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GLUTAMATERGIC INPUT–OUTPUT PROPERTIES OF THALAMIC ASTROCYTES

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Abstract—Astrocytes in the somatosensory ventrobasal (VB) thalamus of rats respond to glutamatergic synaptic input with metabotropic glutamate receptor (mGluR) mediated intracellular calcium ($[Ca^{2+}]_i$) elevations. Astrocytes in the VB thalamus also release the gliotransmitter (GT) glutamate in a Ca^{2+}-dependent manner. The tripartite synapse hypothesis posits that astrocytic $[Ca^{2+}]_i$ elevations resulting from synaptic input releases gliotransmitters that then feedback to modify the synapse. Understanding the dynamics of this process and the conditions under which it occurs are therefore important steps in elucidating the potential roles and impact of GT release in particular brain activities. In this study, we investigated the relationship between VB thalamus afferent synaptic input and astrocytic glutamate release by recording *N*-methyl-D-aspartate (NMDA) receptor-mediated slow inward currents (SICs) elicited in neighboring neurons. We found that Lemniscal or cortical afferent stimulation, which can elicit astrocytic $[Ca^{2+}]_i$ elevations, do not typically result in the generation of SICs in thalamocortical (TC) neurons. Rather, we find that the spontaneous emergence of SICs is largely resistant to acute afferent input. The frequency of SICs, however, is correlated to long-lasting afferent activity. In contrast to short-term stimulus-evoked GT release effects reported in other brain areas, astrocytes in the VB thalamus do not express a straightforward input–output relationship for SIC generation but exhibit integrative characteristics.

Key words: astrocytes, glutamate, SIC, gliotransmission, somatosensory, tripartite synapse.

Glial cells are now considered as active participants in nervous system function (Volterra and Meldolesi, 2005), and the concept of the "tripartite synapse" (Araque et al., 1999) has been advanced to describe the situation where synaptically associated astrocytes act as integral modulatory elements. Astrocytes respond to released neurotransmitters with intracellular calcium $[Ca^{2+}]_i$ elevations (Cornell-Bell et al., 1990; Porter and McCarthy, 1996; Araque et al., 2002; D'Ascenzo et al., 2007). In turn, astrocytic $[Ca^{2+}]_i$ elevations can induce the release of gliotransmitters such as glutamate (Pasti et al., 1997; Kang et al., 1998; Parri et al., 2001; Fellin et al., 2004; Perea and Araque, 2005a), ATP (Pascual et al., 2005; Serrano et al., 2006), and D-serine (Henneberger et al., 2010).

In the ventrobasal (VB) thalamus, astrocytes can display spontaneous calcium oscillations *in vitro*, which consequently lead to excitatory *N*-methyl-D-aspartate (NMDA) receptor-mediated currents in thalamic neurons (Parri et al., 2001). These slow inward currents (SICs) are seen in many brain areas and can occur spontaneously or can be evoked by various methods that induce astrocytic Ca^{2+} increases (Parri et al., 2001; Angulo et al., 2004; Fellin et al., 2004; Perea and Araque, 2005a; Kozlov et al., 2006; D'Ascenzo et al., 2007; Navarrete and Araque, 2008; Shigetomi et al., 2008).

However, despite the known ability of synaptic stimulation to evoke astrocytic $[Ca^{2+}]_i$ elevations and subsequent glutamate release, little is known about the possible physiological roles of SICs and their potential to interact with afferent input in the somatosensory system. To understand these potential roles of astrocytic gliotransmission (GT) release in thalamic function, it is necessary to determine its release properties in relation to afferent activity. We recently found that spontaneous SIC frequency was increased following a period of sustained (>30 min) afferent activity (Pirttimaki et al., 2011). In this study, we sought to determine the dynamic input–output properties of VB thalamus astrocytes by stimulating afferent inputs, which induce metabotropic glutamate receptor (mGluR) mediated $[Ca^{2+}]_i$ elevations (Parri et al., 2010), and recording astrocytic output in the form of SICs in thalamocortical (TC) neurons.

We found that SIC emergence was unaffected by acute synaptic stimulation but was correlated to the duration of long-term afferent stimulation. VB thalamus astrocytes do not therefore release glutamate in a dynamic way in response to afferent activity but display integrative properties that induce long-lasting changes to astrocyte-neuron signaling.

