Astrocytes Discriminate and Selectively Respond to the Activity of a Subpopulation of Neurons within the Barrel Cortex

Sensory information from single whiskers in rodents projects to defined morphological units in the cortex, the barrels. We found that astrocytes selectively respond with an increase in the cytosolic Ca\(^{2+}\) concentration to activation of layer 4 neurons, the input cells of the barrel columns. The neuronal Ca\(^{2+}\) signal spread across barrel column borders mainly in layer 2/3, but the glutamate-mediated astrocyte response stayed restricted to the barrel column. In contrast, when interfering with inhibitory pathways by blocking either purinergic, adenosine or γ-aminobutyric acid\(_A\) receptors, the stimulation activated a Ca\(^{2+}\) response in a much larger astrocyte population no longer restricted to the borders of the barrel column. We also observed spontaneous and evoked Ca\(^{2+}\) activity in the synaptic target cells of layer 4 neurons, the layer 2/3 pyramidal cells, but again, we never recorded Ca\(^{2+}\) responses in astrocytes following activity in this neuronal population. Our data show that astrocytes can discriminate and selectively respond to the activity of a subpopulation of excitatory neurons within a given brain region. This selectivity in the astrocyte response describes a new level of complexity and integration in the reaction of astrocytes to neuronal activity.

Keywords: astrocyte, barrel cortex, calcium, neuron–glia interaction, slice

Introduction

Defining the role for astrocytes in information processing in the mammalian brain has emerged as an important recent topic. A series of studies indicates that astrocytes sense and influence neuronal, in particular synaptic activity. It has long been known, that astrocytes ensheath synapses and are important elements that control the extracellular milieu. It is now well established that Ca\(^{2+}\) signals in astrocytes can be induced by neuronal activity and that, in turn, astrocytes can release neuroactive substances and thereby affect neuronal activity (Kang et al. 1998; Serrano et al. 2006). Glutamate, adenosine triphosphate (ATP) and γ-serine have been established as essential signaling substances for neuron–glia interaction. Studies in the hippocampus have revealed that one given astrocyte can even sense activity from 2 distinct synaptic connections thereby integrating the information from glutamate and acetylcholine releasing pathways (Perea and Araque 2005).

A recent in vivo study demonstrated that sensory input can trigger responses in a defined population of astrocytes. Stimulation of a single whisker triggers astrocyte Ca\(^{2+}\) responses in the cortical representation field, the barrel cortex (Wang et al. 2006). The barrel cortex has been employed for a long time as a model to study the plasticity of sensory input into the mammalian brain (Woolsey and Van der Loos 1970; Petersen 2003) and is now well established that Ca\(^{2+}\) signals in astrocytes can be induced by neuronal activity and that, in turn, astrocytes can release neuroactive substances and thereby affect neuronal activity (Kang et al. 1998; Serrano et al. 2006). Glutamate, adenosine triphosphate (ATP) and γ-serine have been established as essential signaling substances for neuron–glia interaction. Studies in the hippocampus have revealed that one given astrocyte can even sense activity from 2 distinct synaptic connections thereby integrating the information from glutamate and acetylcholine releasing pathways (Perea and Araque 2005).

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Material and Methods

Animals, Preparation of Brain Slices, Stimulation, and Ca\(^{2+}\) Recordings

All experiments were performed according to the guidelines of the German animal protection law. For experiments either 8- to 12-day-old outbred NMRI mice (Charles River, Berlin) or transgenic Cx30−/−/Cx43(fl/fl):GFAP-cre mice were used. Genotyping of mice lacking Cx43 in astrocytes was done according to the instructions of Thesl et al. (2003) and for Cx30 according to Teubner et al. (2003). For slice preparation, mice were decapitated and their brains were removed. To obtain slices containing the barrel cortex, the cerebellum was cut off in an angle of about 30° and the brain was glued onto that plane. We cut semicoronal slices of 250-μm thickness in ice-cold artificial cerebrospinal fluid (ACSF) using a vibratome (HM 650 V, Microm International GmbH, Walldorf, Germany). Staining with Fluor-α-acetoxyxymethylene (Fluo-α-AM) and image recording was performed exactly as described in Peters et al. (2003). In brief, after the preparation, slices were stored

