Supplementary Information

SUSD4 Controls Activity-Dependent Degradation of AMPA Receptor GLUA2 and Synaptic Plasticity

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Supplementary Information:

Table S1

Figures S1 to S11

STAR Methods

Supplementary references

Table Supplementary 1. Behavioral characterization of *Susd4* KO mice. From three month-old *Susd4* KO and *wild type* mice.

	WT		*Susd4* KO	
Physical Characteristics				
Weight (g)	24,1	± 2,91	24,7	± 3,45
Whiskers (% with)	80	%	83,3	%
Palpebral Closure (% with)	0	%	0	%
Piloerection (% with)	20	%	25	%
Sensorimotor Reflexes				
(% subjects displaying "normal response")				
Cage movement	100	%	100	%
Whisker response	100	%	100	%
Eye Blink	100	%	100	%
Ear Twitch	100	%	100	%
Motor Responses				
Open Field Locomotion				
Improvement (number)	21,92	± 2,83	19,42	± 2,03
Distance (cm)	2653,84	± 230,25	2300,68	± 158,47
Speed (cm/s)	12,79	± 0,41	13,06	± 0,30
Time on Center (%)	12,76	± 1,30	11,18	± 1,26

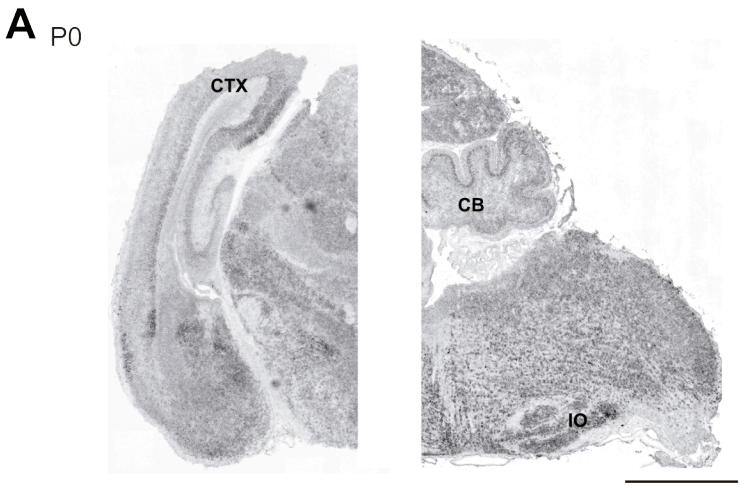

A P0
CTX
CB
IO
B P7
CTX
DG
CA3
CB
IO
C Adult
PCs
CTX
DG
CA3
CB
IO

Figure Supplementary 1. *Susd4* mRNA expression in the developing mouse brain.

(A) *Susd4* mRNA expression was visualized in the brain of wild-type mouse by *in situ* hybridization. Coronal (left) and sagittal (right) sections are presented at postnatal day 0,
(B) postnatal day 7 and
(C) postnatal day 30. *Susd4* expression was found in many regions including the cerebral cortex (CTX), the dentate gyrus (DG) and CA3 regions in the hippocampus (coronal section), the cerebellum (CB), in particular Purkinje cells (PCs), and the inferior olive (IO; sagittal section, right). Scale bars: 250μm (**A**, **B**) and 500μm (**C**).

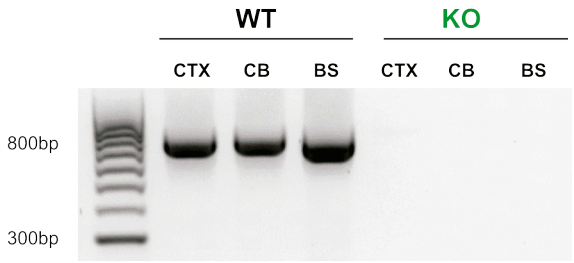

WT
KO
CTX
CB
BS
CTX
CB
BS
800bp
300bp

Figure Supplementary 2. Characterization of *Susd4* knockout mice.

Susd4 expression was assessed by RT-PCR in extracts from cortex (CTX), cerebellum (CB) and brainstem (BS) in control and *Susd4* KO mice.

Footprint Test

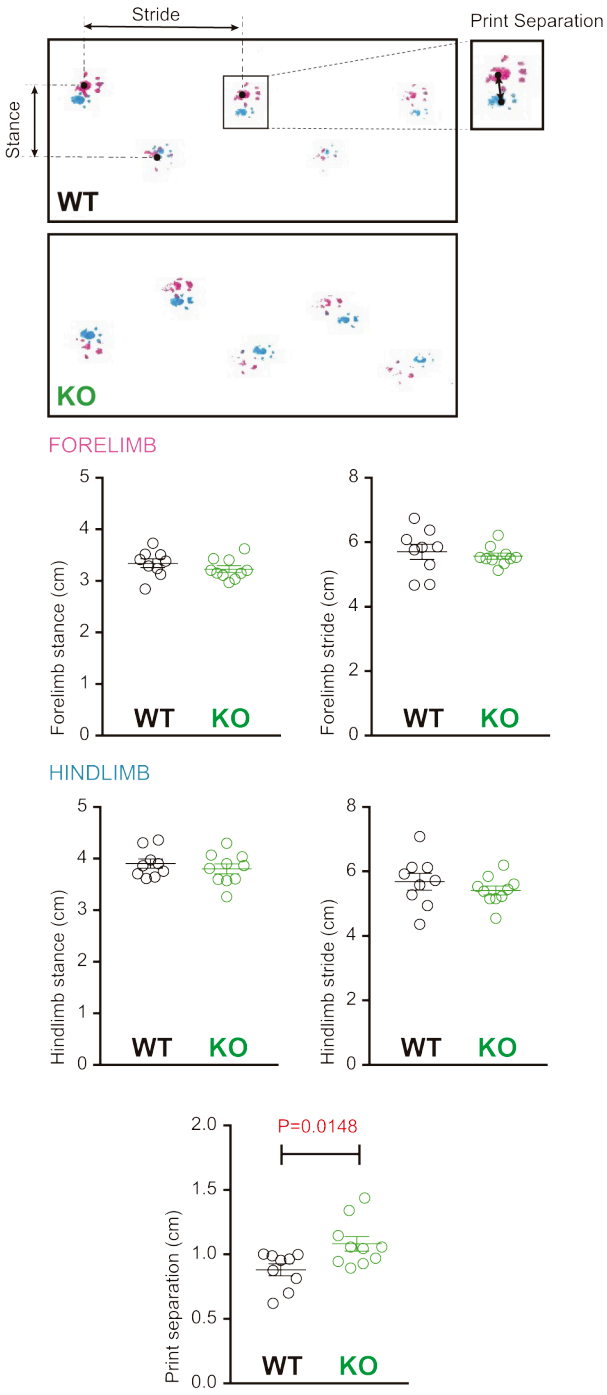


Figure Supplementary 3. Footprint analysis and motor correlations in *Susd4* KO mice.

Footprint patterns of P30 wild-type and KO mice were quantitatively analyzed by measuring stride length for the fore paws (pink) and hind paws (blue), stance length for the forelimbs and hindlimbs, and print separation. Mean ± s.e.m. (WT n=9 and KO n=10 mice; Forelimb stance, unpaired Student's t-test, P=0.3059; Forelimb stride, P=0.5882; Hindlimb stance, P=0.4533; Hindlimb stride, P=0.3580; Print separation, * P=0.0148).

