Supplementary Figures

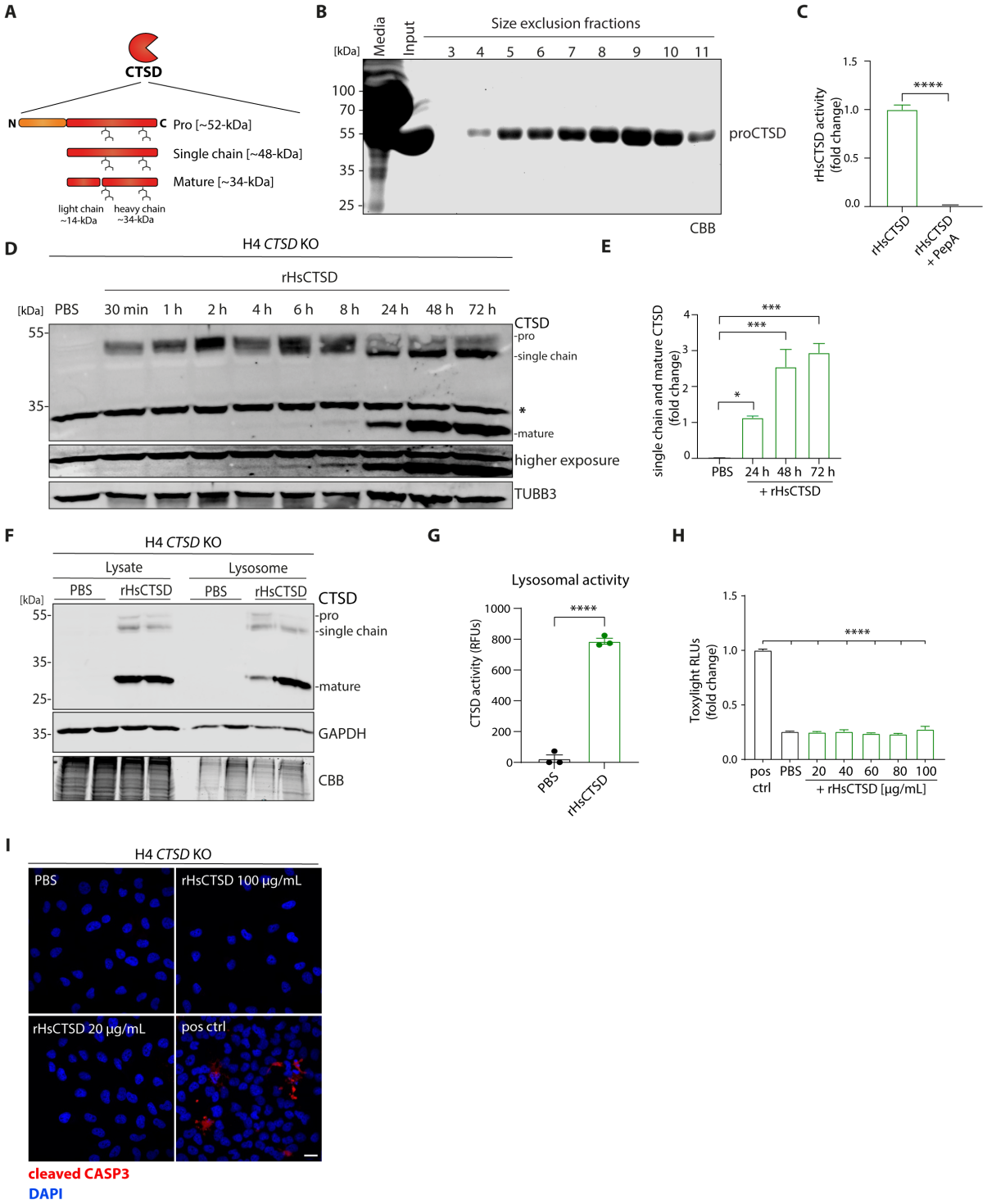


Figure S1. Purification of recombinant proCTSD from HEK cells and functional analysis of H4 *CTSD* KO cells. (**A**) Cartoon illustrating CTSD maturation steps with corresponding molecular

