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Supporting Online Material for

Astrocytes Potentiate Transmitter Release at Single Hippocampal Synapses

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SUPPORTING ONLINE MATERIAL

MATERIALS AND METHODS

Hippocampal slice preparation. Hippocampal slices were obtained from wistar rats (12-17 days old). All the procedures for handling and sacrificing animals followed the European Commission guidelines (86/609/CEE). Animals were anaesthetized and decapitated. The brain was rapidly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF). Slices (350-400 μm thick) were incubated during >1 h at room temperature (21-24° C) in ACSF that contained (in mM): NaCl 124, KCl 2.69, KH_2PO_4 1.25, MgSO_4 2, NaHCO_3 26, CaCl_2 2 and glucose 10, and was gassed with 95% O_2 / 5% CO_2 (pH = 7.3). Slices were then transferred to an immersion recording chamber and superfused at 2 ml/min (the chamber volume was replaced in 10-12 min) with gassed ACSF including 0.05 mM Picrotoxin to block GABA_A receptors. Cells were visualized under an Olympus BX50WI microscope.

Electrophysiology. Simultaneous electrophysiological recordings from CA1 pyramidal neurons and astrocytes located in the *stratum radiatum* were made using the whole-cell patch-clamp technique. Patch electrodes had resistances of 3-10 $\text{M}\Omega$ when filled with the internal solution that contained (in mM) for pyramidal neurons: KGlucuronate 135, KCl 10, HEPES 10, MgCl 1, ATP- Na_2 2 (pH = 7.3); and for astrocytes: KMeSO_4 100, KCl 50, HEPES 10, ATP- Na_2 4 (pH = 7.3). Recordings were obtained with PC-ONE amplifiers. Fast and slow whole-cell capacitances were neutralized and series resistance was compensated (≈ 70 %), and the membrane potential was held at -70 mV. Electrophysiological properties were monitored before and at the end of the experiments. Series and input resistances were monitored throughout the experiment using a -5 mV pulse. Recordings were considered stable when the series and input resistances, resting membrane potential and stimulus artifact duration did not change $> 20\%$. Furthermore, I-V curves and firing pattern before and at the end of the experiments were similar. Cells that did not meet these criteria were discarded. Signals were fed to a Pentium-based PC through a DigiData 1320 interface board. Signals were filtered at 1 KHz and acquired at 10 KHz sampling rate. The pCLAMP 8 software was used for stimulus generation, data display, acquisition and storage. ATP (20 mM in ACSF, pH 7.5–8) was ionophoretically delivered by 5 s duration current pulses using a micropipette located in *stratum radiatum*. Experiments were performed at room temperature (21-24 °C).

Synaptic stimulation. Theta capillaries (2–5 μm tip) filled with ACSF were used for bipolar minimal stimulation. The electrodes were connected to a stimulator S-900 through an isolation unit and placed in the *stratum radiatum* to stimulate Schaffer collateral (SC) afferents. Single pulses (250 μs duration) or paired pulses (50 ms interval), were continuously delivered at 0.5 Hz. The stimulus intensity (10-50 mA) was adjusted to meet the conditions that putatively stimulate single, or very few synapses (c.f., 1,2), and was unchanged during the experiment. The recordings that did not meet these criteria (1,2) and synapses that did not show amplitude stability of EPSCs were rejected. The synaptic parameters analyzed were: synaptic efficacy (mean peak amplitude of all responses including failures); synaptic potency (mean peak amplitude of the successes); probability of release (Pr, ratio between number of successes versus total number of stimuli); and paired-pulse facilitation (PPF = [(2nd EPSC – 1st EPSC)/ 1st EPSC]). Synaptic parameters were determined from 50 stimuli before (basal), during and shortly after astrocyte stimulation, and after stimulus (recovery). Up to four responses per cell were averaged. All the responses were included in data evaluation. Relative values of synaptic parameters were averaged from different cells. To illustrate the time course of astrocyte-induced effects, synaptic parameters were grouped in 20 or 33 s bins. EPSC amplitude was determined as the peak current amplitude (2-6 ms after stimulus) minus the mean baseline current (20-30 ms before stimulus). A response was considered a failure if the amplitude of the current was < 3 times the standard deviation of the baseline current (1.3 - 2.3 pA) and was verified by visual inspection. To obtain τ_{on} and τ_{off} , EPSC time courses were fitted to single exponential curves using the pClamp software. Synapses that in the presence of CPT showed a high Pr that prevented the adequate quantification of a further ATP-evoked Pr increase were excluded. In some cases, CPT application led to epileptiform activity; in such cases the experiments were discarded.

