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Differential Mechanisms of Ca^{2+} Responses in Glial Cells Evoked by Exogenous and Endogenous Glutamate in Rat Hippocampus

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ABSTRACT: The mechanisms of Ca^{2+} responses evoked in hippocampal glial cells in situ, by local application of glutamate and by synaptic activation, were studied in slices from juvenile rats using the membrane permeant fluorescent Ca^{2+} indicator fluo-3AM and confocal microscopy. Ca^{2+} responses induced by local application of glutamate were unaffected by the sodium channel blocker tetrodotoxin and were therefore due to direct actions on glial cells. Glutamate-evoked responses were significantly reduced by the L-type Ca^{2+} channel blocker nimodipine, the group I/II metabotropic glutamate receptor antagonist (*S*)-α-methyl-4-carboxyphenylglycine (MCPG), and the *N*-methyl-D-aspartate (NMDA) receptor antagonist (±)2-amino-5-phosphonopentanoic acid (APV). However, glutamate-induced Ca^{2+} responses were not significantly reduced by the non-NMDA receptor antagonist 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX). These results indicate that local application of glutamate increases intracellular Ca^{2+} levels in glial cells via the activation of L-type Ca^{2+} channels, NMDA receptors, and metabotropic glutamate receptors. Brief (1 s) tetanization of Schaffer collaterals produced increases in intracellular Ca^{2+} levels in glial cells that were dependent on the frequency of stimulation (≥50 Hz) and on synaptic transmission (abolished by tetrodotoxin). These Ca^{2+} responses were also antagonized by the L-type Ca^{2+} channel blocker nimodipine and the metabotropic glutamate receptor antagonist MCPG. However, the non-NMDA receptor antagonist CNQX significantly reduced the Schaffer collateral-evoked Ca^{2+} responses, while the NMDA antagonist APV did not. Thus, these synaptically mediated Ca^{2+} responses in glial cells involve the activation of L-type Ca^{2+} channels, group I/II metabotropic glutamate receptors, and non-NMDA receptors. These findings indicate that increases in intracellular Ca^{2+} levels induced in glial cells by local glutamate application and by synaptic activity share similar mechanisms (activation of L-type Ca^{2+} channels and group I/II metabotropic glutamate receptors) but also have distinct components (NMDA vs. non-NMDA receptor activation, respectively). Therefore, neuron-glia interactions in rat hippocampus in situ involve multiple, complex Ca^{2+}-mediated processes that may not be mimicked by local glutamate application. *Hippocampus 2001;11:132–145.*

KEY WORDS: neuron-glia interactions; L-type Ca^{2+} channels; metabotropic glutamate receptors; NMDA receptors; non-NMDA receptors

INTRODUCTION

Until recently, glial cells were mostly considered passive cells, fulfilling neuronal growth and supportive functions. These roles include promoting neuronal migration by providing physical support (Hatten, 1990), maintaining ionic homeostasis by regulating the extracellular concentration of potassium during neuronal activity (Orkand et al., 1966), and providing neurotransmitter clearance (Rothstein et al., 1996).

The privileged localization of glial cells, tightly surrounding synapses (Smith, 1992; Ventura and Harris, 1999) and neuronal membranes (Smith, 1992), led to the hypothesis that glial cells could directly affect neuronal function and modulate synaptic activity. Many findings support such an active role of glial cells. First, many receptors for neurotransmitters have been identified on glial cells of the central nervous system (CNS) (Kimelberg, 1995; Vernadakis, 1996; Porter and McCarthy, 1997), and glial cells respond to these neurotransmitters (McCarthy and Salm, 1991; Shao and McCarthy, 1994; Porter and McCarthy, 1995; Duffy and MacVicar, 1995; Cai and Kimelberg, 1997). Second, glutamate or neuronal stimulation raises intracellular calcium levels in CNS glial cells (Cornell-Bell et al., 1990; Smith, 1992; Porter and McCarthy, 1995, 1996; Cai and Kimelberg, 1997; Pasti et al., 1997; Carmignoto et al., 1998; Shelton and McCarthy, 1999), often as intracellular calcium oscillations, spreading like waves across glial cells in culture (Cornell-Bell et al., 1990; Smith, 1992; Dani et al., 1992; Venance et al., 1997; Harris-White et al., 1998) and in situ (Pasti et al., 1997). These calcium waves are considered a form of intercellular communication (Smith, 1992) and have been shown to efficiently modulate synaptic activity (Hassinger et al., 1995; Pasti et al., 1997; Newman and Zahs, 1998; Kang et al., 1998; Araque et al., 1998a, 1999).

The types of ionotropic and metabotropic glutamate receptors, as well as Ca^{2+} channels, involved in these Ca^{2+} responses have been described for glial cells in cul-

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ture (MacVicar, 1984; MacVicar and Tse, 1988; Cornell-Bell et al., 1990; MacVicar et al., 1991; McCarthy and Salm, 1991; Shao and McCarthy, 1994; Steinhäuser and Gallo, 1996; Verkhratsky and Kettenman, 1996; Cai and Kimelberg, 1997). However, the responsiveness of glial cells in culture to transmitters, as well as the underlying mechanisms, differ from those of glial cells in situ or acutely isolated (Cai and Kimelberg, 1997; Kimelberg et al., 1997). Indeed, despite a general consensus that glutamate application and neuronal stimulation induce intracellular calcium rises in glial cells in hippocampal slices (Porter and McCarthy, 1995, 1996; Shelton and McCarthy, 1999; Pasti et al., 1997), a lack of glutamate responsiveness has also been reported (Duffy and MacVicar, 1995). In addition, the implication of voltage-dependent calcium channels in glutamate-induced calcium responses (MacVicar, 1984; MacVicar and Tse, 1988; MacVicar et al., 1991; Porter and McCarthy, 1995; Pasti et al., 1997; Carmignoto et al., 1998) and the respective contribution of ionotropic and metabotropic glutamate receptors in the stimulation-induced calcium responses (Porter and McCarthy, 1996; Pasti et al., 1997) also remain controversial for glial cells in situ.

To address the mechanisms that contribute to the responses of hippocampal glial cells to synaptic activity in situ, we characterized the Ca^{2+} responses evoked by Schaffer collateral stimulation and brief local applications of glutamate, using the membrane-permeant fluorescent Ca^{2+} indicator fluo-3AM and confocal microscopy in rat hippocampal slices, and examined the contribution of voltage-dependent calcium channels and different types of glutamate receptors to these responses. Our results suggest that L-type calcium channels and metabotropic glutamate receptors (mGluRs) are involved in both glutamate- and stimulation-induced Ca^{2+} responses, whereas *N*-methyl-D-aspartate (NMDA) glutamate receptors contribute significantly to glutamate-evoked responses, and non-NMDA glutamate receptors contribute to stimulation-induced responses.

MATERIALS AND METHODS

Hippocampal Slices

Transverse hippocampal slices were obtained from young (13–17 days) male Sprague-Dawley rats (Charles River, Montreal, Canada) as described previously (Carmant et al., 1997). Briefly, rats were anesthetized with halothane and decapitated with a guillotine. The brain was rapidly excised and placed in cold (4°–5°C) oxygenated (95% O_2 /5% CO_2) artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, 10 glucose, and 50 μM Trolox. A block of tissue containing the hippocampus was prepared, and transverse hippocampal slices (300 μm thickness) were obtained in ACSF at 4°C using a vibratome (Campden Instruments). Slices were transferred to a container filled with oxygenated ACSF at room temperature and allowed to recover for 1 h.

Confocal Microscopy and Ca^{2+} Imaging

Cells were loaded with the fluorescent Ca^{2+} indicator fluo3-AM (Molecular Probes, Eugene, OR) as described previously (Carmant et al., 1997). Slices were incubated for 1 h at 37°C in oxygenated ACSF containing 30 μM fluo3-AM, 0.5% dimethyl sulphoxide, and 0.02% pluronic acid (Molecular Probes). After the incubation period, slices were returned to oxygenated ACSF at room temperature for at least 1 h. For individual experiments, a slice was placed in a chamber mounted on the stage of an upright laser scanning confocal microscope (Olympus BH5 and BioRad MRC-600). The slice was submerged and continuously perfused with oxygenated ACSF (1–2 ml/min). Using confocal microscopy and a 40× water-immersion objective (0.75 NA), fluorescent glial cells were visually identified in stratum radiatum of the CA1 region, based on their small soma diameter (~10 μm) and their dispersed distribution. The 488-nm excitation line of the laser was attenuated to 1% of maximum power, and emission was detected through a barrier filter with cutoff at 515 nm. Similar ranges of gain (9–10) and black level (4.7–5.3) were used throughout experiments to standardize Ca^{2+} responses. For Ca^{2+} imaging, time-lapse fluorescent images were collected every 645 ms and recorded using MPL software (BioRad). The images were further analyzed off-line using Cfocal software (kindly provided by Dr. M.P. Charlton, University of Toronto). For each time-lapse image, the fluorescence intensity (F) was averaged over the soma area. One hundred to 300 control images were taken prior to glutamate application, and 20 prior to electrical stimulation. Changes in fluorescence (ΔF), induced by glutamate or electrical stimulation, were measured as relative changes from baseline fluorescence and expressed as

$$\%\Delta F/F = [(F_{post} - F_{rest})/F_{rest}] \times 100$$

For each Ca^{2+} response, the peak amplitude and the area under the response, calculated using a trapezoidal rule-based algorithm (SigmaPlot), were determined.

Glutamate was applied locally using a patch micropipette (tip diameter <2 μm) filled with ACSF containing 500 μM L-glutamic acid (Sigma). The pipette was positioned, under visual control, near an identified glial cell. Fluorescence measurements were taken from cells located within approximately 100 μm from the pipette tip. Glutamate was applied by micropressure (10–20 ms; 20–25 psi) using a Picospritzer (Medical Systems, Greenvale, NY). Cells usually responded to glutamate application with responses >50% ΔF/F and with a latency of ~3 s. Therefore, an arbitrary threshold of 10% ΔF/F and onset latency <6.45 s were set for selecting responsive cells. In addition, cells in which the Ca^{2+} response did not recover to baseline were discarded from the analysis.

