PNAS

Supplementary Information for

Neuronal activity-induced, equilibrative nucleoside transporter-dependent, somatodendritic adenosine release revealed by a GRAB sensor

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Supplementary materials and methods

Cell cultures

HEK293T cells were obtained from ATCC (CRL-3216) and verified based on their morphology under the microscope and by their growth curve. HEK293T cells were cultured at 37°C in 5% CO2 in DMEM (Biological Industries) supplemented with 10% (v/v) fetal bovine serum (FBS, CellMax) and 1% (v/v) penicillin-streptomycin (Gibco).

Rat and mouse primary neurons were prepared from 0-day-old (P0) pups (male and female, randomly selected). Hippocampal neurons were dissociated from the dissected brains in 0.25% Trypsin-EDTA (Gibco) and plated on 12-mm glass coverslips coated with poly-D-lysine (1mg/ml, Sigma-Aldrich) in a neurobasal medium (GIBCO) containing 2% B-27 supplement (Gibco), 1% (v/v) GlutaMAX (Gibco), and 1% penicillin-streptomycin (Gibco). Based on glial cell density, after approximately 3 days in culture (DIV 3), cytosine β-D-arabinofuranoside (Sigma) was added to the hippocampal cultures in a 50% growth media exchange at a final concentration of 2 μM.

Primary astrocytes were prepared as previously described (1). In brief, the hippocampi were dissected from P0 rat pups, and the cells were dissociated with trypsin digestion for 10 mins at 37ºC and plated on a poly-D-lysine-coated T25 flask. The plating and culture media contained DMEM supplemented with 10% (v/v) FBS and 1% penicillin-streptomycin. The next day and every 2 days thereafter, the medium was changed. At DIV 7-8, the flask was shaken on an orbital shaker at 180 rpm for 30 min, and the supernatant containing the microglia was discarded; 10 ml of fresh astrocyte culture medium was then added to the flask, which was shaken at 240 rpm for ≥6 h to remove oligodendrocyte precursor cells. The remaining astrocytes were dissociated with trypsin and plated on 12-mm glass coverslips containing culture medium.

Chemicals and drugs

Adenosine (Sigma), adenosine 5'-triphosphate (ATP, sigma), SCH-58261 (Abcam), ZM-241385 (MedChemExpress or Bio-Techne), NBQX (Sigma), D-AP5 (Tocris or MedChemExpress), tetrodotoxin (TTX, absin), $CdCl_2$ (Sigma), POM1 (Santa Cruz), S-(4-nitrobenzyl)-6-thioinosine (NBTI, Santa Cruz), dipyridamole (Santa Cruz), ω-Conotoxin-GVIA (Tocris), ω-Agatoxin IVA (Cayman), Clozapine N-oxide (CNO, Cayman), nimodipine (Cayman), (±)-felodipine (Cayman), oxotremorine-M (Oxo-M, Cayman), Scopolamine (Tocris), U73122 (Tocris), L-glutamate (Sigma), bradykinin (Sangon Biotech Shanghai), and thrombin (Sigma).

Confocal imaging in cultured cells

Solutions containing chemicals or drugs were delivered via a custom-made perfusion system or bath application. The chamber was cleaned thoroughly with Tyrode's solution and 75% ethanol between experiments. The GFP signal ($GRAB_{Ado}$ sensors) was collected using a 525/50-nm emission filter, and the RFP signal (GAP43-jRGECO1a, jRGECO1a, R^{ncp}-iGluSnFR, mCherry, and Calbryte 590,) was collected using a 595/50-nm emission filter, and the BFP signal (EBFP2-iP2A-TeNT) was recorded using a 450/25-nm emission filter. Where applicable, cells were pre-loaded

with the Ca^{2+} dye Calbryte 590-AM (AAT Bio) by incubation at 37ºC for 40 min before imaging. High K^+-containing Tyrode's solution contained (in mM): 79 NaCl, 75 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 10 glucose (pH 7.3-7.4).

Immunofluorescence staining in brain slices

Brains were fixed in 4% PFA for 8 h at room temperature and dehydrated in 30% sucrose solution for 1~2 days. Thin sections (30 μm) were prepared on a freezing microtome (Leica CM1950). Slices were permeabilized in PBS with 0.3% Triton X-100 (PBST) and blocked in 5% BSA in PBST at room temperature for 40 min. Slices were incubated with primary antibodies (anti-HA, 1:1000, #3724, Cell Signaling Technology) at 4°C overnight. Slices were washed three times in PBST and then incubated with fluorescent secondary antibodies (goat anti-rabbit Alexa Fluor 647, 1:500, 111-605-144, Jackson ImmunoResearch Labs) at room temperature for 2 h and then washed three times in PBST. Brain slices were imaged using Zeiss LSM800.

Patch-clamp electrophysiology

For Patch-clamp electrophysiology, the brain slices were transferred to a recording chamber at room temperature for recordings. The cell was identified with differential interference contrast optics. The pipettes (2-6 MΩ) used for the recordings were prepared using a micropipette puller (P1000, Sutter Instrument). For whole-cell recordings, the pipettes were filled with an internal solution that contained the following (mM): 130 K-gluconate, 10 HEPES, 0.6 EGTA, 5 KCl, 3 Na.2ATP, 0.3 Na.3GTP, 4 MgCl2, and 10 Na.2-phosphocreatine. Voltage- and current-clamp recordings were performed with a MultiClamp 700B amplifier (Molecular Devices). The neurons were held at −65 mV. For photostimulation, an optical fiber (200 μm core diameter, NA = 0.22) coupled to a diode-pumped solid-state 633 nm laser was submerged in ACSF and placed ∼500 μm from the recording site. Data were acquired and analyzed using Clampfit 10.0 software (Molecular Devices).

Western blotting

Tissue samples were collected from mice brains. Tissue lysates were prepared in SA buffer (in mM): 20 HEPES; 10 KCl, 1.5 $MgCl_2$, 1 EDTA, 1 EGTA, 2 DTT, and 0.3% (w/v) Chaps with EDTA-free protease inhibitor cocktail (Roche). After denaturation (70 ℃, 10 min), samples were separated on SDS-polyacrylamide gels (prepared through SDS-PAGE Gel Kit, CWBIO) for 30 min at 90 V and 50 min at 140V, then transferred to a hybridization nitrocellulose transfer membrane (Millipore) for 2 h at 200 mA. The solution of 5% non-fat milk (APPLYGEN) in TBST was used for blocking (1 h at room temperature) and subsequent primary antibody incubations (4 ℃, overnight). Anti-HA-tag (rabbit, CST), anti-CD73 (Abclonal), and anti-β-actin (mouse, CWBIO) was used in western blot.

References

1. S. Schildge, C. Bohrer, K. Beck, C. Schachtrup, Isolation and culture of mouse cortical astrocytes. JoVE, e50079 (2013).