Calcium imaging and UV flash photolysis. Astrocytes were identified according to morphological and electrophysiological criteria (c.f., 3,4). In photo-stimulation experiments, only astrocytes with passive electrophysiological properties that are known to be competent for glutamate release through SNARE protein-dependent mechanisms were considered (5,6). To monitor Ca^{2+} levels in single astrocytes, patch pipettes were filled with the internal solution containing 50 μM fluo-4, and when appropriate, 5 mM NP-EGTA. For Ca^{2+} uncaging, train pulses (1 ms duration, 6-15 mW) of UV light (340-380 nm) were delivered at 2 Hz during 5 s to the soma and processes of the recorded astrocyte

(optical window of 15-25 μm diameter) using a flash photolysis system. When pairing the astrocyte Ca^{2+} stimulation with a transient neuronal depolarization (to -30 mV; 5 min), 3 UV-flash trains or ATP pulses (5 s) were applied. Astrocytes were illuminated with a xenon lamp at 490 nm using a monochromator Polychrome V, and were imaged using a CCD camera. Astrocytes were illuminated during 100–150 ms, and images were acquired every 0.5–1 s. The Polychrome V and the CCD camera were controlled by IPLab software, which was used for epifluorescence measurements. Ca^{2+} variations were estimated as changes in the fluorescence signal over baseline ($\Delta F/F_0$) after background subtraction. In some cases, slices were incubated with fluo-4 AM (2–10 μM) for 20–30 min at room temperature. In these conditions, most of the cells loaded were astrocytes (c.f., 4,7). For the cleavage of synaptobrevin 2 by Tetanus toxin (TeNT) astrocytes were intracellularly dialyzed with the light-chain of TeNT for at least 10 min before starting the uncaging experiments, and whole-cell parameters remained stable during that period.

Data are expressed as mean \pm SEM. Results were compared using a two-tailed Student's *t*-test ($\alpha = 0.05$). Statistical differences were established with $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***). Cumulative amplitude plots were analyzed using the Kolmogorov-Smirnov test.

Drugs and chemicals. Light-chain of Tetanus toxin (400 nM dissolved in electrode-filling solution) was generously donated by Dr. J. Blasi.

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Fig. S1. Summary of synaptic transmission parameters evoked by minimal stimulation. Synaptic potency (average amplitude of successful responses) and synaptic efficacy (average EPSC amplitudes including failures). Mean probability of neurotransmitter release (Pr) (n = 34). PPF index (n = 20) and representative averaged EPSCs (50 consecutive trials, including failures) evoked by paired-pulse (50 ms delay) minimal stimulation. Error bars indicate SEM.

Fig. S2. Ca²⁺ uncaging in single astrocytes. (A) Pseudocolor images representing fluorescence intensities of a single astrocyte filled with fluo-4 (50 μ M) and NP-EGTA (5 mM), before (Pre) and after UV-flash stimulation (2Hz, 5s). (B) Astrocyte Ca²⁺ elevations evoked by successive UV-flashes (horizontal lines). Scale bar, 15 μ m.

Fig. S3. Astrocyte stimulation evoked changes in PPF index. (A) Representative mean EPSCs (50 successive stimuli, including failures) evoked by paired-pulse minimal stimulation before (basal) and after astrocyte stimulation, and the scaled trace of the basal EPSC. (B) Summary of PPF index before (basal), after astrocyte stimulation and 2 min later (Recovery; n = 18). **P < 0.01. Error bars indicate SEM.