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Figure 1. Astrocytes selectively respond to stimulation of input into a barrel column, but not to spontaneous neuronal activity in layer 2/3. Subtraction images showing a recording in a slice obtained from a p9 mouse brain under 488 nm illumination. Time points are indicated in each image. Images are background subtracted and highlight reacting cells. (A) Focal electrical stimulation in layer IV leads to an increase in Ca\(^{2+}\) in neurons along the barrel and horizontally across barrel structures in layer 2/3. This response is followed by an astrocytic Ca\(^{2+}\) signal restricted to the stimulated barrel. Bar = 100 μm. (B) Overlay of the regions indicated in (A). The neuronal signal during stimulation (blue) does not overlap with the astrocytic signal appearing 1 s delayed (violet). Note, that astrocytic signaling stops at barrel borders. Bar = 30 μm. (C) Spontaneous neuronal network activity in layer 2/3 is not followed by a Ca\(^{2+}\) signal in astrocytes. (D) Cartoon illustrating the barrel borders, the orientation of the slice as observed in bright-field optics (as shown in Fig. 2A) and the position of the stimulation pipette.

at room temperature for at least 45 min, then incubated with the Ca\(^{2+}\) indicator dye Fluo-4-AM (10 μM Fluo-4-AM, Molecular Probes, Eugene, OR) for 45–60 min at room temperature.

Slices were transferred to a perfusion chamber on an upright microscope ( Axioskop FS, Zeiss, Oberkochen, Germany), fixed in the chamber using a U-shaped platinum wire with a grid of nylon threads and superfused with ACSF at a flow rate of 4 to 6 ml/min at a temperature of 33–34 °C. Substances were applied by changing the perfusate in the following concentrations (mM): tetrodotoxin (TTX) (1), n-methyl-D,-carboxyphenylglycine (S-MCPG) (50), 6-cyano-7-nitroquinoline-2,3-dione (CNQX) (50), MK801 (40), 2-methyl-6-(phenyl-piperidine (MPEP) (100), LY367385 (200), carbeneoxolone (100), N(omega)-nitro-L-arginine (L-NNA) (2000), O-2050 (2), methysergide (30), kynurenic acid (0.5), bicuculline (100), SR 95533 (10), suramin (100), 8-cyclopentylethylphophylline (CPT) (4).

Solutions and Electrodes

The standard ACSF contained 134 mM NaCl, 2.5 mM KCl, 2 mM CaCl\(_2\), 1.3 mM MgCl\(_2\), 21.5 mM NaHCO\(_3\), 1.25 mM K\(_2\)HPO\(_4\), 10 mM glucose and was heated to 32–34 °C for the recording. By continuously gassing the solutions with carbogen, the pH was adjusted to 7.4.

All pipettes were fabricated from borosilicate capillaries ( Hilgenberg, Malsfeld, Germany). Recording pipettes had resistances ranging from 4 to 6 MOhm. The pipette solution for patch-clamp experiments was composed of (in mM): 140 K-gluconate, 2 NaCl, 4 ethylene glycol tetraacetic acid, 2 Na\(_2\)ATP, 2 glucose, 10 TTX, 2 GluCl, 1 nicotine, 0.5 bicuculline, 0.5 kynurenic acid, 0.5 kynurenic acid, 0.5 bicuculline, 0.5 suramin, 0.5 memantine, 0.5 niflumic acid, 0.5 indomethacin, 0.5 hemin, 0.5 coenzyme A, 0.5 ATP, 0.5 creatine phosphate, 0.5 creatine kinase, and 500 SR101.

Results

Astrocytes Respond to Stimulation of the Barrel Input

Coronal slices from young mouse brains (P8–P14) containing the barrel cortex were loaded with the Ca\(^{2+}\)-sensitive dye Fluo-4. The barrels were identified in bright-field optics and a stimulation electrode was placed in layer IV in the center of a barrel (Fig. 1D). Stimulation for 0.3 s with 100 Hz triggered an immediate Ca\(^{2+}\) response in a large number of cells (Fig. 1A), which were identified as neurons (see below). Responding neurons were found to be located within the stimulated barrel in layer IV, but also beyond barrel column borders in layer II/III and V throughout the whole observation area (10x objective, 900 x 720 μm; Figs 1A, 2A). Subsequently, 1 s after stimulation,
following the neuronal response, a different cell population responded with a slower rise in Ca²⁺ and the localization of this group of cells was strongly restricted to the stimulated barrel column (Fig. 1A). We identified these cells as astrocytes (see below). A comparison and overlay of images analyzing the nature of the responding cell types reveals that the rapidly and the slower reacting cells comprise 2 distinct populations, namely immediately responding neurons and delayed activated astrocytes (Fig. 1B). In the presence of 1 μM TTX, Ca²⁺ responses were no longer observed in response to stimulation, neither in neurons nor in astrocytes (Fig. 2C). To verify that the application of 1 μM TTX indeed blocked the propagation of action potentials, we recorded from neurons in layer 2/3. In control conditions, a current injection of 40 pA depolarized the neuronal membrane robustly and triggered action potentials. After application of TTX, action potentials were no longer observed in response to depolarizing pulses (Fig. 2C).