SI Appendix Fig. S1

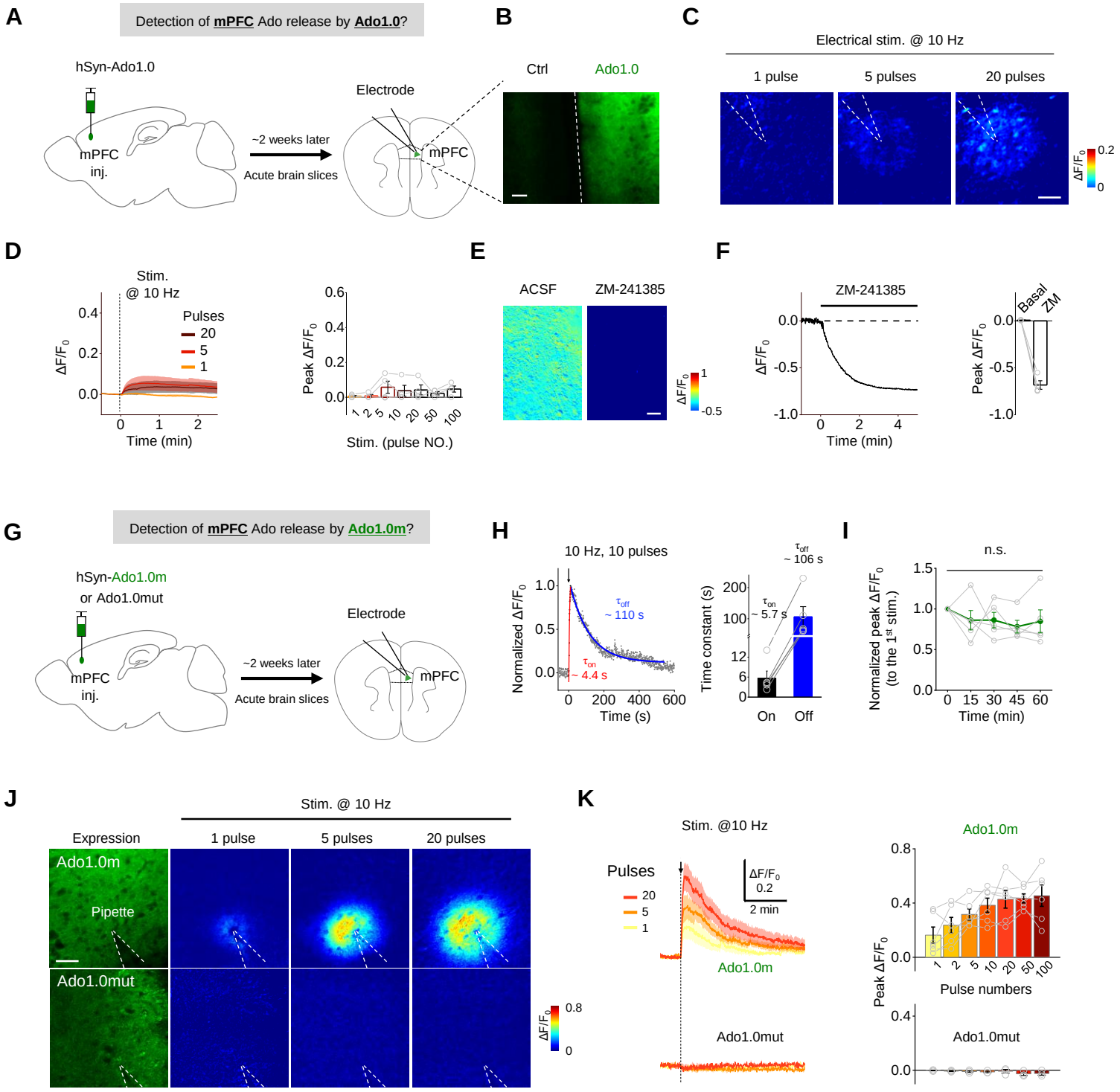

Fig. S1. The characterization of Ado1.0 and Ado1.0m in acute mPFC brain slices, related to Fig. 1.

(A-F) Detecting Ado release with Ado1.0 in the mPFC.

(A) Schematic illustration depicting the expression of Ado1.0 in the mouse mPFC, an acute brain slice containing the mPFC, and the placement of a bipolar stimulating electrode in the mPFC.

(B) Fluorescence images showing the expression of Ado1.0 in the ipsilateral mPFC, with no expression in the contralateral mPFC.

(C) Pseudocolor images of Ado1.0 $\Delta F/F_0$ in response to 1, 5, and 20 electrical pulses at 10 Hz.

(D) Traces (left panels) and group summary (right panels) of Ado1.0 $\Delta F/F_0$ in response to 1, 5, and 20 electrical pulses at 10 Hz; n = 4 slices from 3 mice each.

(E) Pseudocolor images of Ado1.0 $\Delta F/F_0$ in response to control solution (ACSF) or solution containing the $A_{2A}R$ antagonist ZM-241385 (1 μM).

(F) Traces (left panels) and group summary (right panels) of Ado1.0 $\Delta F/F_0$ in control solution (ACSF) or solution containing the $A_{2A}R$ antagonist ZM-241385 (ZM, 1 μM); n = 4 slices from 3 mice.

(G-K) Detecting Ado release with Ado1.0m in the mPFC.

(G) Schematic illustration depicting the expression of Ado1.0m or Ado1.0mut in the mouse mPFC.

(H) Representative traces (left) and group summary (right) of normalized $\Delta F/F_0$ and kinetics (τ_{on} and τ_{off}) in Ado1.0m-expressing neurons in response to 10 electrical stimuli delivered at 10 Hz; n = 5 slices from 3 mice.

(I) $\Delta F/F_0$ was measured in Ado1.0m-expressing neurons in response to repeated trains of electrical stimuli applied every ~15 min and normalized to the first train; n = 5 slices from 4 mice.

(J) Fluorescent images of Ado1.0m- or Ado1.0mut-expressing in the mPFC (left panels) and pseudocolor images of Ado1.0m and Ado1.0mut $\Delta F/F_0$ in response to the indicated number of electrical stimuli delivered at 10 Hz (right panels).

(K) Example traces and group summary of Ado1.0m and Ado1.0mut $\Delta F/F_0$ in response to the indicated number of electrical stimuli delivered at 10 Hz; n = 6 slices from 3 mice per group.

Scale bars represent 50 μm. Summary data are presented as the mean ± SEM. Statistical significance in **(I)** was assessed using a one-way ANOVA followed by Bonferroni's Multiple Comparison Test; n.s. not significant.

SI Appendix Fig. S2

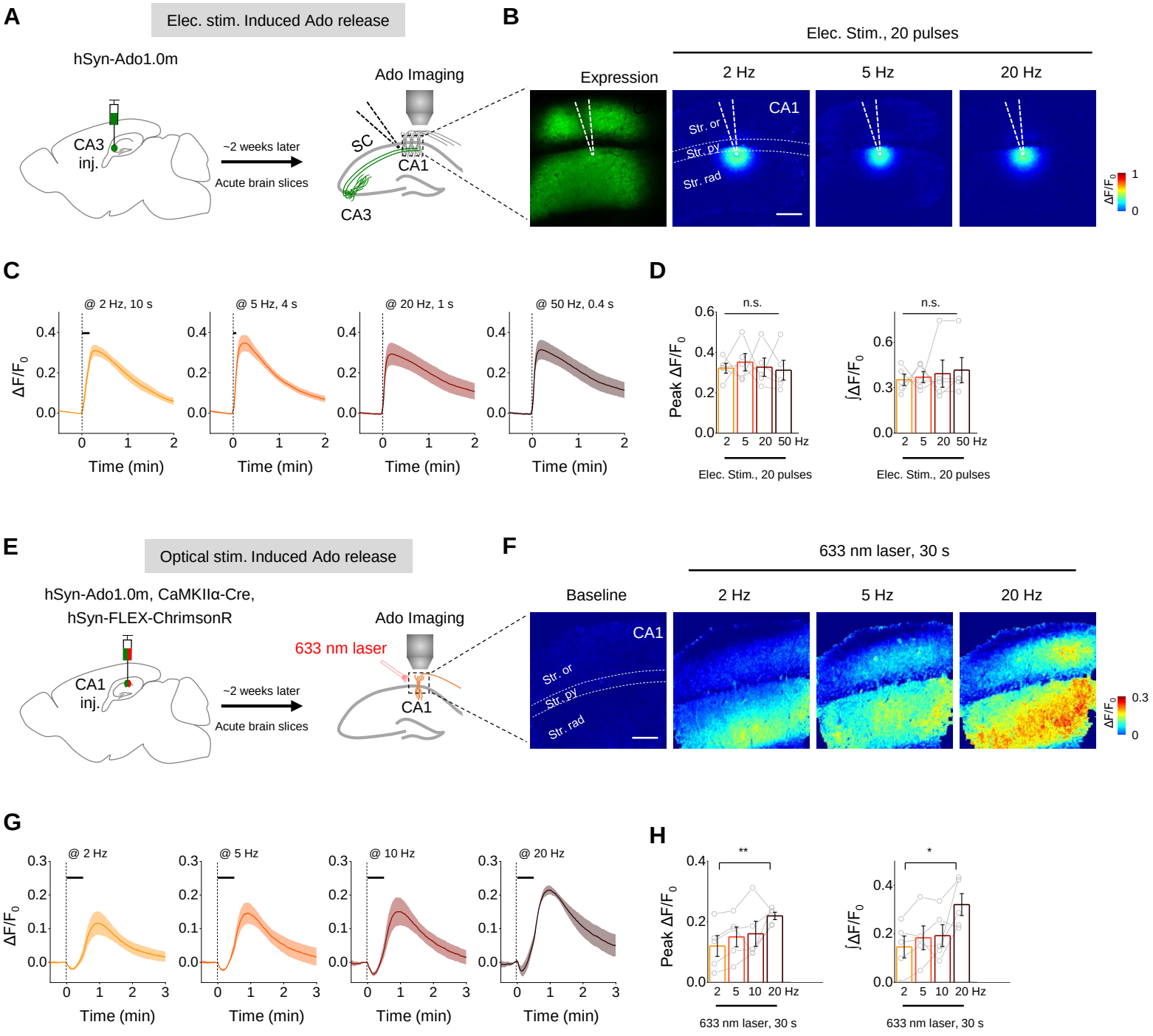

Fig. S2. Imaging local electrical stimuli and 633-nm laser stimuli induced Ado release in CA1, related to Fig. 1.

