Neuron, Vol. 40, 971–982, December 4, 2003, Copyright ©2003 by Cell Press

ATP Released by Astrocytes Mediates Glutamatergic Activity-Dependent Heterosynaptic Suppression

Jing-ming Zhang,[1,3] Hui-kun Wang,[1,3] Chang-quan Ye,[1] Wooping Ge,[1] Yiren Chen,[1] Zheng-lin Jiang,[1] Chien-ping Wu,[1] Mu-ming Poo,[1,2] and Shumin Duan[1,*]

[1]Institute of Neuroscience
Shanghai Institutes for Biological Sciences
Chinese Academy of Sciences
Shanghai, 200031
China

[2]Division of Neurobiology
Department of Molecular and Cell Biology
University of California, Berkeley
Berkeley, California 94720

Summary

Extracellular ATP released from axons is known to assist activity-dependent signaling between neurons and Schwann cells in the peripheral nervous system. Here we report that ATP released from astrocytes as a result of neuronal activity can also modulate central synaptic transmission. In cultures of hippocampal neurons, endogenously released ATP tonically suppresses glutamatergic synapses via presynaptic P2Y receptors, an effect that depends on the presence of cocultured astrocytes. Glutamate release accompanying neuronal activity also activates non-NMDA receptors of nearby astrocytes and triggers ATP release from these cells, which in turn causes homo- and heterosynaptic suppression. In CA1 pyramidal neurons of hippocampal slices, a similar synaptic suppression was also produced by adenosine, an immediate degradation product of ATP released by glial cells. Thus, neuron-glia crosstalk may participate in activity-dependent synaptic modulation.

Introduction

Purine receptors are widely distributed throughout the central nervous system. Adenosine triphosphate (ATP), an endogenous ligand of purinergic receptors, may directly mediate synaptic transmission as a fast neurotransmitter (Edwards et al., 1992; Bardoni et al., 1997), or it may modulate synaptic efficacy as a neuromodulator. Both potentiating (Hugel and Schlichter, 2000; Nakatsuka and Gu, 2000) and suppressing (for review, see Cunha and Ribeiro, 2000) effects of exogenous ATP on synaptic transmission have been reported. There is evidence for vesicular corelease of ATP with acetylcholine (Silinsky and Redman, 1996), norepinephrine (Burnstock, 1995), and GABA (Jo and Schlichter, 1999) from various neurons. In addition, glial cells are also shown to secrete ATP (Queiroz et al., 1997; Guthrie et al., 1999; Cotrina et al., 1998, 2000; Stout et al., 2002). While both ATP release and synaptic modulation by exogenous ATP have been widely reported, the endogenous source of ATP responsible for synaptic modulation has not been clearly identified, nor is it clear how endogenous ATP release is regulated.

There is increasing evidence for bidirectional communication between neurons and glia (for reviews, see Araque et al., 2001; Haydon, 2001). Studies of Schwann cells of both neuromuscular junctions and myelinated axons show that extracellular ATP released from axons acts as an activity-dependent signal between neurons and glia (for review, see Fields and Stevens, 2000). Although astrocytes, the most abundant glial cells in the brain, lack excitability in terms of action potentials, they can trigger Ca^{2+} waves in response to various stimuli, including neuronal activity (Cornell-Bell et al., 1990; Dani et al., 1992; Nedergaard, 1994; Parpura et al., 1994). Extracellular ATP released from glial cells can also trigger interglia propagation of Ca^{2+} waves (Guthrie et al., 1999; Cotrina et al., 1998, 2000) and induce neuronal hyperpolarization (Newman, 2003). Because astrocytes are found to associate closely with neuronal synapses (Grosche et al., 1999; Ventura and Harris, 1999), we thus investigated whether neuronal activity can trigger ATP release from astrocytes and whether astrocyte-derived ATP can modulate synaptic transmission. We found that activation of glutamatergic neurons causes ATP release from astrocytes, which in turn induces both homo- and heterosynaptic suppression through presynaptic P2Y receptors. Similar neuronal activity-dependent heterosynaptic suppression was also observed in CA1 pyramidal neurons of hippocampal slices. In the latter case, synaptic suppression can be attributed mainly to adenosine, which is derived from glia-released ATP through degradation by ectonucleotidase. These results demonstrated that, in addition to transmitter spillover (Isaacson et al., 1993; Vogt and Nicoll, 1999; Mitchell and Silver, 2000; Satake et al., 2000; Li et al., 2001), astrocyte-derived ATP also contributes to heterosynaptic modulation induced by neuronal activity. Our finding provides a clear example of activity-dependent synaptic plasticity in which synaptic modification is mediated by neuron-glia crosstalk.

Results

Suppression of Excitatory Transmission by Endogenous ATP in Hippocampal Cultures

We first examined whether endogenous ATP associated with the basal synaptic activity modulate synaptic transmission in cocultures of hippocampal neurons and astrocytes. Dual whole-cell recording was made from neuronal pairs, and depolarizing pulses (+100 mV, 2 ms, 0.05 Hz) were delivered through the recording pipette to elicit synaptic responses. The property of synaptic connections (glutamatergic or GABAergic) was determined by the time course, reversal potential, and sensitivity of synaptic currents to specific antagonists for $GABA_A$ receptors and non-NMDA receptors, i.e., bicuculline and

*Correspondence: shumin@ion.ac.cn
[3]These authors contributed equally to this work.

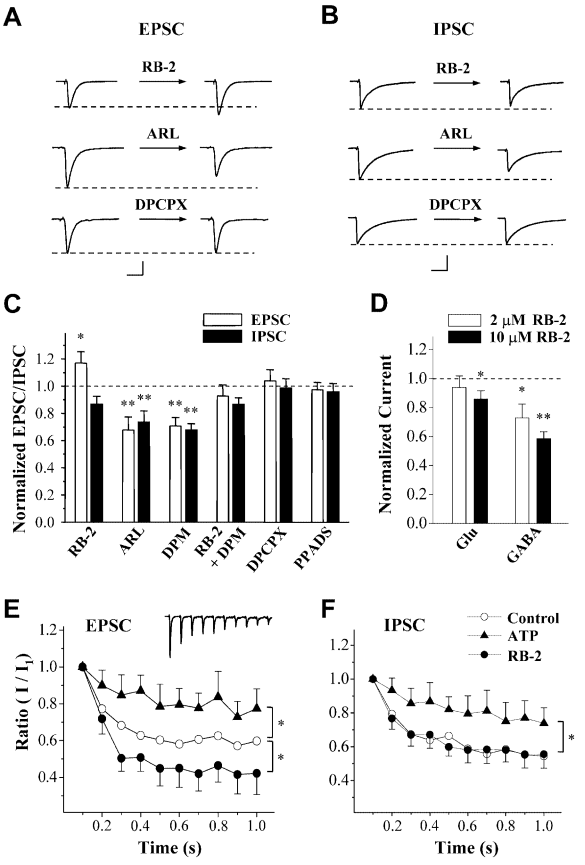

Figure 1. Modification of Synaptic Transmissions by Endogenous ATP

(A and B) Representative traces of EPSCs (A) or IPSCs (B) before (left, control) and after (right) treatments with P2Y receptor antagonist RB-2 (2 μM), A1 receptor antagonist DPCPX (20 nM), or ecto-ATPase inhibitor ARL (100 μM). Scale bars equal 100 (upper traces) or 150 pA, 10 ms in (A) and 100 pA, 25 ms in (B).

(C) Summary of all experiments (n = 9–14; *p < 0.05; **p < 0.01, paired t test) similar to those shown in (A) and (B). Data are normalized by the averaged amplitude of synaptic currents obtained in each neuron before drug application (control).

(D) Effects of RB-2 on membrane currents induced by perfusion with 100 μM glutamate or GABA (n = 8–9; *p < 0.05; **p < 0.01). Data normalized as in (C).

(E and F) Effects of ATP (2 μM) and RB-2 (2 μM) on the extent of synaptic fatigue, as reflected in the amplitude of EPSCs (E) and IPSCs (F) induced by repetitive stimulations (2 ms, 10 Hz for 1 s). Data are normalized by the amplitude of the first postsynaptic current (I_1) (n = 9–11, *p < 0.01). Inset: samples of EPSCs induced by repetitive stimulations.

6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX), respectively.

The effect of endogenously released ATP on excitatory postsynaptic currents (EPSCs) was examined by measuring the effects of bath-applied purinergic receptor antagonists, including A1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), P2X antagonist pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), and P2Y antagonist reactive blue 2 (RB-2). We found that RB-2 (2 μM) increased the amplitude of EPSCs but decreased that of inhibitory postsynaptic currents (IPSCs), while DPCPX (20 nM) and PPADS (10 μM) had no effect on either currents (Figures 1A–1C). In addition, RB-2 at high concentrations (≥2 μM) also reduced the amplitude of currents induced by exogenously applied glutamate or GABA, perhaps due to nonspecific effect on postsynaptic receptors (Figure 1D; see also Nakazawa et al., 1995). This may account for the RB-2-induced suppression of IPSCs and cause an underestimate of RB-2-induced EPSC amplitude increase. At glutamatergic (but not GABAergic) synapses, RB-2 also accelerated the synaptic fatigue induced by a train of 10 depolarizing pulses (at 10 Hz, Figures 1E and 1F). Furthermore, an ectonucleotidase inhibitor dipyridamole (DPM, 10 μM; Connolly and Duley, 2000) decreased the amplitudes of both EPSCs and IPSCs (Figure 1C) and the effect was blocked by RB-2, while it had no effect on exogenous glutamate- or GABA-induced currents (97.4% ± 6.6%, n = 8 and 95.5% ± 7.2%, n = 6, respectively). Similar suppression of EPSCs and IPSCs was also found for an ecto-ATPase inhibitor ARL 67156 (ARL, 100 μM; Westfall et al., 1997). Thus, there is an endogenous ATP-induced tonic synaptic suppression, mediated through P2Y receptors. Furthermore, extracellular ATP was effective in suppressing basal glutamatergic transmission in these cultures and became effective also in modifying GABAergic synapses only when ATP degradation is inhibited, consistent with the expectation of an elevated extracellular ATP concentration and more extensive spread of ATP from the site of release.