**Astrocytes do not Respond to Layer 2/3 Neuronal Network Activity**

Especially in slices obtained from younger mice (P<10), we observed spontaneously occurring increases in the Ca²⁺ level within a large population of neurons (Figs 1A, 3A). This synchronous, spontaneous Ca²⁺ activity was rapid in onset and decayed within 1 s, similar to the neuronal signal in response to stimulation. The spontaneous neuronal activity was restricted to layer 2/3 and did not involve cells in deeper layers. In sharp contrast to electrical stimulation, the spontaneously occurring neuronal Ca²⁺ signal was found to be neither accompanied nor followed by a Ca²⁺ signal in astrocytes.

Spontaneous Ca²⁺-activity in neurons occurred at a higher frequency after application of the GABAA receptor antagonist SR 95531. Under these conditions, we robustly recorded spontaneous responses (n=9 slices, data not shown). Coapplication of TTX for 2 min completely abolished these spontaneously occurring neuronal Ca²⁺ transients (n=13, data not shown).

We also could activate Ca²⁺ signals in this neuronal cell population by placing the stimulation electrode in layer 2/3. After application of the same stimulus as we used in layer 4 (0.3 s, 100 Hz) we observed Ca²⁺ transients in neurons spreading along layer 2/3 to neighboring columns (Fig. 3A). The neuronal activity in layer 2/3 was never followed by an astrocyte Ca²⁺ signal as also observed for the spontaneous neuronal signals. However, astrocytes reacted within the barrel column where the electrode was placed (n=13 slices from 5 animals).

To further characterize the transmitter involved in the layer 2/3 neuronal activity we applied CNQX to block α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate-type glutamate receptors. The astrocyte response within the stimulated barrel column was unaffected. In contrast, the response in layer 2/3 neurons, which spread over neighboring barrel columns, was reduced to 22.5% (standard error of the mean [SEM] 13.0, n=11) after CNQX-application as compared to control (Fig. 3B). This indicates the excitatory nature of the neuronal activity along layer 2/3.

**Neurons and Astrocytes Show Distinct Ca²⁺-Signals**

To characterize the Ca²⁺ responses in the neuronal and the astrocyte population, we recorded images with a frequency of 4 Hz and analyzed the time course in an area outlining the cell soma of astrocytes and neurons (Fig. 2A, B).

Neurons responded within the first image after stimulation (0.005 s) and showed both a very rapid onset and decay of the Ca²⁺ signal. In all neurons analyzed outside the stimulated barrel column, the fluorescence signal dropped to the half-maximal value within less than 2 s (Fig. 4A,B). In contrast in neurons located within the stimulated barrel column, the decay times were often longer. It is assumed that part of the prolonged signal within the barrel column, is due to the underlying Ca²⁺ signals from neighboring astrocytes (Fig. 4A,B). Independent of their location, all neurons displayed comparably smaller amplitudes with an average fluorescence increase of 41% (±3.9%) (Fig. 4C).

The response of astrocytes was strictly confined to those cells located within the stimulated barrel column. The Ca²⁺ signal recorded from astrocyte somata was mostly larger in amplitude and showed different kinetics when compared to the neuronal response (Figs 2B, 4A). The Ca²⁺ transients in astrocytes appeared with a slower rise time, peaked with a delay of 1–2 s after the stimulation and exhibited a longer decay time. The fluorescence signal dropped to the half-maximal amplitude after about 2–17 s (average 8.8 s, Fig. 2A,B). Signals with longer decay times were commonly recorded from astrocytes in deeper tissue layers. We assume that glutamate seems to persist longer in the extracellular space due to the limited diffusion. Moreover, in astrocytes we recorded an 80% (±4.9%) increase in fluorescence, which was about twice of the change in fluorescence observed in neurons (Fig. 4C). The vast majority of responding astrocytes were located in layer 2/3 and 4 and restricted to the barrel column. Only very few cells responded in layer 5.

