Caffeine-induced synaptic potentiation in hippocampal CA2 neurons

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SUPPLEMENTARY METHODS

Hippocampal Slices

Hippocampal slices were prepared from Sprague Dawley rats (postnatal day 14 to 18 or >28 days), as approved by the NIEHS Animal Care and Use Committee. Animals were anesthetized with sodium pentobarbital, decapitated and the brains rapidly removed. Coronal brain slices (340 µm thick) containing the hippocampus were cut using a vibrating blade microtome in ice-cold artificial cerebral spinal fluid (ACSF); sucrose-substituted for NaCl, containing the following (in mM): 240 sucrose, 2.0 KCl, 1 $MgCl_2$, 2 $MgSO_4$, 1 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$ and 10 glucose, which was bubbled with 95% O_2 / 5% CO_2. Freshly-cut slices were placed in a holding chamber containing ACSF at room temperature and allowed to recover for at least one hour prior to experimental use. Following the recovery period, slices were transferred via transfer pipette to a recording chamber where they were bathed continuously with room temperature ACSF unless otherwise noted. Standard ACSF consisted of the following (in mM): 124 NaCl, 2.5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$ and 17 D-glucose. In experiments where caffeine was administered orally to rats *in vivo* prior to whole-cell recordings *in vitro*, the caffeine was first dissolved in water to one of three doses (2 mg/kg, 6.5 mg/kg, or 20 mg/kg) and then administered to rats manually by gavage one hour before dissection.

Electrophysiological Recordings

Whole-cell voltage clamp recordings from hippocampal neurons were made using patch pipettes (3 to 4 MΩ) filled with a solution containing (in mM): 120 K-gluconate, 10 KCl, 3 $MgCl_2$, 0.5 EGTA, 40 HEPES, 2 Na_2-ATP and 0.3 Na-GTP, with pH adjusted to 7.2 by KOH. Stimulating electrodes (cluster-type, from FHC, Bowdoin, ME) were placed in the stratum radiatum in a location intended to stimulate the Schaffer Collaterals. All compounds used for pharmacological experiments were prepared as concentrated stock solutions prior to use (PKI, Enzo Life Sciences, NY, USA; all other compounds purchased from Tocris Biosciences, MO, USA). In experiments where the duration of A_1R-P was tested, DPCPX or caffeine was applied to slices in a separate holding chamber for 5 minutes following the one hour recovery period. Immediately after the 5-min treatment period, slices were returned to the holding chamber containing only standard ACSF for a period of either one, two or >three hours before being transferred to the recording chamber for whole-cell experiments.

Two-Photon Laser Scanning Confocal Microscope Imaging of Dendritic Spines

To determine whether the persistence of A_1R-P in CA2 neurons reflected structural changes at the synapse, we imaged spine-containing segments of secondary and tertiary apical dendrites from CA2 neurons. Cells were dialyzed through patch pipettes with internal solution containing 100 mM Alexa Fluor-594 and were allowed 15 to 20 min following break-in for dye equilibration. Because extensive dialyzing of neurons may prevent expression of plasticity, 5 µM beta-actin was added to the internal solution to prevent washout of A_1R-P induction with DPCPX when assessed 20-min after break-in (Tanaka et al., 2008). Once loaded with dye, images of the neurons were acquired using a Ti-Sapphire laser tuned to 840 nm. Volumetric image-stacks of spine-containing dendrites on CA2 neurons were acquired at 0.5 µm thickness (3 to 4 images per stack for a total imaged volume of ~2 µm).

Computation of Spine Volume

Two photon images of spine-containing dendrites were first deconvolved to minimize contributions from light scattering, using commercially available software (Huygens Pro). Following deconvolution, spine volume was then measured at each 5-min capture with two independent measures using custom-written routines in MATLAB. In the first approach spine volume was approximated using an ellipsoid model ($V = 4/3*r1*r2^2$) and a basic edge detection algorithm to identify the length of spine radii. Fluorescence values were binned along a line segment extending across the horizontal (across spine head) and longitudinal (along spine neck) axis of each spine to approximate r2 and r1, respectively. The value obtained for r2 was used to approximate the third radial distance in the volume as the resolution of our z-axis acquisition (0.5 µm) was inadequate for precise calculation. Gaussian curves were fit to the fluorescence values

along each line and spine diameters were estimated by identifying the points of maximal slope along the gaussian fit (r = d/2). In the second approach spine volume was approximated using a spherical model ($V = 4/3*r^3$). Spine radii were measured based on hand-drawn polygons which were tightly fit to enclose each spine head ($r = \sqrt{(Area/\pi)}$). Volumetric measurements using both approaches were consistently within 10% of each other. All values presented were computed using our radial edge detection method.