Synaptic Suppression by Exogenous ATP

The role of ATP in synaptic suppression was further studied by examining the effects of exogenous ATP in hippocampal cultures. Direct perfusion of ATP (≥2 μM) significantly reduced the amplitude of EPSCs and IPSCs (Figure 2A). The presence of RB-2 (2 μM), P2 receptor antagonist suramin (2 or 10 μM), adenosine A1 receptor antagonist 8-cyclopentyl-1,3-dimethylxanthine (CPT, 100 nM), and DPCPX (20 nM) all diminished ATP-induced suppression, while PPADS (10 μM) and A2 receptor antagonist, 7-dimethyl-1-propargylxanthine (DMPX, 10 μM), had no effect (Figure 2B). The blocking effects of A1 antagonists suggest the involvement of adenosine, which is generated by degradation of ATP through ectonucleotidase (Dunwiddie et al., 1997; Cunha et al., 1998). Perfusion of adenosine (10 μM) suppressed the EPSC amplitude (Figure 2C) but failed to affect the amplitude of IPSCs (95.3% ± 9.1% of control, n = 6, Figure 2C). Furthermore, adenosine-induced suppression can be blocked by CPT or DPCPX, but not by RB-2 (Figures 2C and 2D), suggesting that ATP-induced synaptic suppression is mainly mediated by P2Y receptors. Consistent with the fact that P2Y is a G protein-coupled receptor, we found that ATP-induced suppression of EPSCs was largely prevented by a GTPase inhibitor GDP-β-S (1 mM) applied through the recording pipette or by preincubation of the culture with pertussis toxin (PTX, 100 ng/ml, 12 hr), a G_i/G_o inhibitor (Figure 2B).

Synaptic Suppression by ATP Involves Presynaptic Modulation

Synaptic suppression induced by ATP may reflect either decreased presynaptic transmitter release or reduced postsynaptic receptor responses. We found that inward

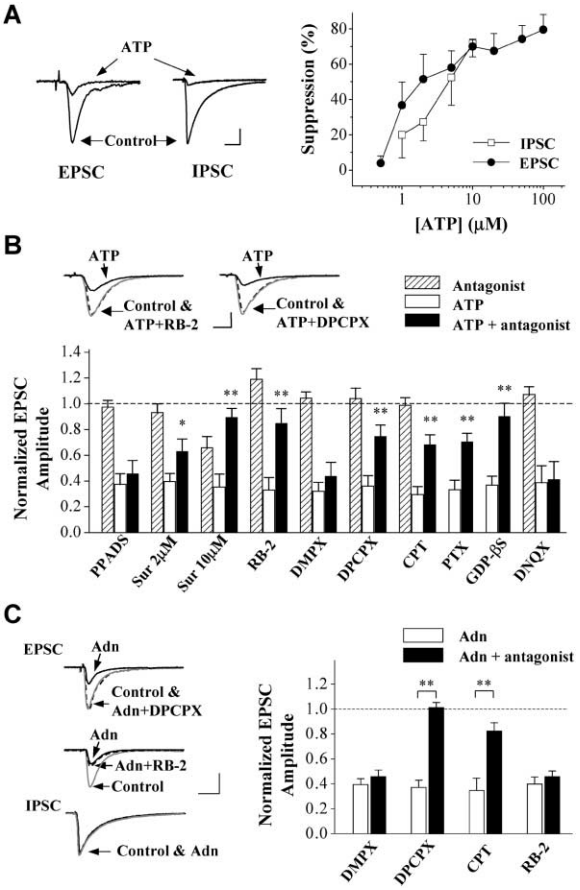

Figure 2. Synaptic Suppression Induced by Exogenous ATP and Adenosine

(A) Modification of glutamatergic and GABAergic synaptic transmission by bath application of ATP. Left: typical EPSCs and IPSCs recorded before and after bath application of ATP (10 μM). Scale bars equal 25 pA, 5 ms for EPSC and 100 pA, 20 ms for IPSC. Right: dose dependence of ATP-induced suppression of EPSCs and IPSCs (n = 6–11 for each data point).

(B) Sensitivity of ATP-induced suppression of EPSCs or IPSCs (for testing the effect of DNQX) to various pharmacological treatments. n = 8–12, *p < 0.05; **p < 0.01. Data normalized as in Figure 1C. Traces on top: typical EPSCs before (gray lines) and after bath application of ATP with (dashed lines) and without antagonists (solid black lines). Scale bars equal 50 pA, 10 ms.

(C) Effects of pharmacological treatments on adenosine-induced synaptic suppression. Left: typical EPSCs (top and middle) and IPSCs (bottom) before (gray lines) and after bath application of adenosine (Adn) with (dashed lines) or without (solid black lines) antagonists. Scale bars: EPSCs, 50 (upper traces) or 100 pA, 10 ms; IPSCs, 100 pA, 25 ms. Right: summarized data as shown in the left panel. n = 9–12, *p < 0.05; **p < 0.01. Data are normalized as in Figure 1C.

currents induced by locally applied glutamate or GABA were not significantly affected by ATP (10 μM, Figure 3A). In the presence of tetradotoxin (TTX, 0.5 μM) and bicuculline (10 μM), ATP (10 μM) significantly decreased the frequency but not the amplitude of spontaneous miniature EPSCs (mEPSCs) (Figure 3B). These results are consistent with suppression of presynaptic transmitter release rather than of postsynaptic responses. Similar effect of ATP on mIPSCs was also observed (data not shown). Furthermore, we found that ATP induced an increase in paired-pulse ratio, consistent with a reduction in presynaptic transmitter release probability (Zucker and Regehr, 2002). Finally, the effects of applied ATP and RB-2 (Figures 1E and 1F) on synaptic fatigue are also consistent with the presynaptic modulation of transmitter release.

Modification of presynaptic Ca^{2+} channels is a potential mechanism for ATP-induced suppression of transmitter release. Since it is difficult to record Ca^{2+} currents from presynaptic nerve terminals of these neurons, we examined the effect of ATP on the somatic Ca^{2+} current. Perfusion of ATP (10 μM) decreased the Ca^{2+} current and the effect was reversible (Figure 3D), suggesting that ATP may indeed reduce transmitter release by inhibiting presynaptic Ca^{2+} channels.

Glutamatergic Activity Induces Heterosynaptic Suppression in Culture

The findings that glutamatergic, but not GABAergic, transmission is potentiated by RB-2, while applied ATP suppressed both synapses (Figures 1–3), suggest that tonic ATP release near glutamatergic synapses that do not affect nearby GABAergic synapses. This led to the question whether endogenous ATP release depends on synaptic activity. One may expect a stronger RB-2-sensitive synaptic suppression after high-frequency synaptic activation. However, because this effect of synaptic activation may be confounded by synaptic fatigue due to high-frequency stimulation (Figures 1E and 1F), we decided to examine the heterosynaptic effect of high-frequency stimulation on recurrent synapses made by the unstimulated neuron.

Dual patch recordings were made from two nearby neurons with reciprocal synaptic connections. High-frequency stimulation was applied to one neuron (cell 1, Figure 4A), and its impact on the efficacy of the recurrent connection from cell 2 to cell 1 (2→1) was examined. An example is shown in Figure 4A (1, glutamatergic; 2, GABAergic). Cell 2 was first stimulated by test pulses (+100 mV, 2 ms, 0.033 Hz) and the IPSCs were recorded in cell 1 for a control period. Each test pulse to cell 2 was then preceded (200 ms to 1 s) by a train of high-frequency stimuli (+100 mV, 2 ms, 10 pulses at 10 Hz) applied to cell 1. The heterosynaptic modulation was estimated by comparing the averaged IPSC amplitude of 2→1 synapse with and without high-frequency stimulation of cell 1. We found that the IPSC amplitude of 2→1 was decreased after repetitive stimulation of cell 1, while EPSCs of 1→2 showed stimulation-induced synaptic fatigue. For four types of recurrent connections (Figures 4B and 4C), we found the heterosynaptic suppression of the recurrent connection was induced only when the stimulated neuron was glutamatergic but not GABAergic, regardless of whether the recurrent connection was glutamatergic or GABAergic. The efficiency of heterosynaptic suppression was time dependent, with maximum suppression found at about 500 ms (70.6% ± 5.1% of control, n = 12), a moderate suppression at 200 ms (81.3% ± 5.8% of control, n = 10), and no significant suppression at 1 s (95.9% ± 4.5% of control, n = 9) after cell 1 stimulation.

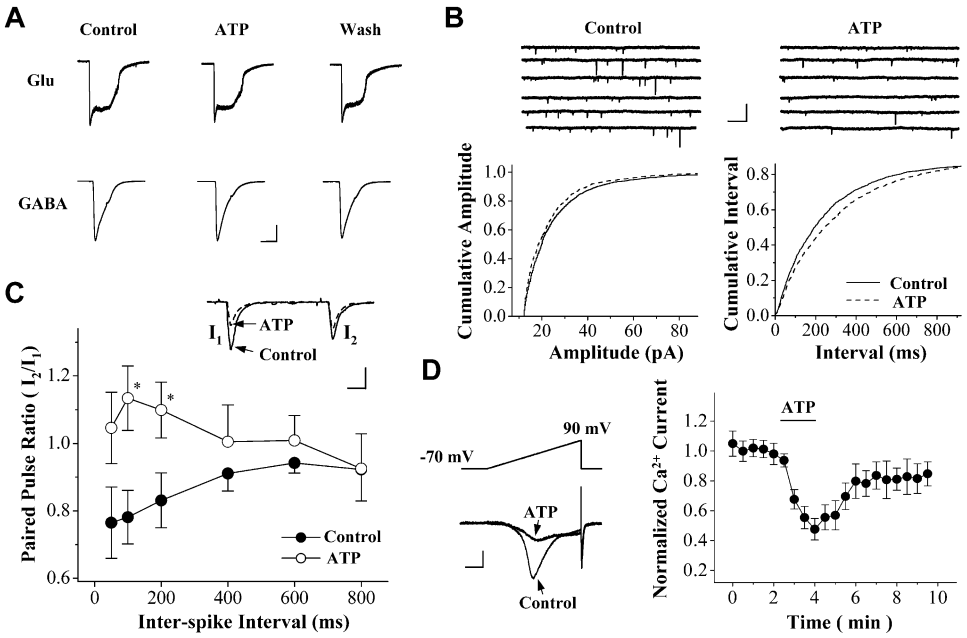

Figure 3. ATP-Induced Synaptic Suppression Involves Presynaptic Modification

(A) Examples of inward currents induced by a brief pulse of glutamate (Glu, 100 μM, upper traces) or GABA (lower traces) before, during, and after perfusion with 10 μM ATP. Scale bars equal 100 (upper traces) or 250 pA, 10 s.

