Figure Supplements

Interneuronal exchange and functional integration of synaptobrevin via extracellular vesicles

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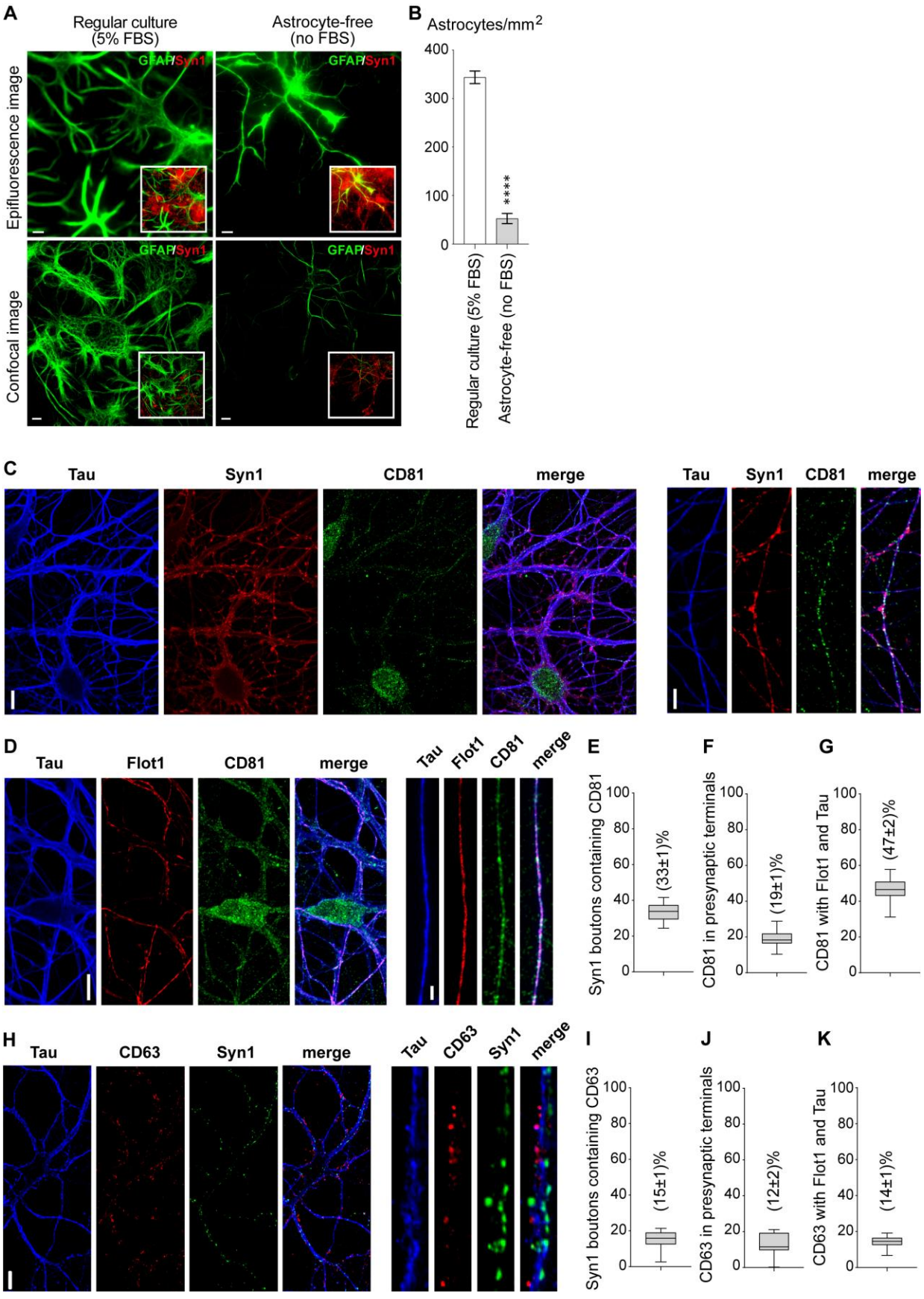
A
Regular culture (5% FBS)
Astrocyte-free (no FBS)
Epifluorescence image
Confocal image
GFAP/Syn1
B
Astrocytes/mm2
400
300
200
100
0
****
Regular culture (5% FBS)
Astrocyte-free (no FBS)
C
Tau
Syn1
CD81
merge
D
Tau
Flot1
CD81
merge
E
Syn1 boutons containing CD81
(33±1)%
F
CD81 in presynaptic terminals
(19±1)%
G
CD81 with Flot1 and Tau
(47±2)%
H
Tau
CD63
Syn1
merge
I
Syn1 boutons containing CD63
(15±1)%
J
CD63 in presynaptic terminals
(12±2)%
K
CD63 with Flot1 and Tau
(14±1)%

Figure S1. Serum-free hippocampal cultures contain minimal levels of astrocytes, Related to Figure 1. (**A**) Representative fluorescence images obtained in an epifluorescence (top) or confocal (bottom) microscope showing GFAP (green, astrocyte marker) and syn1 (red, neuron marker) in hippocampal cultures grown in regular media (4% FBS) or serum-free media. White scale bar = 10 µm. (**B**) Quantification of the number of astrocytes per area (mm^2), data from 6 coverslips. **Hippocampal neurons express CD81 in axons and presynaptic terminals, Related to Figure 2.** (**C**) Representative confocal images of immunofluorescence staining showing Tau (blue), Syn1 (red) and CD81 (green) subcellular localization. Left white scale bar = 10 µm. Left white scale bar = 5 µm. (**D**) Representative confocal images of immunofluorescence staining showing Tau (blue), Flot1 (red) and CD81 (green) subcellular localization. Left white scale bar = 10 µm. Left white scale bar = 2 µm. Flot1, besides being frequently found in secreted vesicles, is a membrane protein associated with several trafficking pathways and with cell adhesion mechanisms (Bodin et al., 2014; Kwiatkowska et al., 2020). In neurons, Flot1 is involved in axonal