SUPPLEMENTARY INFORMATION

Sharma *et al.*, "CSPα Knockout Causes Neurodegeneration by Impairing SNAP-25 Function"

ABBREVIATIONS LIST

Amph	Amphiphysin
AP180	Assembly protein-180
Casp-3^{CL}	Cleaved caspase-3
Clath	Clathrin (light chain)
Cpx	Complexin
CSPα	Cysteine string protein-alpha
DAPI	4',6-Diamidino-2-Phenylindole
DIV	Days *in vitro*
Dyn-1	Dynamin-1
GABAR	GABA receptor
GDI	Guanine-nucleotide dissociation inhibitor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GlyR	Glycine receptor
Hsc70	Heat shock cognate 70
MAP2	Microtubule-associated protein 2
NSF	N-ethylmaleimide sensitive factor
KD	Knockdown
KO	Knockout
PSD-95	Postsynaptic density protein of 95 kDa
SGT	Small glutamine-rich tetratricopeptide repeat-containing protein
shRNA	Small hairpin RNA
SNAP	Soluble NSF attachment protein
SNAP-25	Synaptosome-associated protein of 25 kDa
SNARE	Soluble NSF attachment protein receptor
Syb-2	Synaptobrevin-2
Syj-1	Synaptojanin-1
α-Syn	α-Synuclein
Synt-1	Syntaxin-1
Syp	Synaptophysin
Syt1	Synaptotagmin-1
tau	Microtubule binding protein tau
VAMP2	vesicle-associated membrane protein-2
vGaT	Vesicular GABA transporter
vGluT1	Vesicular glutamate transporter-1
WT	Wild-type