(B) Cumulative distribution of the amplitude (left) and interevent interval (right) of mEPSCs in the presence or absence of 10 μM ATP (n = 9). TTX (0.5 μM) was used to block action potentials. The difference is statistically significant ($p < 0.01$, Kolmogorov-Smirnov Test) for the distribution of interevent interval, but not for the amplitude distribution. Traces on top: typical mEPSC before (left) and after (right) ATP perfusion. Scale bars equal 10 pA, 1 s.

(C) Effects of ATP (5 μM) on paired-pulse ratio (I_2/I_1) of EPSCs at various intervals (n = 6–10 for each data point, (*$p < 0.05$, compared with the control at the corresponding time point). Inset, sample traces showing paired pulse-induced EPSCs before (solid line) and after perfusion of ATP (5 μM, dashed line) at an interpulse interval of 100 ms. Scale bars equal 60 pA, 20 ms.

(D) Effects of ATP on Ca^{2+} currents. Left panel shows the ramp stimulus (upper trace) and Ca^{2+} current recorded (lower traces) before and after ATP (10 μM) application. Right: averaged data from 12 neurons, normalized by the mean amplitude of Ca^{2+} currents obtained in each neuron before ATP application.

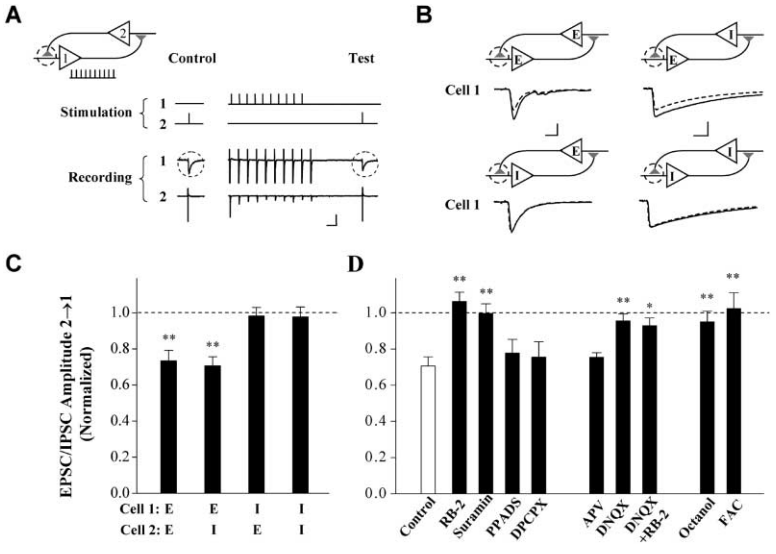

Figure 4. Heterosynaptic Suppressions Induced by Glutamatergic, but not GABAergic, Neuronal Activities

(A) Schematic diagram and example dual patch recordings from two reciprocally connected neurons and stimulation parameters applied. Dashed circles: synaptic responses (cell 2→1) recorded before (control) and after high-frequency stimulation of cell 1. Scale bars equal 100 pA, 100 ms.

(B) Examples of synaptic responses (2→1, dashed circles) in four types of recurrent connections involving glutamatergic (E) and GABAergic (I) neurons, with (dashed lines) and without (solid lines) high-frequency stimulation of neuron 1. Scale bars equal 50 pA, 10 ms (left); 100 pA, 20 ms (right).

(C) Summary of data obtained as shown in (B). Data represent the amplitude of EPSCs or IPSCs at synapse 2→1 after high-frequency stimulation of cell 1, normalized by the mean amplitude of control EPSCs or IPSCs before cell 1 stimulation in each recording. *$p < 0.01$ as compared with control EPSC or IPSCs. n = 10–13 for each group.

(D) Pharmacology of heterosynaptic suppression induced by glutamatergic neuronal activity. Cultures were perfused with one of the following antagonists or toxins before and during high-frequency stimulations: RB-2 (2 μM), suramin (2 μM), PPADS (10 μM), DPCPX (20 nM), APV (10 μM), DNQX (3 μM), octanol (1 mM), and FAC (1 mM). Data are normalized as in (C). n = 9–14 for each group (*$p < 0.05$, **$p < 0.01$, as compared to the control group).

Heterosynaptic Suppression Depends on Activation of P2Y and Non-NMDA Receptors

To determine whether the above heterosynaptic suppression was mediated by ATP, we examined the effect of several purinergic antagonists. Stimulation-induced heterosynaptic suppression was blocked by RB-2 and suramin but was unaffected by PPADS or DPCPX (Figure 4D), consistent with the finding on synaptic suppression induced by exogenously applied ATP (Figure 2B) and the involvement of P2Y receptors.

We have shown that RB-2 treatment increased the amplitude of EPSCs but not IPSCs (Figures 1A–1C) and that RB-2-sensitive heterosynaptic suppression occurred only when glutamatergic but not GABAergic neuron was stimulated (Figures 4B and 4C). Thus, either ATP is coreleased with glutamate or ATP is released by activation of glutamate receptors. We addressed this issue further by examining heterosynaptic suppression of IPSCs in the presence or absence of glutamate receptor antagonists, using GABAergic/glutamatergic cell pairs. We found that DNQX, but not NMDA receptor antagonist APV, blocked the heterosynaptic suppression of IPSCs (Figure 4D). Furthermore, the effect of DNQX and RB-2 on heterosynaptic inhibition were not additive, suggesting that P2Y and non-NMDA receptor are activated sequentially rather than in parallel.

Astrocytes Participate in Synaptic Suppression in Hippocampal Cultures

The findings that RB-2-sensitive heterosynaptic suppression can be blocked by DNQX and that DNQX did not block the suppressive effect of exogenous ATP on IPSCs (Figure 2B) suggests that endogenous ATP involved in synaptic suppression is triggered by glutamatergic receptor activation. In view of the close apposition of astrocytes to neuron (Grosche et al., 1999; Ventura and Harris, 1999), we examined whether astrocytes are involved in ATP-mediated synaptic suppression.

We first used a glia-specific metabolic inhibitor fluoroacetate (FAC; Szerb and Issekutz, 1987; Swanson and Graham, 1994) to interfere with glial cell functions and found that perfusion of FAC (1 mM) abolished heterosynaptic suppression induced by neuronal stimulation. Application of FAC in the absence of neuronal stimulation had no effect on synaptic efficacy (data not shown). Astrocytes are extensively coupled via gap junctions, which are important for propagation of interastrocyte signals (Giaume and Venance, 1998). We found that a gap junction inhibitor octanol (Nedergaard, 1994; Pappas et al., 1996), while it did not affect exogenous ATP-induced synaptic suppression (at 1 mM, data not shown), blocked the high-frequency stimulation-induced heterosynaptic suppression (Figure 4D), suggesting that interastrocyte signaling is essential for the observed synaptic suppression. Finally, we established a culture preparation in which dissociated hippocampal neurons were cultured in glia-conditioned media (GCM, Figure 5A, see Experiment Procedures) in the absence of glial cells. We found that, similar to mixed cultures containing both neurons and glia, exogenous ATP suppressed the amplitude of EPSCs (Figure 5B) and IPSCs in these GCM cultures, an effect that was abolished by RB-2 (data not shown). However, unlike that in mixed cultures, RB-2 alone failed to elevate the EPSC amplitude in GCM cultures, nor did high-frequency stimulation induce any heterosynaptic suppression (Figure 5B). This is consistent with the absence of a tonic presence of ATP and the expectation that repetitive neuronal activity cannot trigger ATP release in the absence of astrocytes. The decrease in the amplitude of EPSCs by RB-2 in GCM cultures (Figure 5B) may be attributed to the nonspecific effect of RB-2 on postsynaptic transmitter receptors (Figure 1D; Nakazawa et al., 1995).

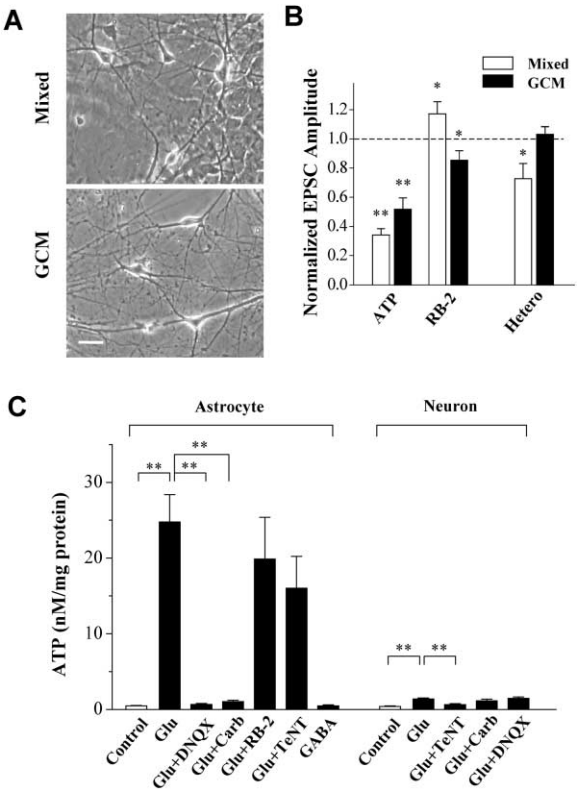

Figure 5. Involvement of Astrocytes in Endogenous ATP-Induced Synaptic Suppression

(A) Phase-contrast images showing mixed and GCM neuronal cultures. Scale bar equals 25 μM.