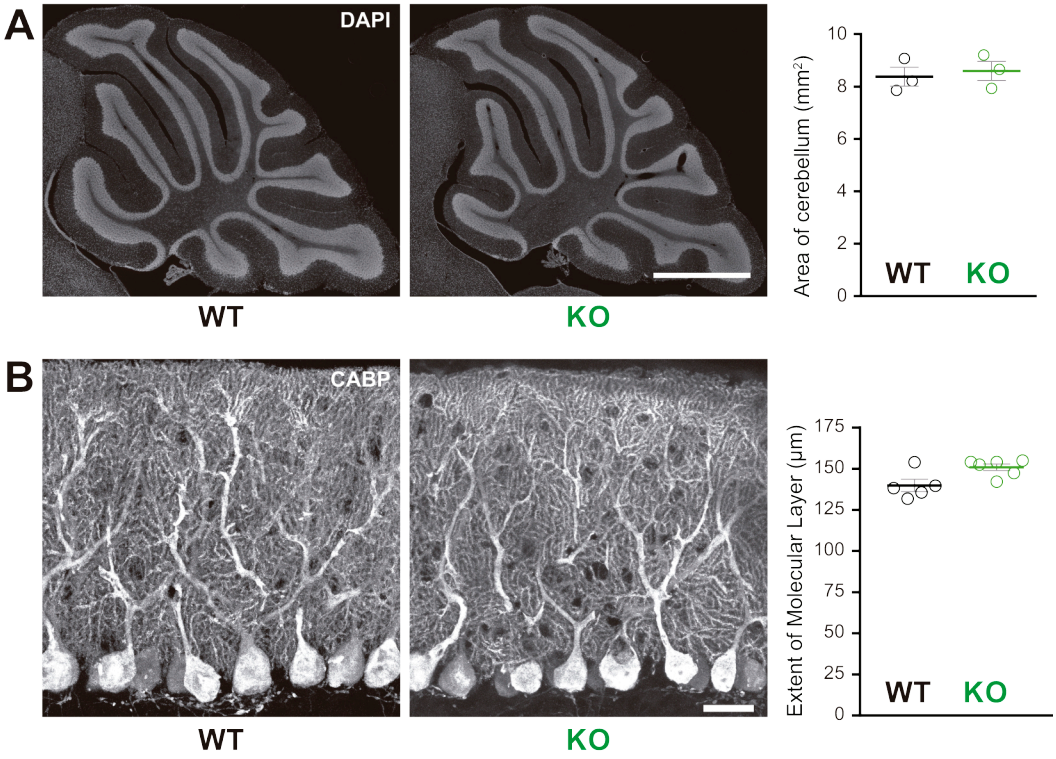
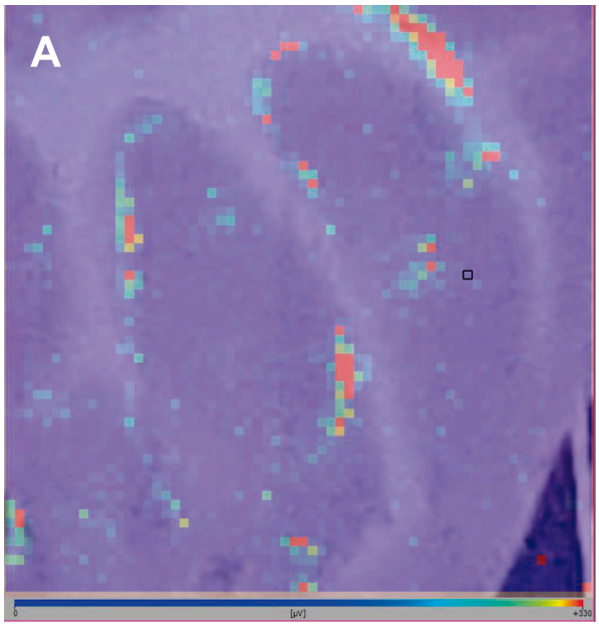
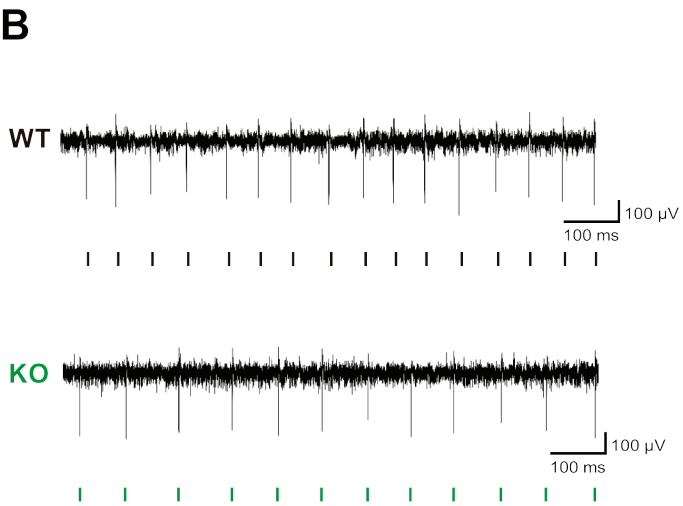

A
DAPI
WT
KO
Area of cerebellum (mm²)
10
8
6
4
2
0
WT
KO
B
CABP
WT
KO
Extent of Molecular Layer (µm)
175
150
125
100
75
50
25
0
WT
KO

Figure Supplementary 4. Normal cerebellar cytoarchitecture in *Susd4* KO mice.

(A) Parasagittal sections of P30 wild-type and KO cerebella were stained with Hoechst

(B) and calbindin protein (CABP) immunostaining and used for quantitative analysis of the mean area of the cerebellum. (Mean ± s.e.m; WT n=3 and KO n=3 mice, unpaired Student's t-test, P=0,4932) and of the height of the molecular layer (Mean ± s.e.m. WT n=5 and KO n=6 mice; unpaired Student's t-test, P=0,1157), respectively. Scale bars: 500μm (**A**) and 30μm (**B**).


A


B
WT
100 µV
100 ms
KO
100 µV
100 ms

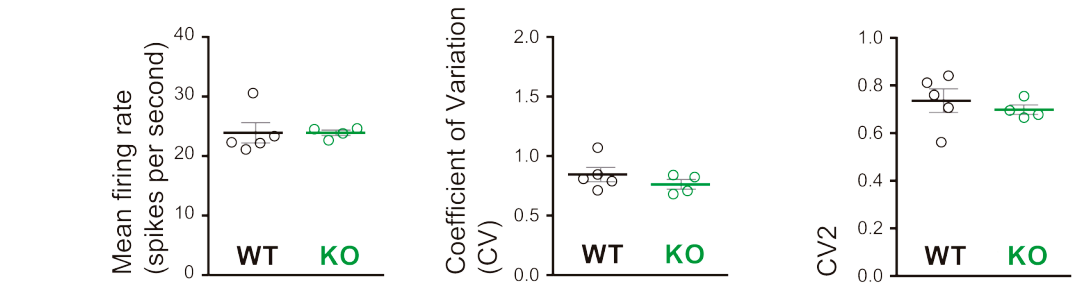

C
Mean firing rate (spikes per second)
40
30
20
10
0
WT
KO
Coefficient of Variation (CV)
2.0
1.5
1.0
0.5
0.0
WT
KO
CV2
1.0
0.8
0.6
0.4
0.2
0.0
WT
KO

Figure Supplementary 5. High density microelectrode array (MEA) analysis of Purkinje cell spiking in acute cerebellar slices from *Susd4* KO compared to WT.

(A) Image of the cerebellar acute slice overlapped with the image of the color map of the MEA recording. Each pixel represents one channel, where the active units are in red.

(B) Representative traces of electrical activity recorded in one channel. Each tick represents one action potential that has been detected and sorted by the Brainwave software.

(C) Histograms of the mean firing rate, coefficient of variation of Inter Spike Intervals (CV) and CV2. (Mean ± s.e.m.; WT n=5 mice and KO n=4 mice; Mean Firing Rate: Mann Whitney test, P=0.2857; Coefficient of Variation: Mann Whitney test, P=0.4127; CV2: Mann Whitney test, P=0.5373).