While the population of responding astrocytes in the somatosensory cortex was confined by the barrel structure and activity spread preferentially to upper layers, a similar stimulus in the motor cortex activated a population of astrocytes in a circular fashion around the point of stimulation (for details see Haas et al. 2006).

**The Slowly Responding Cells are Astrocytes**

To selectively label astrocytes and to distinguish them from Fluo-4 stained neurons, we applied the red dye Sulforhodamine 101 (SR101) to acutely prepared slices. SR101 is preferentially taken up by astrocytes (Nimmerjahn et al. 2004). The slices were costained with Fluo-4. The red-labeled cells always responded with a slow rise of the Ca²⁺ concentration after stimulation (Fig. 5C). We did not detect an overlap between the cell population, which responded simultaneously with the onset of stimulation, and the SR101 labeling. Moreover, astrocytes were never part of the spontaneously responding cell population (Fig. 5A,B).

**The Fast Responding Cells are Neurons**

To characterize the cell population with the fast peaking Ca²⁺ signal we selected single cells for current clamp recordings using the whole-cell patch-clamp technique. When the cells were depolarized by injecting a current pulse, we recorded action potentials in all cells investigated (n=9 cells, n=9 slices, Fig. 5D). We recorded a low spike frequency with strong adaptation in all of these cells, which is typical for glutamatergic regular-spiking pyramidal neurons (Chagnac-Anxitai and Connors 1989b; Porter et al. 2001). During a 200 ms long spike train the spike frequency adaptation ratio was 0.59 (±0.081).
Glutamate is Involved in the Signaling Pathway from Neurons to Astrocytes

To test for the involvement of glutamate in activating astrocytes, in response to neuronal activity emerging from layer 4, we interfered pharmacologically with excitatory, glutamatergic signal transmission. Stimulation in the presence of a cocktail containing MK801, CNQX, and MCPG, to block N-methyl-D-aspartate (NMDA)- and AMPA/kainate-type glutamate receptors, as well as metabotropic glutamate receptors, respectively, largely reduced the Ca\(^{2+}\) signal in both, neurons and astrocytes to 29.8% (SEM 3.1, n = 5; Fig. 6A-C). When we selectively interfered with the signaling of group I metabotropic glutamate receptors (mGluRs), via the application of LY367385 and MPEP, the number of astrocytes responding after stimulation was also reduced to 33.2% (SEM 6.2, n = 8). We thus conclude that astrocytes respond to the excitatory neuronal signaling emerging from layer 4 neurons within a given barrel column.

Figure 2. In the barrel cortex, stimulation in layer IV leads to subsequent Ca\(^{2+}\) increases in neurons and astrocytes. (A) The cartoon illustrates the orientation of the slice and the location of the stimulation pipette. The fluorescence images show consecutive subtraction images highlighting reacting cells in response to a focal electrical stimulation in layer IV in a slice obtained from a P9 mouse brain, recording frequency 4 Hz. To better visualize reacting cells, pixel values of the previous image were subtracted from each image. The resulting images are shown. Increases in fluorescence are displayed by increases in brightness. The time after onset of stimulation is indicated in each image. Immediately after stimulation a population of cells (neurons) reacts with a fast, small Ca\(^{2+}\) signal. A second population (astrocytes) exhibits Ca\(^{2+}\) peaks around 1 s after stimulation. Note that this reaction is restricted to the stimulated barrel column. The last image displays an overlay of all reacting cells (black) with the bright-field image of the slice. Bar = 100 \(\mu\)m. (B) Ca\(^{2+}\) traces from the regions marked in (A). Traces 1 to 3 are examples for neuronal cells reacting fast and with a small amplitude, traces 4–6 for astrocytes reacting delayed with larger amplitudes. The dotted line indicates the onset of stimulation (4 V, 100 Hz, 300 ms). Note the difference in the scale for cells 1–3 and 4–6. To obtain Ca\(^{2+}\) intensities from single cells the tool “AOI analysis in a t-stack” (http://www.mediacy.com/index.aspx?page=ViewSolution&solid=960) was used. (C) Left: Current clamp responses from a pyramidal neuron in layer 2/3. Forty picamperes were injected. Firing of action potentials is completely blocked in the presence of 1 \(\mu\)M TTX. Right: Ca\(^{2+}\) responses are also blocked by TTX. Asterisks indicate the time point of stimulation.
To further verify that the astrocyte response is restricted to a given barrel, we placed a fine stimulation pipette with a resistance of 2–3 MOhm close to the septum, but still within the barrel (Fig. 7A). Similar to the stimulation with larger pipettes the astrocyte response was restricted to the stimulated barrel column. We then moved the electrode to the adjacent barrel and we could activate astrocyte activity within this barrel column, but not in the neighboring one (Fig. 7B). The average fluorescence intensity in the stimulated barrel determined 1 s after the stimulation at the peak of the astrocyte response was 2.9-fold higher compared to the unstimulated neighboring barrel (Fig. 7D).

