Supplementary material

Astrocytes respond to a neurotoxic Aβ fragment with state-dependent Ca^{2+} alteration and multiphasic transmitter release

Abbreviation list:

2-APB – 2-Aminoethoxydiphenyl borate

Aβ – amyloid β

AD – Alzheimer's disease

cAMP – cyclic adenosine monophosphate

CBX - carbenoxolone

CX – connexin

DCPIB –

4-[(2-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1*H*-inden-5-yl)oxy]butanoic acid

EPAC – exchange protein directly activated by cAMP

ER – endoplasmic reticulum

FRET – fluorescence resonance energy transfer

GLT-1 – glutamate transporter 1

GLAST – glutamate aspartate transporter

GPN – glycyl-L-phenylalanine 2-naphthylamide

mGluR – metabotropic glutamate receptor

MPEP – 2-Methyl-6-(phenylethynyl)pyridine

NCX – sodium calcium exchanger

NPPB – 5-nitro-2-(3-phenylpropylamino) benzoic acid

OGB-1 – Oregon green bapta 1

PMCA – plasma membrane Ca^{2+} ATPase

PPADS – pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid

SOC – store-operated channel

SD – standard deviation

TIRFM – total internal reflection fluorescence microscopy

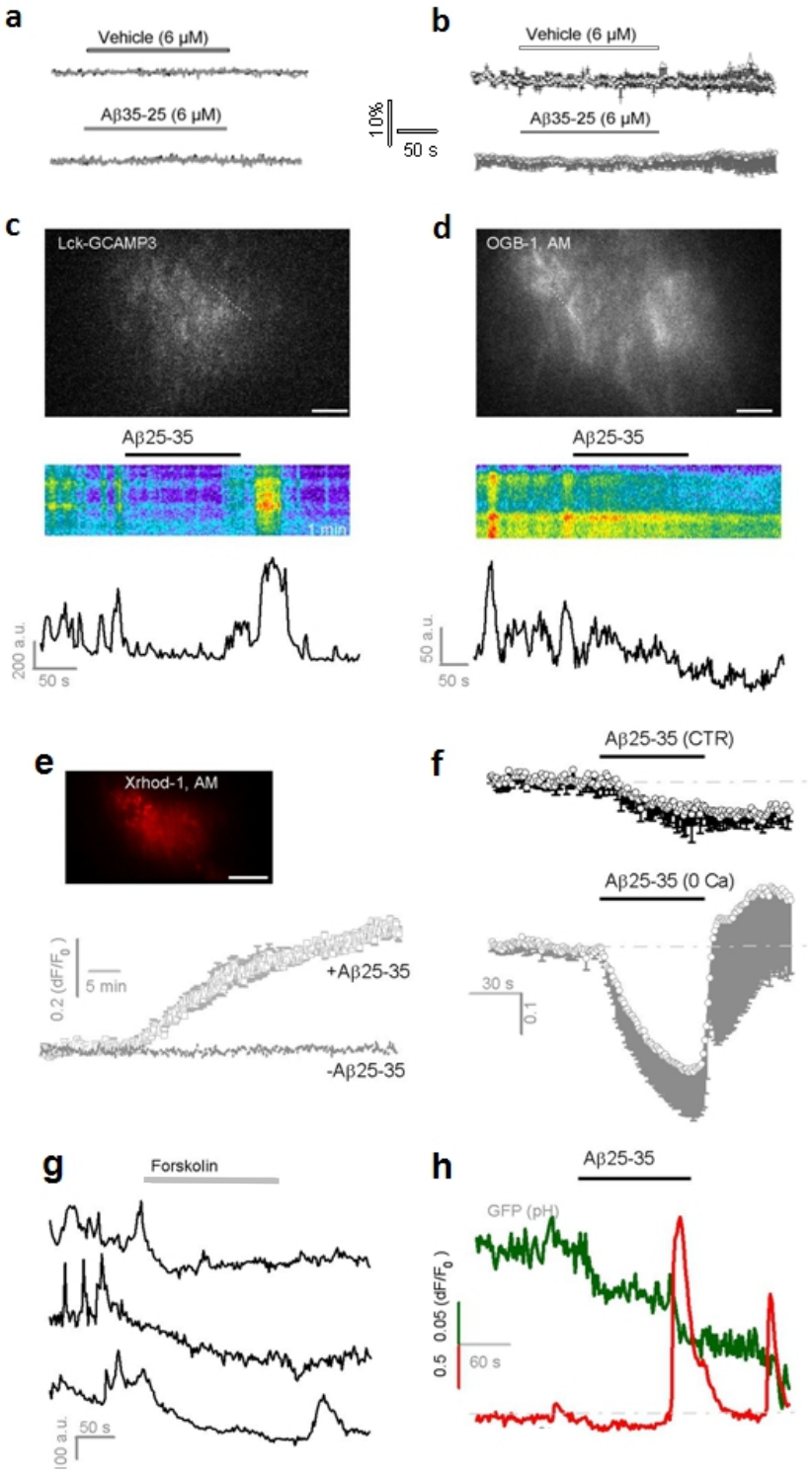

Fig. S1 Effects of Aβ25-35 on astrocyte Ca^{2+} signaling. Cultured astrocytes in basal condition (**a**) and after Aβ preconditioning (**b;** Aβ25-35, 0.5 μM, 2 hr) in response to either vehicle (i.e., equal molar H_2O) or the reversed peptide Aβ35-25 (n = 7 per condition). (**c-d**) Inhibitory effect of Aβ (6 μM) on spontaneous astrocytic Ca^{2+} oscillation. (**e**) Preconditioning with Aβ25-35 (0.5 μM) caused gradual Ca^{2+} overload in astrocytes. The chemical Ca^{2+} dye

Xrhod-1, AM was used for long-term Ca^{2+} imaging (n = 8 cells for each). (**f**) Removing extracellular Ca^{2+} facilitated Aβ25-35-caused Ca^{2+} diminution (n = 9 for CTR, n = 7 for zero external Ca^{2+} condition). (**g**) Inhibition of spontaneous astrocyte Ca^{2+} oscillations by forskolin (100 μM). (**h**) Stepwise decreases in intracellular pH observed in the 'mix' type astrocyte Ca^{2+} response, which was evoked by Aβ25-35 (6 μM) in astrocytes after short-term preconditioning (Aβ25-35 0.5 μM, 0.5 hr). The red Ca^{2+} sensor GECO-R and the pH-sensitive GFP fluorescent protein were co-expressed in cultured astrocytes. During Aβ25-35 application, the initial small diminution in basal Ca^{2+} was likely due to the direct potentiation of PMCA that meanwhile caused H^+ influx thereby decreasing the GFP fluorescence. The delayed Ca^{2+} rise would have also activated PMCA to confine the Ca^{2+} elevation, inducing more H^+ influx to further quench the GFP fluorescence. During the elevation phase, cytosolic Ca^{2+} could also be buffered into ER via the sacro/endoplasmic reticulum Ca^{2+} ATPase. Scale bars, 5 μm.