weights (kDa). The inactive proform of the CTSD enzyme (~52-kDa) contains a pro-peptide (orange) that is cleaved off during maturation, yielding in an active single chain form (~48-kDa). Additionally, mannose residues are attached at two asparagine residues, at position N134, and N263, respectively, through N-glycosylation. The active mature form of CTSD consists of a light chain (~14-kDa) and a heavy chain (~34-kDa). (**B**) Representative Coomassie Brilliant Blue (CBB)-stained SDS-PAGE-gel of recombinant proCTSD after purification and size exclusion chromatography in indicated elution fractions. (**C**) CTSD activity of recombinant proCTSD after combining all fractions positive for proCTSD. To measure enzymatic activity assay, the pH was adjusted to pH 4.5 and aspartyl protease inhibitor pepstatin A (PepA) was added to examine specificity of assay and enzymatic activity of recombinant enzyme (n = 3 independently purified rHsCTSD). (**D**) Representative immunoblot of *CTSD* deficient H4 cells treated with 20 µg/ml rHsCTSD from 30 min to 72 h showing pro- (~ 52-kDa), single chain (~ 48-kDa) and mature (~ 34-kDa) CTSD forms. Additionally, to show all bands that appear as mature CTSD, a higher exposed image is shown. An unspecific band detected by CTSD antibody was present in all samples and labeled with an asterisk (*). (**E**) Quantification of single chain and mature CTSD forms analyzed together as active CTSD in H4 cells deficient in CTSD (*CTSD* KO) treated with 20 µg/ml rHsCTSD for 24, 48 and 72 h. Active CTSD was normalized to TUBB3 and expressed as fold change (n = 3). (**F**) Representative western blot analysis of lysate and lysosomal-enriched fraction of H4 *CTSD* KO cells treated with 20 µg/mL rHsCTSD for 72 h. CTSD pro- and single chain forms (~52-kDa/48-kDa) as well as the mature CTSD (~34-kDa) are enriched in the lysosomal fraction. GAPDH and CBB-stained SDS-Page-gel were shown as loading controls. (**G**) Quantification of CTSD activity of H4 *CTSD* KO enriched lysosomal fractions incubated with 20 µg/mL rHsCTSD for 72 h. Data shows CTSD activity in relative fluorescence units (RFU) (n = 3 independent lysosome-enrichment fractionations) (**H**) ToxiLight cytotoxicity assay of H4 *CTSD* KO cells

treated with 20, 40, 60, 80 and 100 µg/ml rHsCTSD for 72 h, expressed as fold change (measured in triplicates for three independent experiments, n = 3). (**I**) Representative immunofluorescence images of H4 *CTSD* KO cells treated with 20 and 100 µg/mL rHsCTSD for 72 h and stained for cleaved CASP3 (red) as an apoptotic marker. DAPI (blue) was used to stain the nucleus. As a positive control H4 *CTSD* KO cells were incubated with 500 µM H_2O_2 for 4 h. Scale bar: 20 µm. Data represent mean ± SEM. Statistical analyses were performed by using a two-tailed unpaired Student's t-test for (C and G) and a one-way ANOVA together with a Dunnett's multiple comparison test (E and H). Statistical differences are shown toward the PBS treatment (E) and toward positive control for ToxiLight cytotoxicity assay (H). ****$p < 0.0001$, ***$p < 0.001$, *$p < 0.05$.