Electrophysiology

An ultrasmall concentric bipolar electrode (Frederick Haer Co., Brunswick, ME) was placed in the stratum radiatum for electrical stimulation of Schaffer collaterals (100–900 μA, single pulses, 50–65 μs duration). A micropipette filled with ACSF was positioned in the CA1 pyramidal cell layer to record field potentials,

using an Axoclamp-2B amplifier (Axon Instruments, Inc.) in bridge mode. Ca^{2+} responses were evoked by 5–100 Hz trains (1-s duration) and were monitored in glial cells located in stratum radiatum (200 μm away from the stimulation electrode).

For patch-clamp recordings, pipettes were pulled from borosilicate glass (1 mm O.D., A-M Systems) and filled with (in mM) 145 K-methylsulfate, 1 $MgCl_2$, 8 NaCl, 2 ATP, 0.4 GTP, 10 HEPES, 1 EGTA, 0.15% biocytin, and 10–20 μM Oregon green BAPTA-I (Molecular Probes) titrated with KOH to pH 7.2–7.25 and adjusted to 275–285 mOsm (electrode resistance, 4–8 MΩ). Whole-cell recordings were obtained from glial cells using visible light illumination from an area of stratum radiatum, where fluorescent glial cells were identified using confocal microscopy. Current-clamp recordings were made using an Axoclamp-2B amplifier (Axon Instruments) in bridge mode. Signals were filtered at 1 KHz, digitized at 2 KHz (TL-1 Axon Instruments), stored on a PC, and analyzed using pClamp software. The bridge balance was regularly monitored and adjusted using the bridge circuit.

Histology

After whole-cell recordings, slices containing biocytin-filled cells were transferred to a freshly prepared solution of 4% paraformaldehyde in 0.1 M phosphate buffer and fixed for 4–12 h at 4°C. Slices were washed and stored in 0.1 M phosphate buffer for up to 2 weeks, and then embedded in agarose and resectioned at 50–80 μm thickness with a vibratome. The sections were then processed using the Vectastain ABC kit (Vecta Laboratories, Burlingame, CA), followed by nickel-intensification as previously described (Woodhall et al., 1999). Sections were then mounted in D.P.X. medium (distyrene, plasticizer, and xylene mixture) and examined under a light microscope.

Drugs

Chemicals were purchased from Sigma, unless indicated otherwise. Stock solutions were prepared of the NMDA antagonist (±)2-amino-5-phosphonopentanoic acid (APV; 7.7 mM), the non-NMDA antagonist 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX; 2 mM, Tocris-Cookson, Baldwin, MO), the group I/II mGluR antagonist (*S*)-α-methyl-4-carboxyphenylglycine (MCPG; 19 mM, Tocris-Cookson), and the L-type Ca^{2+} channel blocker nimodipine (200 μM, Research Biochemical International), and were stored frozen. On the day of the experiment, antagonists were diluted in ACSF to their final concentration (50 μM APV, 20 μM CNQX, 500 μM MCPG, and 2–4 μM nimodipine) and applied by perfusion. The Na^+ channel blocker tetrodotoxin (TTX; 775 nM) was freshly prepared.

Statistical Analysis

The statistical significance of differences between groups was assessed using one-way ANOVA for differences between three or more groups, and Student's *t*-tests or paired *t*-tests for differences between two groups. The level of significance was set at $P < 0.05$. When normality and variance tests failed, appropriate nonparametric tests (indicated in text) were performed. SigmaStat statistical software (SPSS) was used for all statistical tests. All measures are expressed as mean ± SEM.

RESULTS

Glial Cell Identification

Glial cells and interneurons were loaded by the calcium indicator fluo-3AM, using our procedure (e.g., Carmant et al., 1997). In stratum radiatum, labelled glial cells outnumbered interneurons, and were distinguished by their small soma size (~10 μm) and their widely dispersed distribution. To confirm that the selected cells were glial cells, whole-cell current-clamp recordings were obtained from a sample of cells selected using these criteria (n = 6; Fig. 1A). Consistent with known electrophysiological characteristics of glial cells, these cells had a highly negative resting membrane potential (−71 ± 2.2 mV) and a low input resistance (19 ± 6.6 MΩ) and did not fire action potentials following large depolarizing current injections (Fig. 1B). In contrast, whole-cell recordings obtained from visually identified interneurons in the same layers showed typical neuronal properties (Fig. 1D). Furthermore, visualization of biocytin-filled cells following whole-cell recording from single glial cells revealed an extensive network of labelled glial cells (Fig. 1C), as previously reported (D'Ambrosio et al., 1998). These results suggest that the fluo-3AM labelled cells selected using our criteria were glial cells.

Ca^{2+} Responses Evoked by Local Application of Glutamate

The effects of local application of glutamate on glial cells labelled with the fluorescent Ca^{2+} indicator fluo-3AM were first examined in normal ACSF. Glutamate evoked transient increases in fluorescence in labelled cells, reflecting rises in intracellular Ca^{2+} levels (Fig. 2). In 22 cells examined (n = 11 slices), the mean peak amplitude of glutamate-evoked Ca^{2+} responses was 174 ± 13% ΔF/F, and the mean area under the response was 1,403 ± 164% ΔF/F × s. These Ca^{2+} responses were not due to mechanical stimulation of the cells, since similar local application of ACSF alone failed to evoke Ca^{2+} responses (n = 20 cells; data not shown).

Glutamate-evoked Ca^{2+} responses were stable over time and could be reliably induced in the same cells by repeated glutamate applications at 15–30-min intervals (Fig. 2A). The mean peak amplitude and area under the Ca^{2+} response were not significantly different between a first glutamate application and subsequent applications 15 and 45 min later (n = 22 cells; ANOVA, $P > 0.6$; Fig. 2C). The stability of the glutamate-evoked Ca^{2+} responses allowed comparisons in different pharmacological conditions in the same cell.

To evaluate if glutamate-evoked responses involved direct or indirect actions of glutamate on glial cells, tetrodotoxin (TTX) was used to block voltage-dependent Na^+ channels and activation of neurons by glutamate. Glutamate-evoked Ca^{2+} responses were

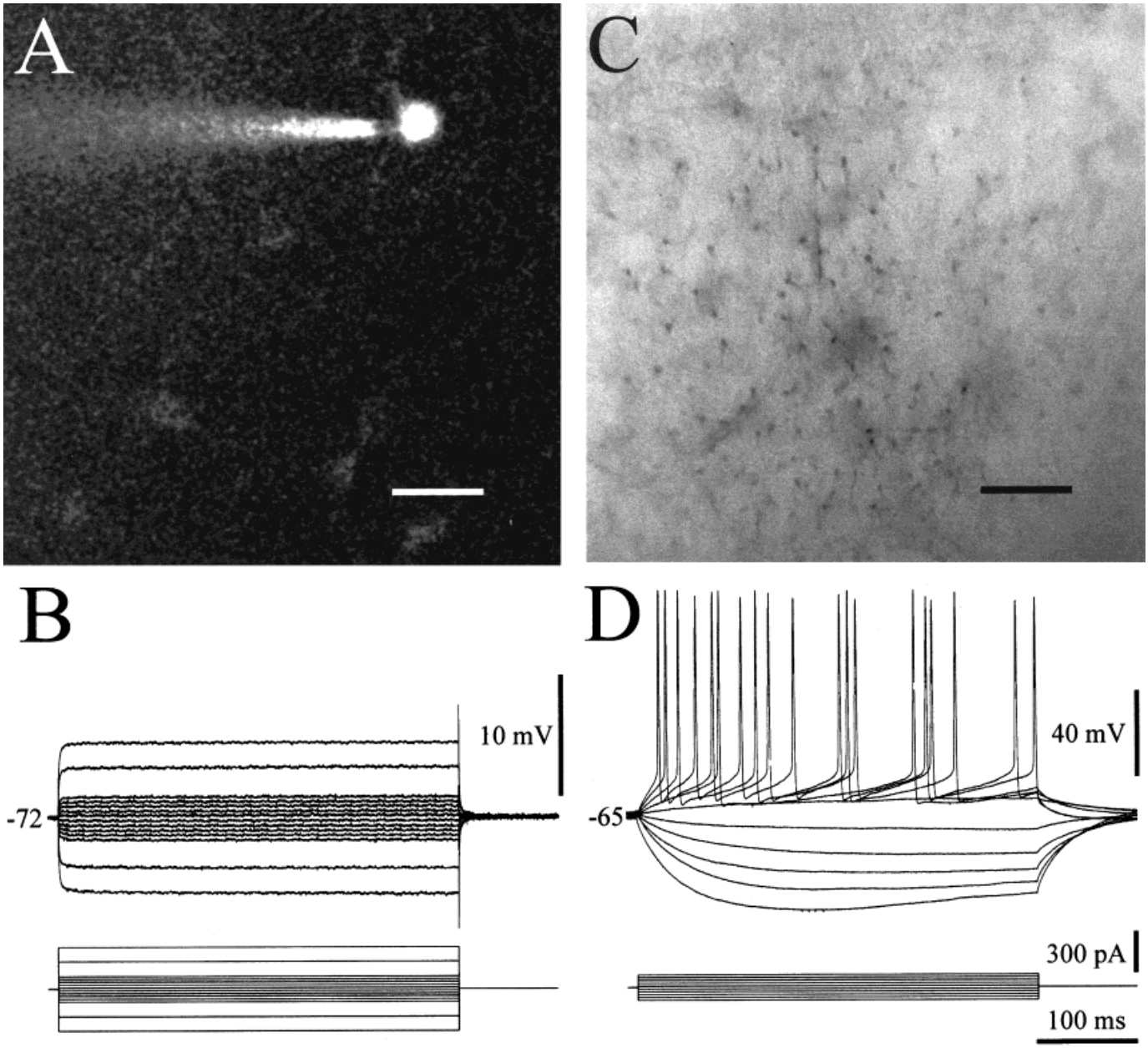

FIGURE 1. Electrophysiological and morphological identification of glial cells. A: Gray-scale confocal image, showing a selected glial cell during whole-cell recording with a patch pipette containing Oregon green BAPTA-1 in a slice labelled with fluo-3AM. B: Whole-cell recording from the cell in A, showing a highly negative resting membrane potential (−72 mV; top traces), a linear I–V profile, and an absence of action potentials following injection of large current pulses (bottom traces). C: Light microscopy image, revealing an extensive intercellular coupling of many glial cells labelled with biocytin following the filling of an individual glial cell during whole-cell recording in A and B. D: Membrane potential responses during whole-cell recording (top traces) from a visually identified interneuron in the stratum radiatum in response to current injection (bottom traces). Typical of neuronal responses, action potentials were elicited by small current injections of 30–90 pA. Scale bar represents 25 μm in A and 145 μm in C.

unaffected during bath application of 775 nM TTX (Fig. 2B). The mean peak amplitude and area under the Ca^{2+} response were not significantly different in control ACSF and TTX (n = 12 cells; Student's paired *t*-tests, $P > 0.8$; Fig. 2C). These results suggest that the glutamate-evoked Ca^{2+} responses were due to direct actions of glutamate on glial cells.