(B) Comparison of synaptic modification induced by ATP, RB-2, and high-frequency stimulation in mixed and GCM cultures. Data normalized as in Figures 1C and 4C (n = 9–20 for each group).

(C) Effects of glutamate (20 μM, 5 min) on ATP release in hippocampal astrocyte cultures and neuronal cultures (GCM). Marked ATP release was induced by glutamate in astrocyte cultures but not in GCM cultures. The glutamate-induced ATP release in astrocytes was blocked by DNQX (10 μM) and Carb (100 μM), while tetanus toxin (100 ng/ml) had little effect. Also shown is the absence of GABA effect (20 μM, 5 min) on ATP release in astrocyte cultures. n = 8–12 for each group (*$p < 0.05$; **$p < 0.01$ in B and C).

Glutamate-Induced ATP Release in Astrocyte Cultures

The above results suggest that glutamate release from neurons may induce ATP release from astrocytes. It has been shown that exogenously applied glutamate induced ATP release in cultured cortical astrocytes (Queiroz et al., 1997). Using a bioluminescence method

(Cotrina et al., 1998), we found that treatment of pure astrocyte cultures with 20 μM glutamate for 5 min resulted in a marked increase in the concentration of ATP found in the culture medium (Figure 5C). The glutamate effect was abolished by DNQX (3 μM) or gap-junction inhibitor carbenoxolone (Carb, 100 μM) (Draguhn et al., 1998) but not by RB-2 (2 μM) or synaptic secretion blocker tetanus toxin (TeNT, 100 ng/ml) (Schiavo et al., 1992). In contrast, the same glutamate treatment of GCM cultures induced only a slight elevation of extracellular ATP concentration, which was blocked by TeNT, but not by DNQX or Carb, consistent with neuronal release of ATP. Finally, treatment of astrocyte cultures with GABA (20 μM, 5 min) did not induce significant ATP release into the medium (Figure 5C).

Adenosine- and ATP-Mediated Synaptic Suppression in Hippocampal Slices

To explore the physiological relevance of the above findings obtained in hippocampal cultures, we further examined whether ATP-mediated synaptic modulation also exists in rat hippocampal slices. Schaffer collaterals were stimulated by extracellular electrodes, and evoked EPSPs or EPSCs were recorded in CA1 pyramidal neurons in the presence of picrotoxin (100 μM) to block $GABA_A$ responses. Consistent with the results found in hippocampal cultures (Figure 2), perfusion of ATP (10 μM) significantly suppressed the amplitude of EPSPs recorded in CA1 neurons (Figures 6A and 6D). However, unlike that found in hippocampal cultures, perfusion of CPT (100 nM), but not RB-2, increased EPSCs in CA1 neurons (Figures 6A, 6B, and 6D), indicating that endogenous adenosine rather than ATP is responsible for the tonic suppression of glutamatergic synapse in the slice preparation. Interestingly, in the presence of ectonucleotidase inhibitor DPM (10 μM), RB-2 significantly increased the EPSP amplitude (Figures 6C and 6D), indicating that endogenous extracellular ATP in the hippocampal slice is normally degraded rapidly and at least part of extracellular adenosine comes from ATP degradation by ectonucleotidase. The results that FAC (1 mM) can block RB-2-induced EPSP increase in the presence of DPM (Figures 6C and 6D) suggest that extracellular ATP in hippocampal slices may indeed be derived from glial cells.

Heterosynaptic Suppression in Hippocampal Slices

To examine whether ATP or adenosine mediates heterosynaptic modulation in hippocampal slices, Schaffer collaterals were stimulated by two stimulating electrodes placed in the *S. radiatum* (Figure 7A). Paired-pulse test (with a 50 ms interval) was used to ensure that the two nonoverlapping inputs to a single CA1 pyramidal neuron were stimulated (Wang et al., 2003). Shortly after (≤2 s) applying a brief train of stimuli (at 10 Hz, 1 s) to one input (S1), we found that EPSPs elicited by the other unstimulated input (S2) were suppressed (Figures 7A, 7B, and 7D). This heterosynaptic suppression was blocked by CPT but not by RB-2, suggesting that adenosine rather than ATP is involved. However, in the presence of DPM (10 μM) or ARL (100 μM), the stimulation-induced heterosynaptic suppression was completely abolished

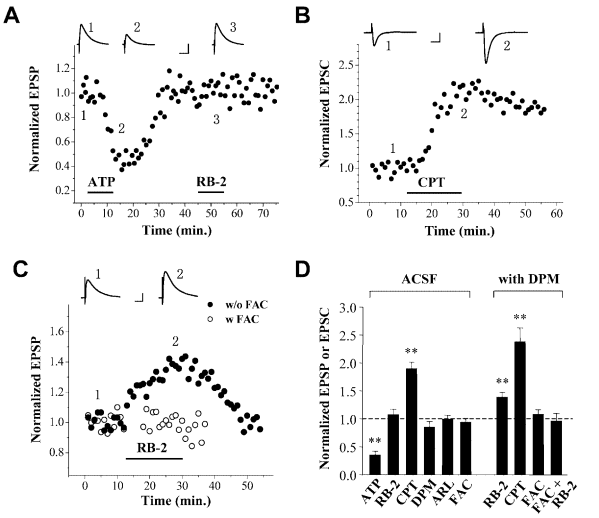

Figure 6. Synaptic Suppression Mediated by ATP and Adenosine in CA1 Neurons of Hippocampal Slices

(A) An example of recording showing that exogenous ATP (10 μM) reversibly suppressed the EPSPs in CA1 pyramidal neurons, while RB-2 (5 μM) had no effect. Sample traces above depict EPSPs before (1) and during bath application of ATP (2) or RB-2 (3). Scale bars equal 150 ms, 6 mV.

(B) An example of recording showing that CPT (100 nM) reversibly enhanced basal EPSCs. Sample traces above depict EPSCs before (1) and during (2) bath application of CPT. Scale bars equal 25 pA, 40 ms.

(C) Effect of RB-2 (5 μM) on EPSP in the presence of DPM (10 μM) with (open circles) or without (filled circles) FAC (1 mM). Sample traces above depict EPSPs before (1) and during (2) bath application of RB-2 without FAC. Scale bars equal 100 ms, 4 mV.

(D) Summary of data as shown in (A)–(C). Data normalized as in Figure 1C (n = 6–10 for each group, *$p < 0.05$; **$p < 0.01$).

by RB-2, although ARL or DPM by itself had no effect on the heterosynaptic suppression (Figure 7D). These results suggest that adenosine, which mediated heterosynaptic inhibition under the normal condition (in the absence of ARL or DPM), is derived from the degradation of ATP, which was released by high-frequency stimulation. We noted that heterosynaptic suppression could still be induced in the presence of glia-specific toxin FAC in the slice preparation, but this suppression was insensitive to CPT (Figure 7D, see Discussion).

Whether heterosynaptic suppression induced in hippocampal slices also requires activation of glutamate receptors was further examined. As shown in Figures 7C and 7D, we found that DNQX (10 μM) blocked activity-induced heterosynaptic suppression, as revealed by the NMDA component of EPSPs recorded in the Mg^{2+}-free solution. In contrast, NMDA receptor antagonist APV (25 μM) had no obvious effect on heterosynaptic suppression under the present stimulation condition (Figures 7C and 7D, see Discussion).

Astrocyte Responses to Neuronal Stimulation in Hippocampal Slices

The result that ATP/adenosine-mediated heterosynaptic suppression induced by Schaffer collateral stimulation is sensitive to DNQX and glia-specific toxin FAC suggests that astrocytes may be activated by glutamate

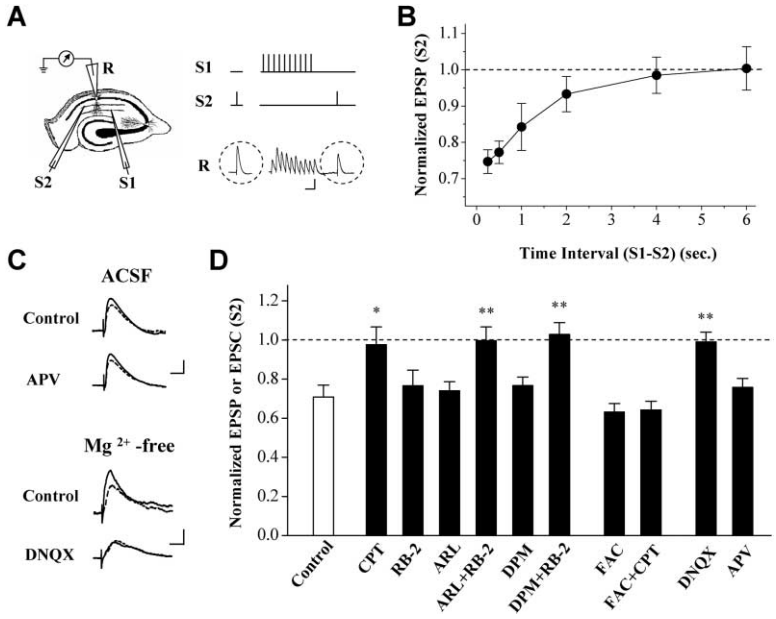

Figure 7. Heterosynaptic Suppression Induced in CA1 Neurons of Hippocampal Slices

(A) Schematic diagram (left) and sample recordings (right) showing the stimulation pattern. The stimulation electrodes were located at about 100 and 200 μm from the cell body layer, respectively, with a lateral separation of 300 μm. Dashed circles indicate the examined synaptic responses induced by S2 stimulation before (left) and 500 ms after (right) S1 stimulation (10 Hz). Scale bars equal 3 mV, 200 ms.