SUPPLEMENTARY FIGURES

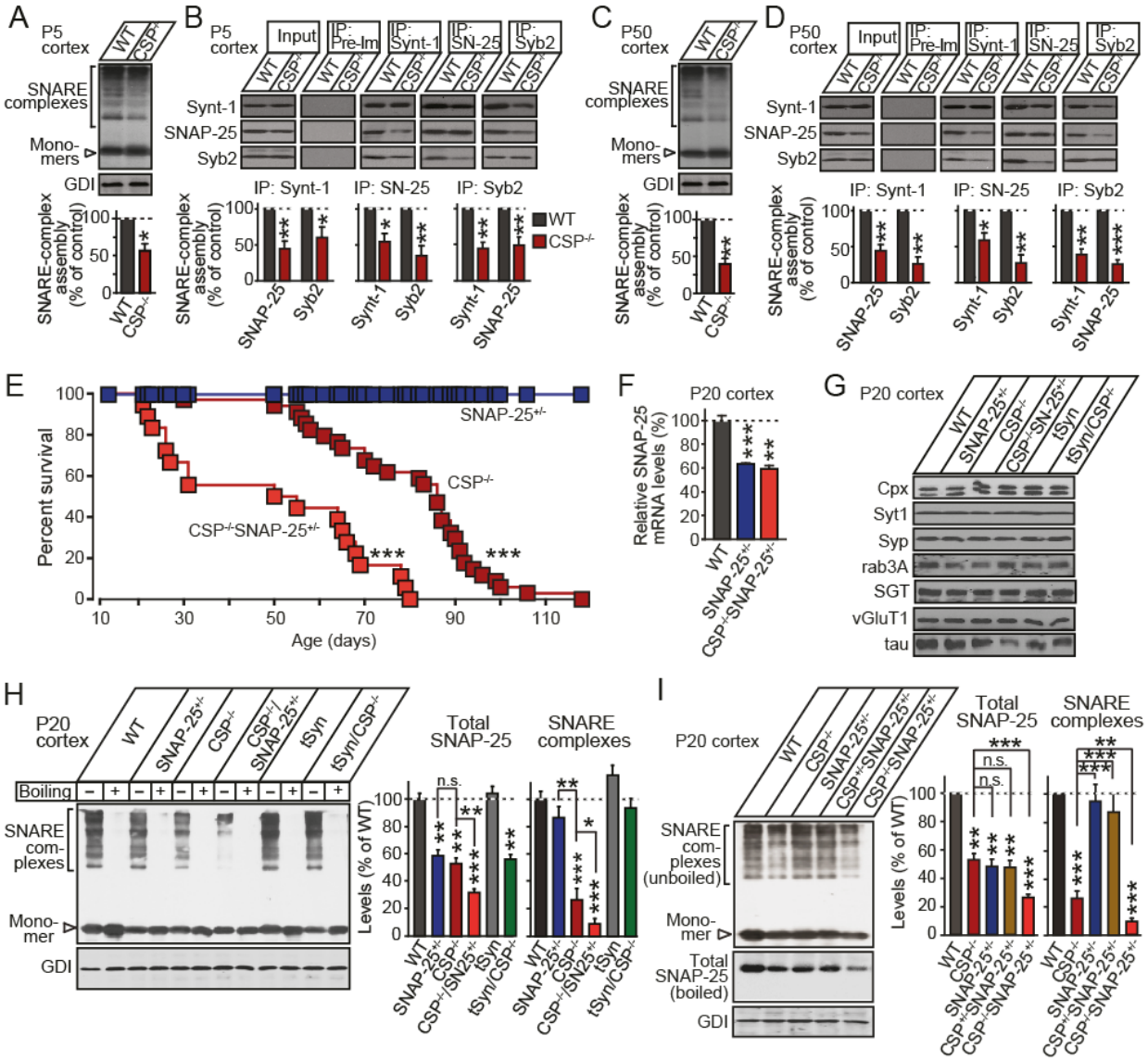

Figure S1. Reduction in SNAP-25 levels accelerates mortality of CSPα KO mice and decreases SNARE-complex assembly.

A-D. SNARE-complex levels in brains from wild-type (WT) and CSPα KO ($CSP^{-/-}$) mice at 5 days (P5; A and B) and 50 days of age (P50; C and D). SNARE-complex levels were measured as high-molecular mass bands immunoreactive to SNAP-25 in unboiled samples, and are shown as percent of WT levels (A and C; n = 3 mice). Independently, SNARE-complex levels were measured by co-immunoprecipitation of SNARE proteins (B and D). Recovered protein (relative to the input) was first normalized to the immunoprecipitated protein, and then to WT (n = 3 mice).

E. Survival of mice with the indicated genotypes as a function of age. Different from Fig. 1C which contains only littermates, this analysis includes non-littermate animals also.

CSPα KO mice with a heterozygous SNAP-25 KO ($CSP^{-/-}SNAP\text{-}25^{+/-}$) perish significantly earlier than CSPα KO mice ($CSP^{-/-}$) (n=18-34 mice; *** = $p<0.001$ by Mantel-Cox test).

F. Measurements of SNAP-25 mRNA levels in the brains from WT, heterozygous SNAP-25 KO ($SNAP\text{-}25^{+/-}$), and compound homozygous CSPα KO/heterozygous SNAP-25 KO mice ($CSP^{-/-}SNAP\text{-}25^{+/-}$) by quantitative RT-PCR. mRNA was isolated from the brains of littermate mice, followed by cDNA preparation. SNAP-25 mRNA levels were normalized to BiP (Grp78) mRNA levels, and then expressed as relative to WT brains (n = 3 mice).

G. Analysis of protein levels in brains from six different genotypes of mice at P20: WT, $SNAP\text{-}25^{+/-}$, $CSP^{-/-}$, $CSP^{-/-}SNAP\text{-}25^{+/-}$, transgenic α-synuclein (tSyn), and tSyn/$CSP^{-/-}$.

H-I. Analysis of SNARE complexes in mouse brains of the indicated genotypes at P20. Representative SNAP-25 and GDI immunoblots, performed on unboiled and boiled brain lysates. Total SNAP-25 levels were quantitated from boiled samples, and normalized to GDI (control). SNARE complexes were measured as high-molecular mass bands immunoreactive to SNAP-25 in the unboiled samples, and are shown as percent of WT levels (n = 4 mice; see also Fig. 1E).

Protein levels were measured by quantitative immunoblotting using ^{125}I-labeled secondary antibody. Data are shown as means ± SEMs, ** = $p<0.01$; *** = $p<0.001$ (assessed by Student's t-test in all experiments except for those shown in panel E which are assessed by the Mantel-Cox test, in each case, each test sample was compared to the control analyzed in the same experiment).