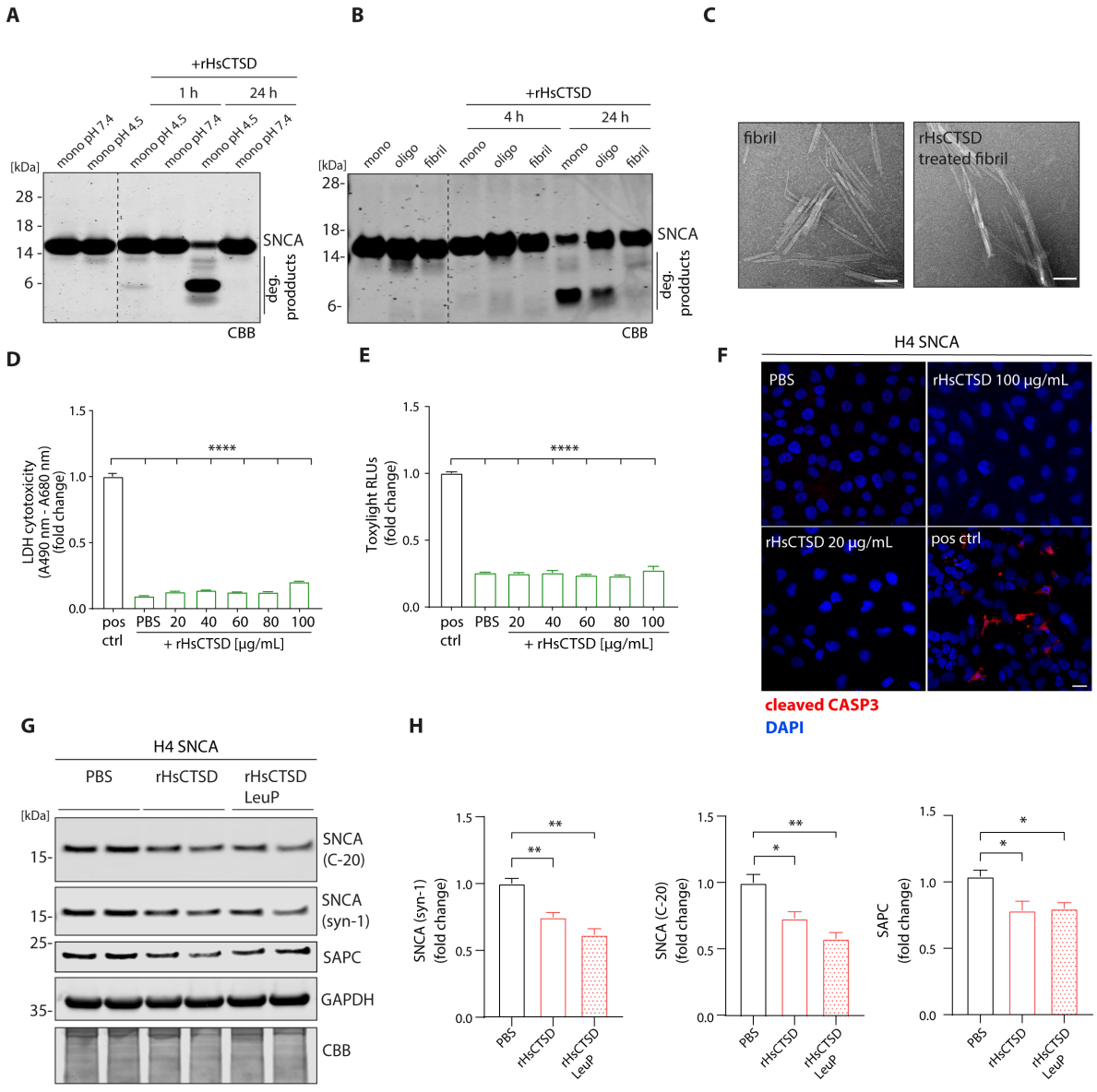


Figure S2. Functional analysis of rHsCTSD *in vitro* and in H4 cells overexpressing WT SNCA. (**A**) Representative CBB-stained SDS-PAGE-gel showing ability of purified enzyme to process recombinant monomeric SNCA. 20 µM SNCA was incubated with 0.3 µM rHsCTSD for 1 h and 24 h at pH 7.4 and pH 4.5 (lysosomal pH). (**B**) Representative CBB-stained SDS-PAGE gel illustrating the capacity of rHsCTSD to cleave and process monomeric as well as oligomeric forms (partly) after 24 h of digesting by rHsCTSD (0.3 µM). Fibrillary forms of SNCA were not processed by rHsCTSD after 24 h. (**C**) Representative negative stain TEM pictures of SNCA fibrils

after incubation with and without rHsCTSD after 24 h. Scale bar: 100 nm. (**D**) LDH cytotoxicity assay and (**E**) ToxiLight cytotoxicity assay of H4 cells (SNCA overexpression) treated with different concentrations of rHsCTSD (20, 40, 60, 80, 100 µg/mL), expressed as fold change (measured in triplicates of three independent experiments, n = 3). (**F**) Immunofluorescence images of H4 cells (SNCA overexpression) incubated with 20 and 100 µg/mL rHsCTSD for 72 h and stained for cleaved CASP3 (red) as an apoptotic marker. DAPI (blue) was used to stain the nucleus. As a positive control, H4 cells were incubated with 500 µM H_2O_2 for 4h. Scale bar: 20 µm**.** (**G**) Representative western blot of H4 cells (SNCA overexpression) treated with PBS, 20 µg/mL rHsCTSD and 20 µg/mL rHsCTSD together with 5 µM Leupeptin (LeuP) for 72 h and stained for SNCA (C-20 and syn-1) and SAPC. GAPDH signal and CBB-stained SDS-gel were used as loading controls. (**H**) Quantification of SNCA (syn-1 and C-20) and SAPC signal were normalized to GAPDH and expressed as fold change (n = 3). All data represent mean ± SEM. Statistical analyses were performed by one-way ANOVA together with Dunnett's multiple comparison test with statistical differences toward the positive control (D and E) and PBS for (H). ****$p < 0.0001$, **$p < 0.01$, *$p < 0.05$.