**Inhibition of GABAergic Transmission Alters the Astrocytic Ca²⁺ Signal**

To determine the role of GABAergic inhibition in the astrocyte Ca²⁺ responses, we pharmacologically blocked GABA A receptors. The classical blocker bicuculline (n = 6) and the more specific GABA A receptor antagonist SR 95531 (n = 7) were used. Both substances had a similar effect: In the presence of the antagonists, the neuronal response increased in amplitude and a larger population of cells was recruited (Fig. 8). Most prominently, the astrocytic response was no longer restricted to the barrel column, but spread throughout the entire observation area. Thus a larger astrocyte population was activated beyond the borders of the barrel column (Fig. 8). As mentioned above, we frequently observed fast spontaneous neuronal network activity in the presence of bicuculline or SR 95331, which was not accompanied by a subsequent astrocyte response (data not shown).

**Inhibition of Purinergic Signaling Modulates the Astrocytic Ca²⁺ Signal**

Purinergic signaling is known to set a certain inhibitory tone at excitatory synapses. Also, ATP released from astrocytes and its degradation products very potently influence synaptic strength (Serrano et al. 2006). We therefore performed experiments in the presence of suramin, a blocker of P2Y type purinergic receptors, and in a different set of experiments in the presence of CPT, a blocker of A1 adenosine receptors.

In the presence of suramin stimulation led to an enhanced neuronal Ca²⁺ signal. Compared to control an increased amount of cells in the neuronal population responded (Fig. 9A, n = 15). Furthermore the consecutive Ca²⁺ signal in astrocytes was also observed in a larger population of cells also including astrocytes in neighboring barrels columns throughout layer 2/3. The stimulation thus recruited more astrocytes reacting to neuronal activity compared to control conditions (Fig. 9A). As Suramin is known to exhibit a number of non-purine-mediated side effects, we used the adenosine receptor CPT to quantify the finding. A similar effect as observed for suramin was observed in the presence of CPT: When compared to control (0.13 ± 0.011 mm²), stimulation in the presence of CPT led to an astrocytic
Coupling via Gap Junctions is not a Prerequisite for Astrocytes to Respond to Electrical Stimulation

Astrocytes in the neocortex are coupled by gap junctions and form a large functional syncytium (Peters et al. 2003; Haas et al. 2006). We thus investigated whether astrocytic gap junction coupling is a prerequisite for the astrocytic Ca\(^{2+}\) response in the barrel cortex and whether the coupling is essential for restricting the responding cell population to a single barrel column. To uncouple astrocytes, we stimulated in the presence of carbenoxolone, a blocker of gap junctions. We found that a similar population of astrocytes responded in the presence of carbenoxolone as compared to a control response. After application of carbenoxolone the average increase in fluorescence intensity in the stimulated barrel column 1 s after the stimulation was reduced to 81.5% (SEM: 11.9), which might be due to side effects of this rather unspecific drug. 

Looking for a more suitable model we also performed experiments in slices from transgenic mice deficient of gap junctional coupling in astrocytes (GFAP-cre, Cx43 flox/flox, Cx30+/− mice, for details see Haas et al. 2006). In these knockout animals we were able to elicit a similar response in the astrocyte population as compared to wildtype animals (Supplementary Fig. S1). We thus conclude that coupling is not a prerequisite for astrocytes to respond to stimulation in layer 4.