Glutamate-evoked Ca^{2+} responses were therefore further characterized in the presence of TTX. Three different types of Ca^{2+} responses were observed. In 92% of cells examined (99/108 cells), local glutamate application evoked a transient, monophasic increase in intracellular Ca^{2+} levels, with a mean onset latency of 3.5 ± 0.14 s, peak amplitude of 145 ± 5% ΔF/F, and area under the response of 1,213 ± 75% ΔF/F × s. In the other cells (9/108), glutamate-evoked responses consisted of an initial rise in Ca^{2+} levels, followed by a sustained plateau with a mean peak amplitude of 191 ± 24% ΔF/F and area under the response of 7,908 ±

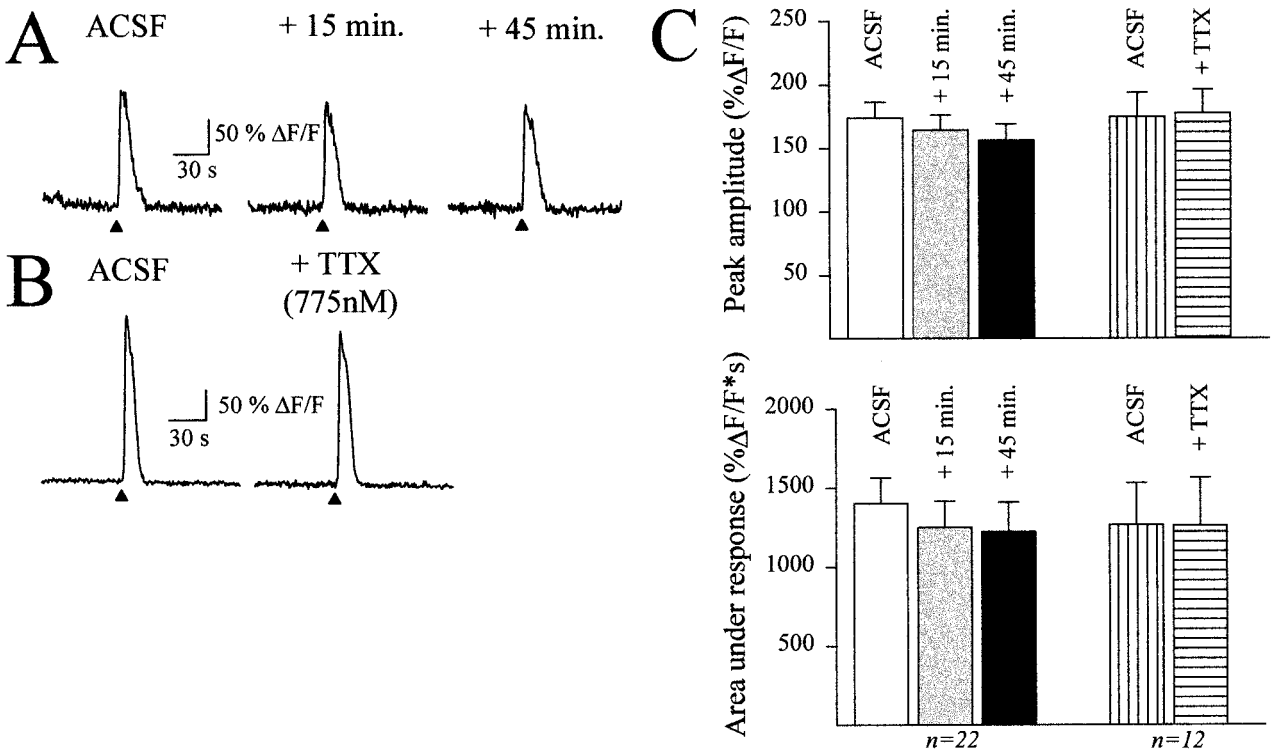

FIGURE 2. Stability of Ca^{2+} responses evoked in fluo-3AM-labelled glial cells by repeated applications of glutamate and insensitivity to tetrodotoxin (TTX). A: Representative example of Ca^{2+} responses evoked in the same cell by repeated glutamate applications (control ACSF, 15 and 45 min later). Ca^{2+} responses were similar after each application. Here and in subsequent figures, the time of application of glutamate is indicated by a triangle. B: In a different cell, the Ca^{2+} response evoked by glutamate in the presence of 775 nM TTX was similar to the response in control ACSF. C: Histograms of the mean peak amplitude (top) and area under Ca^{2+} responses (bottom) for all cells tested. Responses evoked by repeated glutamate applications were not significantly different (bars at left; n = 22 cells), and responses in ACSF were not significantly different from those in TTX (bars at right; n = 12 cells).

1,294% ΔF/F × s. Because of their rare occurrence, cells with plateau type of glutamate-evoked responses (area under Ca^{2+} response >4,000% ΔF/F × s) were excluded from further analysis. Finally, in some cells (n = 10), spontaneous repetitive increases in intracellular Ca^{2+} levels were observed during the control period (before glutamate application). Because spontaneous responses sometimes overlapped in time with evoked responses, these cells were also excluded from further analysis. In two cells, spontaneous Ca^{2+} responses occurred prior to placement of the drug micropipette in the slice, indicating that spontaneous oscillations were not caused by mechanical stimulation of glial cells during placement of the micropipette.

Mechanisms of Glutamate-Evoked Responses

The mechanisms underlying Ca^{2+} responses evoked by glutamate were next examined in the presence of TTX. Control experiments were first performed to verify that repeated glutamate applications evoked stable Ca^{2+} responses in TTX. In 27 cells tested, the mean peak amplitude and area under Ca^{2+} responses evoked by three successive applications of glutamate were not significantly different (Fig. 3A,D; one-way ANOVA, $P > 0.7$).

Since non-NMDA and NMDA receptors have been reported in acutely isolated and in situ hippocampal glial cells (Jabs et al., 1994; Porter and McCarthy, 1995; Seifert et al., 1997; Bezzi et al., 1998; Shelton and McCarthy, 1999), their contribution to glutamate-evoked Ca^{2+} responses was determined using the non-NMDA and NMDA antagonists, CNQX and APV, respectively. In 13 cells (n = 10 slices), the mean peak amplitude and area under Ca^{2+} responses in the presence of 20 μM CNQX were not significantly different from controls (Fig. 3B,D; Student's paired *t*-tests, $P > 0.6$). The lack of effect of CNQX was not due to an ineffective antagonism of non-NMDA receptors, since synaptically evoked field potentials in CA1 pyramidal cells were blocked by this concentration of CNQX (e.g., Fig. 7). These results suggest that Ca^{2+} responses produced in glial cells by local glutamate application did not involve non-NMDA glutamate receptors.

In contrast, in 30 cells tested in the presence of the NMDA antagonist (50 μM APV; Fig. 3C,D; n = 18 slices), the mean peak amplitude of Ca^{2+} responses was significantly reduced by 10% (Student's paired *t*-test, $P < 0.05$), and the area under the response by 22% (Wilcoxon signed rank test, $P < 0.05$). The APV effects were reversible, and Ca^{2+} responses recovered to control level in 16 cells tested (Fig. 3C,D; Student's paired *t*-test, $P > 0.6$). Hence, NMDA receptors contributed significantly to glutamate-evoked Ca^{2+} responses.