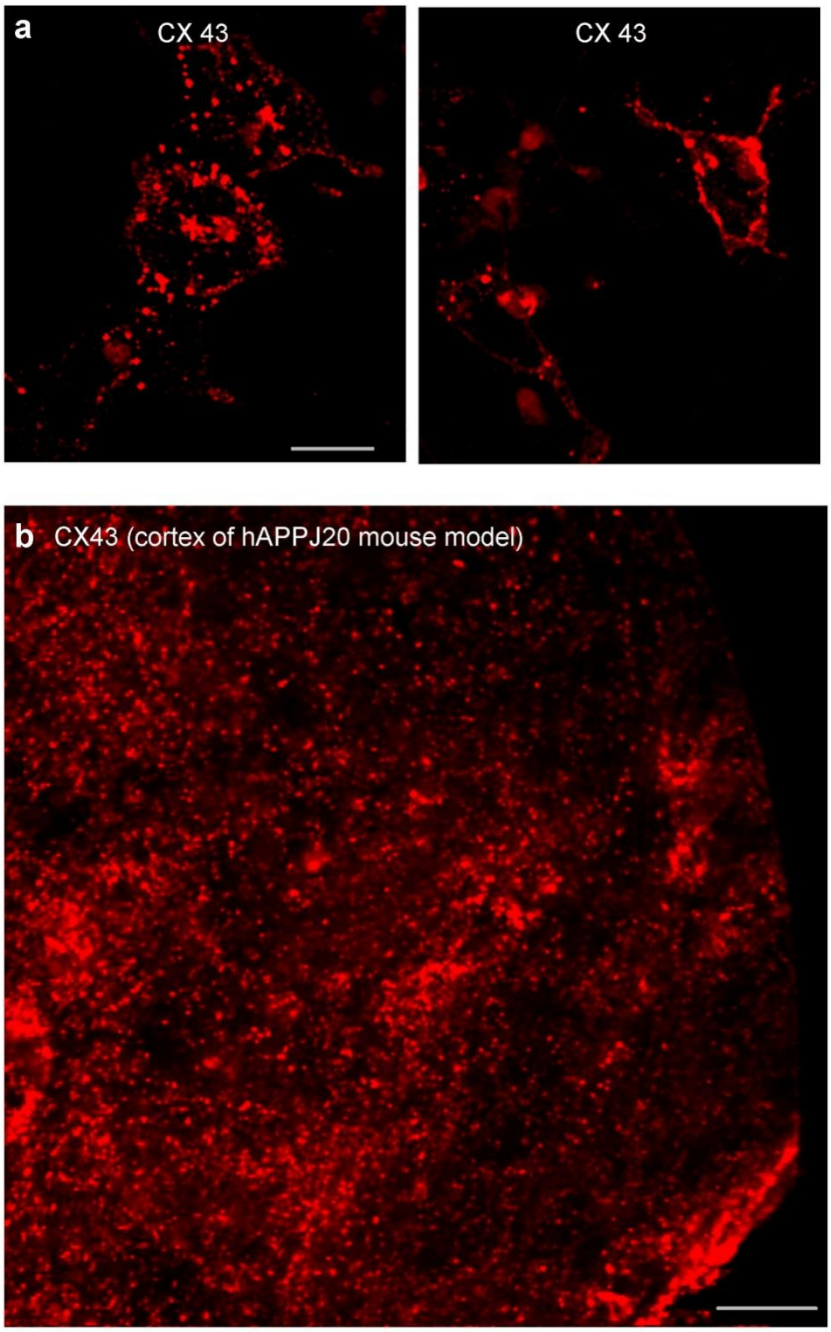

Fig. S2 Immunostaining of connexin 43 in cultured cortical astrocytes (**a**) and in somatosensory cortex of hAPPJ20 AD mouse (~7 month age; **b**). Scale bars, 20 μm.