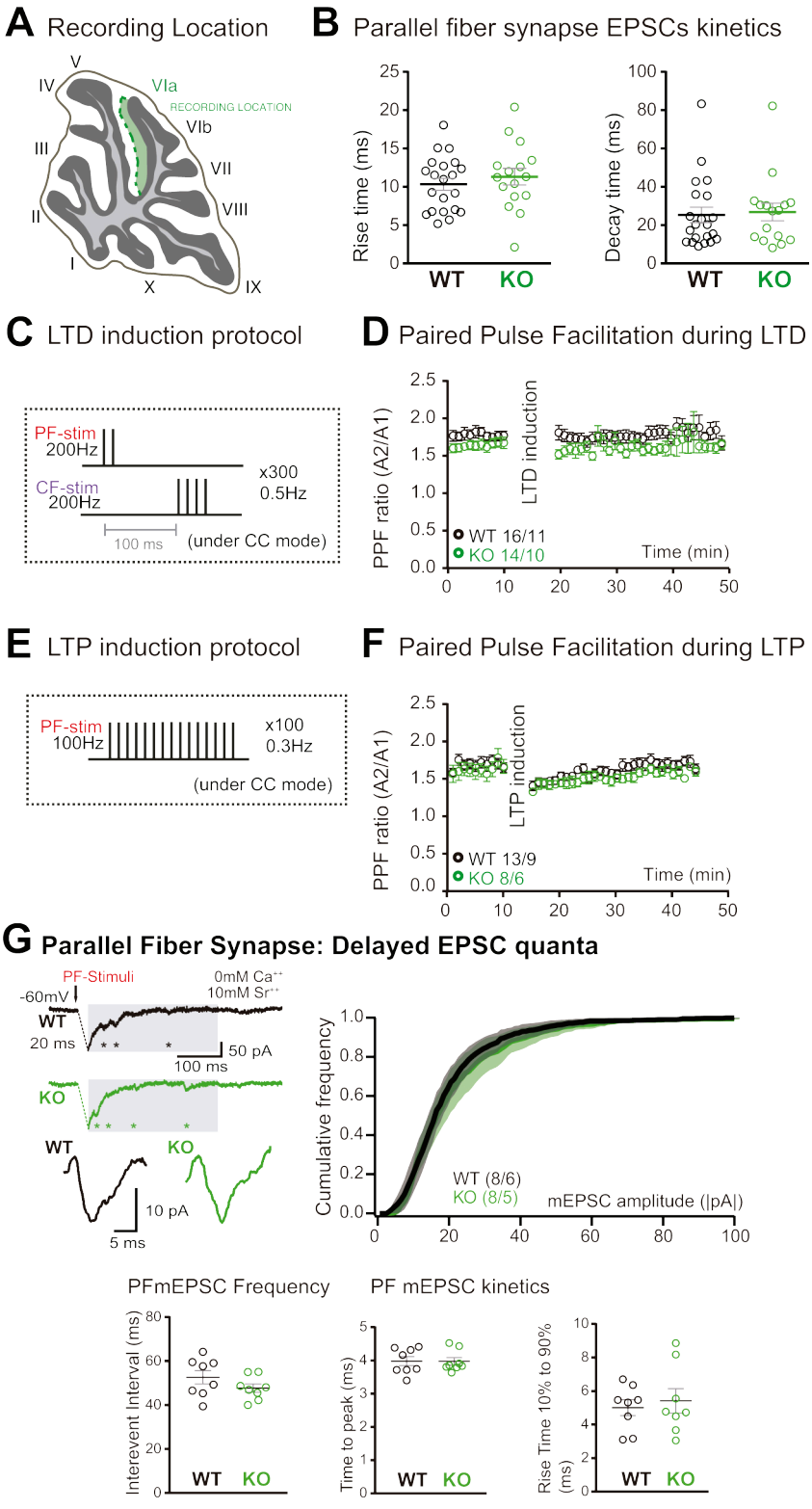

A Recording Location
V
IV
VIa
RECORDING LOCATION
VIb
III
VII
VIII
II
I
X
IX
B Parallel fiber synapse EPSCs kinetics
Rise time (ms)
Decay time (ms)
WT
KO
C LTD induction protocol
PF-stim
200Hz
CF-stim
200Hz
x300
0.5Hz
100 ms
(under CC mode)
D Paired Pulse Facilitation during LTD
PPF ratio (A2/A1)
LTD induction
WT 16/11
KO 14/10
Time (min)
E LTP induction protocol
PF-stim
100Hz
x100
0.3Hz
(under CC mode)
F Paired Pulse Facilitation during LTP
PPF ratio (A2/A1)
LTP induction
WT 13/9
KO 8/6
Time (min)
G Parallel Fiber Synapse: Delayed EPSC quanta
PF-Stimuli
0mM Ca++
10mM Sr++
-60mV
WT
20 ms
50 pA
100 ms
KO
WT
KO
10 pA
5 ms
Cumulative frequency
WT (8/6)
KO (8/5)
mEPSC amplitude (|pA|)
PFmEPSC Frequency
PF mEPSC kinetics
Interevent Interval (ms)
Time to peak (ms)
Rise Time 10% to 90% (ms)
WT
KO

Figure Supplementary 6. Parallel fiber/Purkinje cell synapse EPSCs kinetics, long-term plasticity induction protocols, paired-pulse facilitation ratio and delayed EPSC quanta.

(A) Schematic representation of the recording location, internal lobule VIa of the vermis.

(B) No change in the decay and rise time of Parallel fiber/Purkinje cell synapse EPSCs was induced by *Susd4* deletion. (Mean ± s.e.m.; WT n=21 cells/8 mice and KO n=16 cells/6 mice; Decay time: Mann Whitney test, P=0.7276; Rise time: unpaired Student's t-test, P=0.4570).

(C) Parallel fiber long-term depression induction protocol.

(D) Paired-pulse ratio (A2/A1) measured at 20 Hz (Mean ± s.e.m. WT n=16 cells/11 mice and KO n=14 cells/10 mice, two-way ANOVA with repeated measures, Interaction (time and genotype): P=0.9935, F(39, 1092) = 0.5222).

(E) Parallel fiber long-term potentiation induction protocol.

(F) Paired-pulse ratio (A2/A1) measured at 20Hz (Mean ± s.e.m. WT n=13 cells/9 mice and KO n=8 cells/6 mice, two-way ANOVA with repeated measures, Interaction (time and genotype): P=0.9366, F(39, 741) = 0.6745).

(G) Delayed PF-EPSC quanta were evoked by PF stimulation in the presence of strontium instead of calcium to induce desynchronization of fusion events. Representative sample traces are presented. The cumulative probability for the amplitude shows no difference with *Susd4* loss-of-function. Mean ± s.e.m. (WT n=8 cells, 6 mice and KO n=8 cells, 5 mice; Kolmogorov-Smirnov distribution test, P=0.1667). The individual frequency values for each cell (measured as interevent interval) present no differences between the genotypes. Mean ± s.e.m. (Mann Whitney test, P=0.1913). No change in the time to peak and in the rise time of PF/PC synapse delayed EPSC quanta was induced by *Susd4* deletion. (Mean ± s.e.m. WT n=8 cells/6 mice and KO n=8 cells/5 mice; Time to peak: Mann Whitney test, P=0.6454; Rise time 10% to 90%: unpaired Student's t-test, P=0.6486).

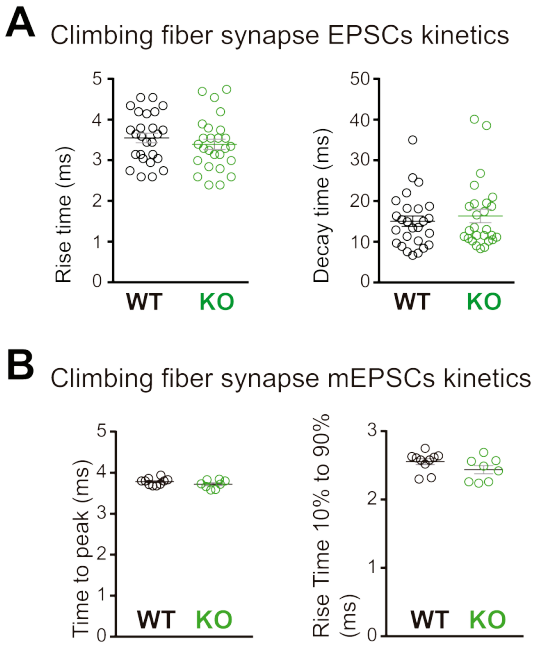

A
Climbing fiber synapse EPSCs kinetics
Rise time (ms)
0
1
2
3
4
5
WT
KO
Decay time (ms)
0
10
20
30
40
50
WT
KO
B
Climbing fiber synapse mEPSCs kinetics
Time to peak (ms)
0
1
2
3
4
5
WT
KO
Rise Time 10% to 90% (ms)
0
1
2
3
WT
KO

Figure Supplementary 7. Kinetics of the climbing fiber/Purkinje cell synapse EPSC.

(A) No change in the rise and decay times of climbing fiber/Purkinje cell synapse EPSCs was induced by *Susd4* deletion. (Mean ± s.e.m. WT n=26 cells/9 mice and KO n=26 cells/7 mice; Rise time: unpaired Student's t-test, P=0.3750; Decay time: Mann Whitney test, P=0.7133).

(B) No change in the time to peak and in the rise time of CF/PC synapse delayed EPSC quanta was induced by *Susd4* deletion. (Mean ± s.e.m. WT n=10 cells/6 mice and KO n=8 cells/3 mice; Time to peak: unpaired Student's t-test, P=0.1692; Rise time 10% to 90%: Mann Whitney test, P=0.0639).