and synaptic growth. We used colocalization with the classical axonal marker Tau plus Flot1 as a means to label axonal organelles involved in the biology of EVs. (**E**) Object-based 3D colocalization quantification of presynaptic boutons (syn1 positive) containing CD81 signal, (**F**) percentage of total CD81 volume in presynaptic terminals and (**G**) percentage of CD81 localizing to axons (labeled with Tau and Flot1). (**H**) Representative confocal images of immunofluorescence staining showing Tau (blue), CD63 (red) and Syn1 (green) subcellular localization. Left white scale bar = 10 µm. Left white scale bar = 5 µm. (**I**) Object-based 3D colocalization quantification of presynaptic boutons (syn1 positive) containing CD63 signal, (**J**) percentage of total CD63 volume in presynaptic terminals and (**K**) percentage of CD63 localizing to axons (labeled with Tau and Flot1). Immunofluorescence analysis was performed using at least 6 coverslips per group (from 3-4 independent cultures), 4-6 images were taken per coverslip.

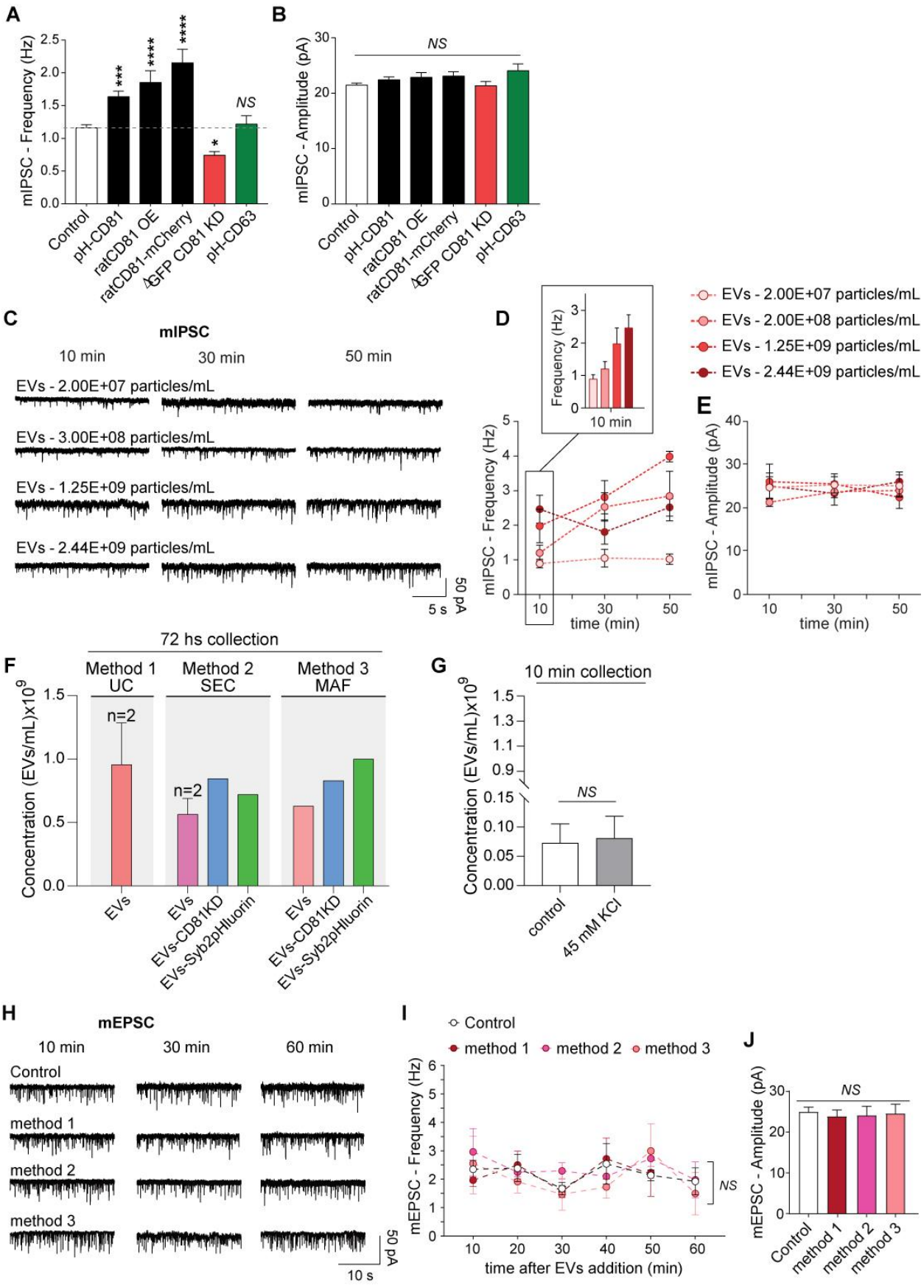
A
mIPSC - Frequency (Hz)
2.5
2.0
1.5
1.0
0.5
0.0
***
****
****
*
NS
Control
pH-CD81
ratCD81 OE
ratCD81-mCherry
ΔGFP CD81 KD
pH-CD63
B
mIPSC - Amplitude (pA)
30
20
10
0
NS
Control
pH-CD81
ratCD81 OE
ratCD81-mCherry
ΔGFP CD81 KD
pH-CD63
C
mIPSC
10 min
30 min
50 min
EVs - 2.00E+07 particles/mL
EVs - 3.00E+08 particles/mL
EVs - 1.25E+09 particles/mL
EVs - 2.44E+09 particles/mL
50 pA
5 s
D
Frequency (Hz)
3
2
1
0
10 min
mIPSC - Frequency (Hz)
5
4
3
2
1
0
10
30
50
time (min)
EVs - 2.00E+07 particles/mL
EVs - 2.00E+08 particles/mL
EVs - 1.25E+09 particles/mL
EVs - 2.44E+09 particles/mL
E
mIPSC - Amplitude (pA)
40
30
20
10
0
10
30
50
time (min)
F
72 hs collection
Method 1
UC
Method 2
SEC
Method 3
MAF
Concentration (EVs/mL)x10^9
1.5
1.0
0.5
0.0
n=2
n=2
EVs
EVs
EVs-CD81KD
EVs-Syb2pHluorin
EVs
EVs-CD81KD
EVs-Syb2pHluorin
G
10 min collection