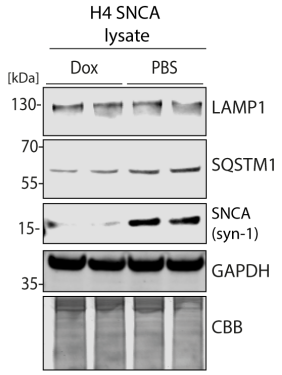
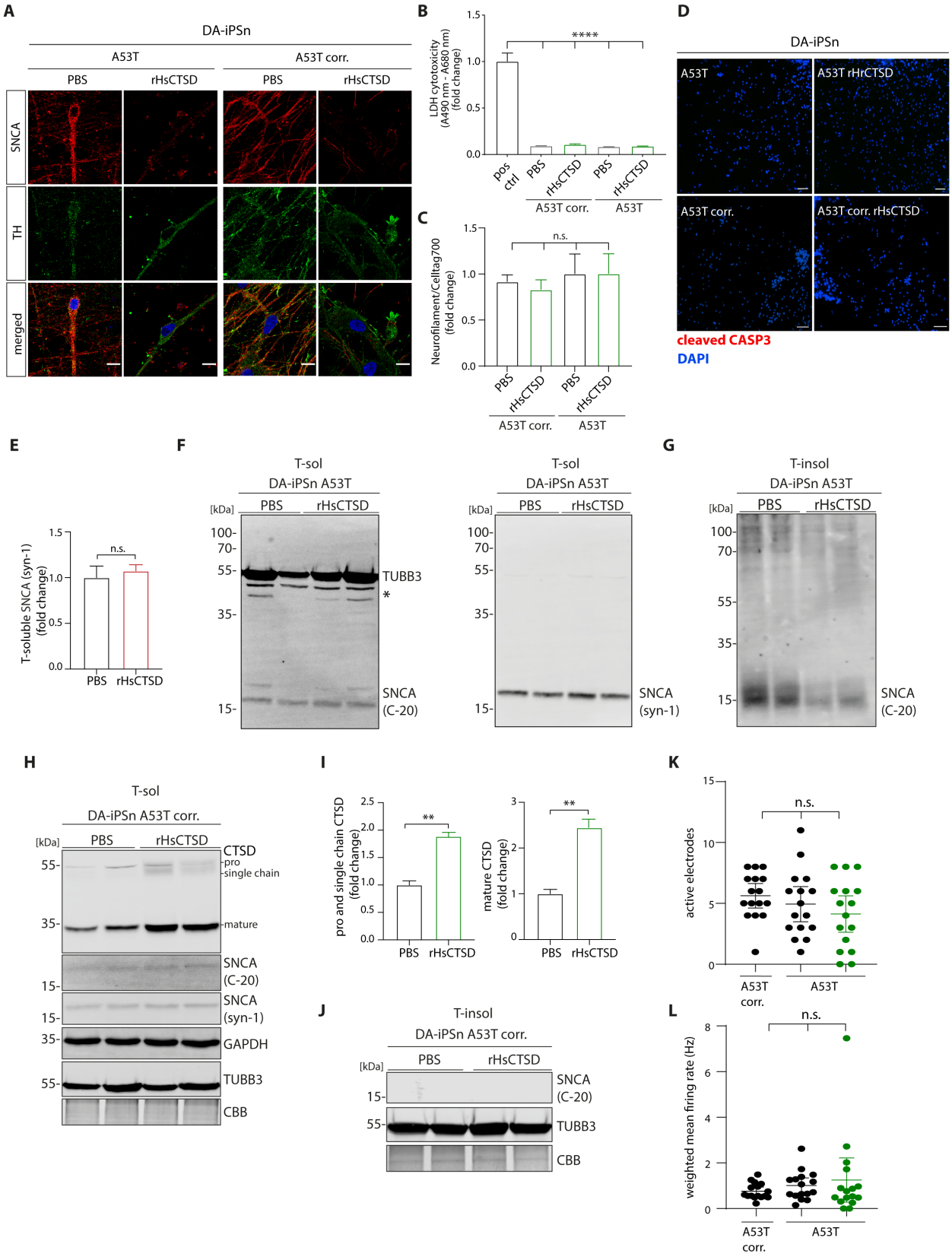


Figure S3. Effects of SNCA on endo-lysosomal and autophagic system in H4 cells overexpressing SNCA. Representative western blot of H4 cells incubated with Dox to decrease SNCA expression and PBS as an untreated control for 72 h. Signal for SNCA was detected by syn-1 and as autophagic markers, LAMP1 and SQSTM1 were used. Corresponding quantifications of LAMP1 and SQSTM1 are shown in Figure 3D. GAPDH and CBB stained SDS-PAGE were used as loading controls.


A
DA-iPSn
A53T
A53T corr.
PBS
rHsCTSD
PBS
rHsCTSD
SNCA
TH
merged
B
LDH cytotoxicity (A490 nm - A680 nm) (fold change)
****
pos ctrl
PBS
rHsCTSD
PBS
rHsCTSD
A53T corr.
A53T
C
Neurofilament/Celltag700 (fold change)
n.s.
PBS
rHsCTSD
PBS
rHsCTSD
A53T corr.
A53T
D
DA-iPSn
A53T
A53T rHrCTSD
A53T corr.
A53T corr. rHsCTSD
cleaved CASP3
DAPI
E
T-soluble SNCA (syn-1) (fold change)
n.s.
PBS
rHsCTSD
F
T-sol
DA-iPSn A53T
[kDa]
PBS
rHsCTSD
100-
70-
55-
35-
15-
TUBB3
*
SNCA (C-20)
T-sol
DA-iPSn A53T
[kDa]
PBS
rHsCTSD
100-
70-
55-
35-
15-
SNCA (syn-1)
G
T-insol
DA-iPSn A53T
[kDa]
PBS
rHsCTSD
100-
70-
55-
35-
15-
SNCA (C-20)
H
T-sol
DA-iPSn A53T corr.
[kDa]
PBS
rHsCTSD
55-
CTSD
pro
single chain
35-
mature
15-
SNCA (C-20)
15-
SNCA (syn-1)
35-
GAPDH
55-
TUBB3
CBB
I
pro and single chain CTSD (fold change)
**
PBS
rHsCTSD
mature CTSD (fold change)
**
PBS
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J
T-insol
DA-iPSn A53T corr.
[kDa]
PBS
rHsCTSD
15-
SNCA (C-20)
55-
TUBB3
CBB
K
active electrodes
n.s.
A53T corr.
A53T
L
weighted mean firing rate (Hz)
n.s.
A53T corr.
A53T