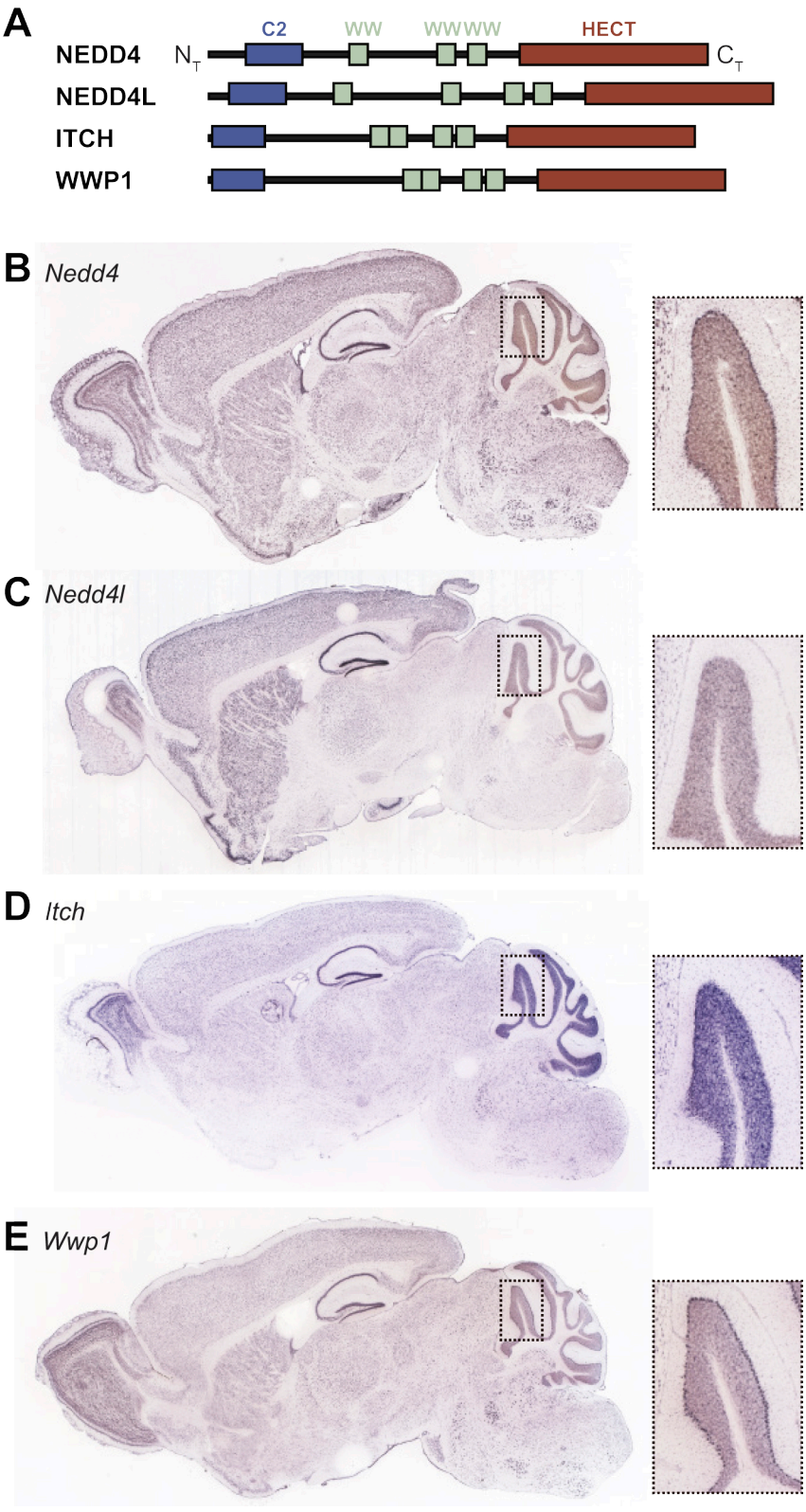
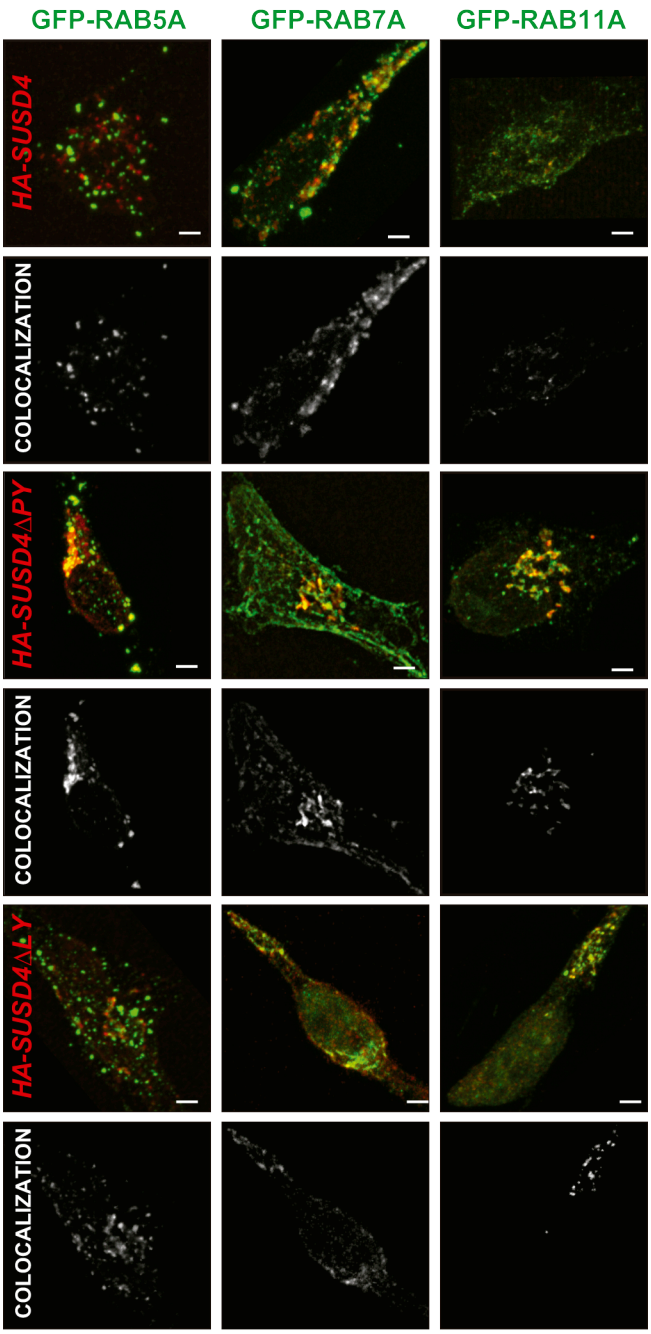

A
C2
WW
WWWW
HECT
NEDD4
NEDD4L
ITCH
WWP1
N
C
B Nedd4
C Nedd4l
D Itch
E Wwp1

Figure Supplementary 8. Expression of HECT ubiquitin ligases in adult mouse brain.

(A) Schematic representation of four SUSD4 interactors: NEDD4, NEDD4L, ITCH and WWP1. Legends: N_T, N-terminus; HECT, Homologous to the E6-AP C-terminus domain; C_T, C-terminus.
(B) Pattern of expression of *Nedd4* (RP_050712_03_C08),
(C) *Nedd4l* (RP_040625_01_G10),
(D) *Itch* (RP_050222_01_H06) and
(E) *Wwp1* (RP_050510_02_E12) mRNA in the adult mouse brain. From Allen Brain Atlas (www.brain-map.org).


GFP-RAB5A
GFP-RAB7A
GFP-RAB11A
HA-SUSD4
COLOCALIZATION
HA-SUSD4ΔPY
COLOCALIZATION
HA-SUSD4ΔLY
COLOCALIZATION

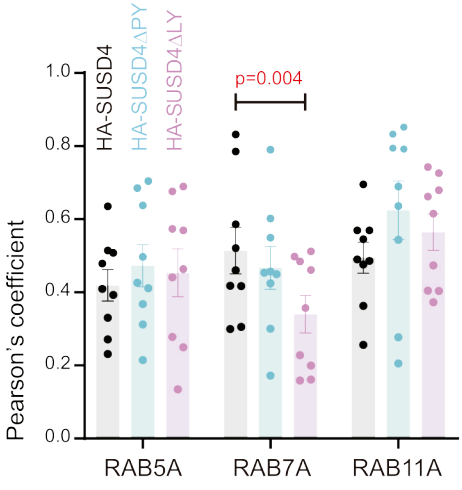

HA-SUSD4
HA-SUSD4ΔPY
HA-SUSD4ΔLY
p=0.004
Pearson's coefficient
1.0
0.8
0.6
0.4
0.2
0.0
RAB5A
RAB7A
RAB11A

Figure Supplementary 9. SUSD4 *in vitro* localization in intracellular compartments.

Representative images of HeLA cells transfected with HA-SUSD4, HA-SUSD4ΔPY or HA-SUSD4ΔLY together with the early endosomal marker (RAB5a-GFP, clathrin coated pits), the late endosomal and lysosomal marker (RAB7a-GFP) or the recycling endosomal marker (RAB11a-GFP). Colocalization between SUSD4 constructs (red; anti-HA) and the endosomal compartment markers (green; anti-GFP) channels was measured using the Pearson's correlation coefficient. Mean ± s.e.m. (n=9 cells from 3 independent experiments).

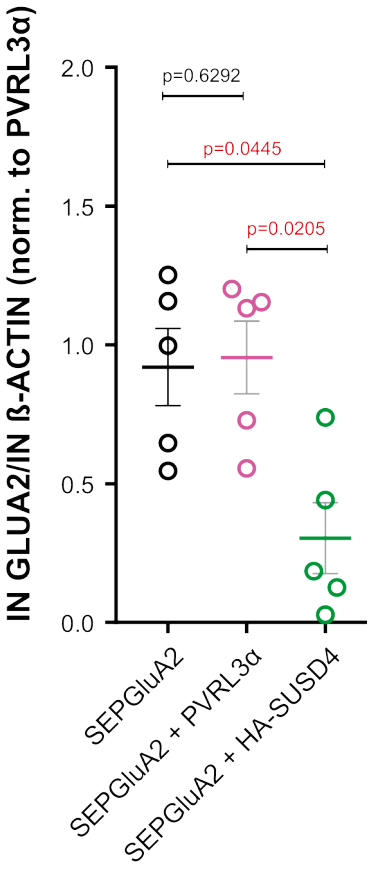

IN GLUA2/IN ß-ACTIN (norm. to PVRL3α)
2.0
1.5
1.0
0.5
0.0
p=0.6292
p=0.0445
p=0.0205
SEPGluA2
SEPGluA2 + PVRL3α
SEPGluA2 + HA-SUSD4

Figure Supplementary 10. SUSD4 overexpression in transfected cells reduces total GLUA2 levels.

HEK293 cells were transfected with SEPGLUA2 alone or with the control transmembrane protein PVRL3 or with HASUSD4. The levels of GLUA2 were quantified. Mean ± s.e.m. (5 independent experiments).