Concentration (EVs/mL)x10^9
1.5
1.3
1.1
0.9
0.15
0.10
0.05
0.00
NS
control
45 mM KCl
H
mEPSC
10 min
30 min
60 min
Control
method 1
method 2
method 3
50 pA
10 s
I
Control
method 1
method 2
method 3
mEPSC - Frequency (Hz)
6
5
4
3
2
1
0
NS
10
20
30
40
50
60
time after EVs addition (min)
J
mEPSC - Amplitude (pA)
30
20
10
0
NS
Control
method 1
method 2
method 3

Figure S2. Endogenous CD81, but not CD63, modulates inhibitory spontaneous neurotransmission, Related to Figure 2. (**A**) Average frequency and (**B**) average amplitude of mIPSC in neurons infected with empty vector (control), different CD81 over expression constructs, CD81 KD or CD63 over expression. Frequency one-way ANOVA: $F=18.65$, $p<0.0001$; Dunnet's post hoc comparisons of all groups vs control: pH-CD81 $p=0.0001$, rat CD81 OE $p<0.0001$, mCherry-CD81 rat $p<0.0001$, ΔGFP CD81KD $p=0.0311$, pH-CD63 $p=0.9932$. Amplitude one-way ANOVA: $F=1.805$, $p=0.1135$. **Dose-dependent effect of EVs on spontaneous release, Related to Figure 3.** (**C**) Representative mIPSC recordings of hippocampal neurons at different time points after addition of increasing concentrations of EVs. (**D**) Time course of frequency and (**E**) amplitude of mIPSC after addition of different concentrations of EVs. Inset: Average mIPSC frequency at 10 min post EVs addition. Data from 2 independent experiments, N is 3-4 per time point per experimental group. (**F**) Average concentration of EVs in the culture media of hippocampal neurons back-calculated for different isolation methods and experimental conditions. **Acute stimulation does not impact EV secretion from hippocampal neurons, Related to Figure 3.** (**G**) Dissociated neurons were incubated in Tyrode's buffer (control) or Tyrode's buffer with 45 mM KCl for 10 min at 37 ^{o}C and then EVs were isolated from the media using method 2 (size exclusion chromatography). Data was analyzed by unpaired t-test ($p=0.8772$). **EVs do not impact excitatory spontaneous neurotransmission, Related to Figure 3.