein-coupled receptors, which stimulate phospholipase C, production of inositol-1,4,5-trisphosphate (InsP₃) and opening of InsP₃ receptors/Ca²⁺ channels residing in the endomembrane that forms the endoplasmic reticulum. Indeed astrocytes in vitro, in situ and in vivo express multiple types of metabotropic receptors linked to a generation of cytosolic Ca²⁺ signals [7–16].

The role for ionotropic receptors in generating astroglial Ca²⁺ signals is much less appreciated despite the widespread expression of these receptors in astrocytes from different brain regions [17–20]. The ionotropic receptors can be activated by neurotransmitters released during on-going neuronal activity [21]. Activation of ionotropic receptors can trigger rapid Ca²⁺ influx either directly (by Ca²⁺ permeation through the receptor pore) or indirectly (by activating other Ca²⁺ permeable channels or affecting Ca²⁺ transporters/exchangers). Many ionotropic receptors expressed in the brain (for example glutamate receptors, nicotinic cholinoreceptors and purinoceptors) allow Ca²⁺ fluxes [22–31], the NMDA receptors being arguably the most Ca²⁺ permeable [32]. Cortical astrocytes express AMPA and NMDA glutamate receptors and P2X₁/₅ purinoceptors [21,33]. The astroglial NMDA receptors are of a peculiar type (molecular identity of which remains unknown) being insensitive to extracellular Mg²⁺-block [21]. Similarly, the P2X₁/₅ receptors can be glia-specific because this type of heteromeric expression has not been observed in other neural cells. Both NMDA and P2X₁/₅ receptors are highly sensitive to their natural agonists (glutamate and ATP) and mediate slowly desensitising currents that can generate long-lasting ion fluxes. Furthermore, both receptors are potentially Ca²⁺ permeable (although their Ca²⁺ permeability has not been characterised in astrocytes) and may generate Ca²⁺ entry.

Here we present in depth analysis of Ca²⁺ permeability of astroglial NMDA and P2X₁/₅ receptors and show that their activation can increase intracellular free Ca²⁺ concentration ([Ca²⁺]ᵢ) when stimulated by both endogenous agonists and synaptic release of neurotransmitters.
2. Materials and methods

2.1. Cortical slice preparation

Experiments were performed on somato-sensory cortex isolated from the brains of transgenic mice expressing enhanced green fluorescent protein (EGFP) under the control of the human glial fibrillary acidic protein (GFAP) promoter (line TgN(GFAP-EGFP);GFE-C-Fki; see Refs. [21,34–36]). Mice (3 months old) were anaesthetized by halothane and then decapitated, in accordance with UK legislation. Slices were prepared using the technique described previously [21]. Brains were rapidly dissected and placed in physiological saline containing (in mM): 135 NaCl, 3 KCl, 1 MgCl₂, 2.4 CaCl₂, 26 NaHCO₃, 1 NaH₂PO₄, 15 glucose, pH 7.4 when gassed with 95% O₂/5%CO₂. Cortical slices (280–300 μm thick) were cut at 4 °C and kept at room temperature for 1–2 h prior to the cell isolation.

2.2. Acute isolation of astrocytes

Astrocytes were acutely isolated using the modified “vibrating ball” technique [21,24,25]. The glass ball (200 μm diameter) was moved slowly some 10–50 μm above the slice surface, while vibrat- ing at 100 Hz (lateral displacements 20–30 μm). The composition of external solution for isolated cell experiments was (mM): 135 NaCl, 2.7 KCl, 2.5 CaCl₂, 1 MgCl₂, 10 HEPES, 1 NaH₂PO₄, 15 glucose, pH adjusted with NaOH to 7.3. The voltage-clamp recordings commenced ~20 min after the isolation.

2.3. Identification of astrocytes

Astrocytes were identified by EGFP fluorescence. For this purpose cells were illuminated at 488 nm and observed at 520 ± 20 nm. Visually identified cells demonstrated electrophysiological signature characteristic of astrocytes (e.g. low input resistance, lack of fast time-dependent conductances, see Ref. [21] for details).