(A) Schematic illustration depicting the strategy used to image acute hippocampal brain slices prepared from mice expressing Ado1.0m in the CA3 region while using an electrode to induce Ado release in the CA1 region.

(B-D) A fluorescence image of the CA1 region showing the expression of Ado1.0m **(B, left)**. Pseudocolor images **(B, right panels)**, traces **(C)**, and group summary **(D)** of Ado1.0m $\Delta F/F_0$ in response to local electrical stimuli with the same number of stimuli (20 pulses) applied at indicated frequencies in the presence of NBQX & AP5; n = 5 slices from 2 mice.

(E) Schematic illustration depicting the strategy used to image acute hippocampal brain slices prepared from mice expressing Ado1.0m and ChrimsonR-mCherry in the CA1 region while using a 633-nm laser to activate the neurons.

(F-H) Pseudocolor images **(F)**, traces **(G)**, and group summary **(H)** of Ado1.0m $\Delta F/F_0$ in response to 633-nm laser stimuli with the same time duration (30 s) at indicated frequencies; n = 5 slices from 2 mice.

Scale bars represent 100 μm. Summary data are presented as the mean ± SEM. The data in **(D)** were analyzed using a one-way ANOVA followed by Bonferroni's Multiple Comparison Test; the difference between the 2 Hz and 20 Hz induced response in **(H)** was analyzed using Student's t-test; **p<0.01; *p<0.05; n.s., not significant.

SI Appendix Fig. S3

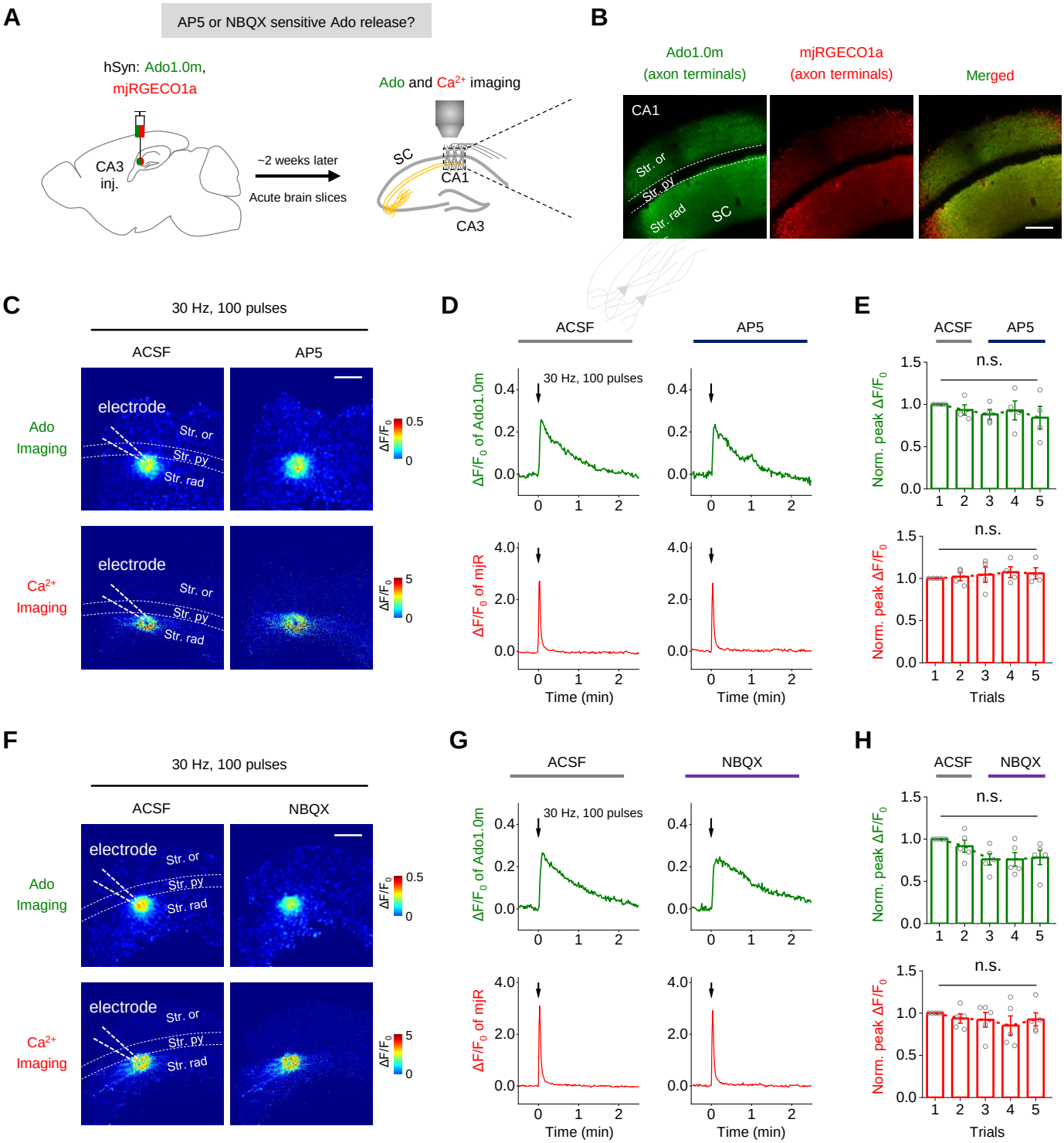

Fig. S3. The effects of NBQX alone and AP5 alone on the Ado1.0m and mjRGECO1a, related to Fig. 1.

(A) Schematic illustration depicting the strategy used to image acute hippocampal brain slices prepared from mice expressing Ado1.0m and membrane-targeted jRGECO1a (mjRGECO1a) in the CA3 region while using an electrode to induce Ado release in the CA1 region.

(B) Fluorescence images of the CA1 region showing Ado1.0m (green) and mjRGECO1a (red) in CA1 regions.

(C-E) Pseudocolor images **(C)**, traces **(D)**, and group summary (E) of Ado1.0m $\Delta F/F_0$ in response to 100 pulses applied at 30 Hz in the absence (ACSF) and presence of D-AP5; n = 5 slices from 3 mice.

(F-H) Pseudocolor images **(F)**, traces **(G)**, and group summary **(H)** of Ado1.0m $\Delta F/F_0$ in response to 100 pulses applied at 30 Hz in the absence (ACSF) and presence of NBQX; n = 4 slices from 3 mice.

Scale bars represent 50 μm. Summary data are presented as the mean ± SEM. Statistical significances in **(E and H)** were assessed using a one-way ANOVA followed by Bonferroni's Multiple Comparison Test; n.s., not significant.