** (**H**) Example traces of mEPSC for each experimental group. (**I**) Time course of mEPSC frequency in control (no EVs addition) and after incubation with EVs isolated by the three different methods. Two-way ANOVA: time factor $F=0.8555$, $p=0.5160$; experimental group factor $F=0.3325$ $p=0.8018$. N is 3-6 neurons per time point per experimental condition. (**J**) Average mEPSC amplitude for hippocampal neurons treated or not with EVs. One-way ANOVA: $F=0.2419$ $p=0.8660$.

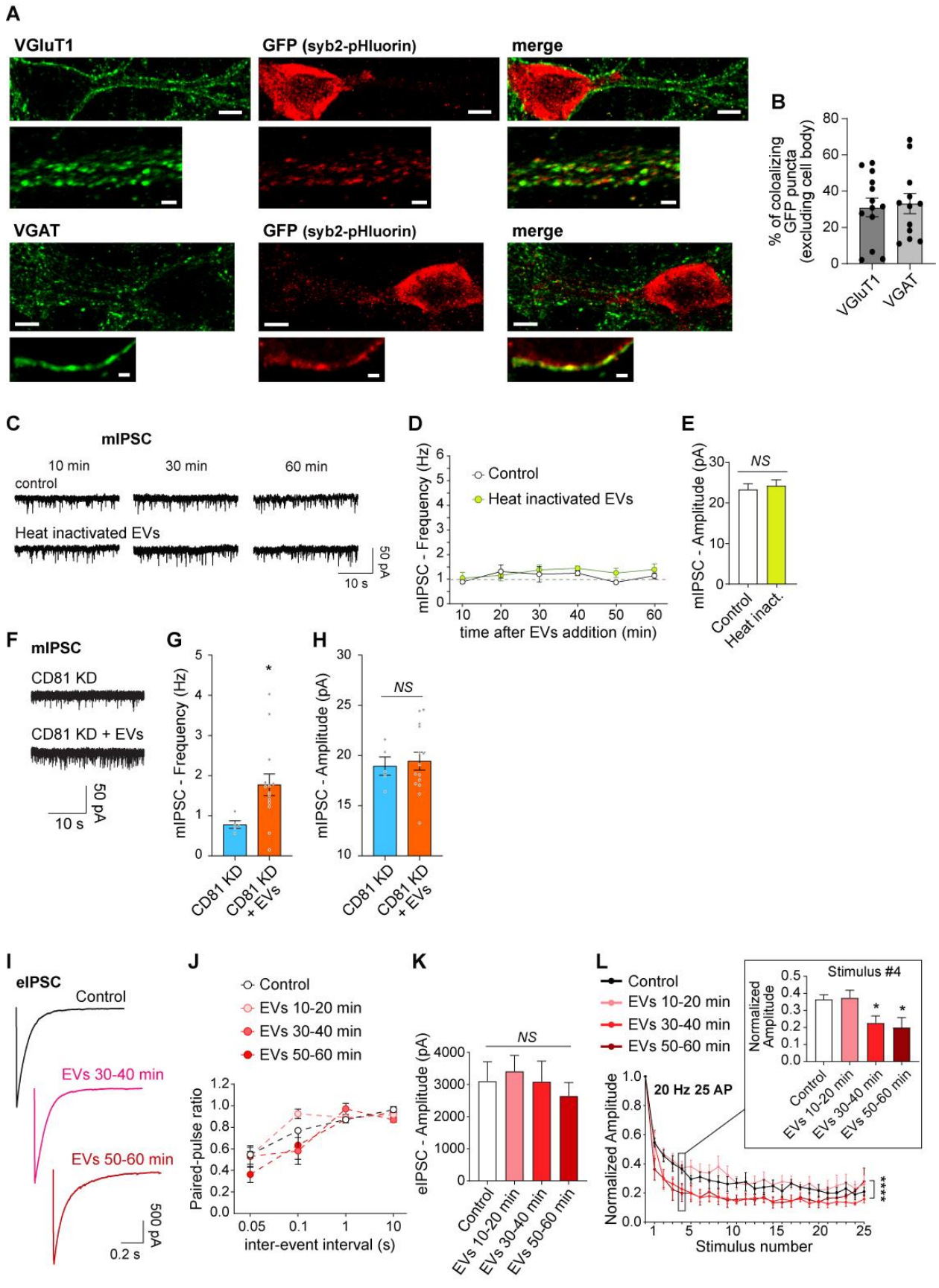
A
VGluT1
GFP (syb2-pHluorin)
merge
VGAT
GFP (syb2-pHluorin)
merge
B
% of coloalizing GFP puncta (excluding cell body)
VGluT1
VGAT
C
mIPSC
10 min
30 min
60 min
control
Heat inactivated EVs
50 pA
10 s
D
mIPSC - Frequency (Hz)
Control
Heat inactivated EVs
time after EVs addition (min)
E
mIPSC - Amplitude (pA)
NS
Control
Heat inact.
F
mIPSC
CD81 KD
CD81 KD + EVs
50 pA
10 s
G
mIPSC - Frequency (Hz)
CD81 KD
CD81 KD + EVs
H
mIPSC - Amplitude (pA)
NS
CD81 KD
CD81 KD + EVs
I
eIPSC
Control
EVs 30-40 min
EVs 50-60 min
500 pA
0.2 s
J
Control
EVs 10-20 min
EVs 30-40 min
EVs 50-60 min
Paired-pulse ratio
inter-event interval (s)
K
eIPSC - Amplitude (pA)
NS
Control
EVs 10-20 min
EVs 30-40 min
EVs 50-60 min
L
Control
EVs 10-20 min
EVs 30-40 min
EVs 50-60 min
20 Hz 25 AP
Normalized Amplitude
Stimulus number
Stimulus #4
Normalized Amplitude
Control
EVs 10-20 min
EVs 30-40 min
EVs 50-60 min

Figure S3, Related to Figure 3. EVs are equally incorporated by excitatory and inhibitory presynaptic terminals. (**A**) Representative confocal images of immunofluorescence staining showing Syn1 (red) and GFP (green) subcellular localization. White scale bar for top images = 10 µm. White scale bar for zoomed-in images = 1 µm. (**B**) Object-based colocalization analysis and quantification of fraction of GFP-positive (syb2-pHluorin) bouton-like objects colocalizing to VGluT1 (glutamatergic terminals) or VGAT (GABAergic terminals). **Heat inactivated EVs do not impact on neurotransmission.