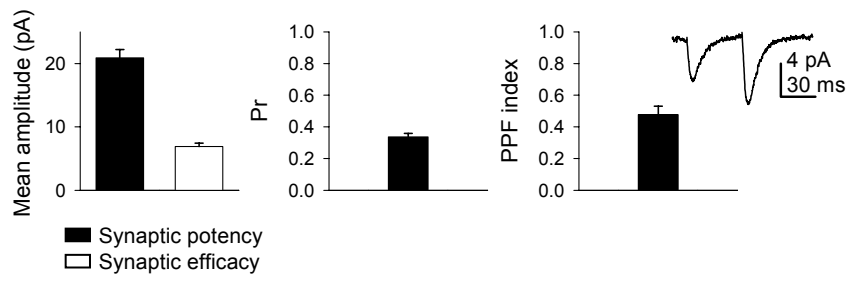
Fig. S4. UV-flash stimulation *per se* did not modify the synaptic transmission. (A) Pseudocolor images representing fluorescence intensities of a fluo-4-filled astrocyte in the absence of NP-EGTA, and synaptic responses (10 consecutive stimuli) obtained from paired whole-cell recordings before and after UV-flash stimulation. Scale bar, 20 μ m. (B) Synaptic efficacy, Pr and synaptic potency over time (bin width, 33 s; n = 9 astrocyte-neuron pairs). Zero time corresponds to the time of UV-flash stimulation. (C) Relative changes of synaptic parameters evoked by UV-flash stimulation (n = 9). Error bars indicate SEM.

Fig. S5. Effects of A1 purinergic receptor antagonist CPT on synaptic transmission. Synaptic parameters (synaptic efficacy, Pr and synaptic potency) normalized to baseline and plotted over time before and after perfusion with CPT (2 μ M) (n = 14). Error bars indicate SEM.

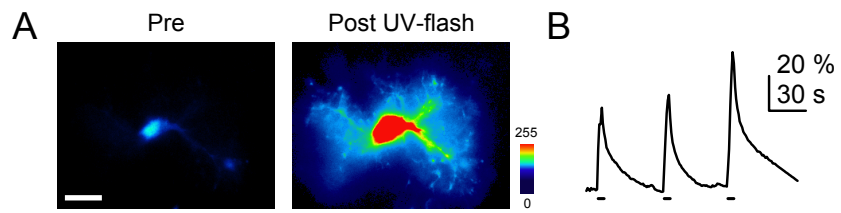
Fig. S6. ATP-evoked astrocyte Ca²⁺ elevations potentiate synaptic transmission through activation of group I mGluRs. Relative changes of synaptic parameters evoked by ATP-induced astrocyte Ca²⁺ elevations in control (top) and after perfusion with MPEP+LY367385 (bottom) (n = 7). *P < 0.05. Error bars indicate SEM.

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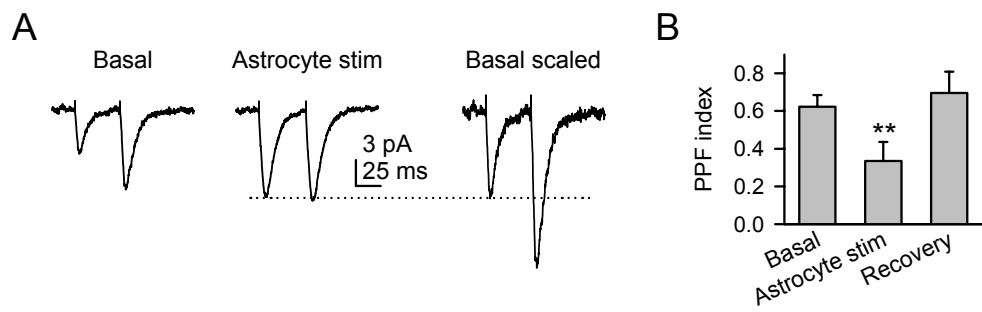
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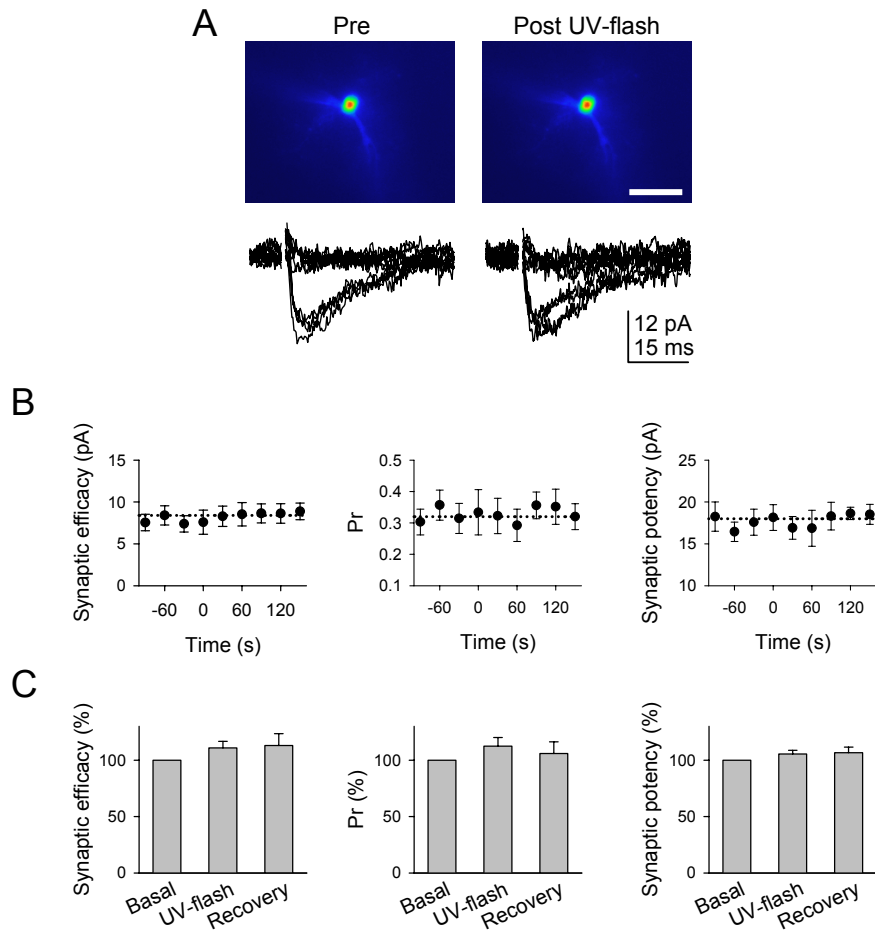
Perea and Araque Fig. S1