5-HT\(_{1/2}\), Cannabinoid-, and Acetylcholine-Receptors and NO-Formation do not Interfere with the Astrocytic Ca\(^{2+}\) Signal after Stimulation

To study the involvement of other transmitter systems, we used specific antagonists to block serotonin- (5-HT\(_{1/2}\)), cannabinoid- (CB), and acetylcholine- (ACH) receptors. Additionally we blocked the formation of nitric oxide (NO) (Fig. S2). To interfere with the production of NO, we stimulated the slice with superpressing with 2 mM L-NNA in the bath solution. We signal also outside the stimulated barrel column, altogether recruiting cells from a larger area (0.18 ± 0.017 mm\(^2\), Fig. 9B).
did not observe a significant change in the number of reacting cells or in the shape of the signal (n = 6 slices). To block 5-HT1/2-receptors we superfused with 30 μM methysergide. Blockade of 5-HT1/2-receptors did also not interfere with the astrocytic Ca\(^{2+}\) signal after stimulation (n = 3 slices). To rule out the involvement of cannabinoid (CB1) receptors, we applied the blocker O-2050 (2 μM). Also this substance did not alter the astrocytic signaling pattern (n = 6 slices). As acetylcholine is a prominent transmitter in the neocortex, we used kynurenic acid in a low concentration (500 nM) to block α7 nicotinic ACh-receptors. The astrocytic signal after stimulation was unchanged in comparison to a previous stimulation and to a washout experiment (n = 5 slices). Interfering with signaling via the reported transmitter pathways thus did not interfere with the astrocytic Ca\(^{2+}\) signal in the barrel cortex.

### Discussion

**Astrocytes in the Barrel Cortex are Activated by Neuronal Activity**

Neuronal circuits in the primary somatosensory cortex integrate sensory information from the whiskers and form a remarkable somatotopic map. Each whisker is represented by a defined morphological area termed barrel (Woolsey and Van der Loos 1970; Petersen and Sakmann 2001; Petersen 2003). In a recent *in vivo* approach, Wang et al. (2006) demonstrated that whisker stimulation elicited Ca\(^{2+}\) transients in astrocytes in the barrel cortex. Using acutely isolated slices, we were able to detect the morphological units of single barrels using a light microscope and could therefore correlate astrocyte activity and somatosensory representation. A role for astrocytes in the development of the somatosensory system has been described (Mitrovic et al. 1994; Irintchev et al. 2005), but their role in information processing within this structure has not been investigated.

We here show that astrocytes selectively respond to neuronal activity emerging from layer 4 in the barrel cortex. Astrocytic and neuronal Ca\(^{2+}\) signals could be clearly distinguished and separated by different kinetics (Fig. 4). Astrocytes were identified by the overlap of Fluor4-fluorescence with the astrocyte specific dye SR101 (Nimmerjahn et al. 2004) and exhibited kinetics in agreement with values determined in previous studies in slices (e.g., Haas et al. 2006). Neuronal Ca\(^{2+}\) signals typically showed faster kinetics than astrocytes (Berger et al. 2007) and we were able to record action potentials from these cells. The lack of an astrocytic Ca\(^{2+}\) signal after applying TTX indicates that astrocytes detect the neuronal activity rather than being activated directly by the stimulation. In accordance with the *in vivo* study (Wang et al. 2006), we observed that mGluR blockers attenuated the astrocytic Ca\(^{2+}\) signal indicating that astrocytes sense and get activated by glutamate, which is released by a subpopulation of neurons stimulated in layer 4. Whisker stimulation in *in vivo* activated astrocytes with a variable delay of up to 9 s after the stimulus (Wang et al. 2006). In contrast, in our *in situ* approach, the electrical stimulation within the barrel triggered astrocyte Ca\(^{2+}\) transients, which peaked with a delay of 1–2 s after stimulation. This indicates that the astrocyte response to activity of layer 4 neurons has a defined delay.

**Astrocytes Selectively Respond to the Activity of Neurons Projecting from Layer 4 to Layer 2/3**

Our data reveal that astrocytes in the barrel cortex selectively respond to the activity of different neuronal cell types. When analyzing the pattern of reacting astrocytes, we found that astrocytes reacted exclusively within the "innervation domain" of spiny stellar neurons projecting from layer 4 to layer 2/3 (Lubke et al. 2003; Feldmeyer et al. 2006). Even when stimulating very close to the septum the astrocyte reaction was confined to the barrel and the column above the stimulated barrel. Excitatory activity in layer 4 spiny neurons spreads to pyramidal cells in layer 2/3 within confined "innervation domains" (Feldmeyer et al. 2002). Layer 2/3 neurons then also project to neighboring barrel columns. We indeed recorded neuronal activity across borders in layer 2/3, but in contrast to neuronal signaling the astrocyte response was restricted to a single "innervation domain" of layer 4 neurons. A similar pattern of neuronal activation after layer 4 stimulation was
observed by Berger et al. (2007). In this study, excitation also started in layer 4, spread in a columnar fashion into layer 2/3 and finally to neighboring columns along the horizontal axonal arboration of the layer 2/3 neurons (Berger et al. 2007).