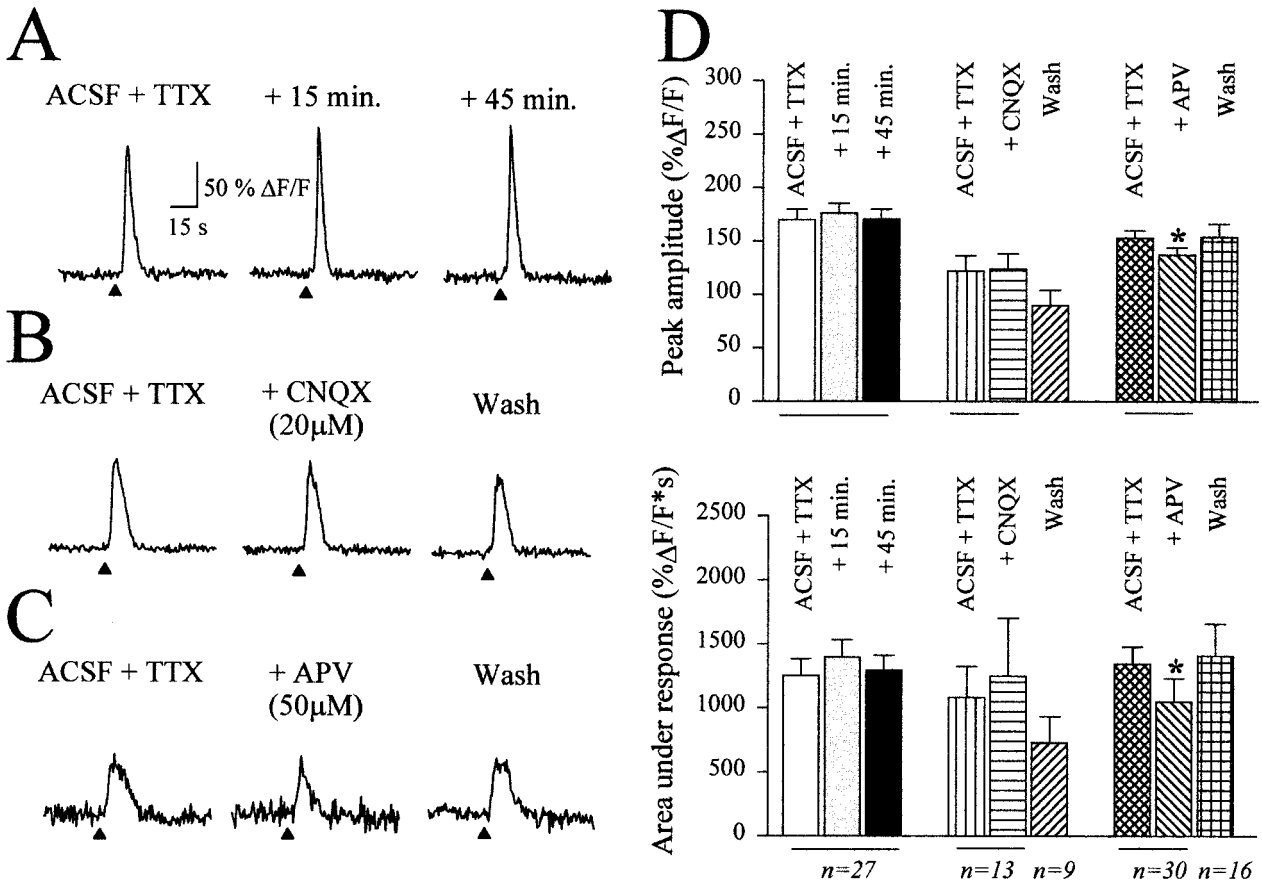

FIGURE 3. Partial antagonism of glutamate-evoked responses by the NMDA antagonist APV. A: Example of similar Ca^{2+} responses evoked in a glial cell by repeated local applications of glutamate in the presence of TTX. B: Example from a different cell of unchanged Ca^{2+} responses evoked by glutamate in the presence of 20 μM CNQX. C: Example from another glial cell of the partial reduction of the glutamate-evoked Ca^{2+} response in the presence of 50 μM APV (center). The antagonism of APV was reversible, and Ca^{2+} responses recovered to control levels after a 30-min washout in TTX (right). D: Histograms of mean peak amplitude (top) and area under Ca^{2+} responses (bottom) for all cells tested, showing unchanged responses with repeated glutamate applications (left bars) or in the presence of CNQX (middle bars), and the partial antagonism of responses in APV (right bars).

Metabotropic glutamate receptors have also been reported in acutely isolated and in situ hippocampal glial cells (Porter and McCarthy, 1995; Pasti et al., 1997; Cai and Kimelberg, 1997; Carmignoto et al., 1998; Shelton and McCarthy, 1999). Therefore, the effects of the group I/II mGluR antagonist MCPG (500 μM) on glutamate-evoked Ca^{2+} responses were also investigated. In 12 cells tested (6 slices), the group I/II mGluR antagonist significantly reduced Ca^{2+} responses, and in some cells completely blocked them (e.g., Fig. 4B). In the presence of MCPG, the mean peak amplitude and area under Ca^{2+} responses were significantly reduced by 49% and 77%, respectively (Fig. 4C; Student's paired *t*-tests, $P < 0.05$). The antagonistic effects of MCPG were reversible, and glutamate-evoked responses recovered to baseline levels following a 30-min washout in TTX (Fig. 4B,C; n = 5 cells, Student's paired *t*-tests, $P > 0.2$). These results indicate that group I/II mGluRs contribute markedly to the glutamate-evoked intracellular Ca^{2+} elevations.

Previous studies reported the presence of voltage-dependent calcium channels in hippocampal glial cells in situ (Porter and McCarthy, 1995; Duffy and MacVicar, 1996). We examined their contribution to glutamate-evoked Ca^{2+} responses, using the L-type Ca^{2+} channel antagonist nimodipine. Glutamate-evoked Ca^{2+} responses were partially blocked in the presence of 2–4 μM nimodipine (Fig. 4A). In 17 cells tested (11 slices), the mean peak amplitude was significantly reduced by 18% (paired Student's *t*-test, $P < 0.05$), and the area under the Ca^{2+} response by 30% (Fig. 4C; paired Student's *t*-test, $P < 0.05$, one-tailed). The partial block by nimodipine was reversible after a 30-min washout of the antagonist (Fig. 4A,C; n = 14 cells, paired Student's *t*-test, $P > 0.1$). These results indicate that voltage-dependent L-type Ca^{2+} channels are partly involved in glutamate-evoked Ca^{2+} responses in glial cells.

Ca^{2+} Responses Evoked by Stimulation of Schaffer Collaterals

To assess if a synaptically released transmitter produces similar responses in glial cells, Schaffer collaterals were electrically stimulated in stratum radiatum. First, the intensity of single-pulse stimulation was adjusted to evoke a field potential and population spike

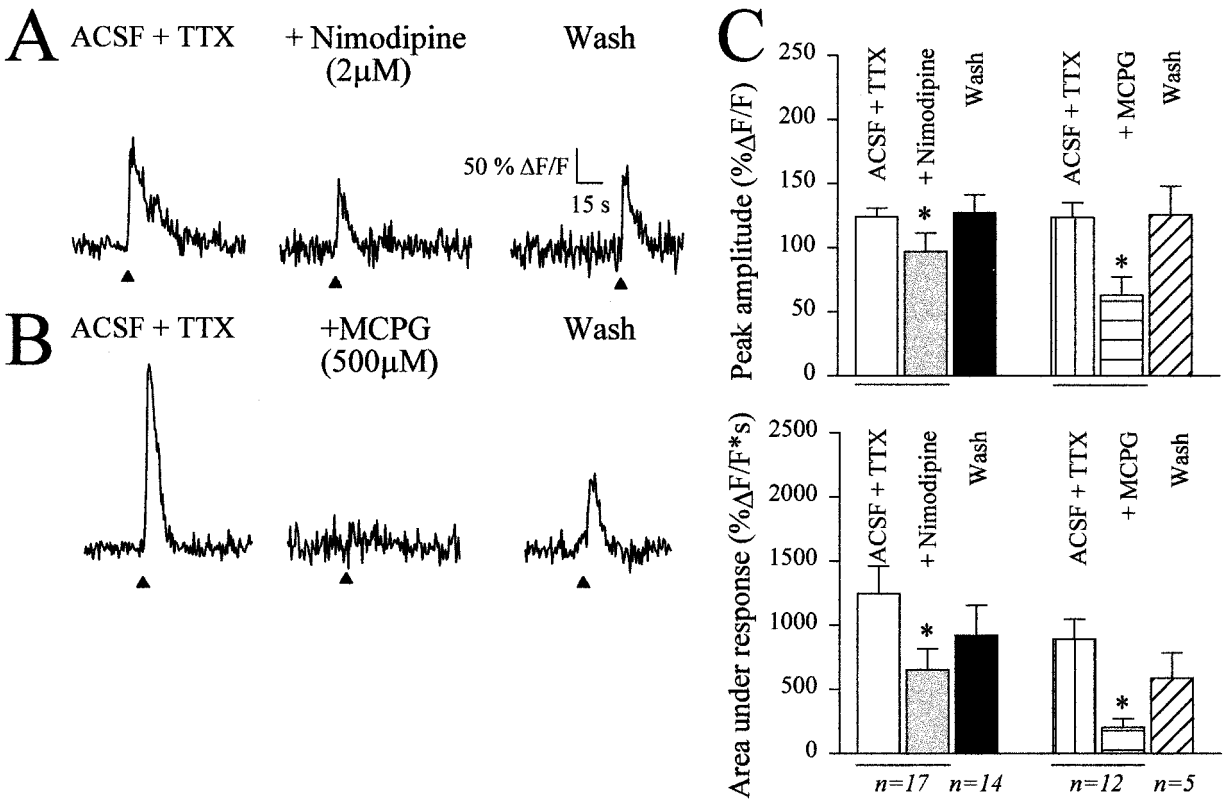

FIGURE 4. Antagonism of glutamate-evoked Ca^{2+} responses by the L-type Ca^{2+} channel antagonist nimodipine and the group I/II mGluR antagonist MCPG. A: Example of glutamate-evoked Ca^{2+} responses in a glial cell that were reversibly reduced in the presence of nimodipine. B: Example of a complete block of glutamate-evoked Ca^{2+} responses by the group I/II mGluR antagonist MCPG in a different glial cell. The block was partially reversible, and glutamate applications evoked Ca^{2+} responses after a 30-min washout in TTX. C: Histograms of mean peak amplitude (top) and area under Ca^{2+} responses (bottom) for all cells tested, showing the reversible antagonism of responses by nimodipine (left) and MCPG (right).

in the stratum pyramidale (e.g., Fig. 7A). A 1-s 100-Hz train was then given at this intensity, and fluorescence changes were examined in fluo-3AM-loaded glial cells in the stratum radiatum. Schaffer collateral stimulation evoked increases in intracellular Ca^{2+} levels in 44% of glial cells (58 cells in 18 slices; e.g., Fig. 7A). Compared to Ca^{2+} responses evoked by glutamate in ACSF, Schaffer collateral-evoked Ca^{2+} responses displayed a significantly smaller mean peak amplitude (125 ± 8% (F/F, Student's *t*-test, $P < 0.05$) and area under the response (538 ± 62% ΔF/F × s, Mann-Whitney rank sum test, $P < 0.05$). Schaffer collateral-evoked Ca^{2+} responses were of the transient monophasic type; plateau-type Ca^{2+} responses were not observed. Spontaneous oscillations in intracellular Ca^{2+} levels were observed in 11 glial cells, which were not considered any further.

Since direct electrical stimulation of glial cells in culture evokes intracellular Ca^{2+} rises (Nedergaard, 1994; Araque et al., 1998b), we used the Na^+ channel blocker tetrodotoxin to verify that stimulation-evoked Ca^{2+} responses were due to activation of Schaffer collaterals. In the presence of TTX, stratum radiatum-evoked field potentials were blocked (Fig. 5A), and tetanization of the stratum radiatum did not evoke Ca^{2+} responses in any glial cells examined (n = 27 cells in 4 slices; Fig. 5A). The block of synaptically evoked field potentials and Ca^{2+} responses by TTX was reversible. After a 30-min washout in ACSF, field potentials and Ca^{2+} responses were evoked by stratum radiatum stimulation in previously unresponsive slices (Fig. 5B). The mean peak amplitude and area under Ca^{2+} responses in these cells (n = 7 cells in 2 slices) were similar to those evoked in the group of cells in ACSF (Student's *t*-test, $P > 0.4$). These results indicate that Ca^{2+} responses evoked in glial cells by stratum radiatum stimulation involve activation of Schaffer collaterals and not direct stimulation of glial cells.