Figure S4. Characterization and functional analyses of DA-iPSn A53T mutant and isogenic control (A53T corr.) lines. (**A**) Representative immunofluorescence images of DA-iPSn A53T and A53T corr. using SNCA antibody LB509 (red) and dopaminergic marker tyrosine hydroxylase (TH; green) (n = 7 individual neurons per group). Scale bar: 10 µm. (**B**) LDH cytotoxicity assay of A53T corr. and mutant treated with and without rHsCTSD for 25 days (measured in triplicates of five independent treatments, n = 5), shown as fold change compared to the positive control. Positive control was provided by the Kit. (**C**) Quantification of neurofilament release to determine neuronal viability and early cell death in DA neurons performed by an in-cell western. Signals were normalized to cell stain (Celltag700) and are displayed as fold change compared to A53T PBS (measured in triplicates of four independent experiments, n = 4). (**D**) Representative immunofluorescence pictures of DA-iPSn A53T corr. and A53T mutant with and without rHsCTSD stained for cleaved CASP3. Scale bar: 100 µm. (**E**) Quantification of soluble SNCA by syn-1 antibody (corresponding western blot is shown in Figure 4D), normalized to GAPDH and expressed as fold change (n = 3). (**F**) Full size western blot membrane from T-sol (Triton-soluble) lysates of DA-iPSn A53T PBS and rHsCTSD treated cells. Left, Membrane was stained with the SNCA C-20 antibody, which was detected by the infrared channel 800. TUBB3 and its unspecific protein signal, which is marked with an asterisk (*), is also shown. Right, the same full size western blot stained with SNCA antibody syn-1 and detected by the infrared channel 680. (**G**) Full western blot membrane from T-insol (Triton-insoluble) lysates of DA-iPSn A53T treated with PBS or rHsCTSD stained with SNCA antibody C-20. (**H**) Representative immunoblot of T-sol lysates of A53T corr. DA-iPSn, stained for CTSD (pro- and single chain forms and mature forms) and SNCA (C-20 and syn-1 antibody). GAPDH, TUBB3 and CBB-stained SDS-gels were used as loading control. (**I**) Quantification of pro-/single chain form and mature CTSD signal intensities, normalized to GAPDH and expressed as fold change (n=3) (**J**) Representative immunoblot of T-

insol lysates of A53T corr. DA-iPSn, indicating the absence of SNCA signal in the corrected line. TUBB3 and CBB-stained SDS-gels were used to control for equal protein loading (n = 3). (**K**) Quantification of active electrodes and (**L**) weighted mean firing rate (Hz) in A53T corr. and A53T before treatment (n = 16, each point represents one active well of a 48-well MEA plate). Green labeled graphs were exposed to rHsCTSD at day 44 of DA neuronal differentiation (Figure 4 J and K). Data represent mean ± SEM. Statistical analyses were performed by one-way ANOVA together with Dunnett's multiple comparison test (A and B). Statistical differences are shown in (B) toward positive control and in (C) towards A53T mutant line with PBS. A two-tailed unpaired Student's t-test was used for (E) and (I) and one-way ANOVA together with a Tukey's comparison test for (K and L). ****$p < 0.0001$, **$p < 0.01$; n.s., not significant.