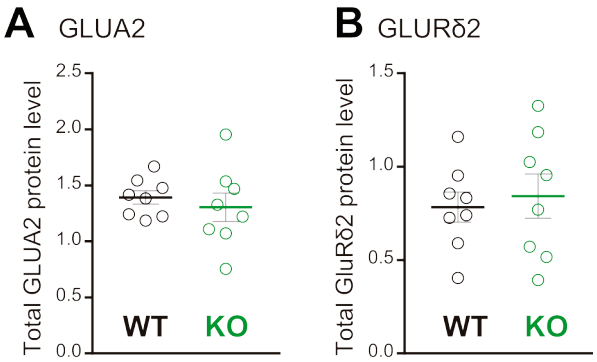

A
GLUA2
Total GLUA2 protein level
2.5
2.0
1.5
1.0
0.5
0.0
WT
KO
B
GLURδ2
Total GluRδ2 protein level
1.5
1.0
0.5
0.0
WT
KO

Figure Supplementary 11. Total GLUA2 levels after modulation of SUSD4 expression.

(A) Mean total protein levels (normalized to β-Actin) of GLUA2 and
(B) GLURδ2 (GRID2) were not changed in cerebellar slices in basal conditions. Mean ± s.e.m. (n=8; unpaired Student's t-test; GLUA2: P=0.5424; GLURδ2: P=0.6821).

STAR Methods

Animals

Susd4 knockout mice were generated and maintained on the C57BL/6J background (generated by Lexicon Genetics Incorporated, The Woodlands, USA). Out of the 8 *Susd4* exons, coding exon 1 (NCBI accession NM_144796.2) and the 5'UTR (NCBI accession BM944003) were targeted by homologous recombination. This resulted in the deletion of a 1.3kb sequence spanning the transcription initiation site and exon 1 (**Figure 1D**). Subsequent genotyping of mice was performed using PCR to detect the wild-type allele (Forward primer: 5' CTG TGG TTT CAA CTG GCG CTG TG 3'; reverse primer 5'GCT GCC GGT GGG TGT GCG AAC CTA 3') or the targeted allele (forward primer 5'TTG GCG GTT TCG CTA AAT AC 3'; reverse primer 5' GGA GCT CGT TAT CGC TAT GAC 3'). The Htr5b-GFP mouse line was used for labeling of climbing fibers (The Gene Expression Nervous System Atlas (GENSAT) Project, NINDS Contracts N01NS02331 & HHSN271200723701C to The Rockefeller University (New York, NY)). Genotyping was performed using the following primers: 5'-TTG GCG CGC CTC CAA CAG GAT GTT AAC AAC-3'; 5'-CGC CCT CGC CGG ACA CGC TGA AC-3'. The L7Cre mouse line was obtained from Jackson laboratories (B6.129-Tg(Pcp2-cre)2Mpin/J ; Stock Number: 004146) and genotyping was performed using the following primers: 5' GGT GAC GGT CAG TAA ATT GGA C 3'; 5' CAC TTC TGA CTT GCA CTT TCC TTG G 3'; 5' TTC TTC AAG CTG CCC AGC AGA GAG C 3'. All animal protocols were approved by the *Comité Regional d'Ethique en Experimentation Animale* (no. 00057.01) and animals were housed in authorized facilities of the CIRB (# C75 05 12).

Antibodies

The following primary antibodies were used: mouse monoclonal anti-CABP (1:1000; Swant, Switzerland, Cat#300), rabbit polyclonal anti-CABP (1:1000; Swant, Cat#CB38), mouse monoclonal anti-GFP (1:1000; Abcam, Cambridge, United Kingdom, Cat#ab1218), rabbit polyclonal anti-GFP (1:1000; Abcam, Cat#ab6556), mouse monoclonal anti-GluR2 (clone 6C4; 1:500; Millipore, Massachusetts, USA, Cat#MAB397 and BD, New Jersey, USA, Cat#556341), rabbit polyclonal anti-GLURδ1/2 (1:1000; Millipore, Cat#AB2285), rat monoclonal anti-HA (1:1000; Roche Life Science, Penzberg, Upper Bavaria, Germany, Cat#11867423001), rabbit monoclonal anti-ITCH (1:1000; Cell Signaling Technology, Massachusetts, USA, Cat#12117), rabbit polyclonal anti-NEDD4 (1:10000; Millipore, Cat#07-049), guinea pig polyclonal anti-VGLUT1 (1:5000; Millipore, Cat#AB5905), guinea pig polyclonal anti-VGLUT2 (1:5000; Millipore, Cat#AB2251) and rabbit polyclonal anti-WWP1 (1:2000; Proteintech, Chicago, USA, Cat#13587-1-AP).

The following secondary antibodies were used: donkey polyclonal anti-Goat Alexa Fluor 568 (1:1000; Invitrogen, California, USA, Cat#A11057), donkey anti-Mouse Alexa Fluor 488 (1:1000; Invitrogen, Cat#R37114), donkey polyclonal anti-Mouse Alexa Fluor 568 (1:1000; Invitrogen, #A10037), donkey polyclonal anti-Rabbit Alexa Fluor 488 (1:1000; Invitrogen, Cat#A21206), donkey polyclonal anti-Rat Alexa Fluor 594 (1:1000; Invitrogen, #A21209), donkey polyclonal anti-Rat Alexa Fluor 568 (1:1000; Abcam, Cat#175475), goat polyclonal anti-Guinea Pig Alexa Fluor 488 (1:1000; Invitrogen, Cat#A110-73), goat polyclonal anti-Guinea Pig Alexa Fluor 647 (1:1000; Invitrogen, Cat#A21450), goat polyclonal anti-Mouse HRP (1:20000; Jackson Immune Research Laboratories, Pennsylvania, USA, Cat#115-035-174), goat polyclonal

anti-rat HRP (1:20000; Jackson Immune Research Laboratories, #112-035-175) and mouse polyclonal anti-rabbit HRP (1:20000; Jackson Immune Research Laboratories, #211-032-171).

The following conjugated antibodies were used: sheep polyclonal anti-digoxigenin alkaline phosphatase (1:2000 - 1:5000; Roche, Cat#11093274910), mouse monoclonal anti-βactin (clone AC-15) HRP (1:25000; Abcam, Cat#ab49900), rabbit polyclonal anti-GFP Alexa Fluor 647 (1:1000; Invitrogen, Cat#A31852), mouse monoclonal anti-GluR2 (clone 6C4) Alexa Fluor 488 (1:1000; Millipore, Cat#MAB397A4) and mouse monoclonal anti-HA (clone 2-2.2.14) DyLight 650 (1:1000; Thermo Fisher Scientific, Cat#26183-D650).

Plasmids

Full-length *Susd4* was cloned into the mammalian expression vector pEGFP-N1 (Addgene, Massachusetts, USA, Cat#6085-1) to express a SUSD4-GFP fusion construct under the control of the CMV promoter (pSUSD44-GFP). An N-terminal HA tag was inserted just after the signal peptide (pHA-Susd4-GFP). pHA-SUSD4 was obtained by removal of the C terminal GFP of pHA-SUSD4-GFP. A truncated form of *Susd4*, HA-SUSD4-ΔC_T, was obtained by inserting a stop codon downstream of the sixth exon, 39bp after the transmembrane domain using PCR on the pHA-SUSD4-GFP plasmid and the following primers: forward primer 5' GCG CTA GCG ATG TAT CCT TAT GAT GTT CCT G3'; reverse primer 5'TAG CGG CCG CTA TTA GGG GGG GAA GTG GGC CTT3'. Other mutant constructs were similarly obtained similarly : the truncated form HA-SUSD4-ΔN_T corresponding to aminoacids 294-490, and the extracellular form of *Susd4* HA-SUSD4-N_T corresponding to aminoacids 2-299. The HA-SUSD4-ΔPY contained a mutation in aminoacids 411 and 414 changing PPAY to APAA, HA-SUSD4-ΔLY in aminoacids 376 and 379 changing LPTY to APTA. Mutagenesis was performed using the QuikChange Lightning Multi site directed mutagenesis kit (agilent, Cat#210513) according to the manufacturer's instructions. The plasmid pIRES2-eGFP (Addgene, Cat#6029-1) was used as transfection control. The plasmid SEPGluA2 (Addgene, Cat#24001) was used to follow GLUA2. The control transmembrane protein PVRL3α was cloned into the mammalian expression vector pCAG-mGFP (Addgene, Cat#14757) to express the protein under the pCAG promoter (pCAG-PVRL3α). Rab4a-GFP, Rab5a-GFP, Rab7a-GFP and Rab11a-GFP were kindly provided by Dr. Bruno Goud.

Viral mediated *in vivo* expression of HA-SUSD4

AAV2 particles were generated using a hSYN-DIO-HA-SUSD4-2A-eGFP-WPRE construct (Vector biolabs, Malvern, USA) and injected stereotaxically in cerebella of adult mice expressing the CRE recombinase in cerebellar Purkinje cells (using the L7Cre mice). In the absence of Cre expression, the transgene is not produced. In the presence of Cre expression, the transgene will be "FLip-EXchanged" leading to expression of the transgene.