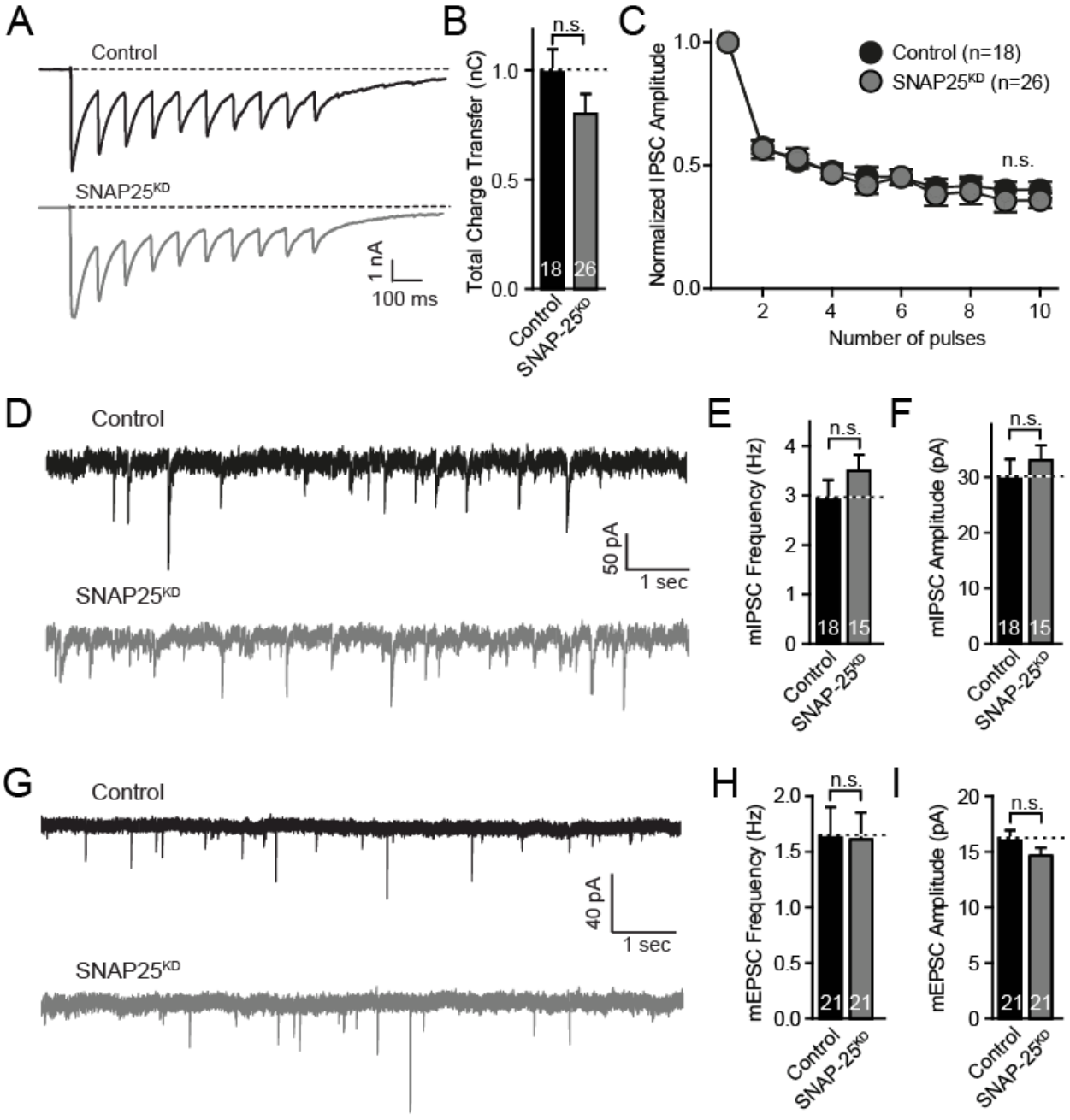

Figure S2. Effect of the SNAP-25 knockdown on synaptic neurotransmitter release in wild-type neurons.

Cultured cortical neurons from wild-type (WT) mice were infected with lentiviruses expressing the SNAP-25 shRNA and mCherry (SNAP-25^{KD}) or mCherry alone (control) on 5 days *in vitro* (DIV5), and used for electrophysiological recordings at 14 days *in vitro* (DIV14).

A-C. Inhibitory postsynaptic currents (IPSC) were evoked by stimulus trains composed of 10 electric pulses delivered at 10 Hz. Representative traces (A), and summary

graphs of the total charge transfer (B) and of the normalized amplitudes of individual synchronous IPSCs (C) are shown. In C, the amplitudes of individual IPSCs were normalized to the amplitude of the first IPSC in the train, and plotted as a function of stimulus number; note that B depicts non-normalized data.

D-I. Effect of the SNAP-25 knockdown on spontaneous 'mini' release in WT neurons. Miniature inhibitory (mIPSCs; D-F) and excitatory postsynaptic currents (mEPSCs; G-I) were measured in control and SNAP-25^{KD} neurons. Representative traces (D and G), and summary graphs of the frequency (E and H) and average amplitudes (F and I) of mIPSCs (D-F) and mEPSCs (G-I) are shown.

Data shown are means ± SEMs; n = 3 independent cultures; numbers in panels (B-I) indicate the number of neurons analyzed. n.s. = not significant ($p>0.05$; using Student's t-test or two-way repeated-measures ANOVA in panel B, in each case comparing each test sample to the control analyzed in the same experiment).