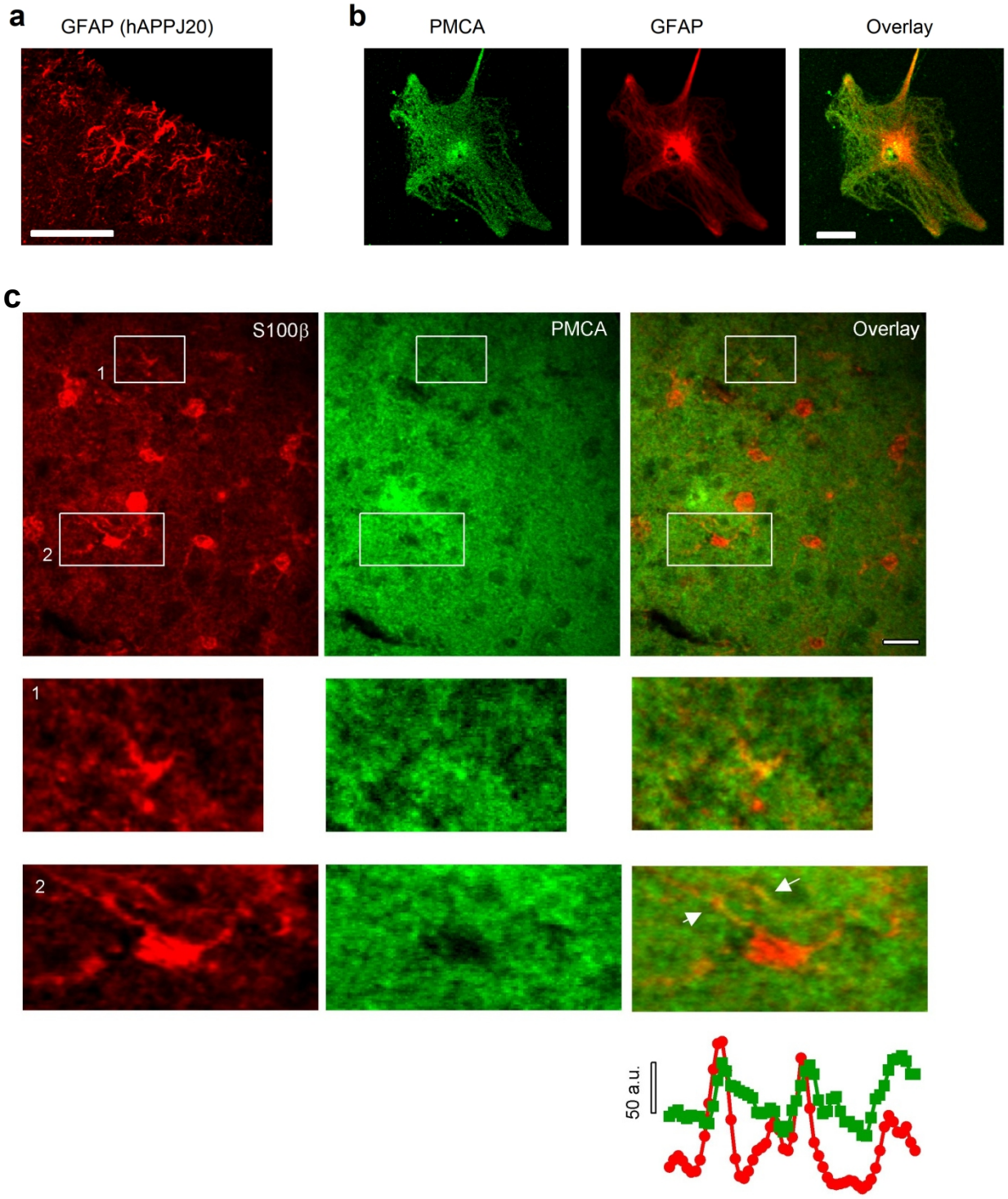

Fig. S3 Immunostaining of PMCA in astrocytes. (**a**) GFAP-positive astrocytes observed by immunostaining in the somatosensory cortex of J20 AD mouse model (~7 month old). Scale bar, 50 µm. (**b**) Immunostaining of a pan PMCA antibody against the astrocyte marker GFAP in cultured cortical astrocytes of wild-type mice. Scale bar, 10 µm. (**c**) PMCA

immunostaining in astrocytes in somatosensory cortex of hAPPJ20 AD mouse model. The diffuse staining pattern is consistent with the membranous expression of PMCA. A subpopulation of PMCA labeling was observed to surround S100β-positive astrocytic somata, and present in S100β-identified processes. Mouse age is ~7 month. Scale bar, 20 μm.

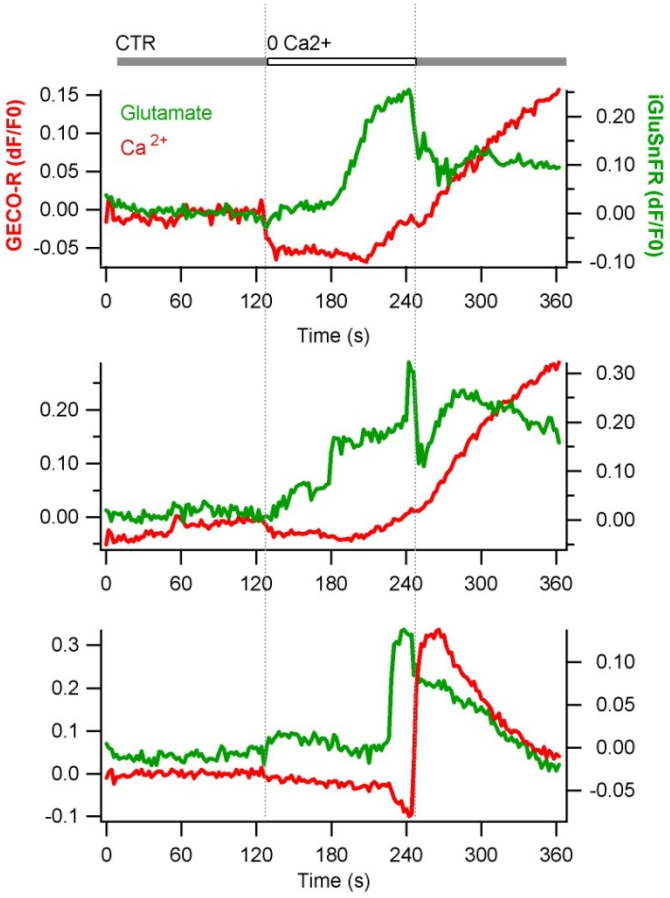

Fig. S4 Astrocyte glutamate release via opened CX hemichannels. CX hemichannels were opened by Ca^{2+}-free solution (open bar). Cytosolic Ca^{2+} and glutamate release were simultaneously imaged with TRIFM, in astrocytes co-expressing the red GECO-R and green iGluSnFR sensor. Glutamate release was observed in the absence of Ca^{2+} elevation. A diminution in cytosolic Ca^{2+} was observed in the phase of 0 Ca^{2+}, indicating the gradual Ca^{2+} efflux due to the reversed ion gradient. Switching back to control solution (1.8 mM Ca^{2+}) re-closed CX hemichannels, causing a transient inhibition for the glutamate release; meanwhile, re-supplying Ca^{2+} elevated Ca^{2+} levels in the cytosol that then sustained another

phase of glutamate release. This result is in line with the co-existence of Ca^{2+}-independent and -dependent glutamate release from astrocytes.