***In situ* hybridization**

Fresh frozen 20µm thick-sections were prepared using a cryostat from brains of *Susd4* WT and KO mice at P0, P7 or P21. The probe sequence corresponded to the nucleotide residues 287-1064 bp for mouse *Susd4* (NM_144796.4) cDNA. The riboprobes were used at a final concentration of 0,05 µg/µL, and hybridization was done overnight at a temperature of 72°C. The anti-digoxigenin-AP antibody (for details see antibodies in supplemental information) was used at a dilution of 1:5000. Alkaline phosphatase detection was done using BCIP/NBT colorimetric revelation (Roche, Cat#11681451001).

Behavioral Study

12-14 weeks old male mice were used in this study. They were housed in groups of 3-5 in standard conditions: 12h. light/dark cycle, with *ad libitum* food and water access. Seven days before the beginning of behavioral test, mice were housed individually to limit inter-houses variability resulting from social relationships. All behavioral test took place in the light cycle.

S.H.I.R.P.A. protocol: Mice performed a series of tests to ensure their general good health and motor performance and habituate them to being manipulated (Crawley, 2006). The test includes observation of appearance, spontaneous behavior, neurological reflexes, anxiety, motor coordination, balance rotarod and muscular strength tests and were performed within five days. Individuals presenting deficits during the S.H.I.R.P.A. protocol were not used for other behavioral tests.

Footprint analysis: The fore and hind paws of mice were dipped in blue and pink non-toxic paint, respectively. Mice were allowed to walk through a rectangular plastic tunnel (9cm W x 57cm L x 16cm H), whose floor was covered with a sheet of white paper. Habituation was done the day before the test. 5 footsteps were considered for the analysis. Footprints were scanned and length measurements were made using ImageJ.

Rotarod: Mice were first habituated to the rotarod apparatus, three days before the acceleration test. The habituation protocol consists of 5 minutes at 4 r.p.m. To evaluate the motor coordination, mice were placed on immobile rotarod cylinders, which ramped up from 0 to 45 rotations per minute in 10 minutes. The timer was stopped when the mouse fell off the cylinder or did a whole turn with it. For a given session, this procedure was repeated three times per day separated by 60 minutes. The session was repeated during five consecutive days.

Whole-cell patch-clamp on acute cerebellar slices

Responses to PF and CF stimulation were recorded in Purkinje cells of the lobule VI in acute parasagittal and horizontal (LTP experiments) cerebellar slices from *Susd4* KO juvenile (from P25 to P35) or adult (~P60) mice. *Susd4* WT littermates were used as controls. Mice were anesthetized using isoflurane 4% and sacrificed by decapitation. The cerebellum was dissected in ice cold oxygenated (95% O_2 and 5% CO_2) Bicarbonate Buffered Solution (BBS) containing (in mM): NaCl 120, KCl 3, $NaHCO_3$ 26, NaH_2PO_4 1.25, $CaCl_2$ 2, $MgCl_2$ 1 and D(+)-glucose 35. 300µm-thick cerebellar slices were cut with a vibratome (Microm 650 V: Thermo Scientific Microm, Massachusetts, USA) in slicing solution (in mM): N-Methyl-D-Glucamine 93, KCl 2.5, NaH_2PO_4 1.2, $NaHCO_3$ 30, HEPES 20, D(+)-Glucose 25, $MgCl_2$ 10, sodium ascorbate 5, Thiourea 2, sodium pyruvate 3, N-acetyl-cystein 1, Kynurenic acid 1 and $CaCl_2$ 0.5 (pH 7.3). Immediately after cutting, slices were allowed to briefly recover at 37°C in the oxygenated sucrose-based buffer (in mM): sucrose 230, KCl 2.5, $NaHCO_3$ 26, NaH_2PO_4 1.25, D(+)-glucose 25, $CaCl_2$ 0.8 and $MgCl_2$ 8. D-APV and minocycline at a final concentration of 50µM and 50nM respectively were added to the sucrose-based buffer. Slices were allowed to fully recover in bubbled BBS at 37°C for at least 40 minutes before starting the experiment. Patch clamp borosilicate glass pipettes with 3-6MΩ resistance were filled with the following internal solutions:

1. Cesium metanesulfonate solution (CsMe solution, for EPSC elicited from CF and PF), containing (in mM) $CsMeSO_3$ 135, NaCl 6, $MgCl_2$ 1, HEPES 10, MgATP 4, Na_2GTP 0.4, EGTA 1.5, QX314Cl 5, TEA 5 and Biocytin 2.6 (pH 7.3).

2. CsMe S-solution (for delayed EPSC quanta events), containing (in mM): $CsMeSO_3$ 140, $MgCl_2$ 0.5, HEPES 10, MgATP 4, Na_2GTP 0.5, BAPTA 10 and Neurobiotin 1% (pH 7.35).

3. Potasium Gluconate solution (KGlu solution, for PF long-term plasticity), containing (in mM): K Gluconate 136, KCl 10, HEPES 10, $MgCl_2$ 1, Sucrose 16, MgATP 4 and Na_2GTP 0.4 (pH 7.35).

Stimulation electrodes with ~5 MΩ resistances were pulled from borosilicate glass pipettes and filled with BBS. Recordings were performed at room temperature on slices continuously perfused with oxygenated BBS. The experiment started at least 20 minutes after the whole-cell configuration was established. The Digitimer DS3 (Digitimer Ltd, Hertfordshire, UK) stimulator was used to elicit CF and PF and neuronal connectivity responses in Purkinje cells. Patch-clamp experiments were conducted in voltage clamp mode (except for the LTP and LTD induction protocols that were made under current clamp mode) using a MultiClamp 700B amplifier (Molecular Devices, California, USA) and acquired using the freeware WinWCP written by John Dempster (https://pureportal.strath.ac.uk/en/datasets/strathclyde-electrophysiology-software-winwcp-winedr). Series resistance was compensated by 60-100% and cells were discarded if significant changes were detected. Currents were low-pass filtered at 2,2kHz and digitized at 20kHz.

CF and PF-EPSC experiments: To isolate the AMPARs current, the BBS was supplemented with (in mM) picrotoxin 0.1, D-AP5 10, CGP52432 0.001, JNJ16259685 0.002, DPCPX 0.0005 and AM251 0.001. CF and PF EPSCs were monitored at a holding potential of -10mV. During CF recordings, the stimulation electrode was placed in the granule cell layer below the clamped cell; CF-mediated responses were identified by the typical all-or-none response and strong depression displayed by the second response elicited during paired pulse stimulations (20Hz). The number of CFs innervating the recorded PC was estimated from the number of discrete CF-EPSC steps. PF stimulation was achieved by placing the stimulation electrode in the molecular layer at the minimum distance required to avoid direct stimulation of the dendritic tree of the recorded PC. The input-output curve was obtained by incrementally increasing the stimulation strength. Peak EPSC values for PF were obtained following averaging of three consecutive recordings, values for CF-EPSC correspond to the first recording. Short-term plasticity experiments were analyzed using a software written in Python by Antoine Valera (http://synaptiqs.wixsite.com/synaptiqs).

PF-Long-term plasticity experiments: Purkinje cells (PCs) were clamped at -60 mV. Each PF-induced response was monitored by a test protocol of paired stimulation pulses (20Hz) applied every 20 seconds. A baseline was established during 10 minutes of paired-pulse stimulation in the voltage clamp configuration. After that, an induction protocol was applied in current-clamp mode with cells held at -60mV. During LTD induction, the PFs were stimulated with two pulses at high frequency (200Hz) and, after 100ms, the CF was stimulated with four pulses at high frequency (200Hz) repeated every 2 seconds for a period of 10 minutes. During LTP induction, the PFs were stimulated with bursts of 15 pulses at high frequency (100 Hz) repeated every 3 seconds for a period of 5 minutes (Binda *et al.*, 2016). Then, PCs were switched to the voltage clamp mode and paired stimulation pulses applied again, lasting 40 minutes. All the data were normalized to the mean baseline. Long-term plasticity was analyzed with the software Igor Pro 6.05 (WaveMetrics INC, Oregon, USA).

PF and CF delayed EPSC quanta events: were detected and analyzed using the software Clampfit 10.7 (Molecular Devices). PF- and CF-delayed EPSC quanta superposed events were discarded manually based on the waveform. A threshold of 10pA for minimal amplitude was used to select the CF events. 100 (PF) and 300 (CF) events for each neuron were studied by analyzing consecutive traces.

High density microelectrode array (MEA) analysis of Purkinje cell spiking in acute cerebellar slices

Experiments were performed on acute cerebellar slices obtained from 3-6 months-old mice in artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 125, KCl 2.5, D(+)Glucose 25, $NaHCO_3$ 25 , NaH_2PO_4 1.25, $CaCl_2$ 2, and $MgCl_2$ 1 and oxygenated (95% O_2 and 5% CO_2). Parasagittal slices (320µm) were cut at 30°C (Huang and Uusisaari, 2013) with a Campden Ci 7000 smz microtome at an advance speed of 0.03mm/s and vertical vibration set to 0.1 – 0.3µm. Slices were then transferred to a chamber filled with oxygenated ACSF at 37°C and allowed to recover for 1h before recordings.