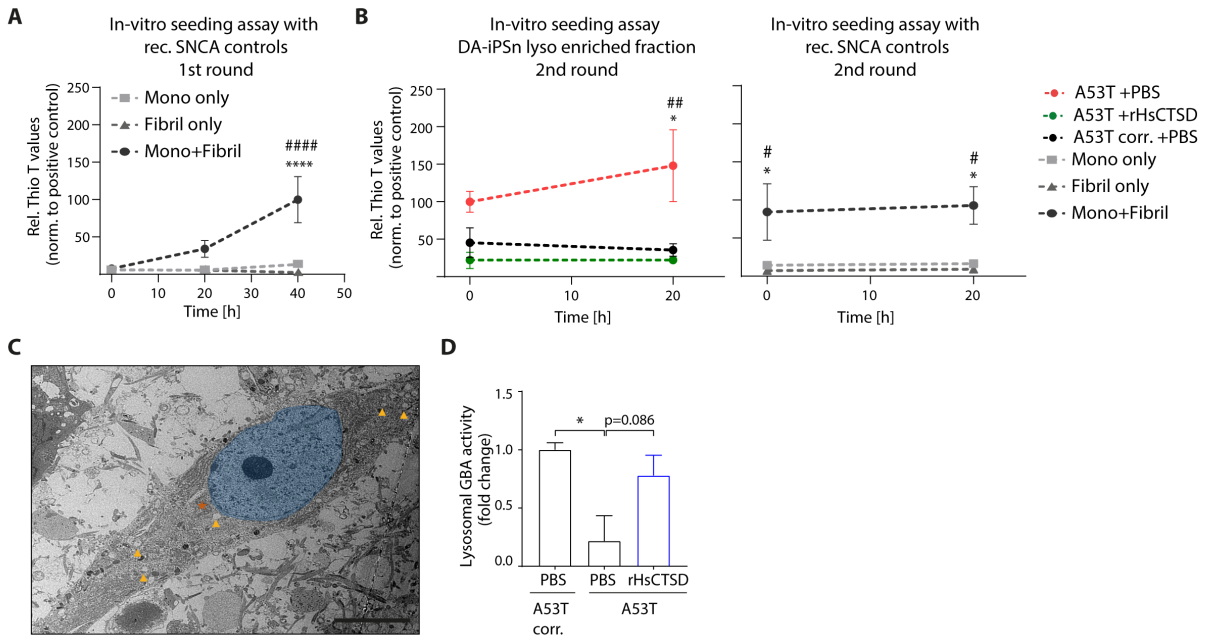

Figure S5. Characterization of lysosomes and autophagic vesicles from DA-iPSn harboring SNCA mutation (A53T). (**A**) Corresponding recombinant controls of first round *in vitro* SNCA seeding assay of DA-iPSn-derived lysosomal enriched fraction shown in Figure 5A. As negative controls purified recombinant SNCA monomers were incubated alone (mono only) and with SNCA fibril alone (fibril only) and as a positive control SNCA monomer was incubated together with SNCA fibril (fibril+mono). Graph shows relative Thio T signal normalized to positive control (fibril+ a-syn mono) (n = 3). (**B**) Analyses of two rounds of *in vitro* SNCA seeding assay of DA-iPSn A53T corr. and A43T mutant with rHsCTSD and without (PBS)-derived lysosomal enriched fraction and corresponding controls (n = 3). (**C**) TEM pictures of ultrastructural analysis of DA iPSn A53T corr. corresponding to Figure 5H. Blue: nucleus, yellow arrows: intracellular vesicles. Exemplary mitochondria is depicted as an orange star. Scale bars: 25 µm. (**D**) Quantification of lysosomal GBA activity in DA-iPSn A53T corr., A53T mutant and mutant treated with rHsCTSD for 21 days expressed as fold change (n = 3). Data represent mean ± SEM. Statistical analyses were performed by using two-way ANOVA with a Tukey's multiple comparison test (A and B). To highlight

statistical differences asterisks (*) were used for A53T corr. vs. A53T and mono only vs. fibril+mono. Diamonds (#) were used for A53T vs. A53T +rHsCTSD and fibril+mono vs. fibril only. For (D) statistical analysis were performed by one-way ANOVA together with a Dunnett's multiple comparison test with statistical differences toward A53T corr.. ****/####$p < 0.0001$, **/##$p < 0.01$, */#$p < 0.05$; n.s., not significant.