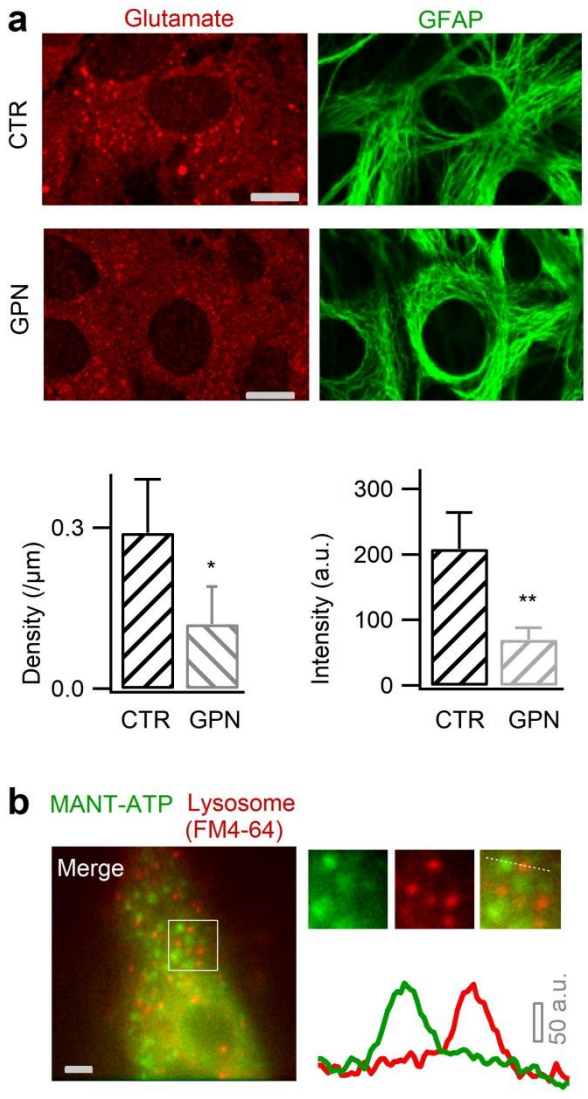

Fig. S5 Astrocyte lysosomes versus glutamate staining and ATP indicator. (**a**) Glutamate immunostaining in cultured astrocytes concentrated in perinuclear vesicular compartments. Punctate staining was reduced by cathepsin C substrate GPN (200 µM), a lysosome-disrupting compound (n = 9 - 11 cells per condition). Scale bar, 5 µm. (**b**) Representative spatial distribution of the fluorescent ATP marker MANT-ATP (50 µM, 1 hr) and the lysosomal marker FM4-64 (6.7 µM, 30 min). Scale bar, 10 µm.