2.4. Electrophysiological recordings

Whole-cell voltage-clamp recordings from isolated astrocytes and astrocytes in situ were made with patch pipettes (5–6 MΩ) filled with intracellular solution (in mM): 110 KCl, 10 NaCl, 10 HEPES, 5 MgATP, 0.2 EGTA, pH 7.35. The membrane potential was clamped at −80 mV unless stated otherwise. Currents were monitored using an Axopatch200B patch-clamp amplifier (Axon Instruments, USA) filtered at 2 kHz and digitized at 4 kHz. Experiments were controlled by PCI-6229 data acquisition board (National Instruments, USA) and WinFluor software (Strathclyde Electrophysiology Software, Glasgow, UK). The [Ca²⁺]i levels were expressed as F₃₄₀/F₃₈₀ ratio after background subtraction. To quantify the average amplitude of Ca²⁺-transients in different age groups, the F₃₄₀/F₃₈₀ ratios were averaged over the whole cell image over two 1 s-long time windows, in the rest (immediately before stimulus) and at the peak of transient; after then the resting F₃₄₀/F₃₈₀ value was subtracted from the peak value.

2.5. In situ recordings

Whole-cell voltage-clamp recordings were made from identified astrocytes situated in the layer II of somato-sensory cortex. All recordings shown were made at temperature of 30–32 °C in the presence of 100 μM picrotoxin, membrane potential was kept at −80 mV unless specifically indicated. Axons originating from layer IV–VI neurones were stimulated at 0.25–0.5 Hz with a bipolar coaxial electrode (WPI, USA) placed in the layer IV approximately opposite the site of recording; stimulus duration was 300 μs. The stimulus magnitude was set 3–4 times higher (typically 2.5–5 μA) than minimal stimulus adjusted to activate the single-axon response in the layer II pyramidal neurones as described in Ref. [38]. The currents evoked in the layer II astrocytes exhibited large amplitude fluctuations, their average amplitude reached 10–20% of maximal current evoked by saturating stimulus (30–40 μA); no noticeable astroglial response was recorded at stimulus intensity below minimal (i.e. threshold intensity for neuronal EPSCs).

2.6. Fluorescent Ca²⁺-imaging

To monitor the cytoplasmic free Ca²⁺ concentration ([Ca²⁺]i), cortical astrocytes were loaded with 100 μM Fura-2 (Ca²⁺ probe equimolarly substituting EGTA in the intracellular saline) through the patch pipette in whole-cell configuration. For Fura-2 excitation, cells were alternately illuminated at wavelengths 340 ± 5 nm and 380 ± 5 nm using OptoScan monochromator (Cairn, Faversham, UK). Fluorescent images were recorded using Olympus BX51 microscope equipped with UPLFL20×/NA0.95 objective and 2 × intermediate magnification and Andor iXon885 EMCCD camera; exposure time was 35 ms at 2 × 2 binning. Astrocyte whole-cell currents were recorded simultaneously; experiments were controlled by WinFluor software (Strathclyde Electrophysiology Software, Glasgow, UK). The [Ca²⁺]i levels were expressed as F₃₄₀/F₃₈₀ ratio after background subtraction. To quantify the average amplitude of Ca²⁺-transients in different age groups, the F₃₄₀/F₃₈₀ ratios were averaged over the whole cell image over two 1 s-long time windows, in the rest (immediately before stimulus) and at the peak of transient; after then the resting F₃₄₀/F₃₈₀ value was subtracted from the peak value.

2.7. Data analysis

All data are presented as mean ± SD, the statistical significance of difference between age groups was tested by one-way ANOVA test, unless indicated otherwise.

2.8. Drugs

Receptor antagonists were from Tocris (Bristol, UK), Other salts and chemicals were from Sigma (Dorset, UK) unless specifically indicated.

3. Results

3.1. Calcium permeability of NMDA and P2X₁/5 receptors in cortical astrocytes

To determine Ca²⁺ permeability of NMDA/P2X receptors we measured the shift in the reversal potential of agonist-evoked currents recorded from acutely isolated astrocytes at two concentration of extracellular Ca²⁺ ([Ca²⁺]₀; 2.5 and 10 mM). The permeability ratios were calculated using the extended Goldman–Hodgkin–Katz (GHK) theory as described previously [23,24,39].