SUPPLEMENTARY REFERENCES

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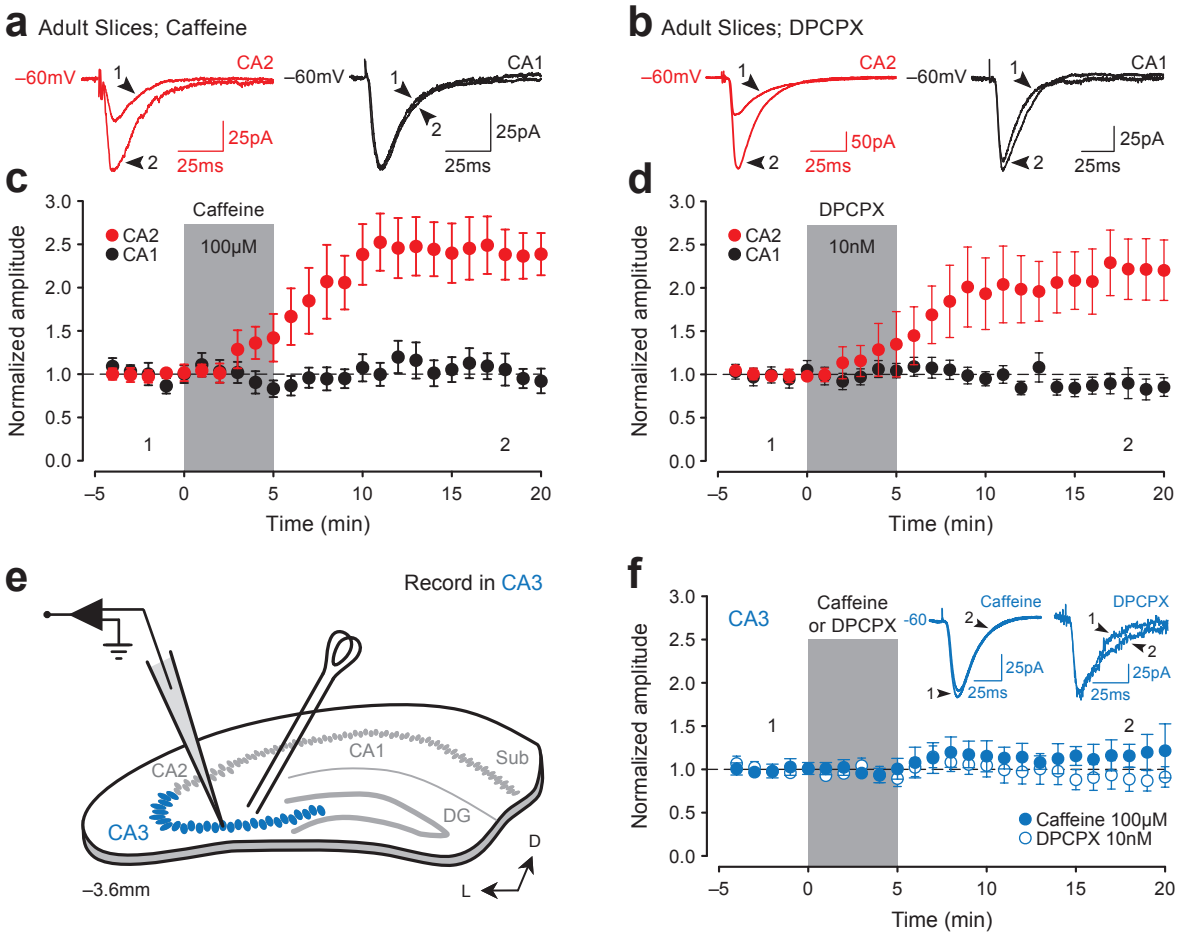
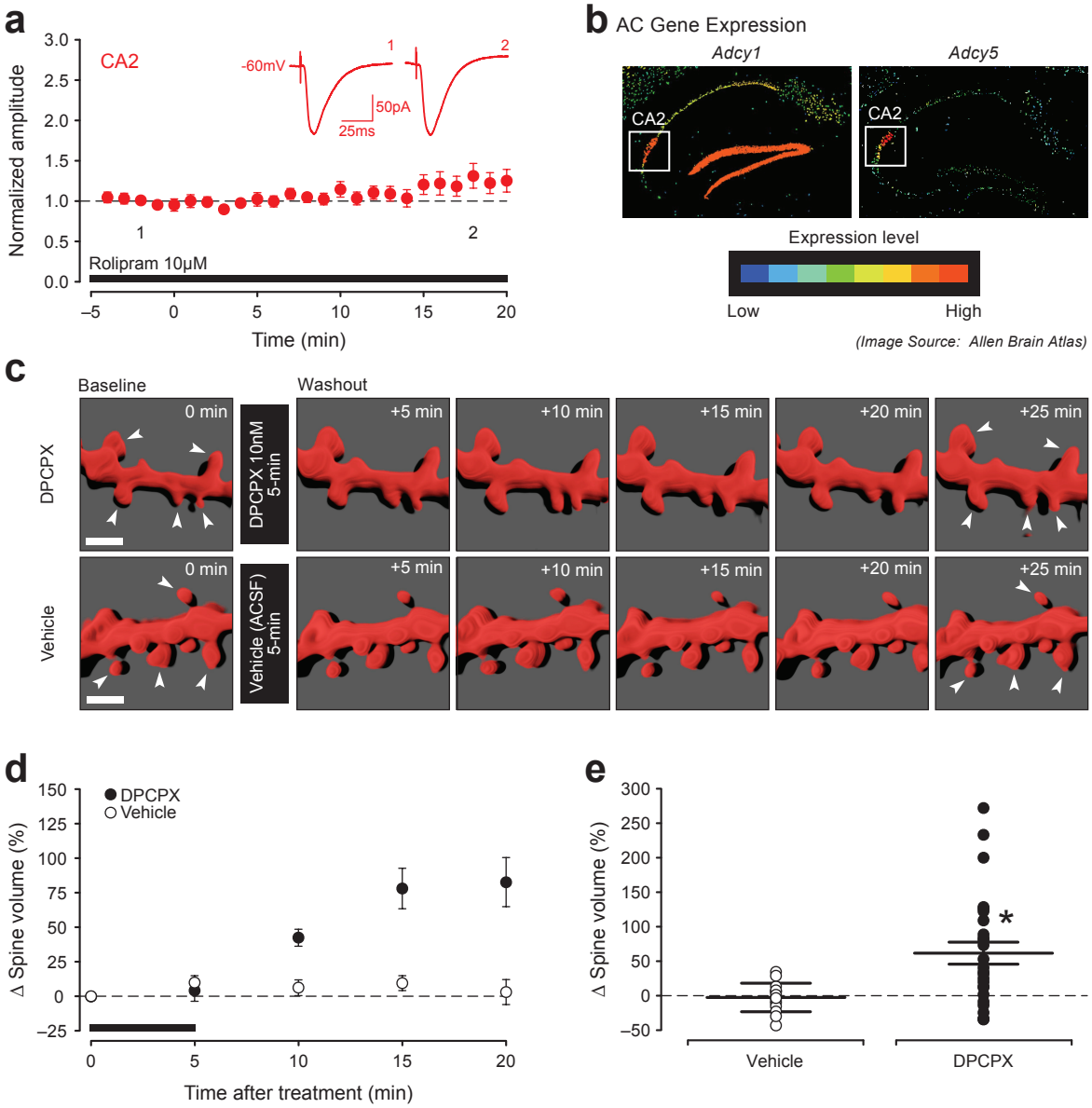