** (**C**) Representative recordings of mIPSC from control and neurons treated with heat inactivated EVs (inactivation was performed via incubation at 95 ^{o}C for 15 min). (**D**) Time course of frequency and (**E**) average amplitude (right) of mIPSC. Frequency data was analyzed with a two-way ANOVA (time factor: F=1.221, p=0.3358; experimental group factor: F=1.505, p=0.2341). N is 3-4 neurons per time point per experimental group. Amplitude data was analyzed using unpaired t-test, p=0.3269. **Incubation with control EVs can revert reduced mIPSC phenotype in CD81 KD neurons.** (**F**) Representative recordings, (**G**) frequency and (**H**) amplitude of mIPSC in CD81 KD hippocampal neurons untreated (control) or treated with EVs for 30-60 min. Data analyzed using unpaired t-test (mIPSC Frequency: p=0.0482; mIPSC Amplitude: p=0.7641). **Neuronal EVs increase evoked probability of release.** (**I**) Representative eIPSC traces from neurons incubated with or without EVs. (**J**) Paired-pulse ratio (two-way ANOVA, interval factor: F=37.80 p<0.0001, group factor: F=2.487 p=0.0720) and (**K**) amplitudes (one-way ANOVA F=0.2092 p=0.8881) of eIPSC at different time points after EVs addition (N is 4-5 for each interval point for each group). (**L**) Normalized eIPSC amplitudes after a train of 25 AP at 20 Hz for control and different incubation times with EVs. Data was analyzed with a two-way ANOVA (stimulation number factor: F=55.80, p<0.0001; experimental group factor: F=46.03, p<0.0001). Multiple comparisons by Sidak's post hoc test revealed p<0.0001 (****) for EVs 30-40 min and 50-60 min vs control, and p=0.0994 (non-significant – NS –) for EVs 10-20 min vs control (N is 4 neurons per group).