SI Appendix Fig. S4

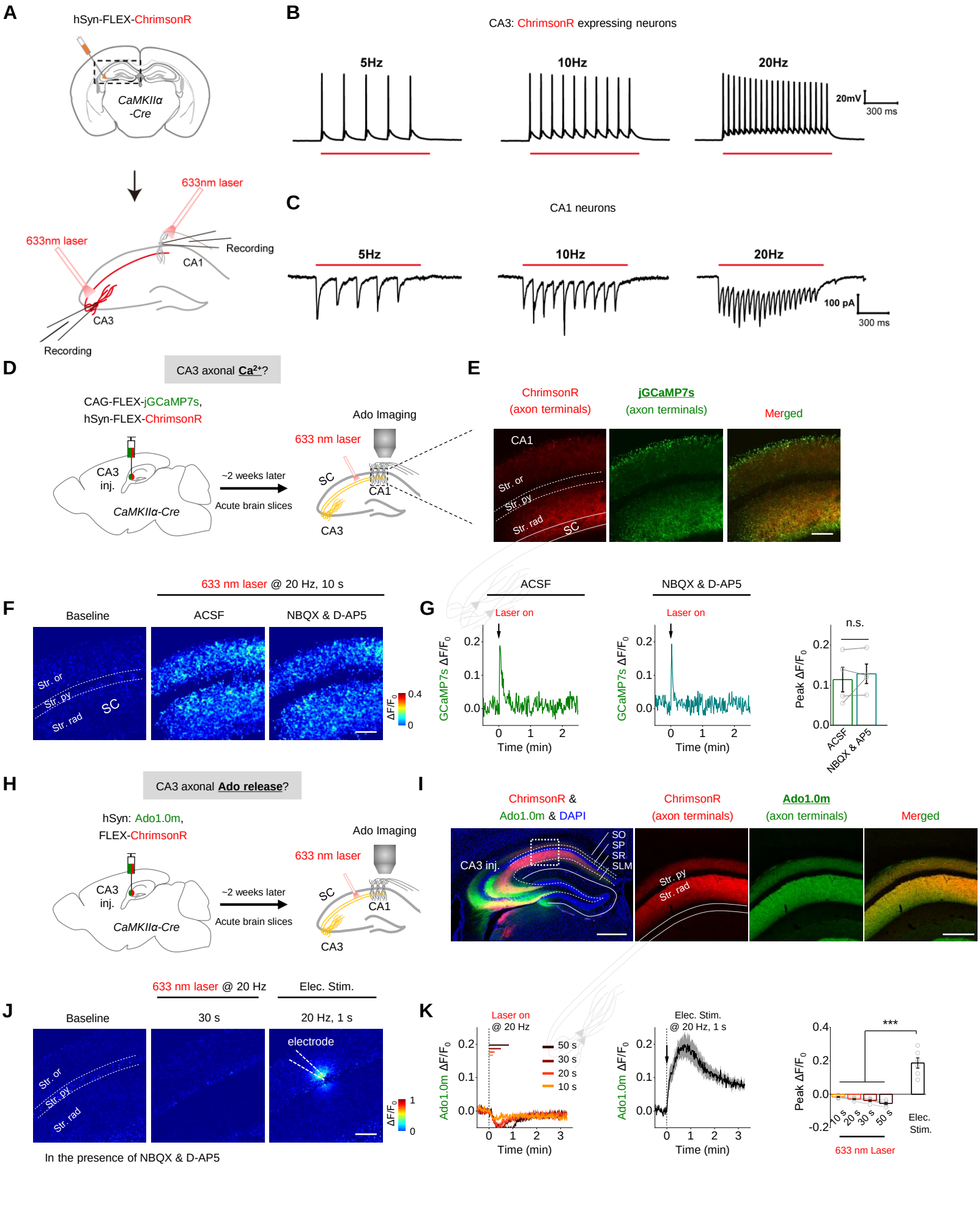

Fig. S4. Optogenetic activation of CA1 axons, related to Fig. 2.

(A) Schematic illustration depicting the strategy used to activate CA3 axon terminals in acute hippocampal brain slices prepared from mice expressing ChrimsonR in the CA3 region while using 633-nm light to activate the CA3 regions or CA3 axonal terminals in the CA1 region.

(B) Electrophysiological recordings at ChrimsonR-expressing CA3 neurons while using a 633-nm laser to activate the CA3 region.

(C) Electrophysiological recordings at CA1 neurons while using a 633-nm laser to activate the CA3 axon terminals.

(D) Schematic illustration depicting the strategy used to image acute hippocampal brain slices prepared from mice expressing jGCaMP7s and ChrimsonR-mCherry in the CA3 region while using a 633-nm laser to activate the axonal terminals in the CA1 region.

(E) Fluorescence images of the CA1 region showing jGCaMP7s (green) and ChrimsonR-mCherry (red) in CA1 regions.

(F and G) Pseudocolor images **(F)** and traces **(G)** of jGCaMP7s $\Delta F/F_0$ in response to 633-nm laser pulses applied at 20 Hz for the indicated duration; n = 4 slices from 2 mice.

(H) Schematic illustration depicting the strategy used to image acute hippocampal brain slices prepared from mice expressing Ado1.0m and ChrimsonR-mCherry in the CA3 region while using a 633-nm laser to activate the axonal terminals (Schaffer Collaterals, SC) in the CA1 region.

(I) Left, Fluorescence images of hippocampus showing the expression of Ado1.0m (green) and ChrimsonR (red). Right, magnified images of the CA1 region showing ChrimsonR-mCherry and Ado1.0m in CA1 regions.

(J and K) Pseudocolor images **(J)**, traces, and group summary **(K)** of Ado1.0m $\Delta F/F_0$ in response to 633-nm laser pulses or local electrical stimuli applied at 20 Hz for the indicated duration within the 10 μM NBQX and 50 μM D-AP5; the solid line is shown in the right panel a linear fit to the data; n = 6 slices from 3 mice.

Scale bars represent 500 μm **(I, left)** and 100 μm **(E, F and J; I, right)**. Summary data are presented as the mean ± SEM. Statistical significance in **(G)** was assessed using Student's t-test; statistical significance in **(K)** was assessed and analyzed using a one-way ANOVA followed by Bonferroni's Multiple Comparison Test; n.s., not significant; ***p<0.001.

SI Appendix Fig. S5

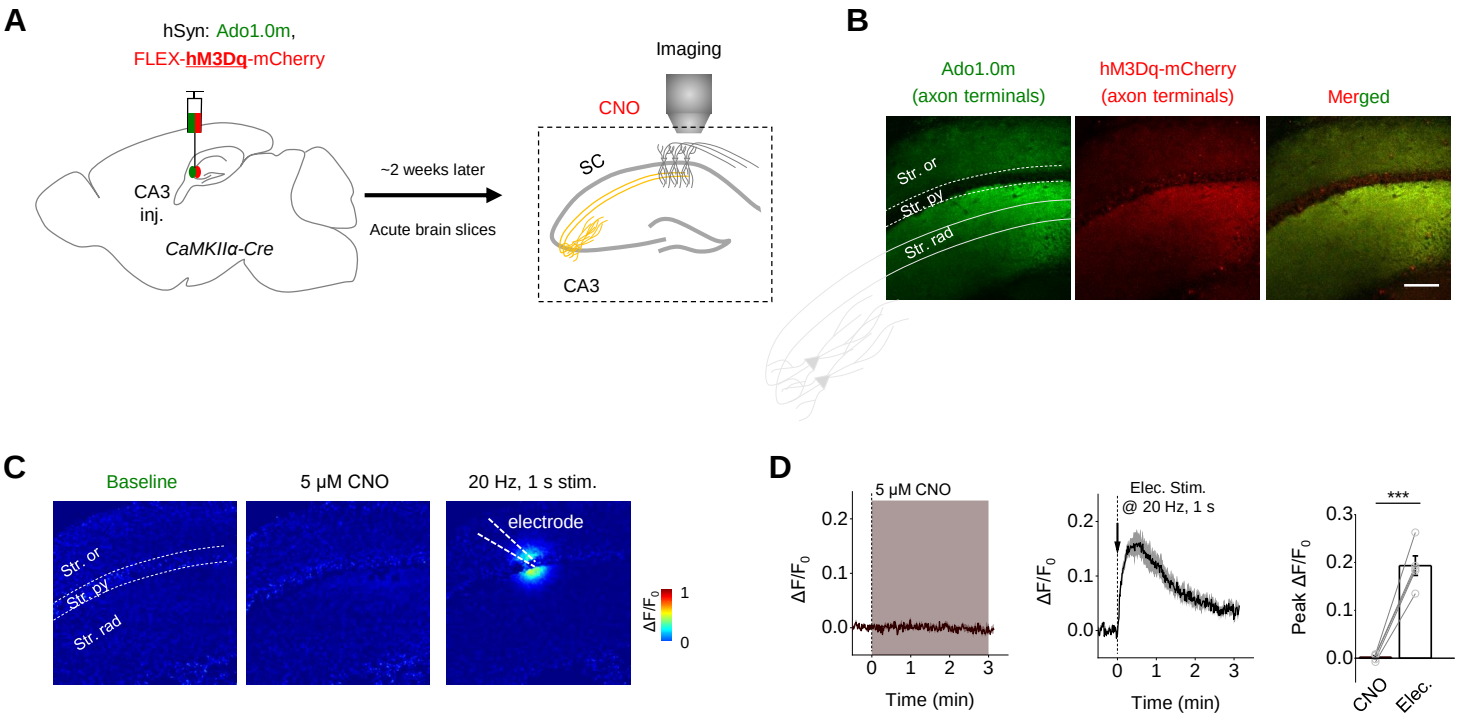

Fig. S5. No detectable chemogenetic activation-induced Ado release in CA3 axons, related to Fig. 2.