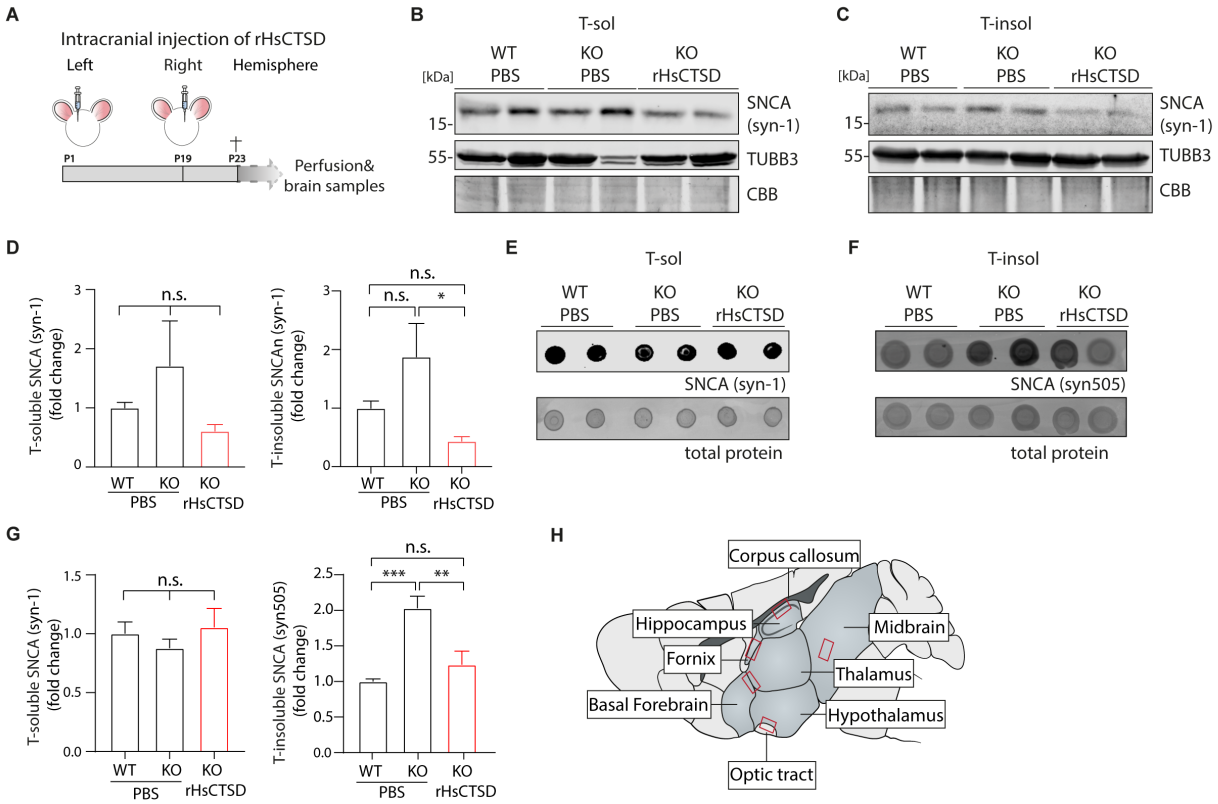

Figure S6. Analysis of SNCA levels within *ctsd* KO animals after treatment with rHsCTSD. (**A**) Cartoon illustrating the time line of intracranial injection (i.c.) time points of 100 µg rHsCTSD (P1 and P19) as well as time point of mice perfusion and sample acquisition (P23). (**B**) Representative immunoblot of T-sol and (**C**) T-insol from whole brain lysates extracted from wildtype (WT) and *ctsd* KO and *ctsd* KO mice i.c. injected with rHsCTSD (KO +rHsCTSD). (**D**) Respective quantification of T-sol (left) and T-insol (right) SNCA levels detected by syn-1 antibody, normalized to TUBB3 and expressed as fold change (n = 4 mice per group). (**E**) Representative dot blot from T-sol whole brain lysates of WT, *ctsd* KO and *ctsd* KO mice i.c. injected with rHsCTSD. Total SNCA was detected by syn-1 antibody and total protein stain (DirectBue71) was used to ensure equal protein load. (**F**) Representative dot blot of T-insol fraction of whole brain lysates of WT, *ctsd* KO and *ctsd* KO mice treated with rHsCTSD. SNCA was detected by syn505 antibody and total protein staining was used as loading control. (**G**) Respective quantification of T-sol SNCA

144 by syn-1 (left) and pathological T-insol SNCA by syn505 (right). SNCA signals were normalized
145 to total protein and expressed as fold change (n = 4 mice per group), compared to WT. (**H**)
146 Schematic representation of the mouse brain in a sagittal cut. The region marked by the red square
147 shows where the confocal images were taken. Data represent mean ± SEM. Statistical analyses
148 were performed by one-way ANOVA together with Tukey's multiple comparison test. *** $p <$
149 0.001, **$p < 0.01$, *$p < 0.05$, n.s., not significant.
150