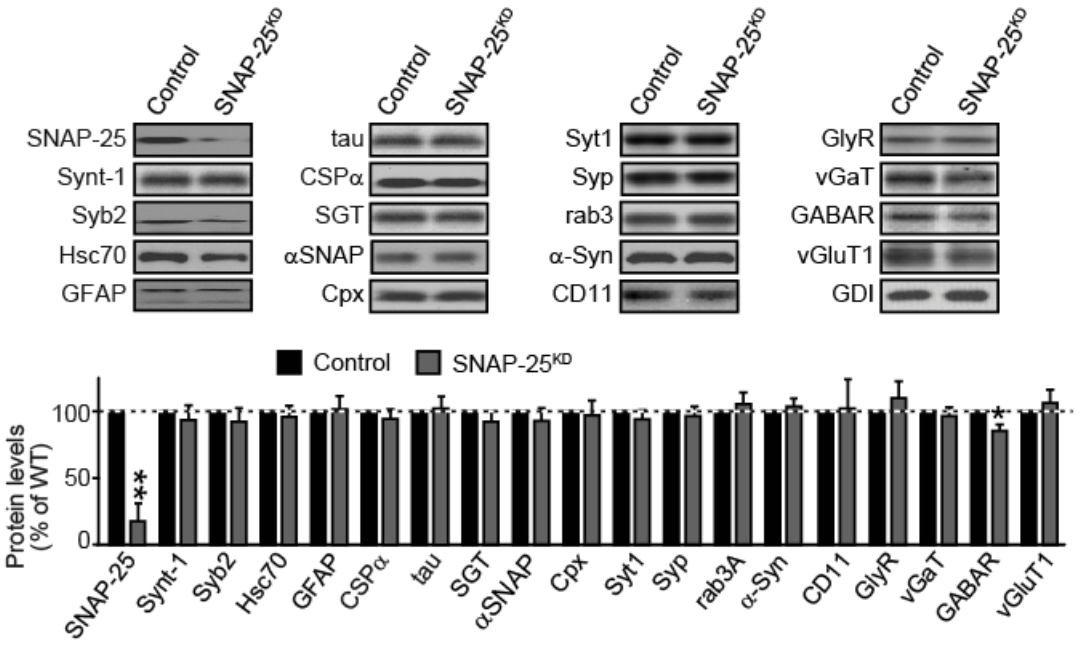

Figure S3. SNAP-25 knockdown reduces SNARE-complex assembly.
Effect of SNAP-25 knockdown (SNAP-25^{KD}) on protein levels in neurons. Cultured cortical neurons from wild-type (WT) mice were infected with lentiviruses expressing SNAP-25 shRNA/mCherry or mCherry alone at 5 days *in vitro* (DIV5). On DIV14, levels of indicated proteins were measured by immunoblotting and normalized to GDI (top, representative immunoblots; bottom, protein levels).

Protein levels were determined using phosphorimager analysis with ^{125}I-labeled secondary antibody (n = 3 cultures). Data are shown as means ± SEMs, * = $p<0.05$, ** = $p<0.01$ (assessed by Student's t-test, comparing each test sample to the control analyzed in the same experiment).