Frequency-Dependence of Schaffer Collateral-Evoked Ca^{2+} Responses

To examine if Ca^{2+} responses evoked by Schaffer collateral stimulation were frequency-dependent, 1-s trains of stimulation were applied at different frequencies (5, 25, and 50 Hz). With stimulation at 5 and 25 Hz, Ca^{2+} responses were not observed (Fig. 6A,B). However, at a stimulation frequency of 50 Hz, Ca^{2+} responses were observed in 59% of glial cells (n = 20 cells). The mean peak amplitude and area under these Ca^{2+} responses were not significantly different from those of responses evoked by 100-Hz stimulation (Fig. 6A,B, Student's *t*-tests, $P > 0.3$). These results indicate that Schaffer collateral-evoked rises in intracellular

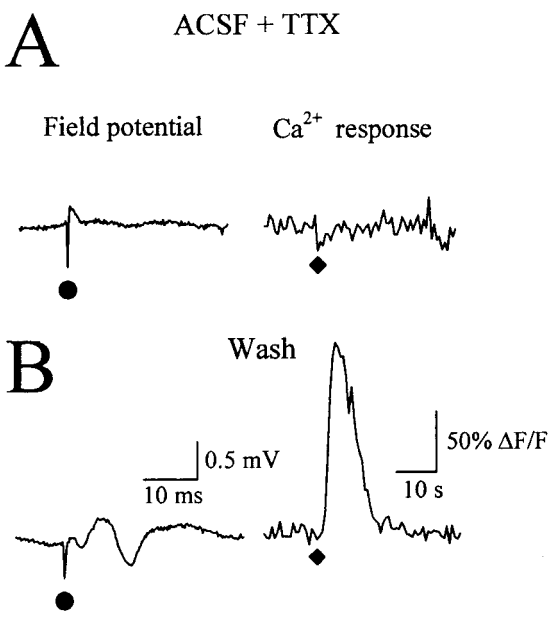

FIGURE 5. Reversible block of Schaffer collateral-evoked field potentials and glial Ca^{2+} responses by tetrodotoxin. A: In the presence of 775 nM TTX, single-pulse stimulation of the stratum radiatum did not evoke a field potential in stratum pyramidale (left trace), and tetanization of the stratum radiatum (100 Hz, 1 s) did not produce an increase in intracellular Ca^{2+} levels in glial cells (right trace). Here and in the following figures, the time of single-pulse stimulation is indicated by a solid circle (field potential), and of tetanization by a solid diamond (Ca^{2+} response). B: In the same slice, after a 40-min washout in ACSF, single-pulse stimulation now evoked a field potential and population spike (left trace). Similarly, tetanization of Schaffer collaterals evoked a typical Ca^{2+} response (right trace) in the glial cell that was previously unresponsive.

Ca^{2+} levels in glial cells are frequency-dependent and are recruited at activation rates of 50 Hz or more.

Mechanisms of Schaffer Collateral-Evoked Ca^{2+} Responses

Schaffer collateral-evoked Ca^{2+} responses exhibited some rundown with repeated stimulation (data not shown), in contrast to glutamate-evoked Ca^{2+} responses. Therefore, to examine the mechanisms involved, stimulation-evoked responses obtained in different pharmacological conditions were compared to responses obtained in the control group described above (100-Hz stimulation in ACSF; n = 58 cells in 18 slices; Fig. 7A,D). We examined the contributions of ionotropic glutamate receptors to Schaffer collateral-evoked responses, using NMDA and non-NMDA antagonists. In the presence of the NMDA antagonist APV (50 μM), single-pulse stimulation of Schaffer collaterals evoked field potentials similar to those in control ACSF (Fig. 7C). Tetanization of Schaffer collaterals elicited Ca^{2+} responses in 51% of glial cells examined in APV (n = 44 cells in 11 slices; Fig. 7C,D). The incidence of responsive cells in APV (mean number of responsive cells per slice) was not significantly different from that in ACSF (Fig. 8C; ANOVA). The mean peak amplitude and area under Ca^{2+} responses in APV were not significantly different from those of responses elicited in ACSF (Fig. 7C,D; multiple comparisons on ranks vs. control group, Dunn's method, $P > 0.05$). These results indicate that Schaffer collateral-evoked Ca^{2+} responses in glial cells do not involve NMDA receptors.

The role of non-NMDA receptors in Schaffer collateral-evoked Ca^{2+} responses was then investigated during application of the non-NMDA antagonist, CNQX (20 μM). Field potentials evoked by single-pulse stimulation were completely blocked in all slices tested in the presence of CNQX (Fig. 7B). In contrast, tetanization of Schaffer collaterals in CNQX evoked Ca^{2+} responses in 44% of glial cells. The mean number of responsive cells per slice was not significantly different from that in control ACSF (Fig. 8C). The Schaffer collateral-evoked Ca^{2+} responses, however, appeared smaller (Fig. 7B). The mean peak amplitude and area under Ca^{2+} responses were significantly reduced by 40% and 60%, respectively, in CNQX (multiple comparisons on ranks vs. control group, Dunn's method, $P < 0.05$; Fig. 7B,D). These results indicate that non-NMDA receptors contribute to Schaffer collateral-evoked rises in intracellular Ca^{2+} levels in glial cells.

To examine the contributions of mGluRs in Schaffer collateral-evoked Ca^{2+} responses, the group I/II mGluR antagonist MCPG was used. In four slices examined, single-pulse stimulation of Schaffer collaterals evoked a field potential and a population spike, as in control ACSF (Fig. 8B). However, tetanization of Schaffer collaterals failed to evoke Ca^{2+} responses in all but 1 of 30 glial cells examined (Fig. 8B,C). This incidence of responsive glial cells in MCPG (mean number of cells responding per slice) was significantly smaller than that of cells in control ACSF (Fig. 8C). This nearly complete block of Schaffer collateral-evoked Ca^{2+} responses in glial cells by MCPG indicates that group I/II mGluRs play an important role in these responses.

Finally, we determined the role of L-type Ca^{2+} channels in Ca^{2+} responses evoked by Schaffer collateral tetanization using nimodipine. Field potentials elicited by single-pulse stimulation of Schaffer collaterals, in slices exposed to nimodipine, were similar to those in slices in ACSF (Fig. 8A). In contrast, tetanization of Schaffer collaterals in the presence of 2 μM nimodipine evoked Ca^{2+} responses in only 2% of the cells examined (1 of 44 cells in 6 slices) (Fig. 8A,C). This incidence of responsive cells in nimodipine was significantly smaller than that in normal ACSF (Fig. 8C). This near-total block by nimodipine suggests that L-type Ca^{2+} channels are activated and participate importantly in Ca^{2+} responses of glial cells evoked by Schaffer collateral tetanization.

To verify that the blocks of Ca^{2+} responses by the antagonists nimodipine and MCPG were not artifactual, similar experiments were performed using their vehicle solutions, ethanol (18 cells in 5 slices) and NaOH (18 cells in 3 slices), respectively. Field potentials and Schaffer collateral-evoked Ca^{2+} responses were unaffected in these solutions. No significant differences were observed in the mean peak amplitude and area under the response for Ca^{2+} responses evoked in these vehicle solutions relative to responses in normal ACSF (ANOVA, $P > 0.1$; data not shown).

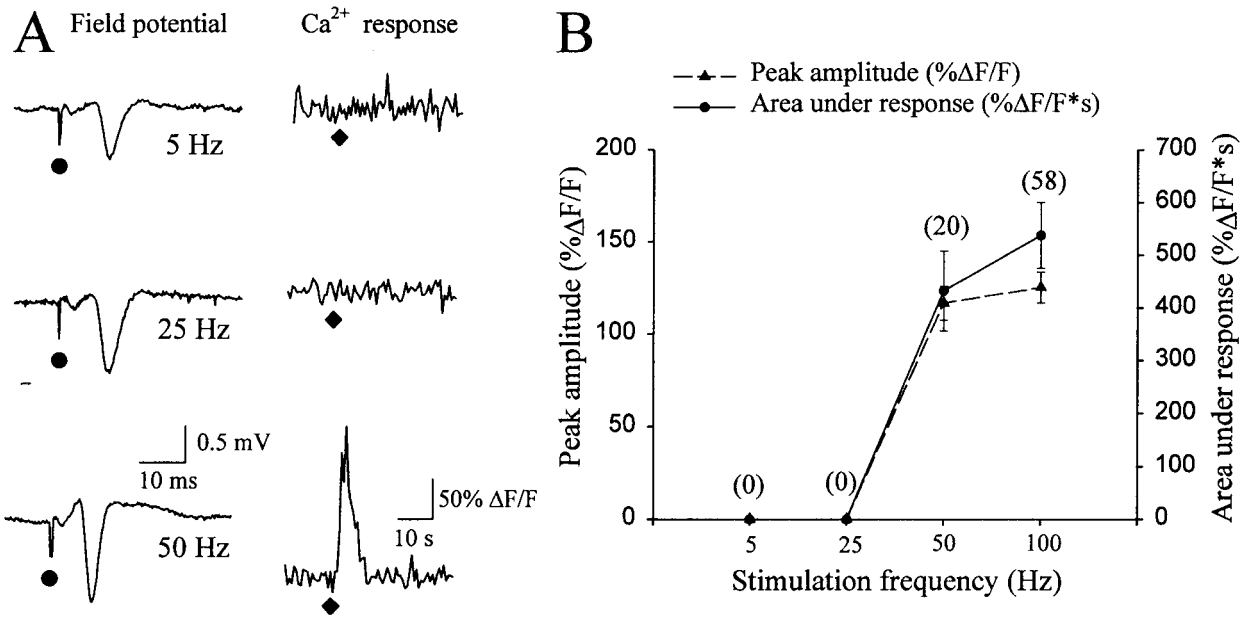

FIGURE 6. Frequency-dependence of Schaffer collateral-evoked Ca^{2+} responses. A: Single-pulse stimulation of Schaffer collaterals evoked field potentials in all slices (left traces). Stimulation of Schaffer collaterals with 1-s trains at 5 and 25 Hz (top and middle traces) failed to evoke Ca^{2+} responses, but stimulation at 50 Hz (bottom trace) evoked large responses (right traces). Examples at each frequency are from different slices. B: Graph of mean peak amplitude (solid triangles, broken line) and area under Ca^{2+} responses (solid circle, solid line) plotted as a function of stimulation frequency, for all cells tested. Ca^{2+} responses were recruited at stimulation frequencies ≥50 Hz, and were similar at 50 and 100 Hz. Number of responsive cells is indicated in parentheses.