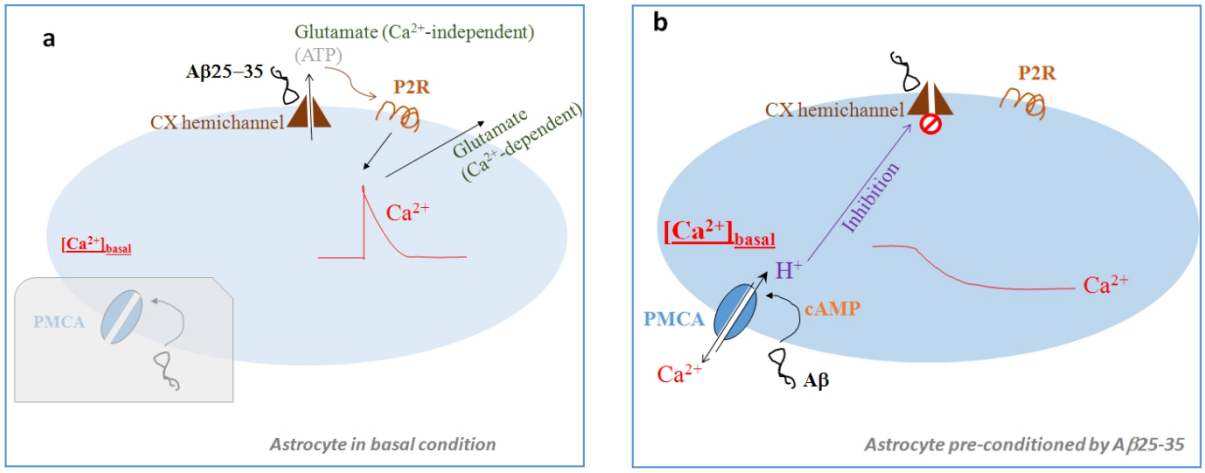

Fig. S6 Astrocyte response to Aβ25-35. (**a**) In astrocyes of basal conditions, cytosolic Ca^{2+} is maintainted at physiological low level (~100 nM) and PMCA shows no strong reaction to Aβ25-35. Meanwhile, Aβ25-35 acts on CX hemichannels leading to the release of glutamate, and likely ATP, in a Ca^{2+}-independent manner. Activation of purinergic P2 receptors then contributes to the intracellular Ca^{2+} elevation that triggers the Ca^{2+}-dependent release of glutamate. Aβ25-35 therefore plays an excitatory role in astrocytes in basal conditions. (**b**) Preconditioning of astrocytes with submicromolar Aβ25-35 peptide leads to gradual intracellular Ca^{2+} overload thereby setting a greater driving tendency for Ca^{2+} efflux. In this situation, PMCA actively reacts to subsequent Aβ25-35 stimulation, extruding cytosolic Ca^{2+} to the extracellular space therefore lowering the intracellular Ca^{2+} level. The coupled H^+ influx then inhibits the opening of CX hemichannels and the intracellular Ca^{2+} elevation. Aβ25-35 hence exerts an inhibitory effect on the inracellular Ca^{2+} level in preconditioned astrocytes.

Table S1 Antibodies used for fluorescence immunostaining

Experiments	Primary antibodies	Secondary antibodies
Single staining		
GFAP in brain slices of hAPPJ20 AD mouse model (Figure 3i)	Rb GFAP, Agilent/DAKO, Cat N° Z0334, 1/1000	Alexa 594 Goat (G) anti-Rb, Invitrogen, Cat N° A11012, 1/1000
Connexin 43 staining in brain slices of hAPPJ20 AD mouse model and in cultured astrocytes (Figure S2)	Rb Connexin 43, Sigma, Cat N° C6219, 1/1500	Alexa 594 G anti-Rb, Invitrogen, Cat N° A11012, 1/1000
Double staining		
GFAP and PMCA in cultured astrocytes (Figure 3j)	Rb GFAP, Agilent/DAKO, Cat N° Z0334, 1/1000 Mouse (M) pan PMCA ATPase Monoclonal Antibody, ThermoFisher Scientific, Cat N° MA3-914, 1/500	Alexa 594 Goat (G) anti-Rb, Invitrogen, Cat N° A11012, 1/1000 Alexa 488 G anti M IgG2A, Invitrogen, Cat N° A-21131, 1/1000
S100β and PMCA co-staining in the cortex of J20 AD mouse (Figure S3)	Rb monoclonal S100β , Abcam, Cat N° ab52642, 1/500 Mouse (M) pan PMCA ATPase Monoclonal Antibody, ThermoFisher Scientific, Cat N° MA3-914, 1/500	Alexa 594 G anti-Rb, Invitrogen, Cat N° A11012, 1/1000 Alexa 488 G anti M IgG2A, Invitrogen, cat N°A-21131, 1/1000

Table S2 Combinations of excitation wavelengths and filters for TIRFM imaging

Fluorophore	Excitation laser line (nm)	Dichroic filter (nm)	Emission filter (nm)
EGFP, pHluorin	488	500LP or PolyX[§]	535(50)BP
GECO-R	568	PolyX[§]	600LP
GFP(nd)-EPAC1(dDEP)-mCherry	488	PolyX[§]	535(50)&600LP[$]
OGB-1	488	PolyX[§]	535(50)BP
OGB-1 and FM4-64	488	PolyX[§]	535(50)&675(50)BP[$]
Xrhod-1	568	PolyX[§]	600LP or 615(45)BP

[§]PolyX – custom dual dichroic mirror with 488/568/NIR reflection bands and low ripple high-transmission elsewhere (AHF Analysentechnik).
[$]*denotes filters for simultaneous view with a custom dual-view device*.