For recording, the slices were placed over a high-density micro electrode array of 4096 electrodes (electrode size, 21 × 21µm; pitch, 42µm; 64 × 64 matrix; Biocam X, 3Brain, Wädenswil, Switzerland), and constantly perfused with oxygenated ACSF at 37C°. Extracellular activity was digitized at 17 kHz and data were analyzed with the Brainwave software from 3Brain. The signal was filtered with a butterworth high-pass filter at 200 Hz, spikes were detected with a hard threshold set at -100µV, and unsupervised spike sorting was done by the software. We selected units with a firing rate between 15 and 100 spikes per second and we excluded units presenting more than 5% of refractory period violation (set to 3ms). Recordings were performed on two slices per animal, each slice containing between 20 and 200 active neurons, and results were then pooled for each animal.

To quantify the average variability in the firing rate, the coefficient of variation (CV) of the ISI (interspike interval in seconds) was calculated as the ratio of the standard deviation (SD) of ISIs to the mean ISI of a given cell. To measure the firing pattern variability within a short period of two ISIs, CV2 was calculated [$CV2 = 2|ISI_{n+1} - ISI_n|/(ISI_{n+1} + ISI_n)$] (Holt and Douglas, 1996).

Affinity-purification of SUSD4 interactors from synaptosome preparations

HEK293H (Gibco, Cat#11631-017) were maintained at 37ºC in a humidified incubator with 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM; containg high glucose and glutamax, Life Technologies, Cat#31966047) supplemented with 10% fetal bovine serum (Gibco, Cat#16141-079), and 1% penicillin/streptomycin (Gibco, Cat#15140122). 10^6 cells were plated per well in a 6-well plate and transfected 24 hours after plating with the indicated plasmids (1µg plasmid DNA per well) using Lipofectamine 2000 (Life Technologies, Cat#11668) according to manufacturer's instructions.

Forty-eight hours after transfection, cells were lysed and proteins were solubilized for 1h at 4°C under gentle rotation in lysis buffer (10 mM Tris-HCl pH7.5, 10 mM EDTA, 150 mM NaCl, 1% Triton X100, 0.1% SDS) supplemented with a protease inhibitor cocktail (1:100; Sigma, Cat#110205) and MG132 (100µM; Sigma, Cat#C2211). Lysates were sonicated for 10 seconds, further solubilized for one hour at 4°C and clarified by centrifugation at 6,000 rpm during 8 minutes. Supernatants were collected, incubated with 5 µg of rat monoclonal anti-HA antibody (for details see antibodies), together with 60 µL of protein G-sepharose beads (Sigma;

Cat#10003D) for 3 hours at 4°C, to coat the beads with the HA-tagged SUSD4 proteins. When SUSD4-GFP was expressed for affinity-pulldowns, GFP-Trap was done according to the instructions of GFP-Trap®_A (Chromotek, New York, USA, Cat#ABIN509397). Coated beads were washed 3 times with 1 mL lysis buffer.
To prepare synaptosome fractions, cerebella from WT mice (P30) were homogenized at 4°C in 10 volumes (w/v) of 10mM Tris buffer (pH7.4) containing 0.32M sucrose and protease inhibitor cocktail (1:100). The resulting homogenate was centrifuged at 800 g for 5min at 4ºC to remove nuclei and cellular debris. Synaptosomal fractions were purified by centrifugation for 20 min at 20,000 r.p.m. (SW41Ti rotor) at 4ºC using Percoll-sucrose density gradients (2-6-10-20%; v/v). Each fraction from the 10–20% interface was collected, washed in 10 mL of a 5 mM HEPES buffer pH 7.4 (NaOH) containing 140 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 1 mM NaH_2PO_4, 5 mM $NaHCO_3$, 10 mM D(+)-Glucose by centrifugation. The suspension was immediately centrifuged at $1x10^4$ g at 4ºC for 10min. Synaptosomes in the pellet were resuspended in 100 µL in lysis buffer (10 mM Tris-HCl pH7.5, 10 mM EDTA, 150 mM NaCl, 1% Tx) supplemented with a protease inhibitor cocktail (1:100) and MG132 (100µM). Lysates were sonicated for 10 seconds, and further incubated for one hour at 4°C. HA-SUSD4 or GFP-SUSD4 coated beads were then incubated with the synaptosomal lysates for three hours at 4°C. Beads were washed three times with lysate buffer supplemented with 0.1% SDS. Bound proteins were eluted for 10 minutes at 75°C using Laemmli buffer (*160mM Tris ph6.8, 4% SDS, 20% glycérol, 0.008% BBP*) with 5% beta mercaptoethanol.

Co-Immunoprecipitation of HA-SUSD4 in HEK293 cells
Proteins from HEK293 cell lysates were solubilized in lysis buffer (1 M Tris-HCl pH8, 10 mM EDTA, 1,5 M NaCl, 1% Tergitol $^{TM.}$ (sigma; Cat#NP40), 2% Na azide, 10% SDS and 10% Na deoxycholate) supplemented with a protease inhibitor cocktail (1%) and MG132 (1%). Then, lysates were sonicated for 15 seconds, further clarified by a centrifugation at 14,000 rpm for 10min. Supernatants were collected and incubated with Dynabeads protein G (life technologies, Cat#10004D) and 28.8 µg of rat monoclonal anti-HA antibody (for details see antibodies) under gentle rotation for 1h at 4°C. Precipitates were washed 3 times in lysis buffer and then eluted by boiling (65°C) the beads 15 min in βME-reducing sample buffer before SDS-PAGE.

Mass spectrometry analysis
Proteins were separated by SDS-PAGE on 10 % polyacrylamide gels (Mini-PROTEAN® TGX™ Precast Gels, Bio-Rad, Hercules USA) and stained with Protein Staining Solution (Euromedex, Souffelweyersheim France). Gel lanes were cut into 5 pieces and destained with 50 mM triethylammonium bicarbonate (TEABC) and three washes in 100% acetonitrile. Proteins were digested in-gel using trypsin (1.2 µg/band, Gold, Promega, Madison USA), as previously described(Thouvenot *et al.*, 2008). Digest products were dehydrated in a vacuum centrifuge.

Nano-flow liquid chromatography coupled to tandem mass spectrometry (NanoLC-MS/MS):
Peptides, resuspended in 3 µL formic acid (0.1%, buffer A), were loaded onto a 15 cm reversed phase column (75 mm inner diameter, Acclaim Pepmap 100® C18, Thermo Fisher Scientific) and separated with an Ultimate 3000 RSLC system (Thermo Fisher Scientific) coupled to a Q Exactive Plus (Thermo Fisher Scientific) *via* a nano-electrospray source, using a 120-min gradient of 5 to 40% of buffer B (80% ACN, 0.1% formic acid) and a flow rate of 300 nl/min.

MS/MS analyses were performed in a data-dependent mode. Full scans (375 – 1,500 m/z) were acquired in the Orbitrap mass analyzer with a 70,000 resolution at 200 m/z. For the full scans, 3 x 10^6 ions were accumulated within a maximum injection time of 60 ms and detected in the Orbitrap analyzer. The twelve most intense ions with charge states ≥ 2 were sequentially isolated to a target value of 1 x 10^5 with a maximum injection time of 45 ms and fragmented by HCD (Higher-energy collisional dissociation) in the collision cell (normalized collision energy of 28%) and detected in the Orbitrap analyzer at 17,500 resolution.

MS/MS data analysis: Raw spectra were processed using the MaxQuant environment ((Cox and Mann, 2008), v.1.5.5.1) and Andromeda for database search (Cox *et al.*, 2011). The MS/MS spectra were matched against the UniProt Reference proteome (Proteome ID UP000000589) of *Mus musculus* (release 2017_03; http://www.uniprot.org) and 250 frequently observed contaminants (MaxQuant contaminants database) as well as reversed sequences of all entries. The following settings were applied for database interrogation: mass tolerance of 7 ppm (MS) and 0.5 Th (MS/MS), trypsin/P enzyme specificity, up to two missed cleavages allowed, only peptides with at least seven amino acids in length considered, and Oxidation (Met) and acetylation (protein N-term) as variable modifications. The "match between runs" (MBR) feature was allowed, with a matching time window of 0.7 min. FDR was set at 0.01 for peptides and proteins.

A representative protein ID in each protein group was automatically selected using an in-house bioinformatics tool (leading v2.1). First, proteins with the most numerous identified peptides are isolated in a "match group" (proteins from the "Protein IDs" column with the maximum number of "peptides counts"). For the match groups where more than one protein ID are present after filtering, the best annotated protein in UniProtKB (reviewed entries rather than automatic ones), highest evidence for protein existence, most annotated protein according to the number of Gene Ontology Annotations (GOA Mouse version 151) is defined as the "leading" protein. Only proteins identified with a minimum of two unique peptides, without MS/MS in control immunoprecipitation and exhibiting more than 4-fold enrichment (assessed by spectral count ratio) in Sushi domain-containing protein 4 (SUSD4) immunoprecipitation, *vs.* control immunoprecipitation, in the two biological replicates, were considered as potential partners of SUSD4 (**Table 1**).