(A) Schematic illustration depicting the strategy used to image acute hippocampal brain slices prepared from mice expressing Ado1.0m and hM3Dq-mCherry in the CA3 region.

(B) Fluorescence images of the CA1 region showing Ado1.0m (green) and hM3Dq-mCherry (red) in CA1 regions.

(C and D) Pseudocolor images **(C)**, traces, and group summary **(D)** of Ado1.0m $\Delta F/F_0$ in response to 5 μM CNO application or local electrical stimuli (scale bar, 100 μm) within the NBQX (10 μM) and D-AP5 (50 μM); n = 5 slices from 2 mice.

Scale bars represent 100 μm. Summary data are presented as the mean ± SEM. Statistical significance in **(D)** was assessed using Student's t-test; ***p ≤ 0.001.

SI Appendix Fig. S6

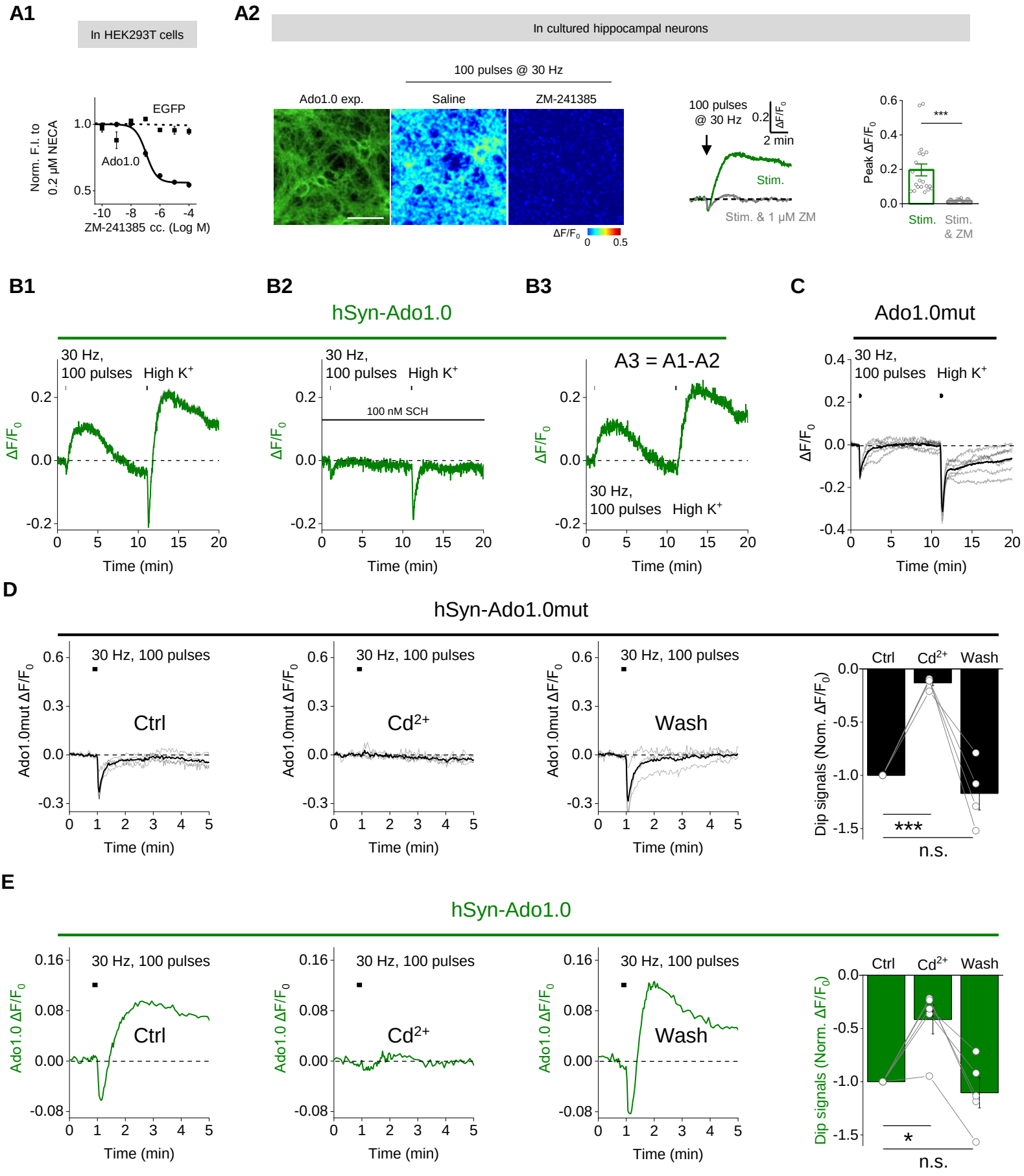

Fig. S6. Specificity of stimulation-induced Ado1.0 $\Delta F/F_0$ in cultured hippocampal neurons, related to Fig. 3.

(A1) Dose-dependent curves for Ado1.0 sensor or EGFP-CAAX control in HEK293T cells in response to ZM-241385. To induce Ado1.0 response, 0.2 μM NECA, an $A_{2A}R$ agonist, was used.

(A2) Traces, peak $\Delta F/F_0$, and summary for Ado1.0 sensor in cultured hippocampal neurons in response to 100 pulses applied at 30 Hz in the absence (ACSF) and presence of ZM-241385 (1 μM); n = 20 ROIs /5 coverslips.

(B) Exemplar traces of $\Delta F/F_0$ measured in Ado1.0-expressing cultured hippocampal neurons in response to 100 field stimuli delivered at 30 Hz and high K^+ in the absence **(B1)** and presence **(B2)** of SCH-58261 (SCH, 100 nM). **(B3)** shows the subtracted trace.

(C) Traces showing Ado1.0mut $\Delta F/F_0$ in response to 100 field stimuli at 30 Hz and high K^+ (n = 6 coverslips).

(D and E) Ado1.0mut **(D)** and Ado1.0 **(E)** $\Delta F/F_0$ were measured in response to 100 field stimuli delivered at 30 Hz before (Ctrl), during, and after (Wash) application of Cd^{2+} (200 μM). Summary data are shown at the right, normalized to control; n = 4-5 coverslips per group.

The scale bar represents 100 μm. Summary data are presented as the mean ± SEM. Statistical significance was assessed by Student's t-test. n.s. not significant, *$p \leq 0.05$, ***$p \leq 0.001$.