(i) NMDA receptors: Application of 30 μM NMDA at a holding potential ~80 mV (in 1 mM extracellular Mg²⁺) induced inward currents in all 21 astrocytes tested (Fig. 1A). Functional properties of NMDA-activated currents (slow desensitisation kinetics, high affinity to glutamate, sensitivity to D-AP5 and lack of


**Fig. 1.** Calcium permeability of astroglial ionotropic receptors. (A and B) I–V curves and examples of current recordings (insets) in response to stimulation with (A) NMDA (30 μM) and (B) ATP (10 μM) at 2.5 and 10 mM [Ca²⁺]o. Increase in [Ca²⁺]o shifts the reversal potentials for both NMDA- and ATP-induced currents indicating their Ca²⁺ permeability. The I–V curves were constructed from 8 (NMDA) and 7 (P2X1/5) independent experiments. The amplitudes of responses were normalized to the value measured at −40 mV, data are presented as mean ± SD. Solid lines show the results of the best polynomial fit (least squares routine). Intersection with zero current axis gives the following values of reversal potential in 2.5 and 10 mM [Ca²⁺]o: 1.9 and 5.1 mV for NMDA-evoked currents and 4.6 mV and 6.7 mV for ATP-evoked currents. The permeability ratio Pca/Pk calculated by extended Goldman–Hodgkin–Katz equation is 3.1 for NMDA receptors and 2.2 for P2X receptors.

Mg²⁺-block) were similar to that reported previously for cortical astrocytes [21]. Although subunit composition of glial NMDA receptors is yet to be studied, their distinct biophysical properties suggest participation of subunits different from NR2A and NR2B [21,40].

The reversal potential of NMDA-evoked current was 1.89 ± 0.98 mV (n = 8) at [Ca²⁺]o 2.5 mM; the reversal potential shifted to 5.1 ± 1.2 mV (n = 8) when [Ca²⁺]o was elevated to 10 mM (Fig. 1B). The permeability ratios Pca/Pk and Pna/Pk calculated from the reversal potentials of NMDA-mediated current were correspondingly 3.1 and 0.89. The slightly lower permeability to Na⁺ compare to K⁺ was reported for cloned NMDA receptors [41].

(ii) P2X1/5 receptors: Application of 10 μM ATP to astrocytes voltage-clamped at −80 mV evoked inward currents in 19 of 20 tested cells. As illustrated in Fig. 1B, ATP induced typical P2X1/5-mediated currents with rapid peak and slowly decaying plateau phase (cf. [33]). The reversal potential of ATP-induced currents sequentially exposed to 2.5 and 10 mM [Ca²⁺]o-containing extracellular solution (Fig. 1B) was 4.6 ± 0.6 mV and 6.7 ± 0.9 mV (n = 7) respectively. Relative permeability of P2X1/5 receptors, calculated from the reversal potential of ATP-evoked currents according the extended GHK theory was: Pca/Pk = 2.2 and Pna/Pk = 0.99.

3.2. **Specific stimulation of NMDA/P2X1/5 Receptors triggers Ca²⁺ signal in individual astrocytes**

(i) NMDA receptors: Glutamate applied to isolated astrocytes at 100 μM induced membrane current response and [Ca²⁺]i transients in all cells tested (n = 13). Selective stimulation of ionotropic responses by application of 30 μM NMDA to isolated astrocytes voltage-clamped at −80 mV also triggered inwards current and caused significant elevation in the intracellular Ca²⁺ concentration (n = 13; Fig. 2A). The amplitude of NMDA-receptor-mediated [Ca²⁺]i elevation was 43 ± 15% of the amplitude of Ca²⁺-transient activated by glutamate. Exposure of astrocytes to 30 μM AMPA triggered rapidly desensitising inward currents but did not significantly affect [Ca²⁺]i (n = 5, data not shown).

(ii) P2X1/5 receptors: Application of broad agonist of P2X and metabotropic P2Y receptors ATP (100 μM) to cortical astrocytes (n = 12) induced membrane currents and cytosolic Ca²⁺ signals. To visualise P2X1/5-mediated changes in [Ca²⁺]i we applied selective P2X agonist, α,β-methyleneATP (α,β-meATP, 10 μM), to isolated cortical astrocytes (Fig. 2B) while simultaneously monitoring membrane currents and [Ca²⁺]i dynamics. Application of α,β-meATP induced both transmembrane inward currents and transient elevation of [Ca²⁺]i in all 12 astrocytes tested. Peak amplitude of α,β-meATP-induced [Ca²⁺]i increase was 48 ± 13% (n = 12) of the peak amplitude of ATP-induced [Ca²⁺]i transient.