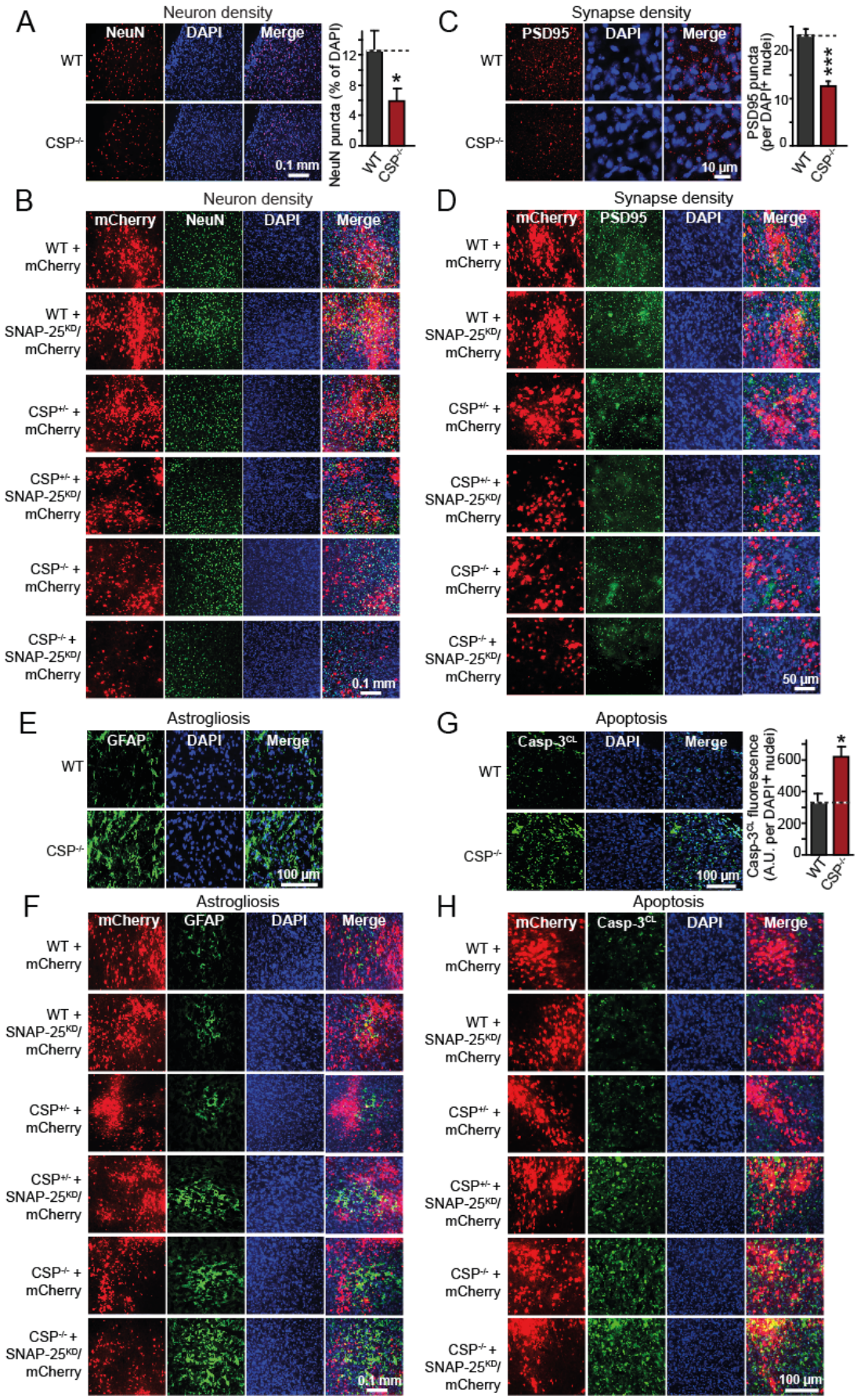
A
Neuron density
NeuN
DAPI
Merge
WT
CSP-/-
0.1 mm
NeuN puncta (% of DAPI)
12
8
4
0
*
WT
CSP-/-
C
Synapse density
PSD95
DAPI
Merge
WT
CSP-/-
10 µm
PSD95 puncta (per DAPI+ nuclei)
20
10
0
***
WT
CSP-/-
B
Neuron density
mCherry
NeuN
DAPI
Merge
WT + mCherry
WT + SNAP-25KD/ mCherry
CSP+/- + mCherry
CSP+/- + SNAP-25KD/ mCherry
CSP-/- + mCherry
CSP-/- + SNAP-25KD/ mCherry
0.1 mm
D
Synapse density
mCherry
PSD95
DAPI
Merge
WT + mCherry
WT + SNAP-25KD/ mCherry
CSP+/- + mCherry
CSP+/- + SNAP-25KD/ mCherry
CSP-/- + mCherry
CSP-/- + SNAP-25KD/ mCherry
50 µm
E
Astrogliosis
GFAP
DAPI
Merge
WT
CSP-/-
100 µm
G
Apoptosis
Casp-3CL
DAPI
Merge
WT
CSP-/-
100 µm
Casp-3CL fluorescence (A.U. per DAPI+ nuclei)
600
400
200
0
*
WT
CSP-/-
F
Astrogliosis
mCherry
GFAP
DAPI
Merge
WT + mCherry
WT + SNAP-25KD/ mCherry
CSP+/- + mCherry
CSP+/- + SNAP-25KD/ mCherry
CSP-/- + mCherry
CSP-/- + SNAP-25KD/ mCherry
0.1 mm
H
Apoptosis
mCherry
Casp-3CL
DAPI
Merge
WT + mCherry
WT + SNAP-25KD/ mCherry
CSP+/- + mCherry
CSP+/- + SNAP-25KD/ mCherry
CSP-/- + mCherry
CSP-/- + SNAP-25KD/ mCherry
100 µm

Figure S4. SNAP-25 knockdown aggravates neurodegeneration.