SI Appendix Fig. S7

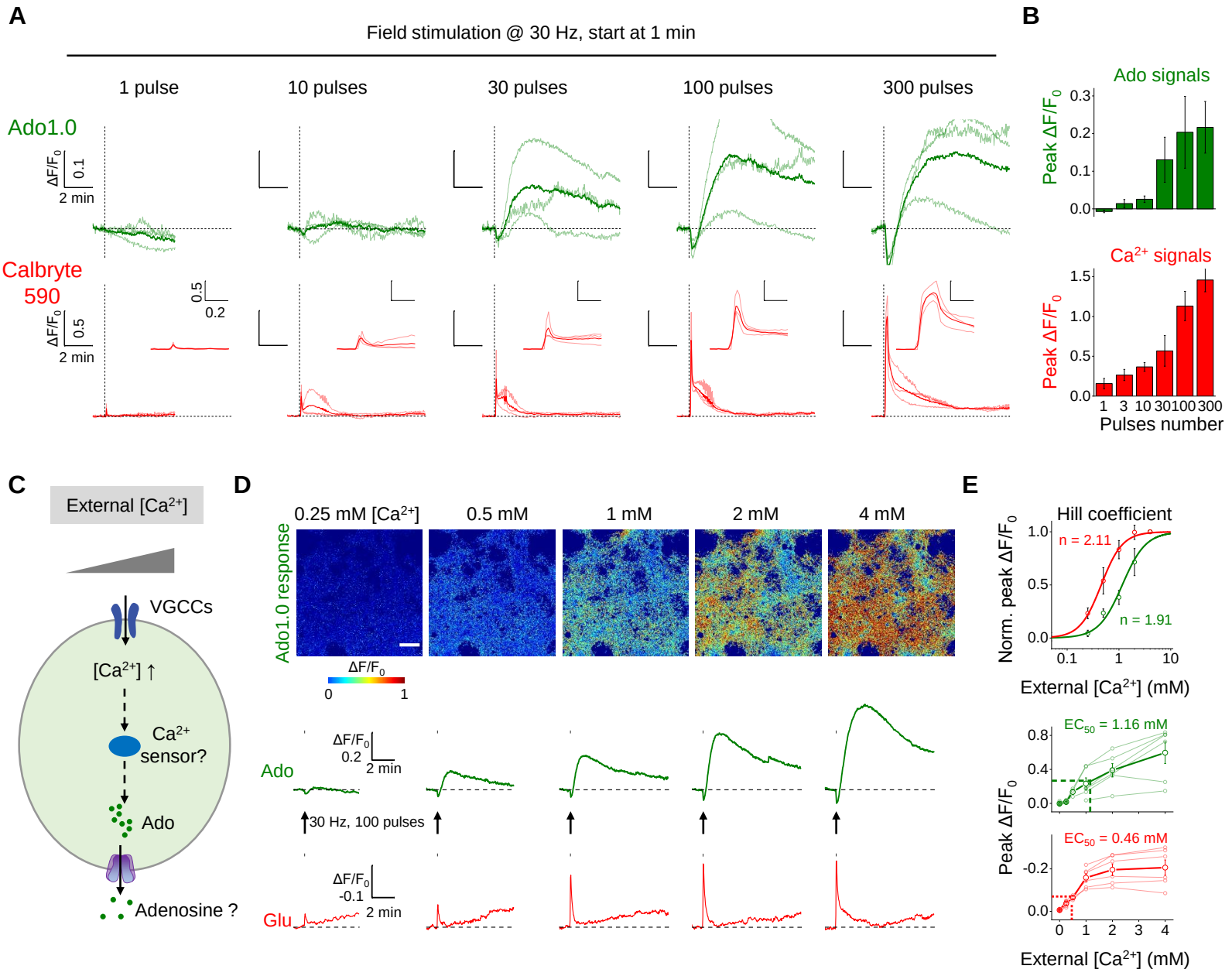

Fig. S7. Simultaneously detecting Ado and Ca^{2+} signals or Ado and glutamate signals, related to Fig. 3 and Fig. 5.

(A and B) Two-color imaging of Ado and Ca^{2+} signals from the same neurons. Traces **(A)** and group summary **(B)** of Ado1.0 and the Ca^{2+} indicator Calbryte 590 $\Delta F/F_0$ in response to the indicated number of field stimuli applied at 30 Hz. In **(A)**, the thin lines represent individual cells, and the thick lines represent the average trace; insets in the lower row meant a magnified view of 0.8-1.5 min of traces; n = 3 coverslips.

(C-E) Ca^{2+} sensitivity differs between Ado release and Glu release. **(C)** Schematic drawing depicting the experimental strategy. Dual-color imaging was used to image Ado and Glu release in various concentrations of extracellular Ca^{2+}. **(D)** Pseudocolor images (top) and traces (bottom) of Ado1.0 and R^{ncp}-iGlu $\Delta F/F_0$ in response to field stimuli (30 Hz, 100 pulses) in the indicated concentrations of extracellular Ca^{2+}. **(E)** Summary of peak Ado1.0 and R^{ncp}-iGlu $\Delta F/F_0$ (n = 6 coverslips); the data in the top panel were normalized to the peak response measured in 4 mM Ca^{2+}, and the data were fitted with a Hill equation (see main text).

Scale bars represent 100 μm. Summary data are presented as the mean ± SEM.

SI Appendix Fig. S8

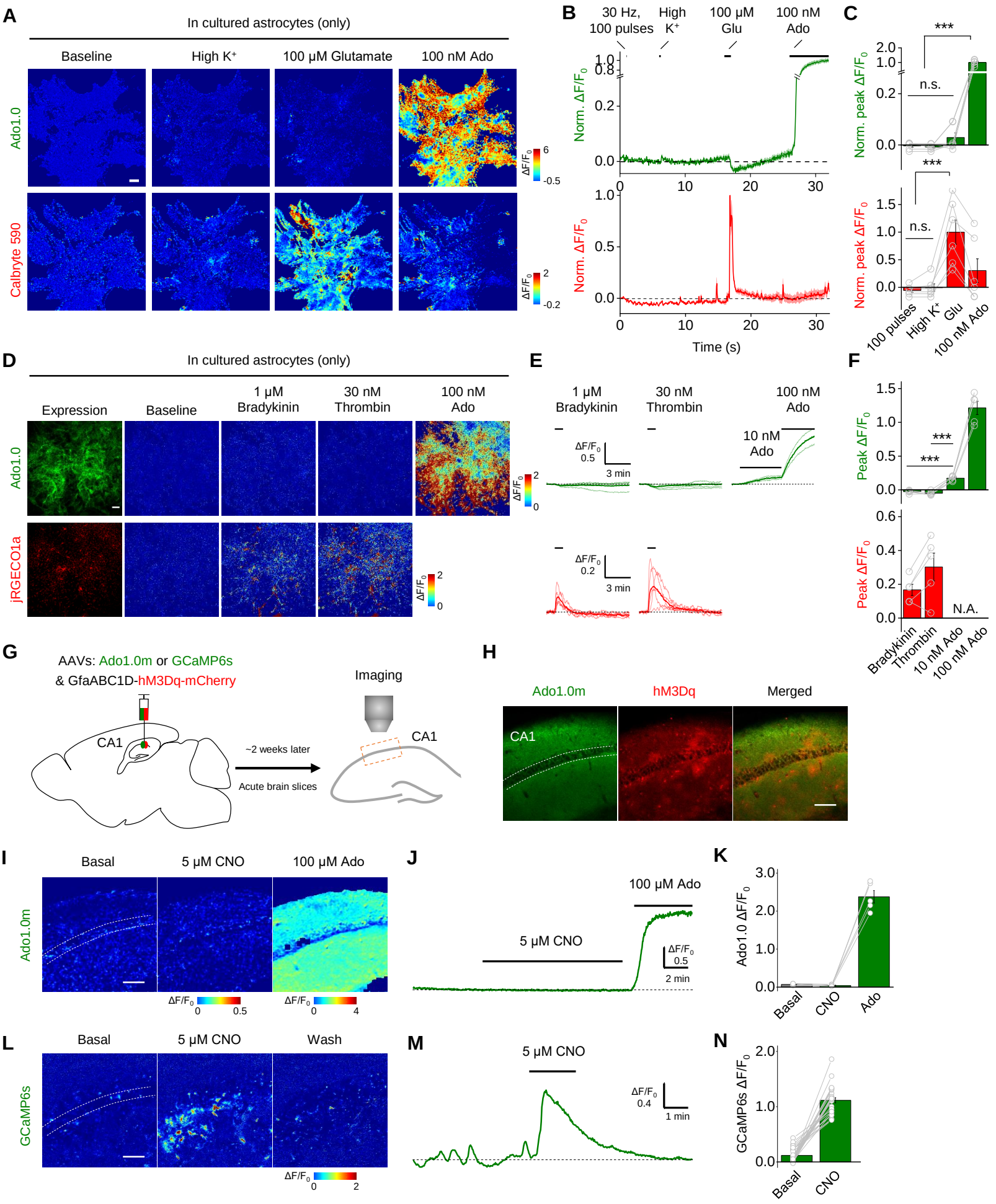

Fig. S8. Not detectable Ado release from astrocytes in cultures and acute brain slices, related to Fig. 3. and Fig. 6.