DISCUSSION

In this study, the glutamate-dependent mechanisms involved in neuron-glia interactions in the rat hippocampus were investigated using Ca^{2+} imaging and confocal microscopy. Our results indicate that common mechanisms responsible for the production of synaptically and glutamate-induced Ca^{2+} responses in glial cells involve group I/II metabotropic glutamate receptors and L-type Ca^{2+} channel activation. In addition, the involvement of ionotropic glutamate receptors differs in the two conditions, with NMDA receptors participating in glutamate-induced Ca^{2+} rises, and non-NMDA receptors contributing to synaptically induced Ca^{2+} responses. Hence, these results indicate that synaptically and glutamate-evoked responses of glial cells involve complex Ca^{2+}-mediated processes and that experimental approaches accurately mimicking physiological conditions must be used to study neuron-glia interactions in situ.

Homogeneity of Glutamate-Induced Ca^{2+} Rises in CA1 Glial Cells

We observed that brief and local applications of glutamate evoked rapid transient Ca^{2+} responses in glial cells, which could be elicited reliably following multiple applications. Only occasionally were sustained or oscillatory Ca^{2+} responses observed. These results differ from previous observations using noncultured (in situ or acutely isolated) hippocampal glial cells where prolonged Ca^{2+} elevations and oscillatory responses were generally reported (Porter and McCarthy, 1995; Cai and Kimelberg, 1997; Shelton and McCarthy, 1999). This difference may be due to the different methods used for drug application. Previous studies of glutamate-induced glial Ca^{2+} responses used prolonged bath application of agonists, whereas brief and local applications were used in the present work. These methods likely result in different physiological consequences. Bath applications may produce more widespread activation of glutamate-dependent mechanisms in many different cell types. Other nonglutamatergic mechanisms may also be activated as a consequence of the widespread application. Bath applications will also produce a more gradual action, owing to the time required for the agonist to diffuse into the slice. Moreover, prolonged bath applications of agonist may lead to receptor desensitization, resulting in the activation of different glial mechanisms (Verkhratsky and Kettenman, 1996). Therefore, bath application may promote the activation of multiple mechanisms in numerous cell types. In contrast, local glutamate application may result in a briefer and more restricted drug application. This latter mode of application may simulate more accurately the synaptic release of glutamate from terminals. However, our results suggest that even local glutamate applications mimic only partially the release of glutamate by electrical stimulation, since their respective Ca^{2+} mechanisms showed some differences. This may be due in part to some activation of non-synaptic receptors by local glutamate application.

Involvement of L-Type Ca^{2+} Channels

Our results suggest a large contribution of L-type Ca^{2+} channels to glutamate- and stimulation-induced Ca^{2+} responses. The identification of L-type Ca^{2+} currents has been demonstrated in astro-

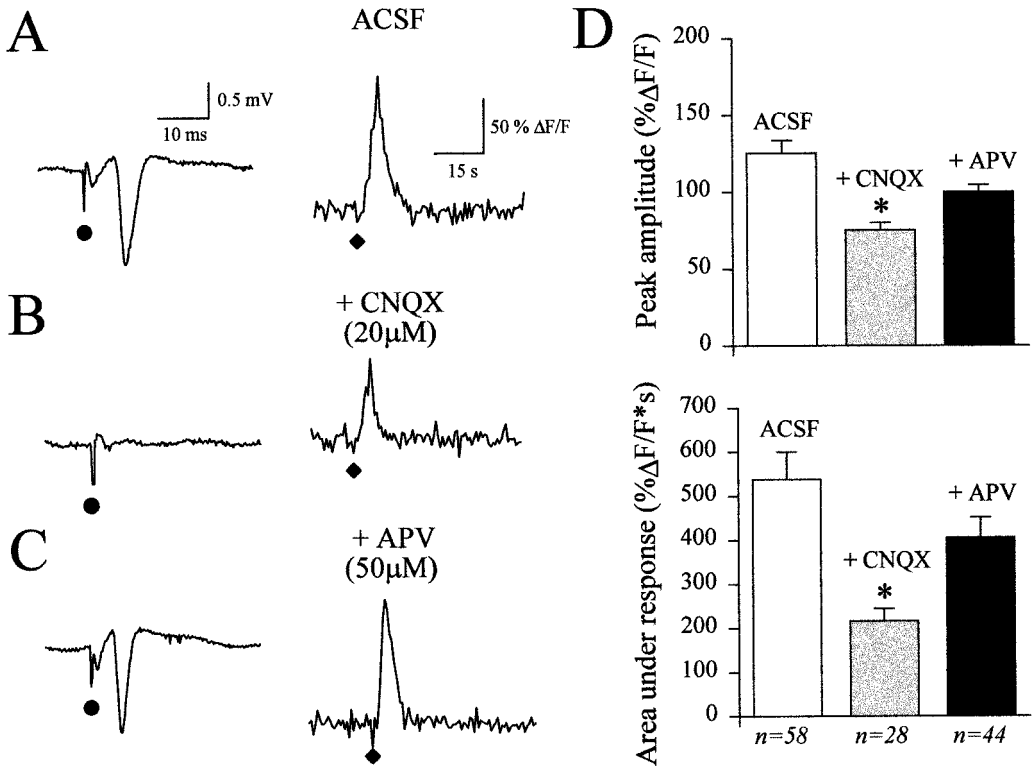

FIGURE 7. Partial antagonism of Schaffer collateral-evoked Ca^{2+} responses by the non-NMDA antagonist CNQX. A: Example of a field potential and population spike evoked by single-pulse stimulation of Schaffer collaterals (left trace) and the Ca^{2+} response evoked by tetanization in a glial cell of the same slice (right trace) in control ACSF. B: Responses in a different slice in the presence of 20 μM CNQX. Field potentials evoked by Schaffer collateral stimulation were blocked (left trace), but a Ca^{2+} response was still evoked in glial cells by tetanization of Schaffer collaterals in the same slice (right trace). The Ca^{2+} response in CNQX was smaller relative to that in control ACSF. C: Responses in another slice in the presence of the NMDA antagonist APV (50 μM). Single-pulse stimulation of Schaffer collaterals evoked a field potential and population spike (left trace). Tetanization elicited a Ca^{2+} response similar to those in control ACSF. D: Histograms of the mean peak amplitude (top) and area under Ca^{2+} responses (bottom) for all cells tested in control ACSF (open bars), CNQX (gray bars) and APV (solid bars). Ca^{2+} responses were significantly reduced in CNQX relative to controls.

cytes in culture (MacVicar, 1984; MacVicar and Tse, 1988; MacVicar et al., 1991), after acute isolation (Barres et al., 1990; Duffy and MacVicar, 1994) and in situ (Porter and McCarthy, 1995; Duffy and MacVicar, 1996). However, it has been argued that the inhibition of astrocytic Ca^{2+} rises observed in glial cells in situ in the presence of L-type Ca^{2+} channel antagonists reflects an indirect effect of partial inhibition of neuronal glutamate release (Carmignoto et al., 1998). However, in our study, glutamate-induced Ca^{2+} responses were not affected by the presence of TTX. Thus, glial Ca^{2+} responses are not dependent on depolarization during action potentials activating neuronal L-type Ca^{2+} channels. Their activation also does not depend on non-NMDA receptor-mediated depolarization, since CNQX did not affect glutamate-induced Ca^{2+} responses. As NMDA receptors are involved in glial Ca^{2+} responses evoked by glutamate, glial cell depolarization mediated by NMDA receptors may lead to activation of voltage-dependent L-type channels and Ca^{2+} entry. Furthermore, nimodipine strongly affected Ca^{2+} responses induced by synaptic stimulation, even though the size and shape of field potentials were not affected. This indicates that the blockade of glial Ca^{2+} responses cannot be explained by a reduction in synaptic transmission. Hence, our results indicate that glial L-type Ca^{2+} channels contribute directly to Ca^{2+} responses induced by both glutamate and synaptic activation.

Involvement of Metabotropic Glutamate Receptors in Glial Ca^{2+} Responses

Similar types of ionotropic and metabotropic glutamate receptors appear present in glial cells in culture, in situ, or after acute dissociation. In general, it is reported that glial cells express functional non-NMDA (Backus et al., 1989; Cornell-Bell et al., 1990; Jensen and Chiu, 1991; Jabs et al., 1994; Porter and McCarthy, 1995; Steinhäuser and Gallo, 1996; Seifert et al., 1997; Bezzi et al., 1998; Shelton and McCarthy, 1999) and metabotropic (Porter and McCarthy, 1995; Pasti et al., 1997; Cai and Kimelberg, 1997; Carmignoto et al., 1998; Shelton and McCarthy, 1999) glutamate receptors, but that they lack NMDA receptors (Porter and McCarthy, 1995; Steinhäuser and Gallo, 1996; Pasti et al., 1997; Cai and Kimelberg, 1997; Carmignoto et al., 1998; Shelton and McCarthy, 1999). Our results also stress the importance of metabotropic receptors in the regulation of Ca^{2+} processes in glial cells, as indicated by the large reduction of Ca^{2+} responses by the group I/II metabotropic glutamate receptor antagonist MCPG. This sup-