A,C,E & G. Changes in neuron density (A), synapse density (C), astrogliosis (E), and caspase-3 cleavage as a measure of apoptosis (G) in CSPα KO ($CSP^{-/-}$) mouse cortex. Representative images are shown of fixed brain sections from 50 day old (P50) wild-type (WT) and $CSP^{-/-}$ mice, immunostained for the neuronal marker NeuN (red; A), the synaptic marker PSD95 (red, C), the astroglial marker glial fibrillary acidic protein (GFAP; green, E), and the apoptosis marker cleaved caspase-3 ($Casp\text{-}3^{CL}$; green, G). Sections are counterstained with the general nuclear stain DAPI (blue). NeuN-positive puncta (A), PSD95-positive puncta (C) and $Casp\text{-}3^{CL}$-positive pixels (G) were quantitated as a function of DAPI puncta in the same image.

B,D,F & H. Effect of the SNAP-25 knockdown on neuron and synapse density in WT, $CSP\alpha^{-/+}$ and $CSP^{-/-}$ mouse brains. Stereotactic injection of lentiviruses at P1 in WT, $CSP\alpha^{-/+}$ and $CSP^{-/-}$ mouse cortex were used to express mCherry as control or the SNAP-25 shRNA with mCherry ($SNAP\text{-}25^{KD}$/mCherry). At P50, neuron density (B), synapse density (D), astrogliosis (F), and apoptosis (H) were visualized and quantitated in the infected areas as described above. See Fig. 4 for quantitations.

Data are shown as means ± SEMs, * = $p<0.05$; *** = $p<0.001$ (using Student's t-test, comparing each test sample to the control analyzed in the same experiment).

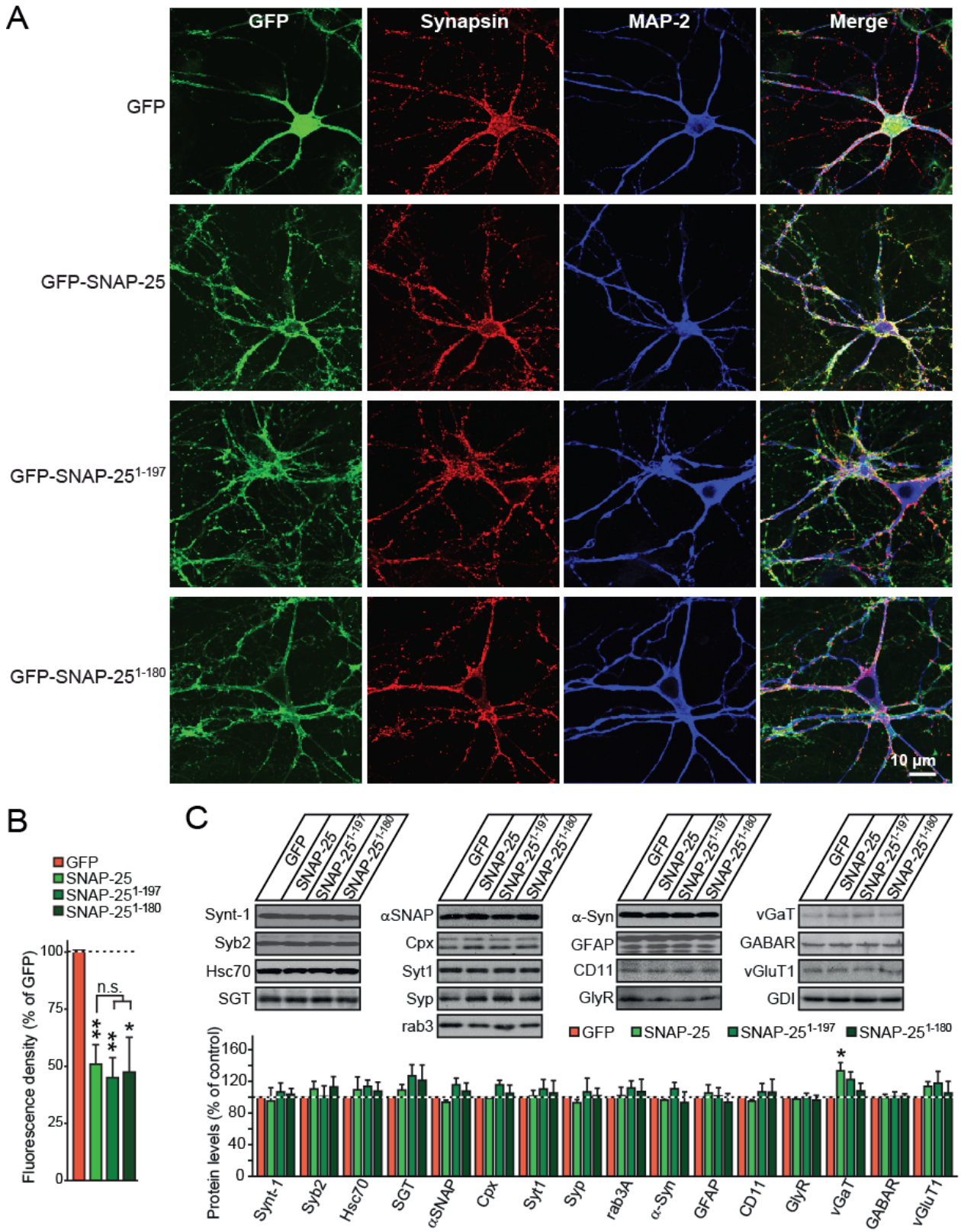
A
GFP
Synapsin
MAP-2
Merge
GFP
GFP-SNAP-25
GFP-SNAP-25 1-197
GFP-SNAP-25 1-180
10 µm
B
GFP
SNAP-25
SNAP-25 1-197
SNAP-25 1-180
Fluorescence density (% of GFP)
100
75
50
25
0
n.s.
**
**
*
C
GFP
SNAP-25
SNAP-25 1-197
SNAP-25 1-180
Synt-1
Syb2
Hsc70
SGT
αSNAP
Cpx
Syt1
Syp
rab3
α-Syn
GFAP
CD11
GlyR
vGaT
GABAR
vGluT1
GDI
Protein levels (% of control)
160
120
80
40
0
*
Synt-1
Syb2
Hsc70
SGT
αSNAP
Cpx
Syt1
Syp
rab3A
α-Syn
GFAP
CD11
GlyR
vGaT
GABAR
vGluT1