(B) Time course of heterosynaptic suppression induced by a train of high-frequency (10 Hz) stimulation, as shown in (A). Abscissa: time interval between the termination of S1 and the start of S2 stimulation. Ordinate: relative amplitude of EPSPs induced by S2 stimulation, normalized by the mean amplitude of control EPSPs (before S1 stimulation) obtained in each neuron (n = 8–13).

(C) Heterosynaptic suppression (dashed lines) induced as shown in (A) was blocked by DNQX (bottom), but not by APV (top).

(D) Summarized data showing the sensitivity of heterosynaptic suppression to various pharmacological treatments as in (C). Data represent amplitude of EPSCs or EPSPs evoked by S2 preceded by S1 stimulation as shown in (A), normalized by the mean amplitude of control EPSCs or EPSPs without S1 stimulation in each recording (n = 5–8 in each group; *$p < 0.05$; **$p < 0.01$, as compared to the control group).

that was released by neuronal activity. To test this idea, we examined whether Schaffer collateral stimulation that induced heterosynaptic suppression also can induce astrocyte responses, as monitored directly by patch clamp recording and fluorescence Ca^{2+} imaging. Astrocytes are identified by their small size, low membrane resistance (10–20 MΩ; see also Lüscher et al., 1998; Bergles et al., 2000), and absence of action potentials in response to membrane depolarizations (Figures 8A and 8B). Schaffer collateral stimulation induced a significant membrane depolarization in astrocytes, with a fast and a slow component (Figure 8C). Although only a mild glial depolarization was induced by a single pulse of stimulation, the depolarization accumulated upon repetitive stimulation (10 Hz, 1 s; Figure 8C). Application of DNQX significantly inhibited glial depolarization induced by Schaffer collateral stimulation, whereas APV had little effect (Figures 8C and 8D). Thus, the fast component appeared to result from the direct action of glutamate on astrocyte non-NMDA receptors, while the slow component is caused mainly by elevation of extracellular K^+ concentration evoked by increased neuronal activity. In addition, activation of glutamate transporters of glial cells by neuronal glutamate release may also contribute to glial depolarization (Bergles and Jahr, 1997; Lüscher et al., 1998).

The Schaffer collateral stimulation-induced glial responses were also detected by fluorescence Ca^{2+} imaging. We first confirmed that glial cells are selectively labeled by Calcium Green-1 AM (Porter and McCarthy, 1996). All labeled cells examined by the patch clamp recording (n = 11) exhibited astrocyte characteristics in accordance to the electrophysiological criteria described above (Figures 8A and 8B). We found that single Schaffer collateral stimulation did not induce detectable intracellular Ca^{2+} changes in the glial cells (data not shown). However, a train of 10 Hz stimuli induced significant Ca^{2+} increase in 9/29 glial cells examined. Furthermore, Schaffer collateral stimulation-induced Ca^{2+} increases were largely abolished by treatment with DNQX (Figure 8E).

Finally, we directly measured ATP release from hippocampal slices in response to perfusion of glutamate that mimics high-frequency neuronal activity, and the ectonucleotidase inhibitor DPM (10 μM) was added to reduce ATP degradation. We found that perfusion of glutamate (250 μM) markedly increased ATP release, and the release was blocked by DNQX (10 μM), FAC (1 mM), or Carb (100 μM), but not by TeNT (100 ng/ml). The pharmacological properties of glutamate-induced ATP release in the slice preparation (Figure 8F) are thus similar to that found in astrocyte cultures (Figure 5C).

Discussion

By using cultured hippocampal neurons and pharmacological manipulations in vitro, we are able to identify a pathway for endogenous ATP release that may account for a tonic form and an activity-induced form of synaptic suppression. The findings that P2Y receptor antagonist RB-2 increased the amplitude of EPSCs but not that of IPSCs (Figures 1A–1C) and that high-frequency stimulation of glutamatergic but not GABAergic neurons caused RB-2-sensitive heterosynaptic suppression (Figures 4B and 4C) led to the notion that endogenous ATP release is related to glutamatergic synaptic activity. A potential ATP source is the corelease of ATP with glutamate. However, four lines of evidence suggest that astrocytes rather than neurons provide the primary source of ATP for both the tonic- and activity-dependent synaptic suppression in cultured neurons. First, non-NMDA receptor antagonist DNQX, while not affecting ATP-induced syn-

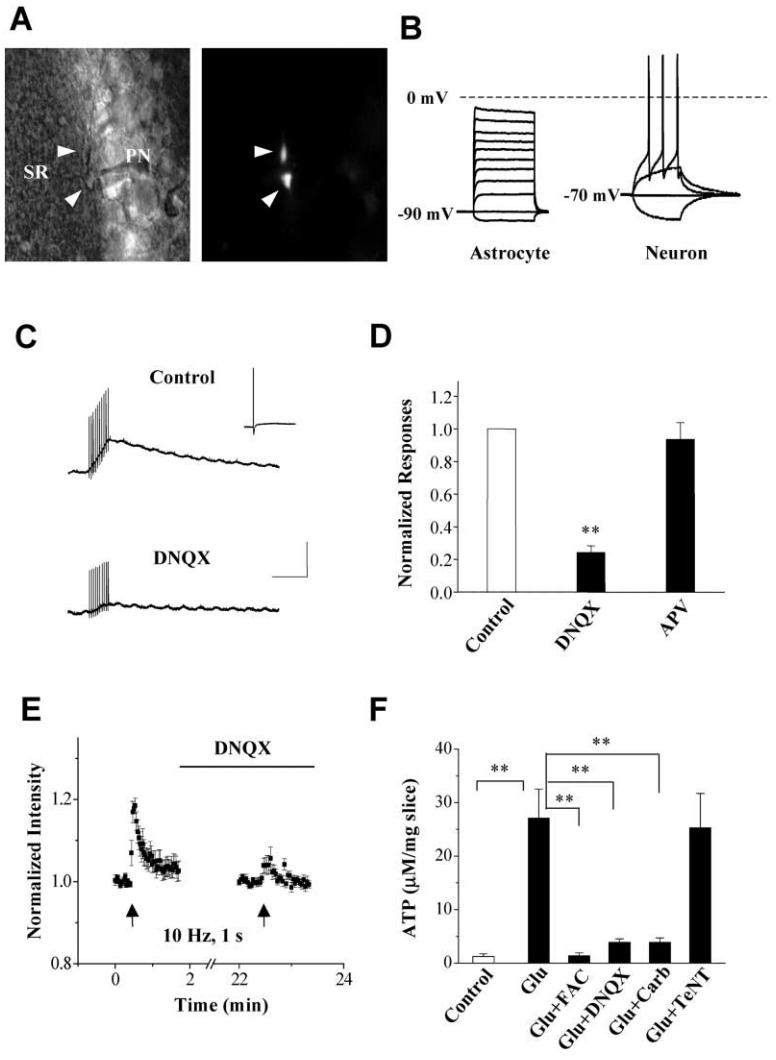

Figure 8. Neuronal Activity-Induced Glial Responses and ATP Release in Hippocampal Slices

(A) DIC (left) and fluorescence (right) images of the CA1 area of hippocampal slices. Arrows indicate two astrocytes near pyramidal neuronal layer (PN), preferentially labeled by Calcium Green-1 dye (right panel). SR, *s. radiatum*. Scale bar equals 10 μm.

(B) Example patch recordings from an astrocyte (left traces) and a pyramidal neuron (right) showing characteristic membrane potential changes in response to depolarizing steps (100 ms).

(C) Example recordings from a hippocampal astrocyte showing membrane depolarization induced by 10 Hz Schaffer collateral stimulation in the absence (top trace) and presence (bottom trace) of DNQX (10 μM). Upward deflections are stimulation artifacts. Inset: slight membrane depolarization recorded from the same astrocyte induced by a single pulse of Schaffer collateral stimulation. Scale bars equal 2 mV, 2 s (2 mV, 500 ms for the inset).

(D) Summary of data as illustrated in (C). The amplitude of the glial membrane potential/current responses at the end of 10 Hz Schaffer collateral stimulation train in the presence of DNQX (10 μM) or APV (25 μM) was normalized by the control values observed in the absence of the drug ($n = 5$ for each group. $^*p < 0.001$, compared to the control).

(E) Changes in the Ca^{2+} signal in astrocytes induced by 10 Hz Schaffer collateral stimulation in the absence (left) or presence (right) of DNQX (10 μM). Astrocytes were preferentially loaded with Calcium Green-1-AM. The fluorescence intensity was normalized with the averaged intensity observed during the control period (before stimulation) in each cell. Arrow marked the time of stimulation ($n = 9$ for each group).

(F) Pharmacology of glutamate (250 μM)-induced ATP release in hippocampal slices. ($n = 10$ for each group, $^{**}p < 0.001$, as compared to the control).

aptic suppression (Figure 2B), blocked RB-2-sensitive heterosynaptic suppression induced by stimulation of the glutamatergic neuron (Figure 4D), inconsistent with a corelease of ATP with glutamate. Second, heterosynaptic suppression can be inhibited by gap junction inhibitor octanol and glia-specific toxin FAC (Figure 4D), both of which had no effect on the suppressive action of exogenously applied ATP on EPSCs or IPSCs (data not shown). Third, in astrocyte-free GCM cultures, RB-2 did not increase basal glutamatergic transmission and there was no heterosynaptic suppression, although direct perfusion of ATP still suppressed the synaptic transmission (Figure 5B). Finally, treatment with glutamate, but not GABA, in pure astrocyte cultures induced marked ATP release that can be blocked by DNQX, FAC, or Carb, but not by RB-2 or TeNT, while the same treatment of glutamate in pure neuron cultures induced only a slight ATP release that is sensitive to TeNT but not to DNQX (Figure 5C). Astrocytes are known to express non-NMDA receptors, while it remains debatable whether they also express functional NMDA receptors (Stenhäuser and Gallo, 1996). To account for all the observations made in cultured hippocampal neurons, we propose the following sequence of events (Figure 9): glutamatergic neuronal activity results in neuronal secretion of glutamate, which activates non-NMDA receptors in the nearby astrocytes, leading to ATP release from these cells. The released ATP in turn activates presynaptic P2Y receptors in nearby neurons and causes homo- and heterosynaptic suppression.