3.3. **NMDA and P2X1/5 receptors contribute to astroglial Ca²⁺ signalling induced by synaptic stimulation in situ**

Stimulation of neuronal afferents originating from layers IV–VI induced transmembrane ion currents in the astrocytes (Fig. 3, see also Ref. [21]). Astroglial responses can be identified as glial synaptic currents (GSCs), directly associated with synaptic transmission, because (i) treatment of slices with 1 μM TTX completely abolished glial responses, (ii) latency time of glial responses was the same as latency of synaptic current evoked in the neighbouring neurones (1.5–2.5 ms) and (iii) the amplitude of the glial response increased dramatically upon the short train of high-frequency stimulation (HFS).

The GSCs were accompanied with [Ca²⁺]i transients (Fig. 3) in all 22 cells tested. The synaptic origin of astrocytic Ca²⁺-transients was confirmed by their considerable enhancement by HFS-train (Fig. 3). Incubation of cortical slices with selective AMPA receptor blocker CNQX (30 μM) did not affect GSC-associated [Ca²⁺]i transients (n = 6, data not shown). In contrast, antagonists of P2X receptors were ineffective.
2. P2X and NMDA receptor-mediated Ca\(^2+\) signalling in acutely isolated cortical astrocytes. Acutely isolated astrocytes were loaded with Fluo-4 via a patch pipette. Fluorescent images were recorded simultaneously with transmembrane currents evoked by application of 100 \(\mu\)M glutamate or 30 \(\mu\)M NMDA (A); 100 \(\mu\)M ATP or 10 \(\mu\)M \(\alpha,\beta\)-methyleneATP (B). Transient currents are represented as the \(\Delta F/F_0\) ratio averaged over the cell soma. Holding potential was –80 mV. (A) The upper row shows the images of Fluo-4-loaded astrocyte at rest (left) and EGFP fluorescence image from the same cell taken prior to the establishment of whole-cell configuration. The images in the middle and low panels were taken at the peak of the \([Ca^{2+}]_i\) response; representative \([Ca^{2+}]_i\) transients and glial currents are shown on the right. (B) Similarly to (A) the upper panel shows Fluo-4 image of the cell at rest; images in the middle and low panels were taken at the peak of the response; representative \([Ca^{2+}]_i\) transients and glial currents are shown on the right.

and NMDA receptors partially inhibited synaptically induced (by HFS-train) Ca\(^{2+}\) responses in all 16 astrocytes tested (Fig. 3).

Selective NMDA receptor blocker D-AP5 (30 \(\mu\)M) decreased amplitude of synaptically induced astroglial \([Ca^{2+}]_i\)-transients (Fig. 3A and C) by 34.2 ± 9.9\% \((n = 4)\). Slice exposure to 5 \(\mu\)M of compound UBP141 (selective against receptors containing NR2D subunit [42,43]) reduced the amplitude of synaptically evoked [Ca\(^{2+}\)]_i transients by 29.2 ± 8.4\% \((n = 4); \text{ Fig. 3C}, \text{ traces are not shown})]. Bath application of selective antagonist of P2X\(_1\) and P2X\(_5\) subunits NF449 [44] at 10 nM concentration decreased amplitude of synaptically-activated Ca\(^{2+}\)-transients by 37.7 ± 12.3\% \((n = 4, \text{ Fig. 3B and C})\) whereas broad P2X/Y antagonist PPADS (10 \(\mu\)M) inhibited Ca\(^{2+}\)-responses by 52.8 ± 14.1\% \((n = 4, \text{ Fig. 3C}, \text{ traces are not shown})\).