Figure S5. SNAP-25 overexpression increases SNARE-complex assembly in CSPα KO neurons.

A. Localization of GFP-tagged wild-type (GFP-SNAP-25) and truncated SNAP-25 (GFP-SNAP-25^{1-197} and GFP-SNAP-25^{1-180}) in CSPα KO ($CSP^{-/-}$) neurons. Cultured cortical neurons from newborn $CSP^{-/-}$ mice were infected with lentiviruses expressing GFP alone or the indicated GFP-tagged versions of SNAP-25 at 7 days *in vitro* (DIV7), and analyzed by immunofluorescence at DIV14 (green, GFP; red, synapsin; blue, MAP2).

B. $CSP^{-/-}$ neurons were infected with lentiviruses expressing GFP, GFP-SNAP-25, GFP-SNAP-25^{1-197} and GFP-SNAP-25^{1-180} as in (A), and the GFP fluorescence density was quantitated as a way to measure the expression levels of the GFP-SNAP-25 fusion proteins per cell. To calculate the fluorescence density in each image, the total GFP positive pixel count was normalized to the total fluorescent area, and plotted as percent of GFP alone.

C. Effect of SNAP-25 overexpression in $CSP^{-/-}$ neurons on the levels of selected neuronal proteins. Cultured cortical neurons from $CSP^{-/-}$ mice were infected with the indicated lentiviruses as described above. At DIV14, the levels of syntaxin-1 (Synt-1), synaptobrevin-2 (Syb2), Hsc70, SGT, αSNAP, complexin (Cpx), synaptotagmin-1 (Syt1), synaptophysin (Syp), rab3A, α-synuclein (α-Syn), GFAP, CD11, glycine receptor (GlyR), vesicular GABA transporter (vGaT), GABA receptor (GABAR) and vesicular glutamate transporter 1 (vGluT1) were measured by immunoblotting (top, representative blots; bottom, protein quantitations with ^{125}I-labeled secondary antibodies; values are normalized to GDI as an internal standard).

Data are shown as means ± SEMs; n = 3 cultures (10 images were quantitated from each culture in B); n.s. = not significant ($p>0.05$); * = $p<0.05$; ** = $p<0.01$ (using Student's t-test, comparing each test sample to the control analyzed in the same experiment).