The finding that glutamatergic, but not GABAergic, synaptic activity is tonically suppressed by endogenous ATP in cultured neurons (Figures 1A–1C) suggests that glutamate release associated with spontaneous synaptic activity is capable of inducing homosynaptic suppression of glutamatergic synapses, but had no heterosynaptic effect, possibly due to a more limited ATP release from nearby astrocytes. This is consistent with the observation that GABAergic transmission became suppressed when degradation of ATP is inhibited by ARL or DPM (Figures 1B and 1C), since more extensive heterosynaptic effect of ATP is expected. While DPM inhibits both adenosine uptake (Cunha et al., 1998) and ectonucleotidase (Connolly and Duley, 2000), ARL is a specific ecto-ATPase inhibitor (Westfall et al., 1997).

We found that both glutamatergic and GABAergic syn-

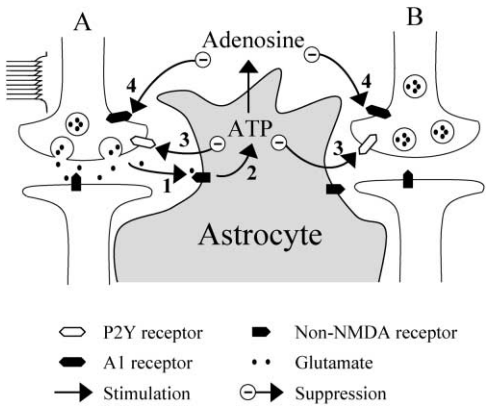

Figure 9. A Model for Activity-Dependent Homo- and Heterosynaptic Suppression Mediated by ATP Release from Astrocytes

Glutamate released resulting from high-frequency activity of glutamatergic neurons (A) activates non-NMDA receptors on astrocytes (1), which evokes ATP release from astrocyte (2). In mixed cultures, ATP released by astrocytes directly induces homo- (A) and heterosynaptic (B) suppression via activation of presynaptic P2Y receptors (3). In more intact brain tissue, due to a high level of ectonucleotidase activity, astrocyte-released ATP is quickly degraded to adenosine, which induces synaptic suppression via presynaptic A1 receptors (4).

apses are inhibited by bath application of ATP (Figure 2A), suggesting that a common mechanism may underlie ATP-induced synaptic suppression. Analysis of the ATP effects on the paired-pulse ratios and miniature synaptic current, together with the absence of ATP effect on exogenous glutamate- or GABA-induced neuronal responses, suggest a pre- rather than postsynaptic mechanism (Figure 3). Furthermore, the inhibition of ATP on neuronal Ca^{2+} currents (Figure 3D, see also Currie and Fox, 1996) is consistent with the idea that ATP acts by reducing presynaptic Ca^{2+} influx, leading to a reduced transmitter release.

Extracellular ATP is known to be degraded rapidly by ectonucleotidase (Dunwiddie et al., 1997; Cunha et al., 1998). It remains debatable whether ATP modulates synaptic transmission directly by activating P2 receptors (Mendoza-Fernandez et al., 2000; Trendelenburg and Bultmann, 2000) or indirectly by generating adenosine through its degradation by ectonucleotidases (Dunwiddie et al., 1997; Cunha et al., 1998). In cultured hippocampal neurons, we found that both ATP and adenosine markedly suppressed EPSCs and ATP-induced synaptic suppression can also be blocked by A1 receptor antagonists (Figures 2B and 2C). However, unlike ATP, adenosine did not affect IPSCs (Figure 2C, see also Deuchars et al., 2001). Furthermore, adenosine-induced suppression of EPSCs was not blocked by RB-2 (Figure 2C), suggesting that ATP and adenosine may not affect the same receptor populations and that at least part of the ATP effects is through its direct action on P2Y receptors in cultured neurons. The finding that the basal synaptic transmission was potentiated by RB-2 but not by DPCPX (Figures 1A–1C) indicates that endogenous ATP modulates transmission mainly through its direct action on P2Y receptors in these neurons.

Interestingly, in hippocampal slices, the basal synaptic transmission induced by stimulation of Schaffer collaterals was potentiated by CPT but not by RB-2, indicating that endogenous adenosine rather than ATP is responsible for the tonic synaptic suppression in the slices. However, in the presence of DPM, RB-2 significantly increased EPSPs (Figure 6). Similarly, heterosynaptic suppression induced in hippocampal slices was sensitive to CPT and insensitive to RB-2, but became sensitive to RB-2 in the presence of DPM or ARL (Figure 7D). In addition, we found that DNQX, rather than APV, blocked heterosynaptic suppression (Figures 7C and 7D) and that FAC blocked both the synaptic potentiation induced by RB-2 in the presence of DPM (Figures 6C and 6D) and the CPT-sensitive heterosynaptic suppression (Figure 7D). Taken together, these results suggest that at least part of the endogenous adenosine that mediates synaptic suppression in the hippocampal slice is derived from the degradation of ATP, which is released from glia as a result of glutamatergic neuronal activity. This idea is supported by the findings that Schaffer collateral stimulation, which induced heterosynaptic suppression, evoked significant astrocyte responses (membrane depolarization and intracellular Ca^{2+} increase) that can be blocked by DNQX (Figures 8C–8E). Furthermore, in the presence of DPM, direct perfusion of glutamate increased ATP release in hippocampal slices (Figure 8F). The main source of glutamate-induced ATP release in slices is likely to be glial cells rather than neurons, since its pharmacological properties are similar to those found for glutamate-induced ATP release from astrocyte cultures (Figures 5C and 8F).

Our results are consistent with previous reports that, in hippocampal slices, heterosynaptic suppression was induced by extracellular high-frequency stimulation and that the suppression was sensitive to A1 receptor antagonists (Mitchell et al., 1993; Manzoni et al., 1994). It is likely that ectonucleotidase activity in slice preparations is much higher than that in cell cultures so that sufficient concentration of adenosine can be created from degradation of extracellular ATP. Rapid dilution in the less restricted extracellular space of the culture may also limit the extent of adenosine build-up that is necessary to induce synaptic suppression. It is also likely that ATP-mediated synaptic suppression is more important in transient modification of synaptic activity within the local region around the activated synapses, while adenosine exerts a more sustained synaptic modification following more extensive and synchronized neuronal activity.

We found that in the presence of FAC, Schaffer collateral stimulation still induced heterosynaptic suppression, but this suppression was not blocked by CPT (Figure 7D). This CPT-insensitive heterosynaptic suppression in the presence of FAC is thus not mediated by ATP-adenosine pathway. Since the capacity of glial cells for clearing extracellular glutamate and K^+ will be decreased by FAC, high-frequency stimulation may result in excessive extracellular accumulation of glutamate and K^+, leading to extensive neuronal depolarization and suppression of synaptic transmission. Further work is needed to clarify the exact cause of CPT-insensitive heterosynaptic depression.

Prolonged heterosynaptic suppression (lasting for 5 to 30 min) induced by the tetanus stimulation (100 Hz) in hippocampal slices was reported to be mediated by adenosine and blocked partly by APV (Manzoni et al.,

1994). We found that the NMDA receptor-dependent prolonged heterosynaptic suppression (see Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/40/5/971/DC1) could be induced only by tetanus stimulation with much higher intensity than that used here (10 Hz stimulation) for inducing heterosynaptic suppression (lasting for a few seconds; Figure 7B). Similarly, we found that intracellular Ca^{2+} elevation and membrane depolarization in astrocytes induced by tetanus stimulation of Schaffer collateral were much less sensitive to DNQX than glial responses induced by 10 Hz stimulation (Supplemental Figure S2). Since the two input pathways frequently overlapped under elevated intensity of tetanic stimulation, as shown by paired pulse test (data not shown), prolonged heterosynaptic suppression may involve homosynaptic posttetanic short-term or long-term depression that depends on NMDA receptor activation. Tetanus stimulation may cause more glutamate spillover to activate extrasynaptic NMDA receptors, result in more extensive neuronal excitation, and induce more ATP/adenosine release due to enhanced glutamate-mediated neuron-glia interaction, and therefore, evokes prolonged heterosynaptic suppression that is sensitive in part to the NMDA receptor antagonist.