Gene Ontology analysis: The statistically enriched gene ontology (GO) categories for the 28 candidats proteins were determined by Cytoscape (v3.6) plugin ClueGO v2.5.3 (Bindea *et al.*, 2009). The molecular function category was considered (release 18.12.2018, https://www.ebi.ac.uk/GOA), except evidences inferred from electronic annotations. Terms are selected by different filter criteria from the ontology source: 3-8 GO level intervals, minimum of 4 genes per GO term and 10% of associated genes/term. A two-sided hypergeometric test for enrichment analysis (Benjamini-Hochberg standard correction used for multiple testing) was applied against the whole identified protein as reference set. Other predefined settings were used. Each node representing a specific GO term is color-coded based on enrichment significance (p-value). Edge thickness represents the calculated score (kappa) to determine the association strength between the terms.

Chemical LTD in cerebellar acute slices

300 µm-thick parasagittal cerebellar slices were obtained from P31-P69 WT and *Susd4* KO mice following the same protocol as for patch-clamp recordings. Slices were then incubated for 2h at 37°C in oxygenated BBS with or without proteasome (50µM MG132) and protease inhibitors (100µg/mL Leupeptine, Sigma, Cat#11017101001). Chemical LTD was induced by incubating the slices for 5 minutes at 37°C in BBS containing 50 mM K^+ and 10 µM Glutamate, followed by a recovery period in BBS for 30 minutes at 37°C; in presence or not of inhibitors. Slices were then homogenized in lysis buffer, containing: 50mM Tris-HCl, 150mM NaCl, 0.1% SDS, 0.02% Na Azide, 0.5% Na Deoxycholate, 1% NP-40 and protease inhibitor cocktail (1:100). Homogenates were incubated 45 minutes at 4°C, then sonicated and centrifuged at 14000 r.p.m. for 10 minutes at 4°C. Supernatants were then heated at 65°C in 2X sample buffer prior to western blot analysis for detection of GLUA2 and GRID2.

Immunofluorescence

Labeling of primary hippocampal neurons: Hippocampi were dissected from E18 mice embryos and dissociated. $1.2x10^5$ neurons were plated onto 18 mm diameter glass cover-slips precoated with 80 µg/mL poly-L-ornithine (Sigma, Gothenburg, Sweden, Cat#P3655) and maintained at 37ºC in a 5% CO_2 humidified incubator in neurobasal medium (Gibco, Massachusetts, USA, Cat#21103049) supplemented with 2% B-27 supplement (Gibco, Cat#17504044) and 2mM Glutamax (Gibco, Cat#35050-038). Fresh culture medium (neurobasal medium supplemented with 2% B-27, 2mM L-glutamine (Gibco, Cat#A2916801) and 5% horse serum (Gibco, Cat#26050088) was added every week for maintenance of the neuronal cultures.

Hippocampal neurons at days in vitro 13 (DIV13) were transfected using Lipofectamine 2000 and 0.5µg plasmid DNA per well. After transfection, neurons were maintained in the incubator for 24h, then fixed with 100% methanol for 10 minutes at -20ºC. After rinsing with PBS, non-specific binding sites were blocked using PBS containing 4% donkey serum (DS, Abcam, Cat#ab7475) and 0.2% Triton X-100 (Tx; Sigma, Cat#x100). Primary and secondary antibodies were diluted in PBS 1% DS / 0.2% Tx and incubated one hour at room temperature. Three PBS 0.2% Tx washes were performed before and after each antibody incubation. Nuclear counterstaining was performed with Hoechst 33342 (Sigma, Cat#H6024) for 15 min at room temperature.

Labeling of brain sections: 30µm-thick parasagittal sections were obtained using a freezing microtome and brains obtained after intracardiac perfusion of mice with 4% PFA in PBS solution. Sections were washed three times for five minutes in PBS, then blocked with PBS 4% DS for 30 minutes. The primary antibodies were diluted in PBS, 1% DS, 1% Triton X-100. The sections were incubated in the primary antibody solution overnight at 4ºC and then washed three times for five minutes in PBS 1% Tx. Sections were incubated in the secondary antibody, diluted in PBS 1% DS 1% Tx solution, for 1 hour at room temperature. The sections were then incubated for 15 minutes at room temperature with the nuclear marker Hoechst 33342 (Thermo Fisher Scientific, Cat#H1399) in PBS 0.2% Tx. Finally, the sections were washed three times for five minutes in PBS 1% Tx, recovered in PBS and mounted with Prolong Gold (Thermo Fisher Scientific, Massachusetts, USA, Cat#P36934) between microscope slides and coverslips (Menzel-gläser, Brunswick, Germany, Cat#15165252).

RT-PCR and quantitative RT-PCR

For standard RT-PCR, total RNA was isolated from the cortex, cerebellum and brainstem of 2-month-old *Susd4*$^{-/-}$ kockout mice and *Susd4*$^{+/+}$ control littermates, using the RNeasy mini kit

(Qiagen, Venlo, Netherlands, Cat#74104). Equivalent amounts of total RNA (100 ng) were reverse-transcribed according to the protocol of SuperScript® VILO™ cDNA Synthesis kit (Life Technologies, California, USA, Cat#11754-250) according to manufacturer's instructions. The primers used were forward 5'TGT TAC TGC TCG TCA TCC TGG3' and reverse 5'GAG AGT CCC CTC TGC ACT TGG3'. PCR was performed with an annealing temperature of 61°C, for 39 cycles, using the manufacturer's instructions (*Taq* polymerase; New England Biolabs, Massachusetts, USA, Cat#M0273S). Quantitative PCR was performed using the TaqMan universal master mix II with UNG (applied biosystems, Cat# 4440038) and the following TaqMan probes: *Rpl13a* (#4331182_Mm01612986_gH) and *Susd4* (#4331182_Mm01312134_m1).

Western Blot analysis

Proteins were resolved by electrophoresis on a 4-12% NuPAGE Bis-Tris-Gel according to Invitrogen protocols, then electrotransferred using TransBlot® Transfer Medium (Bio-Rad) to PVDF membrane (Immobilon-P transfer membrane, Millipore, Cat#IPVH00010). Membranes were blocked in PBS supplemented with Tween 0.2% (PBST) and non-fat milk 5% and incubated with primary antibodies in PBST- milk 5%. After washing in PBST, membranes were incubated with Horseradish Peroxidase-conjugated secondary antibodies in PBST-milk 5%. Bound antibodies were revealed using Immobilon Western (Millipore, Cat#WBKLS) or Western Femto Maximum Sensitivity (Thermo Fisher Scientific, Cat#34095) chemiluminescent solutions and images acquired on a Fusion FX7 system (Vilber Lourmat, Île-de-France, France).

Image acquisition and quantification

In situ hybridization images were acquired using an Axio Zoom. V16 (Zeiss, Oberkochen, Germany) microscope equipped with a digital camera (AxioCam HRm) using a 10x objective (pixel size 0,650µm). Immunofluorescence image stacks were acquired using a confocal microscope (SP5, Leica, Wetzlar, Germany), using a 63x objective (1,4NA, oil immersion, pixel size: 57nm for cell culture imaging, pixel size: 228nm for 63x; 76nm, 57nm, 45nm for higher magnifications for *in vivo* imaging). The pinhole aperture was set to 1 Airy Unit and a z-step of 200 nm was used. Laser intensity and photomultiplier tube (PMT) gain was set so as to occupy the full dynamic range of the detector. Images were acquired in 16-bit range.

Deconvolution was performed for the VGLUT1 images with Huygens 4.1 software (Scientific Volume Imaging) using Maximum Likelihood Estimation algorithm from Matlab. 40 iterations were applied in classical mode, background intensity was averaged from the voxels with lowest intensity, and signal to noise ratio values were set to a value of 25.

VGLUT1 and VGLUT2 puncta were analyzed using the Matlab software and a homemade code source (Dr. Andréa Dumoulin). The number, area and intensity of puncta were quantified using the mask of each puncta generated by the Multidimensional Image analysis software (MIA) from Metamorph. For each animal, puncta parameters were measured from four equidistant images within a 35-image stack at 160 nm interval, acquired from three different lobules (n=12).

Statistical analysis

Data from all experiments were imported in Prism (GraphPad Software, California, USA) for statistical analysis, except for electrophysiology data that were imported to Igor Pro 6.05 (WaveMetrics INC) for statistical analysis.

In the case of two column analyses of means, the differences between the two groups were assessed using two-tailed Student's t-test. Normality of populations were assessed using D'Agostino &

Pearson, Shapiro-Wilk and Kolmogorov-Smirnov normality tests. When groups did not fit the normal distribution, the Mann-Whitney non-parametric test was used. For the rotarod behavioral test (two variables, genotype and trial), two-way repeated measures ANOVA followed by Bonferroni post hoc test was performed. The two-tailed Student's one sample t-test (when normality criterion was met) or the two-tailed Wilcoxon Signed Rank Test was used to compare ratios to a null hypothesis of 1 for biochemical experiments or 100 for long-term plasticity (Fay, 2013). The two-tailed Mann-Whitney test was used to compare two populations of ratios. Differences in cumulative probability were assessed with the Kolmogorov-Smirnov distribution test, and differences in distribution were tested using the chi-squared test.

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