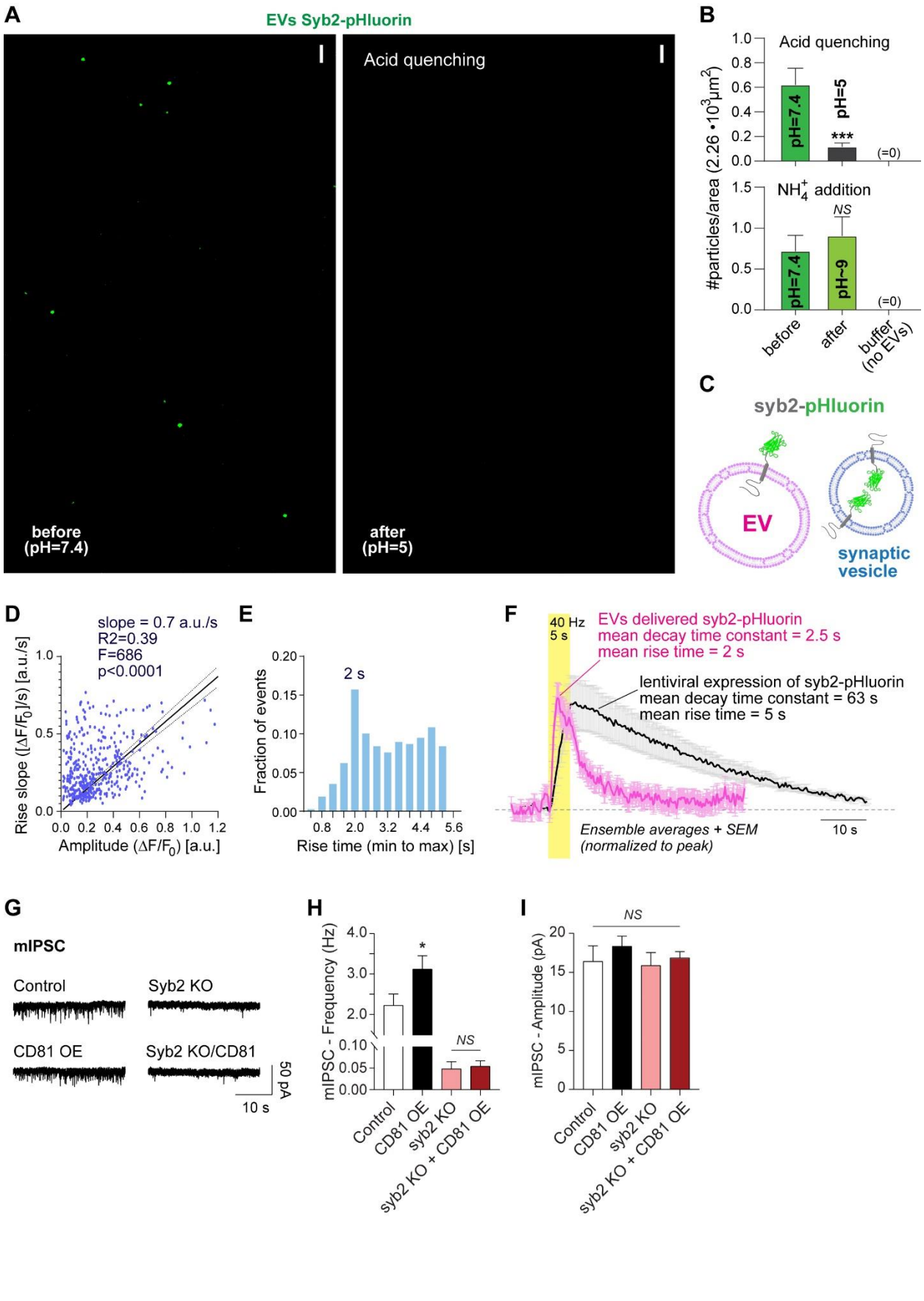
A
EVs Syb2-pHluorin
Acid quenching
before
(pH=7.4)
after
(pH=5)
B
Acid quenching
#particles/area (2.26 •10^3µm^2)
pH=7.4
pH=5
***
(=0)
NH4+ addition
NS
pH=7.4
pH~9
(=0)
before
after
buffer
(no EVs)
C
syb2-pHluorin
EV
synaptic
vesicle
D
slope = 0.7 a.u./s
R2=0.39
F=686
p<0.0001
Rise slope ([ΔF/F0]/s) [a.u./s]
Amplitude (ΔF/F0) [a.u.]