Perpendicular to the barrel columns, neurons form large networks within layer 2/3 (Tucker and Katz 2003). These networks exhibit spontaneous oscillations driven by synchronized excitation and inhibition (Chagnac-Amitai and Connors 1989b). In our preparation we observed, that astrocytes do not respond to this type of neuronal signaling, even when the same cells within the "innervation domain" had reacted before. The selective reaction of astrocytes upon activity of layer 4 neurons is also supported by the observation that kainate- and NMDA-receptor antagonists, which would prevent activation of cells in layer 2/3, did not modify the astrocytic Ca\(^{2+}\) response in vivo (Wang et al. 2006).

Furthermore we analyzed the type of neurons involved in the spontaneous signaling in detail and determined the pattern of action potentials in neurons exhibiting the spontaneous Ca\(^{2+}\) activity in layer 2/3. The firing pattern of these neurons was found to be typical for pyramidal cells (Chagnac-Amitai and Connors 1989b). These cells are interconnected by excitatory synapses and it was recently reported that each layer 2/3 cells interacts with several hundred neurons by excitatory, glutamatergic connections (Feldmeyer et al. 2006). Our data demonstrate that astrocytes in the barrel cortex do not respond to spontaneous signaling of layer 2/3 pyramidal cells, which is driven by glutamatergic activity. Astrocytes were silent although they had shown a glutamate-mediated Ca\(^{2+}\) transient in response to the stimulation of layer 4 glutamatergic connections before. Also, after increasing the frequency of the network oscillations that are controlled by GABA\(_A\) receptor antagonists, we still did not observe an astrocytic reaction to this type of signaling. Moreover we were able to activate this layer 2/3 population of neurons with our stimulation paradigm and blocked this reaction with an AMPA/kainate receptor antagonist. Once again the astrocytes did not react to this type of glutamatergic transmission in layer 2/3, but astrocytic responses were restricted to the barrel column where the stimulation had occurred. Therefore, astrocytes do not only discriminate between spontaneous neuronal activity and activity elicited by an artificial stimulation but between 2 types of excitatory connections.

It was shown before that extensive whisker stimulation leads to an increase in astrocytic glutamate transporters expression and furthermore in the astrocytic ensheathment of excitatory synapses selectively in a stimulated barrel column (Genoud et al. 2006). We have not observed a different density of astrocytes or an obvious difference in astrocyte morphology, which could explain the selectivity in detecting neuronal activity. We speculate that a differential distribution of mGluR
When applying GABAAR antagonists, we observed that neuronal response in the barrel cortex increased by 42% (0.13 mm², SEM 0.011) to 0.18 (SEM 0.017); receptors also leads to a broadening of the signal. The area containing reacting cells in white). Bar = 100 μm. The cartoon in the inset shows the orientation of the slice and the location of the stimulation pipette. (A) Suramin application leads to a spread of the Ca²⁺ signal in both astrocytes and neurons across barrel borders. When compared to control, in the presence of suramin astrocytes and neurons also respond in neighboring barrels. Upper images show the neuronal reaction during the stimulation, lower images the astrocyte reaction 1 s after the stimulation (after background subtraction, reacting cells in white). Bar = 100 μm. The cartoon in the inset shows the orientation of the slice and the location of the stimulation pipette. (B) Histogram displaying the total area containing reacting astrocytes under control conditions and after application of CPT (4 μM), an A1 adenosine receptor antagonist. Blockade of A1 receptors also leads to a broadening of the signal. The area containing reacting astrocytes increased by 42% (0.13 mm², SEM 0.011) to 0.18 (SEM 0.017); P < 0.01 (paired t-test, n = 6 slices).}