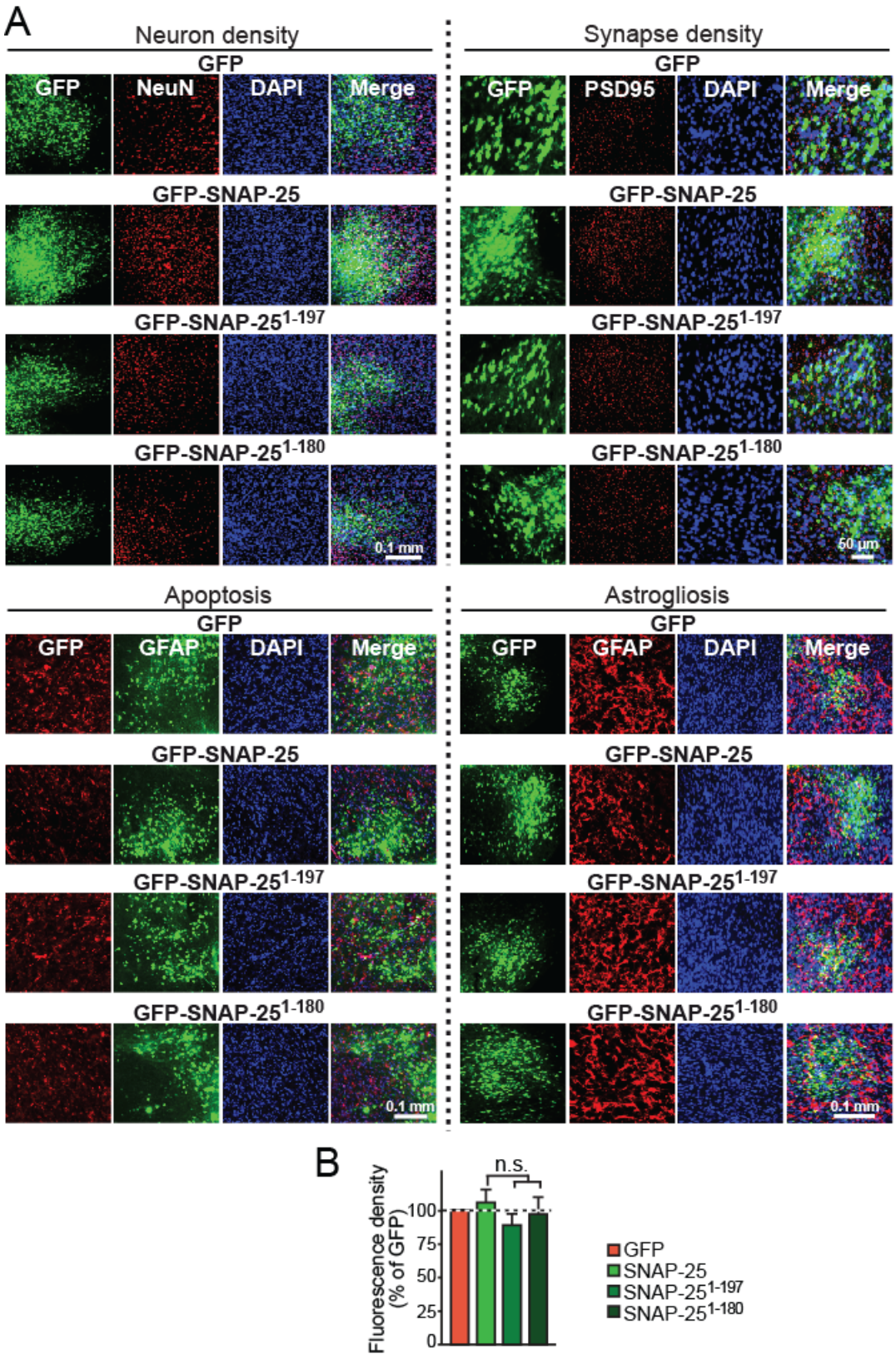
A
Neuron density
Synapse density
GFP
GFP
NeuN
DAPI
Merge
PSD95
GFP-SNAP-25
GFP-SNAP-25$^{1-197}$
GFP-SNAP-25$^{1-180}$
0.1 mm
50 μm
Apoptosis
Astrogliosis
GFAP
B
n.s.
Fluorescence density (% of GFP)
100
75
50
25
0
SNAP-25
SNAP-25$^{1-197}$
SNAP-25$^{1-180}$

Figure S6. SNAP-25 overexpression rescues neurodegeneration in CSPα KO mice.

A. Lentiviruses expressing GFP or GFP-SNAP-25 wild-type and truncated versions (GFP-SNAP-25$^{1-197}$ and GFP-SNAP-25$^{1-180}$) were injected into the cortices of neonatal littermate CSPα KO (CSP$^{-/-}$) mice. Brain sections were immunostained for the indicated proteins at P50. For quantitations of neuron loss (top left), synapse density (top right), and apoptosis (bottom left), see Fig. 6. Representative images from glial fibrillary acidic protein (GFAP; bottom right) stained sections are shown as an indication of astrogliosis in the infected cortical areas.

B. In CSP$^{-/-}$ cortices injected with lentiviruses expressing GFP, GFP-SNAP-25, GFP-SNAP-25$^{1-197}$ and GFP-SNAP-25$^{1-180}$ as in (A), GFP fluorescence density was quantitated as a measure of the GFP-SNAP-25 fusion protein expression levels per cell. To calculate the fluorescence density in each image, the total GFP positive pixel count was normalized to the total fluorescent area, and plotted as percent of GFP alone.

Data are shown as means ± SEMs; n = 3 brains (10 images from each brain); n.s. = not significant ($p>0.05$, using Student's t-test, comparing each test sample to the control analyzed in the same experiment).