E
2 s
Fraction of events
Rise time (min to max) [s]
F
40 Hz
5 s
EVs delivered syb2-pHluorin
mean decay time constant = 2.5 s
mean rise time = 2 s
lentiviral expression of syb2-pHluorin
mean decay time constant = 63 s
mean rise time = 5 s
Ensemble averages + SEM
(normalized to peak)
10 s
G
mIPSC
Control
Syb2 KO
CD81 OE
Syb2 KO/CD81
50 pA
10 s
H
mIPSC - Frequency (Hz)
*
NS
Control
CD81 OE
syb2 KO
syb2 KO + CD81 OE
I
NS
mIPSC - Amplitude (pA)
Control
CD81 OE
syb2 KO
syb2 KO + CD81 OE

Figure S4. Acid quenching of fluorescence in EVs containing syb2-pHluorin, Related to Figure 4. (**A**) Representative confocal images of a suspension of EVs containing syb2-pHluorin, before (left) and after (right) quenching of fluorescence using pH=5. (**B**) Top: Quantification of the number of fluorescent particles per unit area at physiological (before) and acidic pH (after quenching). Data comes from 2 experiments, ~80 pictures per group per experiment were taken (Mann-Whitney test, p=0.0001). Bottom: Number of fluorescent particles at physiological (before) and basic pH (after 50 mM NH_4^+). Data from 2 experiments, 80 pictures per group per experiment (Mann-Whitney test, p=5776). (**C**) Schematic representation of syb2-pHluorin orientation in extracellular vesicles (top) and synaptic vesicles (bottom). **Analysis of fluorescence rise time and decay time in EV-delivered syb2-pHluorin, Related to Figure 4.** (**D**) Rise slope of fluorescence plotted as a function of peak amplitude, each point represents one presynaptic bouton. Linear regression with error margins is superimposed. (**E**) Histogram of absolute rise time of the fluorescence peaks. (**F**) Comparison of the average fluorescence response obtained after 40 Hz (5 s) stimulation for syb2-pHluorin expressed via lentivirus infection and syb2-pHluorin incorporated from EVs. Traces are shown as average ± standard deviation and normalized to the peak (maximum) value. Via lentiviral infection virtually all synaptic vesicles would contain the probe and thus the rise time in fluorescence is equal to the stimulation duration, i.e. synaptic vesicle fuse during the whole duration of the high frequency train. Since EV-delivered syb2-pHluorin only labels a small fraction of the total pool of SVs, the faster rise time indicates a tendency to fuse before the unlabeled vesicles supporting a higher probability of release for synaptic vesicles receiving the extra syb2 copies. **CD81 overexpression cannot increase spontaneous inhibitory release in syb2 KO neurons, Related to Figure 5.** (**G**) Representative recordings of mIPSC in control and syb2 KO neurons with or without overexpression of CD81. (**H**) Average frequency and (**I**) average amplitude of mIPSC. Data comes from 5-7 neurons per experimental group. One-way ANOVA of frequency: $F=39.69$, $p<0.0001$; Dunnet's post hoc test: control vs CD81 OE $p=0.04$, syb2 KO vs syb2 KO + CD81 OE $p>0.9999$. One-way ANOVA of amplitude: $F=0.5919$, $p=0.6271$.