(A-C) Dual-color imaging of Ado1.0 (upper panels) and Calbryte 590 (Cal 590, bottom panels) in response to 100 field stimuli at 30 Hz, high K^+ (75 mM KCl), 100 μM glutamate (Glu), or 100 nM Ado, in cultured astrocytes. Exemplar pseudocolor images **(A)**, exemplar traces **(B)**, and summary data **(C)** are shown; n = 6 coverslips each.

(D-F) Dual-color imaging of Ado1.0 (upper panels) and NES-jRGECO1a (bottom panels) in response to 1 μM bradykinin, 30 nM thrombin, 10 nM Ado or 100 nM Ado, in cultured astrocytes.; confocal or pseudocolor images **(D)**, traces **(E)**, and group summary **(F)** are shown; n = 5 coverslips. N.A., not available.

(G-N) Imaging of Ado1.0m and GCaMP6s responses in acute brain slices. To activate astrocytes, hM3Dq-mCherry was expressed driven by a GfaABC1D promoter; clozapine N-oxide (CNO, 5 μM) was used; to image extracellular Ado, Ado1.0m was expressed driven by a hSyn promoter; to image astrocytic Ca^{2+}, GCaMP6s was expressed driven by GfaABC1D promoter; n = 5 brain slices from 2 mice for Ado1.0m group; n = 29 astrocytes from 2 mice for GCaMP6s group.

Scale bars: 100 μm. Summary data are presented as the mean ± SEM. The data in **(C)** and **(F)** were analyzed using Student's t-test; ***p<0.001; n.s., not significant.

SI Appendix Fig. S9

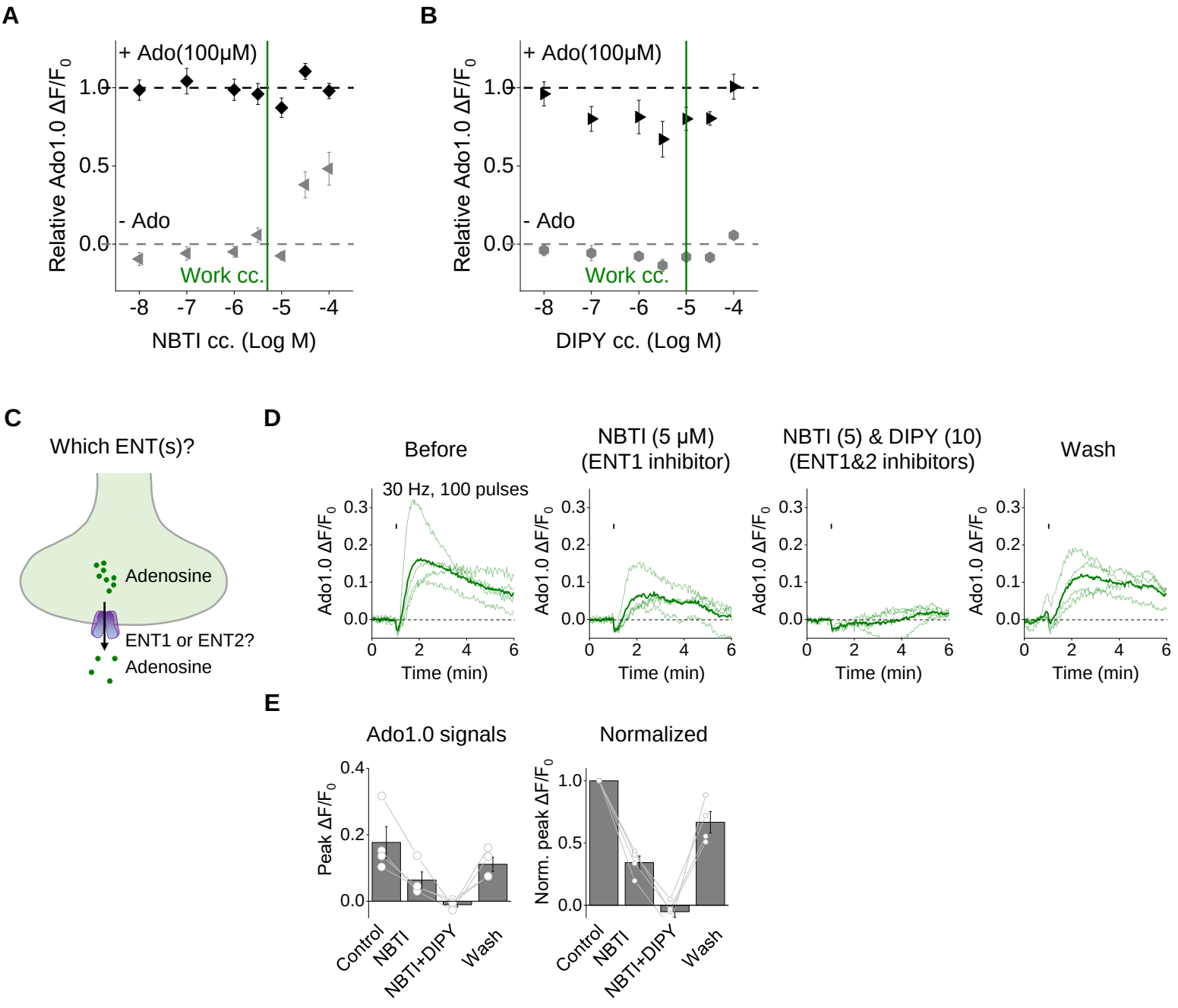

Fig. S9. Both ENT1 and ENT2 contribute to the activity-dependent Ado release, related to Fig. 4.

(A and B) S-(4-nitrobenzyl)-6-thioinosine (NBTI) and dipyridamole (DIPY) did not affect the responses of Ado1.0 at the concentrations of use.

(C) Schematic drawing depicting the putative mechanism by which equilibrative nucleoside transporters (ENTs) mediate Ado release in neurons.

(D and E) Traces **(D)** and group summary **(E)** of Ado1.0 $\Delta F/F_0$ in response to field stimuli (30 Hz, 100 pulses, indicated by black tick marks) before (control), during, and after (wash) application of the ENT1 blocker NBTI (5 μM) and/or the ENT1/2 blocker dipyridamole (DIPY, 10 μM); n = 4 coverslips.

Summary data are presented as the mean ± SEM.