**Figure 9.** Interfering with purinergic signaling relieves the restriction of the astrocyte reaction to barrel borders. (A) Suramin application leads to a spread of the Ca²⁺ signal in both astrocytes and neurons across barrel borders. When compared to control, in the presence of suramin astrocytes and neurons also respond in neighboring barrels. Upper images show the neuronal reaction during the stimulation, lower images the astrocyte reaction 1 s after the stimulation (after background subtraction, reacting cells in white). Bar = 100 μm. The cartoon in the inset shows the orientation of the slice and the location of the stimulation pipette. (B) Histogram displaying the total area containing reacting astrocytes under control conditions and after application of CPT (4 μM), an A1 adenosine receptor antagonist. Blockade of A1 receptors also leads to a broadening of the signal. The area containing reacting astrocytes increased by 42% (0.13 mm², SEM 0.011) to 0.18 (SEM 0.017); P < 0.01 (paired t-test, n = 6 slices).

on astrocyte processes or a selective ultrastructural interaction with defined synaptic regions may explain the selectivity of the astrocytic response.

**Inhibition Limits the Propagation of the Astrocyte Ca²⁺-Response**

When applying GABAAR antagonists, we observed that neuronal activity in response to stimulation in layer 4 spread laterally, crossing the border of the stimulated barrel column, and propagated into the neighboring columns. We assume that by a reduced neuronal inhibition, a larger population of layer 4 cells is recruited. Similar results come from a study using voltage sensitive dyes (Petersen and Sakmann 2001). Intracortical inhibition plays an important role in shaping sensory cortical receptive fields and feed-forward inhibition can prevent targeted cortical neurons from reaching the threshold during weak input (Swadlow and Gusev 2002). The astrocyte Ca²⁺-response to this enhanced layer 4 neuronal signaling involves a much larger population of astrocytes across all layers and borders of barrel columns. This finding demonstrates that the astrocytic reaction is not determined by morphological limitations. We also observed an increased frequency and extent of spontaneous neuronal activity in layer 2/3 after applying GABAAR antagonists. An astrocyte response to this type of neuronal activity remained absent supporting our view that astrocytes discriminate and selectively respond to the activity of a subpopulation of neurons.

Furthermore we found that interfering with purinergic and adenosine receptors leads to a comparable effect as interfering with GABAergic signaling. This leads to the assumption that purines have a prominent role in shaping the balance of excitation and inhibition within the barrel columns. A2 receptors are involved in the modulation of neuronal firing by adenosine (Phillis 1990) and thalamocortical excitation is regulated by presynaptic adenosine A1 receptors (Fontanez and Porter 2006). This provides a mechanism by which increased adenosine levels can directly reduce cortical excitability (Fontanez and Porter 2006).

**Astrocytic Function in the Barrel Cortex**

To date, there is no evidence how the astrocyte response may feed back on neuronal network activity in the somatosensory cortex. Our finding that astrocytes discriminate and selectively respond to the activity of a subpopulation of neurons, describes an important step in the definition of astrocytes as modulators of synaptic transmission. A very interesting aspect is our observation that purinergic transmission has a strong inhibitory effect on excitatory synaptic transmission in the somatosensory cortex. Recent work provides strong evidence that adenosine-mediated inhibition at glutamatergic synapses is due to tonic ATP release from astrocytes and subsequent degradation of ATP to adenosine in the extracellular space (Pascual et al. 2005; Serrano et al. 2006). We have previously shown in the mouse cortex that astrocyte Ca²⁺ activity is correlated with a release of ATP (Haas et al. 2006). Thus, purinergic signaling could be a mean how astrocytes contribute to the extent and duration of excitatory neuronal signaling as well as to mechanisms of synaptic plasticity.

In yet a completely different aspect, it is well accepted that astrocytes modulate plastic changes in the somatosensory cortex, since tenascin-deficient animals show a reduced plastic response in the somatosensory cortex after whisker deprivation (Cybulskia-Klosowicz et al. 2004). During the development in the first postnatal days, astrocytes secrete the extracellular matrix glycoprotein tenascin which delineates the barrel boundaries, but these boundaries also form in tenascin-deficient animals (Mitrovic et al. 1994; Irintchev et al. 2005). Thus, other mechanisms are much likely involved in the plasticity of cortical whisker representation: The purinergic system of glial signaling is a strong candidate as interfering with purinergic signaling modulates excitatory neuronal signal transmission in the barrel cortex.

**Supplementary Material**

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.
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