EXPERIMENTAL PROCEDURES

Slice preparation

Horizontal slices of VB thalamus were prepared as described previously (Parri et al., 2001) from 12–23-day-old male Wistar rats. All procedures were in accordance with UK Home Office legislation: Animals (Scientific procedures) Act 1986. After removal, the brain was placed in ice cold modified artificial cerebrospinal fluid (aCSF) of composition (mM) NaCl 126, $NaHCO_3$ 26, KCl 1, KH_2PO_4 1.25, $MgSO_4$ 5, $CaCl_2$ 1, glucose 10, pyruvate 5, ascorbic acid 0.3, and indomethacin 0.45. Slices were then maintained at room temperature (23–25 °C) in this solution for a recovery period of 1 h before experimental use.

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Abbreviations: CT, corticothalamic; GT, gliotransmission; Lem, Lemniscal; mGluR, metabotropic glutamate receptor; NMDA-R, *N*-methyl-D-aspartate receptor; PSC, post synaptic current; SIC, slow inward current; SSP, spindle stimulation pattern; TC, thalamocortical; VB, ventrobasal; $[Ca2^+]_i$, intracellular calcium.

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Solutions

The standard recording aCSF used in this study was (in mM): NaCl 126, $NaHCO_3$ 26, KCl 2.5, KH_2PO_4 1.25, $MgSO_4$ 1, $CaCl_2$ 2, and glucose 10, unless otherwise stated. As we (Parri et al., 2001) and others have done previously in attempting to enhance NMDA-R mediated current detection, whole cell voltage clamp recordings were conducted in 0-Mg^{2+} (at room temperature), unless otherwise stated. Slices were perfused with the 0-Mg^{2+} solution in the recording chamber. Pharmacological compounds were included in the aCSF as stated in text. Chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. Tetrodotoxin (TTX) was obtained from Ascent Scientific (Weston-super-Mare, UK). Fura-2 A.M., Fluo-4 A.M., Alexa-hydrazide 564, and Pluronic F-127 were obtained from Invitrogen (Carlsbad, CA, USA).

Electrophysiology

The recording chamber and manipulators were mounted on a moveable top plate platform (MP MTP-01, Scientifica, UK). Patch clamp recordings were made using pipettes (2–4 MΩ) containing an internal solution of composition (in mM): $KMeSO_4$ 120, HEPES 10, EGTA 0.1, Na_2ATP 4, GTP 0.5. Currents were recorded using a Multiclamp 700B amplifier, digitized with a Digidata 1440A, and acquired and analysed using pCLAMP (Molecular Devices, CA, USA). Voltage clamp recordings were made at −60 mV, and recordings in which there was a ≥20% change in access resistance during the experiment were excluded from analysis. SICs were analysed using the Event Detection protocols in the Clampfit routine of pCLAMP. Events were accepted as SICs if their amplitude was >20 pA and their time to peak was >20 ms. Data were exported to Sigmaplot (Jandel) for further analysis and plotting.

Synaptic stimulation

Synaptic stimulation was achieved with a computer-controlled constant current isolated stimulator (STG1002, Multichannel Systems, Germany) and bipolar electrodes, which were placed typically >200 μm from the recorded neurons. Sensory stimulation was achieved by placing a bipolar electrode on the medial lemniscus (Lem), and corticothalamic (CT) afferents were stimulated by a bipolar electrode on the internal capsule. Stimulation protocols were written in the STG1002 interface software. "Protocols" were composed of sequences of "Episodes" of stimulation. After a 5–10 min baseline recording, a stimulation protocol was delivered to one pathway at a time or to both simultaneously. Stimulus episodes were separated by approximately 60-s inter-stimulus intervals. Different stimulus amplitudes ranging from 0.1 mA to 3 mA were tested using trains of 2 ms pulses for 1 s at 50 Hz in randomized order. Different stimulus durations ranging between 2 ms and 10 s were tested at 50 Hz by changing the number of pulses within the train (using sub-maximal stimulus amplitude determined from the evoked EPSC, I_{75}). Different frequencies were tested by generating trains of pulses at frequencies between 1 Hz and 500 Hz using constant number of stimuli with I_{75} stimulus amplitudes. A single "spindle stimulation pattern" (SSP) consisted of 22 spikes in duration of 728 ms (Rosanova and Ulrich, 2005). Mean spike rate was 30 Hz, grouped to initial 10 Hz bursts followed by tail of decreasing tonic frequency. In some experiments the SSP was repeated 30 times every 0.6 Hz to mimic the grouping of spindles by the slow (0.6–0.8 Hz) oscillation. Sustained stimulation protocol (10–20 stimuli at 50 Hz every 10 s) as previously described (Pirttimaki et al., 2011) was applied for 30–120 min.