Elevation of astrocyte Ca^{2+} can modulate synaptic transmission through glutamate release (for review, see Araque et al., 2001), but whether ATP is involved and whether this modulation is neuronal activity dependent are unknown. Although ATP can mediate interastrocytes Ca^{2+} wave propagation (Cotrina et al., 1998, 2000; Guthrie et al., 1999; Stout et al., 2002) and adenosine derived from ATP released by glial cells can induce neuronal hyperpolarization (Newman 2003), the physiological trigger for the release of ATP from astrocytes remains unknown. Our finding of ATP release from astrocytes induced by glutamatergic synaptic activity suggests a mechanism of ATP release that mediates interastrocyte Ca^{2+} wave propagation. In this respect, it is interesting to note that exogenous or neuron-released glutamate has been reported to induce Ca^{2+} waves in astrocytes (Cornell-Bell et al., 1990; Dani et al., 1992; Porter and McCarthy, 1996; for review, see Araque et al., 2001) and astrocytic Ca^{2+} waves can suppress synaptic transmission (see Supplemental Figures 3B and 3C at http://www.neuron.org/cgi/content/full/40/5/971/DC1; see also Araque et al., 2001). We also found that high-frequency stimulation used for inducing heterosynaptic suppression evoked Ca^{2+} elevation in neighboring astrocytes (Supplementalal Figure S4) and that mechanical stimulation of astrocytes induced RB-2-sensitive synaptic suppression as well as Ca^{2+} wave propagation among astrocytes (Supplemental Figure S3). Stimulation of Schaffer collaterals in hippocampal slices induced DNQX-sensitive depolarization and intracellular Ca^{2+} elevation in astracytes (Figures 8C–8E). Furthermore, glutamate induces ATP release from astrocytes (Figures 5C and 8F; see also Queiroz et al., 1997) and neuronal synapses are wrapped by processes of astrocytes (Grosche et al., 1999; Ventura and Harris, 1999). The concentration of ATP reported to trigger interastrocyte Ca^{2+} wave propagation (Guthrie et al., 1999) is in the micromolar range, similar to that found to cause synaptic suppression in the present study (Figures 2A and 6A). Our findings in hippocampal slices (Figures 6–8) suggest that within the intact neuronal tissue, ATP released by glial cells may modulate synaptic activity through its catabolic product adenosine. Taken together, these findings support the notion that glutamate and ATP/adenosine act as activity-dependent signaling molecules in neuron-glia communication, resulting in astrocyte Ca^{2+} wave and synaptic modulation. Thus, through the mediation of astrocytes, neuronal activity can influence not only the activated synapse, but also other adjacent synapses (Figure 9). Such neuron-glia crosstalk may represent an integral part of activity-dependent plasticity of neural network.

Experimental Procedures

Cell Cultures

Primary hippocampal cultures were prepared as described (Fitzsimonds et al., 1997). In brief, E18 rat hippocampi were dissected and dissociated by 0.125% trypsin, plated on poly-D-lysine (PDL)-coated glass coverslips at a cell density of 60,000/ml in the solution of Dubecco's Modified Eagle Medium (DMEM) containing 10% heat-inactivated fetal bovine serum and 10% Ham's F-12 (all from GIBCO). The cultures were maintained at 37°C in a 5% CO_2 incubator. The culture medium was changed with the same solution 24 hr after plating and then half-changed once every week. The use and care of animals in this study follows the guideline of the Shanghai Institutes for Biological Sciences Animal Research Advisory Committee.

To prepare pure neuron cultures supplemented with glia-conditioned medium (GCM), the neurons were prepared in the similar manner except that cytosine arabinoside (10 μM) was added into cultures 24 hr after plating to block the proliferation of glial cells and the cultures were maintained in the Neurobasal Medium (GIBCO) containing 2% B27 (GIBCO). To provide GCM to the neurons, one coverslip prepared for pure neuronal cultures and three coverslips plated confluently with astrocyte cultures (10–14 days, see below) were cultured side by side in a single 35 mm dish. Neurons were used 8–14 days after the plating.

Primary cultures of astrocytes were prepared as described previously (Duan et al., 1999, 2003) with some modifications. In brief, hippocampi were prepared from 1-day-old rat and were dissociated by trypsin (0.125%), plated on PDL-coated glass coverslips, cultured with Eagle's MEM (GIBCO) containing 10% FBS, and maintained at 37°C in a 5% CO_2 humidified incubator. Astrocytes formed a confluent layer 10–14 days after plating and were used for ATP assay and for coculturing with pure neuron cultures to provide GCM.

Slice Preparation

Hippocampal slices were prepared as described previously (Kato et al., 1993; Wang et al., 2003). Sprague-Dawley rats (~14–22 days old) were anaesthetized with sodium pentobarbital. After decapitation, hippocampal formation was dissected rapidly and placed in ice-cold oxygenated (95% O_2/5% CO_2) artificial CSF (aCSF) containing (in mM): 119 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, and 11 glucose (pH 7.4). Transverse slices (400 μm thick) were cut with a tissue chopper (MalLWAIN, Mickle Lab) and maintained in an incubation chamber for at least 2 hr at room temperature before recording. During the experiments, individual slices were transferred to a submersion recording chamber and continuously perfused with the above extracellular solution (~4.0–5.0 ml/min) at 28°C–29°C. Slices were visualized with an Olympus microscope (DX50WI, Olympus) using infrared video microscopy and differential interference contrast optics.

Electrophysiology

Whole-cell perforated patch recordings were made from a pair of cultured hippocampal neurons. The patch electrodes were made from borosilicate glass capillaries (B-120-69-15, Sutter Instrument) with a resistance in the range of 4–7 MΩ. The pipettes were tip-filled with internal solution and then back-filled with internal solution containing 200 μg/ml amphotericin B (Sigma). The internal solution contained (in mM): 136.5 K-Gluconate, 17.5 KCl, 9 NaCl, 1 $MgCl_2$,

10 HEPES, and 0.2 EGTA (pH 7.20). The bath was constantly perfused with fresh recording medium containing (in mM): 145 NaCl, 3 KCl, 10 HEPES, 3 $CaCl_2$, 10 glucose, and 2 $MgCl_2$ (pH 7.30) at room temperature (20°C–24°C). Neurons were visualized using a phase-contrast inverted microscope (IX70, Olympus). Recordings were made with two Axopatch 200B patch-clamp amplifiers and 1320A interface (Axon Instruments). Series resistance was in the range of 20–40 MΩ and was compensated to 60%–70%. For assaying synaptic connectivity, each neuron was stimulated at a low frequency (0.05 or 0.033 Hz) by 2 ms step depolarization from −70 to +30 mV in voltage-clamp mode, and the responses of the other neuron as well as autaptic responses in the stimulated neuron itself were recorded. For evoked EPSC or IPSC, 6–8 consecutive traces were averaged and the mean amplitude was calculated. Signals were filtered at 2 kHz using amplifier circuitry, sampled at 10 kHz, and analyzed using Clampex 8.0 (Axon Instruments). Miniature EPSC or IPSC was analyzed by MiniAnal (Synaptosoft, Inc.).

Standard whole-cell recordings were used to measure voltage-gated Ca^{2+} currents with the intracellular solution containing (in mM) 135 CsCl, 3 $MgCl_2$, 10 TEA-Cl, 10 HEPES, 10 EGTA, 3 Mg-ATP, and 0.3 Na-GTP and the external solution containing (in mM) 60 CsCl, 50 NaCl, 20 TEA-Cl, 20 $BaCl_2$, 10 HEPES, 2 $MgCl_2$, 10 Glucose, 2 4-amino-pyradine (4-AP), and 0.0005 tetrodotoxin (TTX). For studying the effects of intracellular loading of GDP-βs, conventional whole-cell recordings were used and data were collected at least 20 min after breaking-in to allow full exchange between cytoplasm and pipette solutions.

For recordings from hippocampal slices, all experiments were carried out in the presence of $GABA_A$ antagonist picrotoxin (100 μM). Whole-cell patch recordings were made from pyramidal neurons in the cell body layer of CA1. Recording pipettes were filled with a solution containing (in mM): 120 K-Gluconate, 30 KCl, 1 $MgCl_2$, 10 HEPES, 4 ATP-Mg, 0.3 GTP, 10 phosphocreatine, and 0.2 EGTA (pH 7.3). EPSPs were induced by constant current pulses (~20–40 μA, 200 μs, 0.033 Hz) through a glass pipette (tip diameter 2–3 μm) that was filled with extracellular solution and placed at the *s. radiatum* (Wang et al., 2003). The membrane potential was maintained at −70 mV by injection of a constant current (<60 pA).

Calcium Imaging in Slices

Hippocampal slices were prepared as described above and incubated in ACSF containing 0.06% pluronic acid and 10 μM Calcium Green-1-AM (Molecule Probes) for 40–60 min at room temperature. The glial cells were preferentially loaded with the dye, as indicated by electrophysiological criteria (Figure 8A; see also Porter and McCarthy, 1996). The dye fluorescence was imaged with 488 nm excitation and a 500 nm long-pass barrier filter, using a Zeiss LSM 510 confocal microscope.

Extracellular ATP Measurements

The concentration of extracellular ATP was quantified by bioluminescence method using luciferase-luciferin test as previously described (Cotrina et al., 1998). The culture media of astrocyte or incubation medium for slices were replaced with recording medium (see before). Glutamate (20 μM) was added 5 min before samples were taken for ATP assay. Sample (100 μl) was added into 100 μl ATP Assay Mix (Sigma) containing luciferase-luciferin buffer. The luminescence was measured by a FLUOstar luminometer (BMG). A calibration curve was obtained from standard ATP samples and the luminescence of normal HBS was measured as the background ATP level. For measurement of ATP release in acute hippocampal slices, slices were incubated in 200 μl oxygenized aCSF with or without 250 μM Glutamate for 12 min. When needed, antagonists were added to aCSF 20–60 min before Glut incubation. The aCSF was then collected and ATP was assayed as described above.

Acknowledgments

We thank Dr. Qian Hu for the help on assistance in Ca^{2+} imaging and Xiaoyuan Zhu for cell culture preparation. This work was supported by a grant from Major State Basic Research Program of China (G200077800) and by Shanghai Science and Technology Development Foundation.

Received: October 21, 2002
Revised: February 19, 2003
Accepted: October 7, 2003
Published: December 3, 2003

References

Araque, A., Carmignoto, G., and Haydon, P.G. (2001). Dynamic signaling between astrocytes and neurons. Annu. Rev. Physiol. *63*, 795–813.

Bardoni, R., Goldstein, P.A., Lee, C.J., Gu, J.G., and MacDermott, A.B. (1997). ATP P2X receptors mediate fast synaptic transmission in the dorsal horn of the rat spinal cord. J. Neurosci. *17*, 5297–5304.

Bergles, D.E., and Jahr, C.E. (1997). Synaptic activation of glutamate transporters in hippocampal astrocytes. Neuron *19*, 1297–1308.

Bergles, D.E., Roberts, J.B., Somogyi, P., and Jahr, C.E. (2000). Glutamatergic synapses on oligodendrocyte precursor cells in the hippocampus. Nature *405*, 187–191.