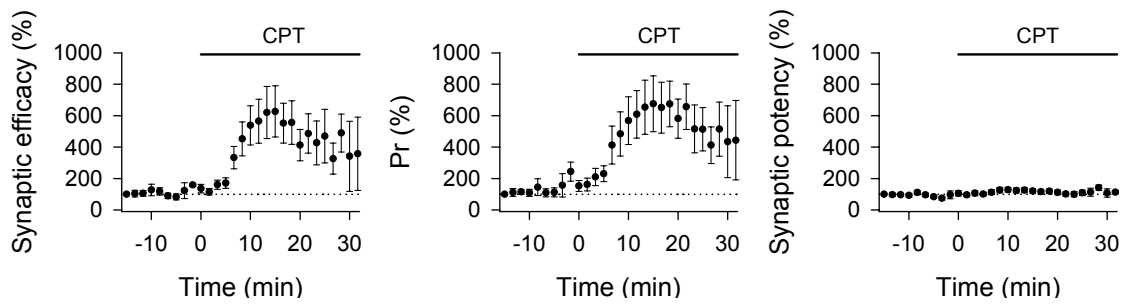
Perea and Araque Fig. S2



Perea and Araque Fig. S3

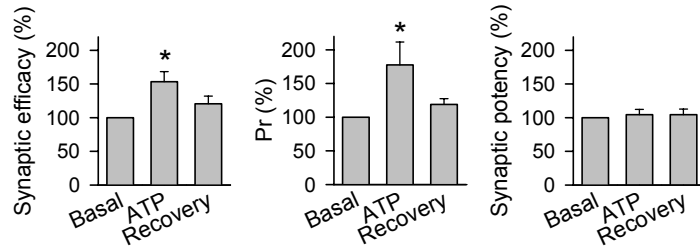


Perea and Araque Fig. S4

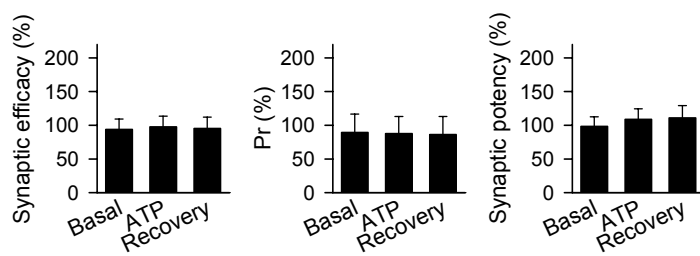


Perea and Araque Fig. S5

Control



MPEP + LY367385



Perea and Araque Fig. S6