Fluorescence imaging

After a recovery period of 1 h, slices were loaded with Fluo4 A.M. (Molecular Probes, Eugene, OR, USA) or Fura-2 A.M. by incubating for 40–60 min at 30 °C with 10 μM of the indicator dye and 0.01% pluronic acid. Under these conditions, astrocytes are preferentially loaded (Parri et al., 2001). For astrocytic identification, slices were also loaded with 1 μM Sulforhodamine 101 (SR101), according to *in vitro* methods of Kafitz et al. (Kafitz et al., 2008). Approximately 40 astrocytes (39.7±2.39, n=4 slices) could be identified in focus in the VB slice, though this is likely a lower estimate of the number in the visible slice, for example, due to SR101 loading variation. All imaging experiments were performed in aCSF containing Mg^{2+} (1 mM) to record astrocytic responses in physiological conditions. Experiments on imaging with Fura-2 A.M. in Mg^{2+}-containing and Mg^{2+}-free conditions showed that astrocytic Ca^{2+} elevation responses for the same cells were greater in Mg^{2+}-free (0.077±0.005, 340/380 ratio change) than in Mg^{2+}-containing aCSF (0.05±0.004 ratio change, n=3 slices, 55 cells, P<0.01). In patch-clamp experiments designed to maximize SIC detection with Mg^{2+}-free aCSF, it would therefore be expected that an increased astrocyte response would also result in a greater probability of detecting any afferent–astrocyte–SIC relationship. Combined patch-clamp and imaging experiments (Fig. 1G) were conducted in Mg^{2+}-free aCSF.

The imaged field size was 444 μm×341 μm. The recording chamber and manipulators were mounted on a motorized moveable bridge (Luigs and Neumann, Germany). Fluorescent dyes were excited using an Optoscan monochromator system (Cairn, UK), fitted to a Nikon FN1 upright microscope. Images were acquired for a duration of 0.05–0.1 s every 1–5 s using an Orca-ER CCD camera (Hamamatsu). The short stimulation protocol consisted of trains of 2 ms pulses for 1–2 s at 50 Hz delivered to Lem or CT. Responses were recorded for at least 1 min post stimulation. Acquisition was controlled by Simple PCI software (Hamamatsu).

Statistics

All quantitative data in the text and figures are presented as mean±SEM. Significance was calculated using unpaired or paired Student's t-test as appropriate. Linear correlations (r^2) were tested using Pearson Rank correlation. Statistical significance in the figures is indicated as: * P<0.05, ** P<0.01, or *** P<0.005.

RESULTS

VB thalamus astrocytes and neurons respond to synaptic stimulation

Patch clamp recordings from VB thalamus TC neurons revealed spontaneous SICs at low frequencies (~0.001 Hz), which have been previously shown to be $[Ca^{2+}]_i$ dependent and TTX-insensitive (Fig. 1A) (Parri et al., 2001; Pirttimaki et al., 2011). The presence of D-AP5 (50 μM) abolished SICs (Ctrl: 16 SICs; D-AP5: 0 SICs; n=6 neurons; paired Student's t-test P=0.003) (Fig. 1B).

Stimulations of Lemniscal and CT inputs at 50 Hz of 1–2 s duration elicited Ca^{2+} elevations which could be detected at the astrocyte soma (Fig. 1C, D). On average 24.5±2.87 astrocytes responded to Lemniscal afferent stimulation (n=4 slices) and 24.25±4.65 (n=8) to CT stimulation within the imaged area of 444 μm×341 μm (Fig. 1E). The relative intensity change of the Ca^{2+} fluorescence was not different between Lemniscal and CT stimulation (Lem 12.04±0.97ΔF%; CT 10.7±0.4ΔF%; n=99, 194 responses, respectively; Student's t-test P=0.14) nor between 1-s and 2-s long stimulation (1 s 11.53±0.58ΔF%; 2 s 10.8±0.6ΔF%; n=149, 144, respectively; P=0.4; Fig. 1F), indicating that a maximal cellular $[Ca^{2+}]_i$ elevation was