SI Appendix Fig. S10

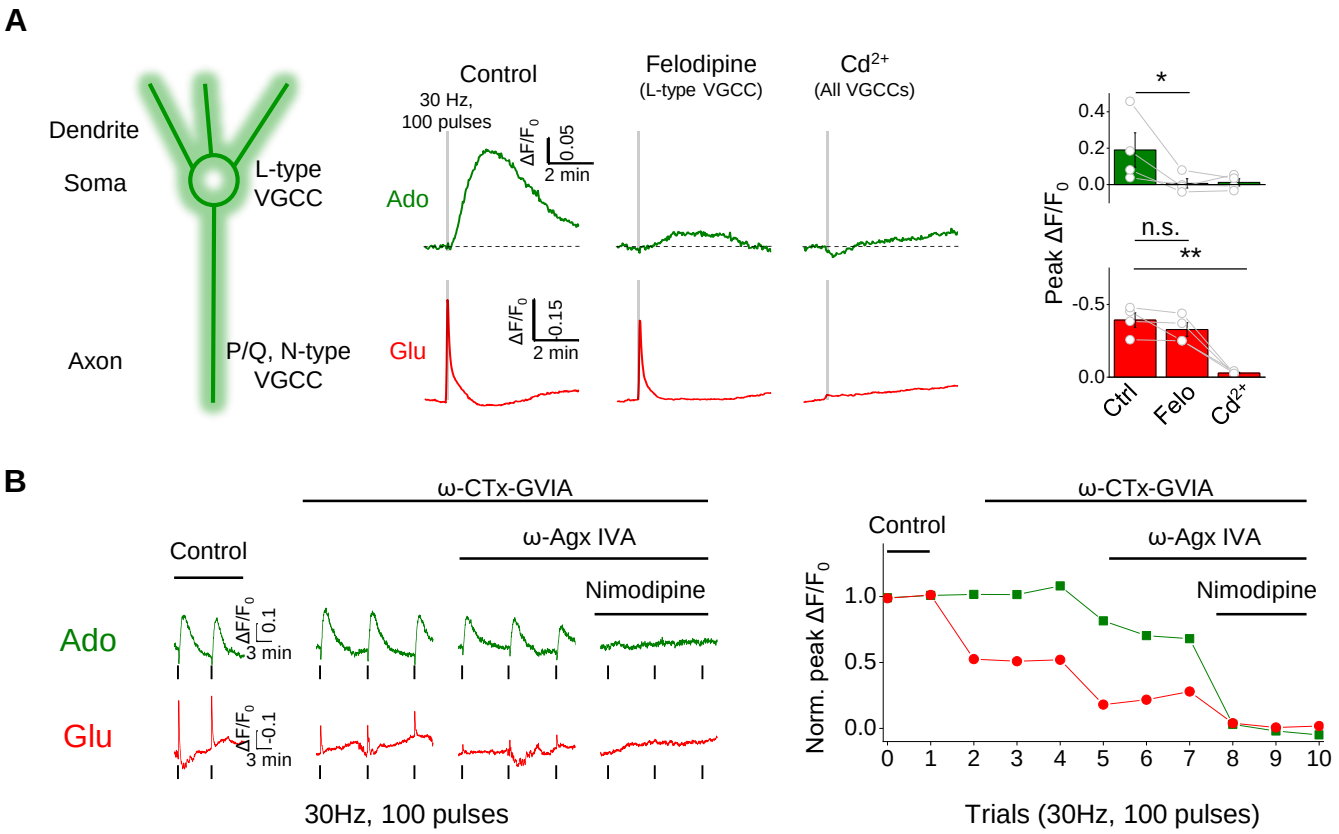

Fig. S10. Calcium sources of activity-dependent Ado release, related to Fig. 5.

(A) Blocking L-type voltage-gated calcium channels (VGCCs) inhibit Ado release. Left panel: cartoon illustrating that L-type VGCCs are expressed primarily in the somatodendritic compartment, while P/Q-type and N-type VGCCs are expressed primarily in the axon. Middle panels: example traces of Ado1.0 and R^{ncp}-iGlu $\Delta F/F_0$ in response to field stimuli (30 Hz, 100 pulses) delivered before (control), during, and after (wash) application of either the L-type VGCC blocker felodipine (Felo, 10 μM) or Cd^{2+} (200 μM). Right: group summary of Ado1.0 (top panel) and R^{ncp}-iGlu (bottom panel) $\Delta F/F_0$ in response to field stimuli; n = 4 coverslips each. The data in the control group are the same as in Fig. 4D.

(B) Example traces and normalized peak Ado1.0 and R^{ncp}-iGlu $\Delta F/F_0$ in response to field stimuli (30 Hz, 100 pulses) applied in the presence of ω-CTx-GVIA (1 μM), ω-Agx-IVA (0.3 μM), and nimodipine (10 μM) to block N-type, P/Q-type, and L-type VGCCs, respectively (the same coverslip was sequentially treated with the indicated blockers).

(C) Confocal images of cultured hippocampal neurons co-expressing Ado1.0 (green) and mCherry-tagged CaMKIIα-hM3Dq (red).

The scale bar represents 100 μm. Summary data are presented as the mean ± SEM. Statistical significance was assessed by a two-tailed Student's t-test. n.s. not significant, *p ≤ 0.05, **p ≤ 0.01.

SI Appendix Fig. S11

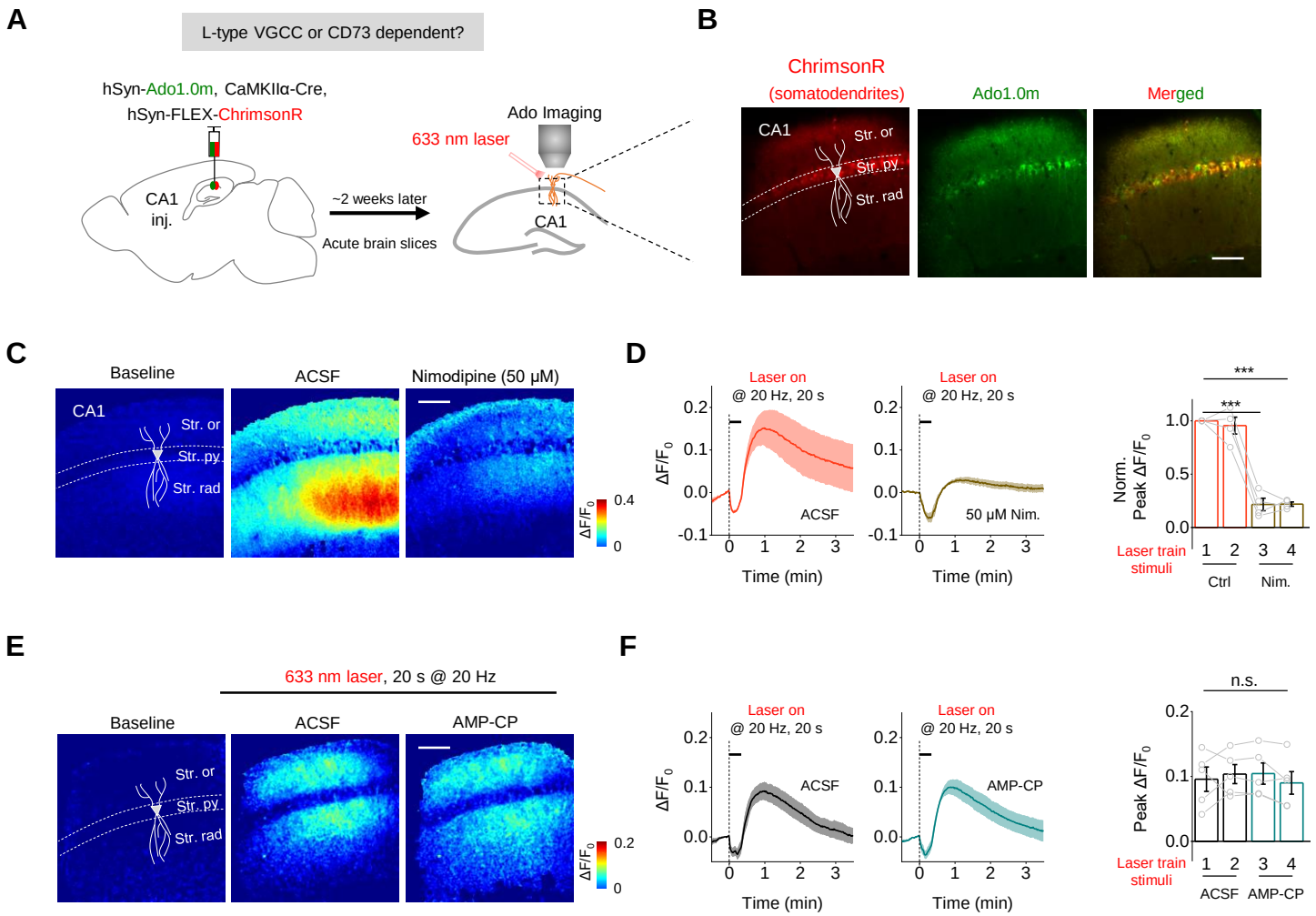

Fig. S11. L-type VGCC dependent and CD73 independent Ado release in hippocampal brain slices, related to Fig. 6.

(A) Schematic illustration depicting the strategy used to image acute hippocampal brain slices prepared from mice expressing Ado1.0m and ChrimsonR in the CA1 region while using a 633-nm laser to activate the CA1 region.

(B) Florescence images showing ChrimsonR-mCherry (red) and Ado1.0m (green) in CA1 regions.

(C and D) Images **(C)**, traces, and group summary **(D)** of Ado1.0m $\Delta F/F_0$ in response to 633-nm laser pulses applied at 20 Hz for 20 s in the absence (Ctrl) and presence of 50 μM nimodipine (Nim); n = 4 slices from 2 mice.

(E and F) Pseudocolor images **(E)**, traces, and group summary **(F)** of Ado1.0m $\Delta F/F_0$ in response to 633-nm laser pulses applied at 20 Hz for 20 s in the absence (ACSF) and presence of a CD73 inhibitor, α,β-Methyleneadenosine 5'-diphosphate (AMP-CP, 10 μM); n = 5 slices from 2 mice.

Scale bars represent 100 μm. Summary data are presented as the mean ± SEM. Statistical significance was assessed by a one-way ANOVA followed by Bonferroni's Multiple Comparison Test; ***$p \leq 0.001$; n.s. not significant.