Burnstock, G. (1995). Noradrenaline and ATP: cotransmitters and neuromodulators. J. Physiol. Pharmacol. *46*, 365–384.

Connolly, G.P., and Duley, J.A. (2000). Ecto-nucleotidase of cultured rat superior cervical ganglia: dipyridamole is a novel inhibitor. Eur. J. Pharmacol. *397*, 271–277.

Cornell-Bell, A.H., Finkbeiner, S.M., Cooper, M.S., and Smith, S.J. (1990). Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. Science *247*, 470–473.

Cotrina, M.L., Lin, J.H., Alves-Rodrigues, A., Liu, S., Li, J., Azmi-Ghadimi, H., Kang, J., Naus, C.C., and Nedergaard, M. (1998). Connexins regulate calcium signaling by controlling ATP release. Proc. Natl. Acad. Sci. USA *95*, 15735–15740.

Cotrina, M.L., Lin, J.H., Lopez-Garcia, J.C., Naus, C.C., and Nedergaard, M. (2000). ATP-mediated glia signaling. J. Neurosci. *20*, 2835–2844.

Cunha, R.A., and Ribeiro, J.A. (2000). ATP as a presynaptic modulator. Life Sci. *68*, 119–137.

Cunha, R.A., Sebastiao, A.M., and Ribeiro, J.A. (1998). Inhibition by ATP of hippocampal synaptic transmission requires localized extracellular catabolism by ecto-nucleotidases into adenosine and channeling to adenosine A1 receptors. J. Neurosci. *18*, 1987–1995.

Currie, K.P., and Fox, A.P. (1996). ATP serves as a negative feedback inhibitor of voltage-gated Ca^{2+} channel currents in cultured bovine adrenal chromaffin cells. Neuron *16*, 1027–1036.

Dani, J.W., Chernjavsky, A., and Smith, S.J. (1992). Neuronal activity triggers calcium waves in hippocampal astrocyte networks. Neuron *8*, 429–440.

Deuchars, S.A., Brooke, R.E., and Deuchars, J. (2001). Adenosine A1 receptors reduce release from excitatory but not inhibitory synaptic inputs onto lateral horn neurons. J. Neurosci. *21*, 6308–6320.

Draguhn, A., Traub, R.D., Schmitz, D., and Jefferys, J.G.R. (1998). Electrical coupling underlies high-frequency oscillations in the hippocampus in vitro. Nature *394*, 189–192.

Duan, S., Anderson, C.M., Stein, B.A., and Swanson, R.A. (1999). Glutamate induces rapid upregulation of astrocyte glutamate transport and cell-surface expression of GLAST. J. Neurosci. *19*, 10193–10200.

Duan, S., Anderson, C.M., Keung, E.C., Chen, Y., and Swanson, R.A. (2003). P2X7 receptor-mediated release of excitatory amino acids from astrocytes. J. Neurosci. *23*, 1320–1328.

Dunwiddie, T.V., Diao, L., and Proctor, W.R. (1997). Adenine nucleotides undergo rapid, quantitative conversion to adenosine in the extracellular space in rat hippocampus. J. Neurosci. *17*, 7673–7682.

Edwards, F.A., Gibb, A.J., and Colquhoun, D. (1992). ATP receptor-mediated synaptic currents in the central nervous system. Nature *359*, 144–147.

Fields, R.D., and Stevens, B. (2000). ATP: an extracellular signaling molecule between neurons and glia. Trends Neurosci. *23*, 625–633.

Fitzsimonds, R.M., Song, H.J., and Poo, M.M. (1997). Propagation of activity-dependent synaptic depression in simple neural networks. Nature *388*, 439–448.

Giaume, C., and Venance, L. (1998). Intercellular calcium signaling and gap junctional communication in astrocytes. Glia *24*, 50–64.

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

Guthrie, P.B., Knappenberger, J., Segal, M., Bennett, M.V., Charles, A.C., and Kater, S.B. (1999). ATP mediates glial calcium wave propagation. J. Neurosci. *19*, 520–528.

Haydon, P.G. (2001). GLIA: listening and talking to the synapse. Nat. Rev. Neurosci. *2*, 185–193.

Hugel, S., and Schlichter, R. (2000). Presynaptic P2X receptors facilitate inhibitory GABAergic transmission between cultured rat spinal cord dorsal horn neurons. J. Neurosci. *20*, 2121–2130.

Isaacson, J.S., Solis, J.M., and Nicoll, R.A. (1993). Local and diffuse synaptic actions of GABA in the hippocampus. Neuron *10*, 165–175.

Jo, Y.H., and Schlichter, R. (1999). Synaptic corelease of ATP and GABA in cultured spinal neurons. Nat. Neurosci. *2*, 241–245.

Kato, K., Clifford, D.B., and Zorumski, C.F. (1993). Long-term potentiation during whole-cell recording in rat hippocampal slices. J. Neurosci. *53*, 39–47.

Li, H., Chen, A., Xing, G., Wei, M.L., and Rogawski, M.A. (2001). Kainate receptor-mediated heterosynaptic facilitation in the amygdala. Nat. Neurosci. *4*, 612–620.

Lüscher, C., Malenka, R.C., and Nicoll, R.A. (1998). Monitoring glutamate release during LTP with glial transportor currents. Neuron *21*, 435–441.

Manzoni, O.J., Manabe, T., and Nicoll, R.A. (1994). Release of adenosine by activation of NMDA receptors in the hippocampus. Science *265*, 2098–2101.

Mendoza-Fernandez, V., Andrew, R.D., and Barajas-Lopez, C. (2000). ATP inhibits glutamate synaptic release by acting at P2Y receptors in pyramidal neurons of hippocampal slices. J. Pharmacol. Exp. Ther. *293*, 172–179.

Mitchell, S.J., and Silver, R.A. (2000). Glutamate spillover suppresses inhibition by activating presynaptic mGluRs. Nature *404*, 498–502.

Mitchell, J.B., Lupica, C.R., and Dunwiddie, T.V. (1993). Activity-dependent release of endogenous adenosine modulates synaptic responses in the rat hippocampus. J. Neurosci. *13*, 3439–3447.

Nakatsuka, T., and Gu, J.G. (2000). ATP P2X receptor-mediated enhancement of glutamate release and evoked EPSCs in dorsal horn neurons of the rat spinal cord. J. Neurosci. *21*, 6522–6531.

Nakazawa, K., Inoue, K., Ito, K., Koizumi, S., and Inoue, K. (1995). Inhibition by suramin and reactive blue 2 of GABA and glutamate receptor channels in rat hippocampal neurons. Naunyn Schmiedebergs Arch. Pharmacol. *351*, 202–208.

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

Newman, E.A. (2003). Glial cell inhibition of neurons by release of ATP. J. Neurosci. *23*, 1659–1666.

Pappas, C.A., Rioult, M.G., and Ransom, B.R. (1996). Octanol, a gap junction uncoupling agent, changes intracellular [H^+] in rat astrocytes. Glia *16*, 7–15.

Parpura, V., Basarsky, T.A., Liu, F., Jeftinija, K., Jeftinija, S., and Haydon, P.G. (1994). Glutamate-mediated astrocyte-neuron signaling. Nature *369*, 744–747.

Porter, J.T., and McCarthy, K.D. (1996). Hippocampal astrocytes in situ respond to glutamate released from synaptic terminals. J. Neurosci. *16*, 5073–5081.

Queiroz, G., Gebicke-Haerter, P.J., Schobert, A., Starke, K., and von Kugelgen, I. (1997). Release of ATP from cultured rat astrocytes elicited by glutamate receptor activation. Neuroscience *78*, 1203–1208.

Satake, S., Saitow, F., Yamada, J., and Konishi, S. (2000). Synaptic activation of AMPA receptors inhibits GABA release from cerebellar interneurons. Nat. Neurosci. *3*, 551–558.

Schiavo, G., Benfenati, F., Puolain, B., Rossetto, O., Polverino de Laureto, P., DasGupta, B.R., and Montecucco, C. (1992). Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. Nature *359*, 832– 835.

Silinsky, E.M., and Redman, R.S. (1996). Synchronous release of ATP and neurotransmitter within milliseconds of a motor nerve impulse in the frog. J. Physiol. *492*, 815–822.

Stenhäuser, C., and Gallo, V. (1996). News on glutamate receptors in glial cells. Trends Neurosci. *19*, 339–345.

Stout, C.E., Costantin, J.L., Naus, C.C., and Charles, A.C. (2002). Intercellular calcium signaling in astrocytes via ATP release through connexin hemichannels. J. Biol. Chem. *277*, 10482–10488.

Swanson, R.A., and Graham, S.H. (1994). Fluorocitrate and fluoroacetate effects on astrocyte metabolism in vitro. Brain Res. *664*, 94–100.

Szerb, J.C., and Issekutz, B. (1987). Increase in the stimulation-induced overflow of glutamate by fluoroacetate, a selective inhibitor of the glial tricarboxylic cycle. Brain Res. *410*, 116–120.

Trendelenburg, A.U., and Bultmann, R. (2000). P2 receptor-mediated inhibition of dopamine release in rat neostriatum. Neuroscience *96*, 249–252.

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

Vogt, K.E., and Nicoll, R.A. (1999). Glutamate and gamma-aminobutyric acid mediate a heterosynaptic depression at mossy fiber synapses in the hippocampus. Proc. Natl. Acad. Sci. USA *96*, 1118–1122.

Wang, Z., Xu, N., Wu, C., Duan, S., and Poo, M. (2003). Bidirectional changes in spatial dendritic integration accompanying long-term synaptic modifications. Neuron *37*, 463–472.

Westfall, T.D., Kennedy, C., and Sneddon, P. (1997). The ecto-ATPase inhibitor ARL 67156 enhances parasympathetic neurotransmission in the guinea-pig urinary bladder. Eur. J. Pharmacol. *25*, 169–173.

Zucker, R.S., and Regehr, W.G. (2002). Short-term synaptic plasticity. Annu. Rev. Physiol. *64*, 355–405.