Supplementary Figure 1. A_1R antagonism potentiates EPSCs in CA2 of young adult rats, and does not effect responses in CA3. Brief 5-min application of caffeine (100 μM; **a** and **c**) or DPCPX (10 nM; **b** and **d**) induces a lasting potentiation of synaptic responses in area CA2 (red circles), but not in area CA1 (black circles), in slices taken from young adult rats (4–6 weeks old). Time of antagonist application marked by the gray bar in (**c**) and (**d**). Representative synaptic currents in (**a**) and (**b**) are from the time points marked by corresponding numbers in (**c**) and (**d**). Additionally, bath-application of caffeine or DPCPX has no effect on the amplitude of EPSCs recorded from neurons in area CA3 of the hippocampus in juvenile rats. Schematic diagram highlighting the placement of stimulating and recording electrodes in CA3 is shown in (**e**) and mean synaptic responses before, during and after 5-min application of either caffeine (100μM, filled circles) or DPCPX (10 nM, open circles) are shown in (**f**).



Supplementary Figure 2. Inhibition of phosphodiesterase has no effect on EPSCs in CA2, and brief exposure to DPCPX increases spine volume in CA2 neurons. Continuous perfusion of the phosphodiesterase inhibitor rolipram (10 μM; black bar) has no effect on basal synaptic transmission in area CA2 (**a**), and is similar to what was shown by Barad and colleagues (1998). Inset traces taken from the time-points indicated by the numbers. Pseudo-color images of mouse brain *in situ* hybridizations for *Adcy1* and *Adcy5* genes in hippocampus (**b**). Adenylyl cyclases 1, 5 and 6 show strong expression in the CA2 region of the hippocampus (*Adcy6*, **not shown**). Images adapted from the Allen Brain Atlas (see Lein et al., 2007). The persistence of A_1R-P in CA2 neurons is associated with structural changes at the synapse. Images of spine-containing dendrites in CA2 taken at 5-min intervals following bath-application of either DPCPX or vehicle for 5-min (**c**; scale bars = 2 μm). Spine volume is increased by DPCPX treatment but not by vehicle (see also Korkotian & Segal, 1999). Mean change in spine volume over time (in **d**) for spines indicated by white arrows in (**c**). Average change in spine volume for all CA2 neurons at 20-min post-treatment with DPCPX (n = 32 spines) or vehicle (n = 24 spines) (**e**). Note; *, $P < 0.05$. Similar results of smaller magnitude were observed with caffeine (not shown).