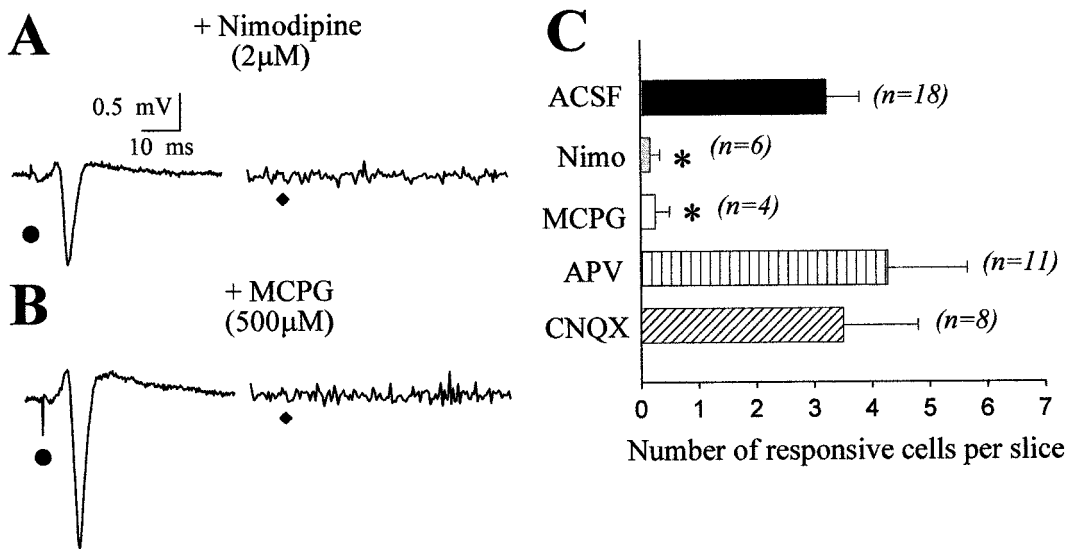

FIGURE 8. Near-complete block of Schaffer collateral-evoked Ca^{2+} responses by the L-type Ca^{2+} channel antagonist nimodipine and the group I/II mGluR antagonist MCPG. A: Responses in a slice exposed to 2 μM nimodipine. Single-pulse stimulation of Schaffer collaterals evoked a field potential and population spike (left trace), as in control ACSF. However, in the same slice, tetanization of Schaffer collaterals failed to evoke a Ca^{2+} response (right trace). B: Responses in another slice exposed to 500 μM MCPG. Single-pulse stimulation of Schaffer collaterals evoked a field potential (left trace). In the same slice, tetanization of Schaffer collaterals did not elicit any Ca^{2+} elevation in glial cells (right trace). C: Histograms of the mean number of glial cells per slice which responded to tetanization of Schaffer collaterals with an increase in intracellular Ca^{2+} levels. The number of responsive cells was significantly smaller in nimodipine (gray bar) and MCPG (open bar) than in control ACSF (solid bar), but was unchanged in APV (bar with vertical stripes) and CNQX (bar with diagonal stripes). The number of slices tested in each group is indicated in parentheses.

ports previous findings which identified functional mGluRs in hippocampal glial cells in situ (Porter and McCarthy, 1995; Pasti et al., 1997; Carmignoto et al., 1998; Shelton and McCarthy, 1999) and the expression of mGluR3 and mGluR5 mRNA in acutely isolated hippocampal astrocytes (Schools and Kimelberg, 1999). Field potentials were not significantly affected by MCPG, indicating that it is unlikely that the blockade of Ca^{2+} responses was an indirect consequence of a decrease of synaptic activity, and probably represents a direct effect on glial mGluRs. This assumption is further supported by our observation that MCPG considerably reduced glutamate-induced Ca^{2+} rises when synaptic activity was already blocked by TTX. Although L-type Ca^{2+} channels, as well as NMDA and non-NMDA receptors, also appear involved in glial Ca^{2+} responses, their role appears more minor than that of mGluRs, since the most robust block of glial Ca^{2+} responses was obtained using a mGluR antagonist.

Role of Non-NMDA Ionotropic Glutamate Receptors in Glial Ca^{2+} Responses

Consistent with previous studies, our results show that non-NMDA ionotropic receptors are involved in synaptically activated Ca^{2+} responses of glial cells (Porter and McCarthy, 1996). This may be due to a direct effect on glial non-NMDA receptors, or to an indirect effect because of a reduction of neuronal synaptic responses, as revealed by the block of field EPSPs. Although the non-NMDA antagonist CNQX reduced the magnitude of stimulation-induced Ca^{2+} increases, it did not impair the ability of glial cells to respond, as indicated by the unchanged incidence of responsive cells. Thus, non-NMDA receptors appear important but not necessary for the activation of Ca^{2+} responses in glial cells.

Unlike in previous reports (Porter and McCarthy, 1995; Shelton and McCarthy, 1999), Ca^{2+} responses elicited by glutamate were not affected by the non-NMDA antagonist CNQX. The differences may be related to the techniques used. The studies identifying non-NMDA glial receptors used bath application of selective agonists, whereas we used local application of glutamate in the presence of specific antagonists. As mentioned earlier, bath application of agonists will produce a widespread activation of several neuronal and glial elements, resulting in a more general activation of multiple and complex glutamate mechanisms. Furthermore, bath application of glutamate is more likely to have effects related to uptake. Finally, glutamate is an endogenous neurotransmitter, and its use in combination with antagonists may reflect more closely the contribution of different receptor types in situ.

Role of NMDA Glutamate Receptors in Glial Ca^{2+} Responses

Our results indicate that NMDA receptors contribute to glutamate-induced but not synaptically activated glial Ca^{2+} responses. It is generally believed that glial cells do not have functional NMDA receptors, since NMDA-induced Ca^{2+} responses were not observed in hippocampal glial cells from mature rats (Shelton and McCarthy, 1999). When observed, NMDA responses were attributed to indirect glutamate release following activation of neuronal NMDA receptors, because responses could be antagonized

by TTX (Shao and McCarthy, 1997). However, NMDA responses in glial cells appear developmentally regulated. TTX-resistant NMDA-induced Ca^{2+} responses were reported in glial cells in hippocampal slices of young rats (<12 days; Porter and McCarthy, 1995) but not of mature rats (Shelton and McCarthy, 1999). In addition, in rat spinal cord, NMDA-induced currents in glial cells gradually decline during development (Ziak et al., 1998). In the present study, the findings that NMDA receptors are involved in glutamate-induced but not synaptically activated glial Ca^{2+} responses indicate that hippocampal glial cells of juvenile rats (13–17 days) have functional NMDA receptors, but these were not activated under the physiological conditions of synaptic stimulation tested. It may be interesting to evaluate in future experiments if these APV-sensitive glutamate-evoked glial Ca^{2+} responses disappear during development. In further support of functional NMDA receptors in glial cells in juvenile slices, preliminary evidence indicates that bath application of NMDA induces Ca^{2+} responses in glial cells that are partially but not completely reduced in TTX, supporting both direct and indirect NMDA effects in glial cells (Haddjeri et al., 2000). Finally, it is also possible that NMDA receptors responsible for glutamate-induced Ca^{2+} responses in glial cells are localized at extrasynaptic sites, and thus would not be accessible to synaptically released glutamate.

The mechanisms of NMDA-induced Ca^{2+} rises in glial cells remain to be elucidated. Conceivably, both direct influx through NMDA channels, as well as indirect actions via cell depolarization and the opening of voltage-dependent Ca^{2+} channels, could contribute. However, the Mg^{2+} block of NMDA channels should not be functional for significant Ca^{2+} influx to occur without activation of non-NMDA channels (e.g., Bardoni et al., 2000). Also, an indirect NMDA action via voltage-dependent Ca^{2+} channels does not appear well supported by our observations, since NMDA antagonism was less effective in reducing Ca^{2+} responses than was an L-type Ca^{2+} channel antagonist.

Functional Properties of Glial Ca^{2+} Responses Evoked by Schaffer Collateral Stimulation

Our observations of Ca^{2+} elevations in glial cells evoked by Schaffer collateral stimulation demonstrate the existence of neuron-glia interactions in the rat hippocampus. These results are consistent with the few studies which demonstrated that glial cells in culture (Dani et al., 1992) and in situ (Porter and McCarthy, 1996; Pasti et al., 1997; Grosche et al., 1999) respond to synaptic activity with a Ca^{2+} increase. Similar to responses induced by local application of glutamate, synaptically activated Ca^{2+} responses were transient, and no prolonged or oscillatory responses were observed. This indicates that local application of glutamate may mimic in part physiological transmitter release (i.e., a brief and high level of neurotransmitter).

The active contribution of glial cells to the regulation of neuronal function will be directly related to the properties of glial responses and their sensitivity to synaptic activity. In the present study, we found that glial Ca^{2+} responses could not be elicited with stimulation frequencies of 25 Hz or less, indicating that a high level of glutamate is required to activate these Ca^{2+}-dependent mechanisms in glial cells. This is consistent with previous observations of Ca^{2+} responses induced only with sustained stimulation at moderate frequency (Dani et al., 1992) or brief and intense stimuli (Porter and McCarthy, 1996; Pasti et al., 1997). The frequency dependence of synaptically elicited glial Ca^{2+} responses further suggests that these responses are unlikely to involve synaptically activated depolarizing glutamate transporter currents, since they are evoked in glial cells with low-frequency stimulation (Bergles and Jahr, 1997), whereas glial Ca^{2+} responses appeared at higher frequencies of stimulation (50–100 Hz). However, experiments with antagonists of glutamate transporter currents could be useful in directly testing their involvement.

Another important characteristic of stimulation-induced Ca^{2+} responses was the rundown of responses with successive episodes of stimulation. This indicates that Ca^{2+}-dependent glial mechanisms involved in neuron-glia interactions may not be reactivated readily. It is unlikely that the rundown was due to receptor desensitization, since repeated local glutamate applications reliably induced Ca^{2+} responses. Interestingly, Ca^{2+} responses induced by local application of glutamate were significantly larger than those elicited by synaptic activity, suggesting that different elements may be involved in the production of both types of glial Ca^{2+} responses. A possible explanation is that other neurotransmitters are likely to be released along with glutamate during high-frequency stimulation. For instance, neurotransmitters such as peptides, ATP, or adenosine may be released by stratum radiatum stimulation (Jonzon and Fredholm, 1985). In addition, stimulation in the stratum radiatum will activate GABA interneurons. The release of GABA during repetitive stimulation may contribute to, or interact with, Ca^{2+} responses in glial cells, since Ca^{2+} responses are produced in hippocampal glial cells by $GABA_A$ receptor-mediated Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Fraser et al., 1995), and by activation of $GABA_B$ receptors (Kang et al., 1998).

Functional Implications for Neuron-Glia Interactions

The results presented in this study, and those of others (Robitaille, 1998; reviewed in Araque et al., 1999), clearly present glial cells as active elements that detect neuronal activity and respond to synaptic transmission. Because glial cells can release various neuromodulatory substances and because the synthesis of these substances is often Ca^{2+}-dependent (Martin, 1992), a functional consequence of synaptically induced Ca^{2+} elevation in glial cells may be the production of such neuromodulatory substances and consequent effects on neuronal properties and synaptic activity. In fact, there is recent evidence of calcium-induced neurotransmitter release by glial cells (Bezzi et al., 1998). Furthermore, the frequency-dependence of synaptically evoked Ca^{2+} responses indicates that Ca^{2+}-dependent glial neuromodulatory mechanisms in situ may only be recruited during repetitive activity or with higher levels of transmitter release. Evidently, other non-Ca^{2+}-mediated neuron-glia interactions may also occur independently under other conditions. The rundown of synaptically evoked glial Ca^{2+} responses suggests that these processes may not be capable of rapid reactivation. An interesting possibility is that Ca^{2+} elevation in

glial cells may result in long-lasting effects. Indeed, our data demonstrate the critical role of mGluR activation in these responses, and hence, a likely involvement of cascades of long-lasting events controlled by G-protein activation.