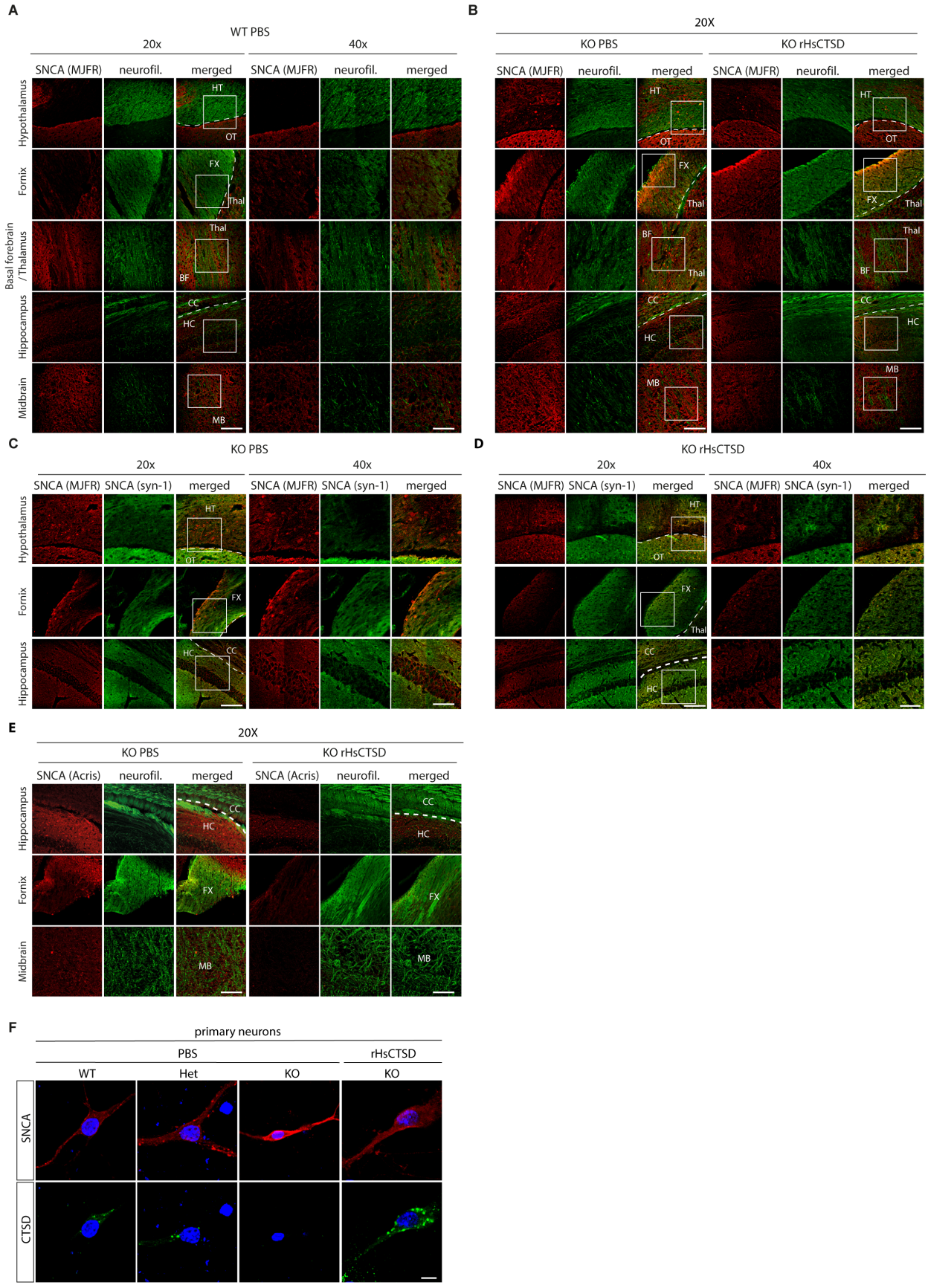
A
WT PBS
20x
40x
SNCA (MJFR)
neurofil.
merged
Hypothalamus
Fornix
Basal forebrain / Thalamus
Hippocampus
Midbrain
HT
OT
FX
Thal
BF
CC
HC
MB
B
20X
KO PBS
KO rHsCTSD
C
KO PBS
SNCA (syn-1)
D
KO rHsCTSD
E
SNCA (Acris)
F
primary neurons
PBS
rHsCTSD
WT
Het
KO
SNCA
CTSD

Figure S7. Immunofluorescence images of WT mouse brain and *ctsd* KO mouse brain treated with PBS or rHsCTSD and stained for SNCA. (**A**) Representative confocal microscopy images of WT mouse brain treated with PBS. Pathology-associated SNCA was detected by the conformation-specific antibody MJFR-14-6-4-2 (red) and co-stained for neurofilament (green). The following brain regions are shown: Hypothalamus (HT), fornix (FX), basal forebrain/thalamus (BF/Thal), hippocampus (HC) and midbrain (MB). OT indicate the optical tract and CC the corpus callosum. The white dotted line indicates boundary between regions. The region marked by the white square in 20x is magnified in 40x. Images were taken with the 20x (scale bar: 100 µm) and 40x (scale bar: 50 µm) magnifications. (**B**) Representative confocal microscopy images of mouse brain from *ctsd* KO treated with PBS or 100 µg of rHsCTSD. The corresponding higher magnified (40x) images were marked by the white square and are shown in Figure 6F. Images were taken with the 20x (scale bar: 100 µm). The same antibodies and brain regions were used as shown in Figure 6F and S7A. (**C**) Representative immunofluorescence images *ctsd* KO mice treated with PBS and (**D**) with rHsCTSD stained with MJFR-14-6-4-2 (red) and co-stained for syn-1 (green). The images are 20x magnified (scale bar: 100 µm) and the marked white square indicate the 40x magnification (scale bar: 50 µm). Immunofluorescence images show the hypothalamus (HT), fornix (FX) and hippocampus (HC) of the mouse brain. The white dotted line indicates boundary between regions and OT stands for optical tract whereas CC for the corpus callosum. (**E**) Representative immunofluorescence microscopy images of *ctsd* KO hippocampus (HC), fornix (FX) and midbrain (MB) mouse brain areas i.c. injected with PBS or rHsCTSD. Brain areas are stained for SNCA by Acris antibody (red) and neurofil. (green). Mouse brain images were taken with a 20x magnification (scale bar: 100 µm). (**F**) Corresponding immunofluorescence stainings of primary neurons from WT, Het, KO and KO +rHsCTSD animals are shown in Figure 6L (merged). SNCA (syn-1) is shown in red, CTSD in green and nucleus in blue. Scale bar: 10 µm.