4. Discussion

4.1. Ca permeability of astroglial ionotropic receptors

(i) NMDA receptors: implications for subunit composition. The \(P_{Ca}/P_{monovalent}\) ratio for astroglial NMDA receptors appeared to be substantially lower compared to relative Ca\(^{2+}\) permeability determined for neuronal NMDA receptors \((P_{Ca}/P_{monovalent} \sim 10; [39,45,46])\). This relatively low Ca\(^{2+}\) permeability may indicate the presence of NR3 subunit in the astroglial receptor heteromer. Indeed, the incorporation of NR3 subunit into the NMDA receptor lowers their Ca\(^{2+}\) permeability and significantly reduces Mg\(^{2+}\) sensitivity [45–47]. The \(P_{Ca}/P_{monovalent}\) value, which we determined in astrocytes, is in a good agreement with permeability ratio determined for NR3 subunit-containing receptors \((P_{Ca}/P_{monovalent} \sim 2.2 [45,46])\). Furthermore, the lack of Mg\(^{2+}\)-block observed in NMDA receptors of cortical astrocytes in our present and previous experiments [21] is compatible with di-meric NR1/NR3 or tri-heteromeric NR1/NR2/NR3 receptor compositions [46,48,49]. The astroglial NMDA receptor-mediated currents were inhibited by D-AP5, MK-801 and UBP141 ([21] and present paper), that indicates the presence of NR2C or NR2D subunits. Therefore the most probable composition of astroglial NMDA receptor is the tri-heteromeric assembly including 2 NR1, NR2C/D and NR3 subunits. Incidentally, the NR2C, NR2D and NR3 subunits are expressed in rodent oligodendrocytes that are also in a possession of functional NMDA receptors with weak Mg\(^{2+}\) sensitivity [40].

(ii) P2X\(_{1/5}\) receptors. The relative Ca\(^{2+}\) permeability of astroglial P2X\(_{1/5}\) receptors was close to that determined for heterologously expressed P2X\(_{1/5}\) receptors \((P_{Ca}/P_{monovalent} \sim 1.5 [50])\) and slightly higher than reported for cloned P2X\(_{1/5}\) heteromeric receptors \((P_{Ca}/P_{monovalent} \sim 1.1 [51])\). The somewhat lower Ca\(^{2+}\)-permeability values reported previously could be due to extremely high concentration (112 mM) of extracellular Ca\(^{2+}\) and employment of voltage-ramp protocols for measuring the reversal potential used in these studies.
Fig. 3. Synaptically induced ionotropic Ca\(^{2+}\) signals in protoplasmic cortical astrocytes in situ. (A and B) Cortical layer II astrocytes were loaded with Fura-2 in situ via patch pipette. Fluorescent images were recorded simultaneously with glial currents evoked by neuronal afferent stimulation in the presence of CNQX (control) and after application of 30 \(\mu\)M D-AP5 (A) and 10 nM NF449, selective antagonist of P2X receptors (B). Representative images (pseudo-colour, pipette image subtracted) and glial synaptic currents (right column) were recorded from two different cells before (images on the top) and at the peak of the response. \([\text{Ca}\(^{2+}\)]\) transients (middle columns) are expressed as \(F_{340}/F_{380}\) ratio. Note significant enhancement of glia currents and Ca\(^{2+}\) transients upon high-frequency (HFS) stimulation. (C) Average inhibitory effect of antagonists of NMDA (D-AP5, UBP141) and P2 (NF449, PPADS) receptors on the amplitudes of \([\text{Ca}\(^{2+}\)]\) responses in cortical astrocytes. Data are presented as mean ± SD for 4 cells for each drug. Note significant inhibitory effect of subunit selective antagonists UBP141 (NR2C and D subunit-containing NMDA receptors) and NF449 (P2X\(_1\) and P2X\(_{1/5}\) receptors).

4.2. NMDA and P2X\(_{1/5}\) Receptors contribute to astroglial Ca\(^{2+}\) signals triggered by physiological stimulation

Despite the fact that Ca\(^{2+}\) permeability of astroglial NMDA and P2X receptors is not as high as in neurones, they both can mediate Ca\(^{2+}\) influx large enough to trigger substantial increases in [Ca\(^{2+}\)]. Both receptors are fully available at resting astrocyte potential thus ensuring the highest possible electro-driving force, and both receptors have slow desensitisation prolonging the period of Ca\(^{2+}\) entry. Importantly, the amplitudes of ionotropic receptor-mediated [Ca\(^{2+}\)] transients are comparable with those mediated by activation of metabotropic Ca\(^{2+}\) signalling pathways. When studying astroglial Ca\(^{2+}\) signalling activated by synaptic stimulation in situ we found that substantial (up to 50%) part of [Ca\(^{2+}\)] transients was blocked by ionotropic receptor antagonists, suggesting that both NMDA and P2X\(_{1/5}\) receptors provide significant Ca\(^{2+}\) entry.