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REFERENCES

Araque A, Parpura V, Sanzgiri RP, Haydon PG. 1998a. Glutamate-dependent astrocyte modulation of synaptic transmission between cultured hippocampal neurons. Eur J Neurosci 10:2129–2142.

Araque A, Sanzgiri RP, Parpura V, Haydon PG. 1998b. Calcium elevation in astrocytes causes an NMDA receptor-dependent increase in the frequency of miniature synaptic currents in cultured hippocampal neurons. J Neurosci 18:6822–6829.

Araque A, Parpura V, Sanzgiri RP, Haydon PG. 1999. Tripartite synapses: glia, the unacknowledged partner. Trends Neurosci 22:208–215.

Backus KH, Kettenman H, Schachner M. 1989. Pharmacological characterization of the glutamate receptor in cultured astrocytes. Neurosci Res 22:274–282.

Bardoni R, Magherini PC, MacDermott AB. 2000. Activation of NMDA receptors drives action potentials in superficial dorsal horn from neonatal rats. Neuroreport 11:1721–1727.

Barres BA, Koroshetz WJ, Chun L, Corey D. 1990. Ion channel expression by white matter glia: the type-1 astrocyte. Neuron 5:527–544.

Bergles DE, Jahr CE. 1997. Synaptic activation of glutamate transporters in hippocampal astrocytes. Neuron 19:1297–1308.

Bezzi P, Carmignoto G, Pasti L, Vesce S, Rossi D, Rizzini BL, Pozzan T, Voltera A. 1998. Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. Nature 391:281–285.

Cai Z, Kimelberg HK. 1997. Glutamate-receptor-mediated calcium responses in acutely isolated hippocampal astrocytes. Glia 21:380–389.

Carmant L, Woodhall G, Ouardouz M, Robitaille R, Lacaille JC. 1997. Interneuron-specific Ca^{2+} responses linked to metabotropic and ionotropic glutamate receptors in rat hippocampal slices. Eur J Neurosci 9:1625–1635.

Carmignoto G, Pasti L, Pozzan T. 1998. On the role of voltage-dependent calcium channels in calcium signaling of astrocytes *in situ*. J Neurosci 18:4637–4645.

Cornell-Bell AH, Finkbeiner SM, Cooper MS, Smith SJ. 1990. Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. Science 247:470–473.

D'Ambrosio R, Wenzel J, Schwartzkroin PA, McKhann GM II, Janigro D. 1998. Functional specialization and topographic segregation of hippocampal astrocytes. J Neurosci 18:4425–4438.

Dani JW, Chernjavsky A, Smith S. 1992. Neuronal activity triggers calcium waves in hippocampal astrocytes networks. Neuron 8:429–440.

Duffy S, MacVicar BA. 1994. Potassium-dependent calcium influx in acutely isolated hippocampal astrocytes. Neuroscience 61:51–61.

Duffy S, MacVicar BA. 1995. Adrenargic calcium signaling in astrocyte networks within the hippocampal slice. J Neurosci 15:5535–5550.

Duffy S, MacVicar BA. 1996. In vitro ischemia promotes calcium influx and intracellular calcium release in hippocampal astrocytes. J Neurosci 16:71–81.

Fraser DD, Duffy S, Angelides KJ, Perez-Velazquez JL, Kettenmann H, MacVicar BA. 1995. $GABA_A$/benzodiazepine receptors in acutely isolated hippocampal astrocytes. J Neurosci 15:2720–2732.

Grosche J, Matyash V, Möller T, Verkhratsky A, Reichenbach A, Kettenman H. 1999. Microdomains for neuron-glia interaction: parallel fiber signaling to Bergmann glial cells. Nat Neurosci 2:139–143.

Haddjeri N, Lacaille JC, Robitaille R. 2000. $GABA_B$-mediated activation of glial cells in NMDA-induced presynaptic depression in the rat hippocampus. Soc Neurosci Abstr 26:891.

Harris-White ME, Zanotti SA, Frautschy SA, Charles AC. 1998. Spiral intercellular calcium waves in hippocampal slice cultures. J Neurophysiol 79:1045–1052.

Hassinger TD, Atkinson PB, Strecker GJ, Whalen LR, Dudek FE, Kossel AH, Kater SB. 1995. Evidence for glutamate-mediated activation of hippocampal neurons by glial calcium waves. J Neurobiol 28:159–170.

Hatten ME. 1990. Riding the glial monorail: a common mechanism for glial-guided neuronal migration in different regions of the developing mammalian brain. Trends Neurosci 13:179–184.

Jabs R, Kirchhoff F, Kettenman H, Steinhauser C. 1994. Kainate activates Ca^{2+}-permeable glutamate receptors and blocks voltage-gated K^+ currents in glial cells of mouse hippocampal slices. Pflugers Arch 426: 310–319.

Jensen AM, Chiu SY. 1991. Differential intracellular calcium responses to glutamate in type 1 and type 2 cultured brain astrocytes. J Neurosci 11:1674–1684.

Jonzon B, Fredholm BB. 1985. Release of purines, noradrenaline, and GABA from rat hippocampal slices by field stimulation. J Neurochem 44:217–224.

Kang J, Jiang L, Goldman SA, Nedergaard M. 1998. Astrocyte-mediated potentiation of inhibitory synaptic transmission. Nat Neurosci 1:683–692.

Kimelberg HK. 1995. Receptors on astrocytes—what possible functions? Neurochem Int 26:27–40.

Kimelberg HK, Cai Z, Rastogi P, Charniga CJ, Goderie S, Dave V, Jalonen TO. 1997. Transmitter-induced calcium responses differ in astrocytes acutely isolated from rat brain and in culture. J Neurochem 68:1088–1098.

MacVicar BA. 1984. Voltage-dependent calcium channels in glial cells. Science 226:1345–1347.

MacVicar BA, Tse FWY. 1988. Norepinephrine and cyclic adenosine 3′:5′-cyclic monophosphate enhance a nifedipine-sensitive calcium current in cultured astrocytes. Glia 1:359–365.

MacVicar BA, Hochman D, Dealy MJ, Weiss S. 1991. Modulation of intracellular Ca^{2+} in cultured astrocytes by influx through voltage-activated Ca^{2+} channels. Glia 4:448–455.

Martin DL. 1992. Synthesis and release of neuroactive substances by glial cells. Glia 5:81–94.

McCarthy KD, Salm AK. 1991. Pharmacologically-distinct subsets of astroglia can be identified by their calcium response to neuroligands. Neuroscience 41:325–333.

Nedergaard M. 1994. Direct signaling from astrocytes to neurons in cultures of mammalian brain cells. Science 263:1768–1771.

Newman EA, Zahs KR. 1998. Modulation of neuronal activity by glial cells in the retina. J Neurosci 18:4022–4028.

Orkand RK, Nichols JG, Kuffler SW. 1966. Effect of nerve impulses on the membrane potential of glial cells in the CNS of amphibia. J Neurophysiol 29:788–806.

Pasti L, Volterra A, Pozzan T, Carmignoto G. 1997. Intracellular calcium oscillations in astrocytes: a highly plastic, bidirectional form of com-

munication between neurons and astrocytes *in situ*. J Neurosci 17: 7817–7830.

Porter JT, McCarthy KD. 1995. GFAP-positive hippocampal astrocytes in situ respond to glutamatergic neuroligands with increases in $[Ca^{2+}]_i$. Glia 13:101–112.

Porter JT, McCarthy KD. 1996. Hippocampal astrocytes in situ respond to glutamate released from synaptic terminals. J Neurosci 16:5073–5081.

Porter JT, McCarthy KD. 1997. Astrocytic neurotransmitter receptors *in situ* and *in vivo*. Prog Neurobiol 51:439–455.

Robitaille R. 1998. Modulation of synaptic efficacy and synaptic depression by glial cells at the frog neuromuscular junction. Neuron 21:847–855.

Rothstein JD, Dykes-Hoberg M, Pardo CA, Bristol LA, Jin L, Kunci RW, Kanai Y, Hediger MA, Wang Y, Schielke JP, Welty DF. 1996. Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. Neuron 16:675–686.

Schools GP, Kimelberg HK. 1999. mGluR3 and mGluR5 are the predominant metabotropic glutamate receptor mRNAs expressed in hippocampal astrocytes acutely isolated from young rats. J Neurosci Res 58:533–543.

Seifert G, Zhou M, Steinhäuser C. 1997. Analysis of AMPA receptor properties during postnatal development of mouse hippocampal astrocytes. J Neurophysiol 78:2916–2923.

Shao Y, McCarthy KD. 1994. Plasticity of astrocytes. Glia 11:147–155.

Shao Y, McCarthy KD. 1997. Response of Bergmann glia and granule neurons in situ to N-methyl-D-aspartate, norepinephrine, and high potassium. J Neurochem 68:2405–2411.

Shelton MK, McCarthy KD. 1999. Mature hippocampal astrocytes exhibit functional metabotropic and ionotropic glutamate receptors in situ. Glia 26:1–11.

Smith S. 1992. Do astrocytes process neural information? Prog Brain Res 94:119–136.

Steinhäuser C, Gallo V. 1996. News on glutamate receptors in glial cells. Trends Neurosci 19:339–345.

Venance L, Stella N, Glowinski J, Giaume C. 1997. Mechanism involved in initiation and propagation of receptor-induced intercellular calcium signaling in cultured rat astrocytes. J Neurosci 17:1981–1992.

Ventura R, Harris K. 1999. Three-dimensional relationships between hippocampal synapses and astrocytes. J Neurosci 19:6897–6906.

Verkhratsky A, Kettenman H. 1996. Calcium signalling in glial cells. Trends Neurosci 19:346–352.

Vernadakis A. 1996. Glia-neuron intercommunications and synaptic plasticity. Prog Neurobiol 49:185–214.

Woodhall G, Gee CE, Robitaille R, Lacaille JC. 1999. Membrane potential and intracellular Ca^{2+} oscillations activated by mGluRs in hippocampal stratum oriens/alveus interneurons. J Neurophysiol 81: 371–382.

Ziak D, Chvatal A, Sykova E. 1998. Glutamate-, kainate- and NMDA-evoked membrane currents in identified glial cells in rat spinal cord slice. Physiol Res 47:365–375.