4.3. Role of ionotropic receptors in fast neuronal–glial signalling at a synaptic level

The present paper and our previous studies [21,33] demonstrate that synaptic stimulation activates ionotropic receptors in cortical astrocytes. This in turn indicates the existence of fast neuronal–glial signalling at the synaptic level. Activation of ionotropic receptors results in (i) local depolarisation of perisynaptic processes and (ii) generation of localised ion fluxes; both can be physiologically relevant for reciprocal signalling between neuronal and glial compartments in the tripartite synapse [52].

The degree of local depolarisation in astroglial processes remains unknown; if anything it is certainly limited by high K\(^+\) permeability of astrocyte membrane. Nonetheless even small depolarisations can contribute to various signalling pathways. For example recent data have shown that membrane depolarisation can enhance signals mediated by metabotropic receptors and in particular metabotropic glutamate and P2Y receptors [53]. Thus, the synergy between ionotropic and metabotropic pathways can shape the local signalling events in the perisynaptic glial processes. Rapid ion fluxes generated by activation of ionotropic receptors represent another physiologically important signalling mechanism (Fig. 4). First, in this paper we found that Ca\(^{2+}\) permeable NMDA and P2X\(_{1/5}\) receptors significantly contribute to astroglial Ca\(^{2+}\) signals. For a long time the primary role in glial Ca\(^{2+}\) signalling was assigned to metabotropic pathway. In particular, calcium signals arising from activation of metabotropic receptors are believed to control exocytic release of gliotransmitters. Several lines of evidence supported this hypothesis. It was found that astroglial [Ca\(^{2+}\)] transients generated by local Ca\(^{2+}\) uncaging, mechanical stimulation or bath application of metabotropic agonists modulated synaptic activity in Schaffer collateral-CA1 neuronal synapses arguably through activation of gliotransmission [13,54–56]. Recently, however, the role of metabotropic receptors/InsP\(_3\)-induced Ca\(^{2+}\) signalling in astroglial physiology was questioned. In experiments on mice with genetically modified Ca\(^{2+}\) signalling pathways in astrocytes (which either overexpressed Mas-related gene A1, MrgA1, metabotropic recep-
tor normally present only in sensory neurones, or did not express type 2 InsP3 receptors \([5,57,58]\)] has demonstrated that neither enhancement nor inhibition of astroglial metabotropic Ca\(^{2+}\) signaling affects synaptic transmission in hippocampus. Our data show that ionotropic receptors are capable of generating Ca\(^{2+}\) signals and can be instrumental for controlling local glutamatergic transmission together with metabotrophic pathways.

Second, activation of glutamate and purinoceptors triggers substantial Na\(^{+}\) entry, which may result in [Na\(^{+}\)]\(_{i}\) transients in astroglia; these [Na\(^{+}\)]\(_{i}\) transients indeed were observed in several types of astroglial cells following synaptic stimulation \([59–61]\). Sodium ions can represent an alternative astroglia-specific signalling system, in particular because [Na\(^{+}\)]\(_{i}\) fluctuations control glial-neuronal lactate shuttle \([62,63]\). In this context rapid focal [Na\(^{+}\)]\(_{i}\) transients arising from activation of ionotropic receptors can control local support of active synapses with the energy substrate and thus influence synaptic transmission. In addition [Na\(^{+}\)]\(_{i}\) transients regulate the activity of glutamate transporter and control the Na\(^{+}\)/Ca\(^{2+}\) fluxes through sodium–calcium exchanger \([60,64]\).

All in all our data demonstrate that astroglial ionotropic receptors can be involved in rapid neuronal–glial signalling at the synaptic level.

**Conflict of interest**

There are no conflicts of interest.

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