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Supplemental Information

The Complement Regulator *Susd4* Influences Nervous-System Function and Neuronal Morphology in Mice

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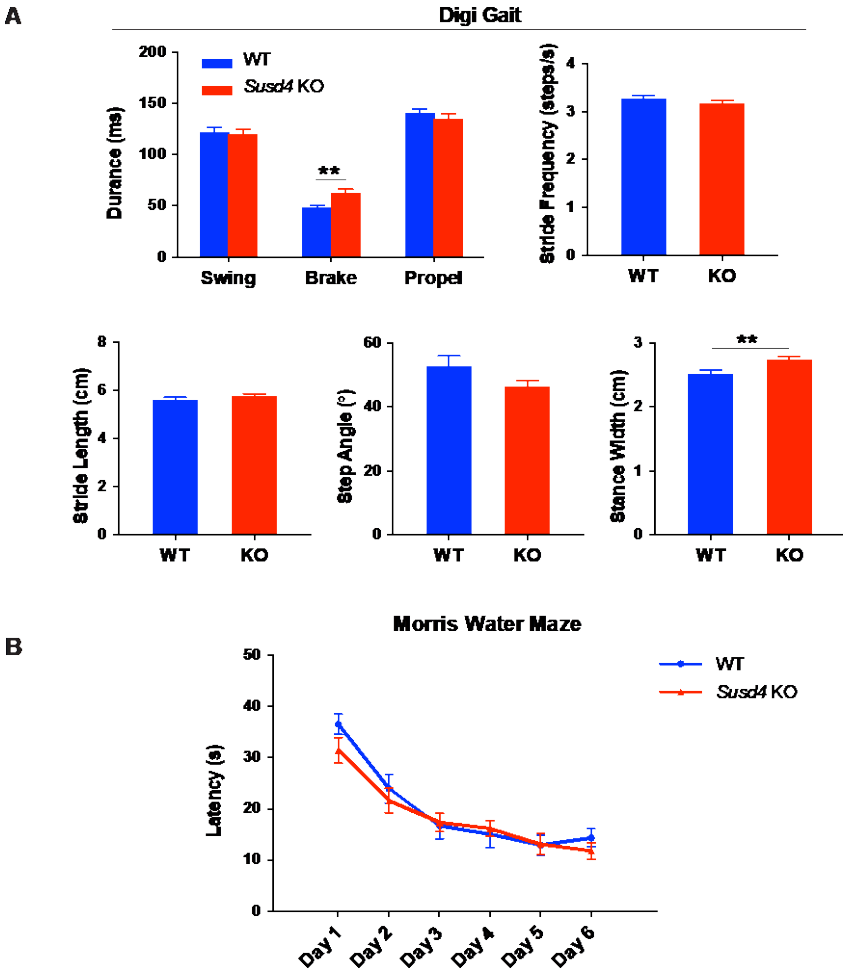

Figure S1. Behavioral phenotyping of *Susd4* KO Mice, Related to Figure 1.
(A) DigiGait test. Swing, brake, and propel of a stride, stride frequency, stride length, step angle and stance width of hind limbs were analyzed. *Susd4* KO, n=17; WT, n=11. (B) Morris water maze test. Latency to find the hidden platform was recorded for consecutive 6 training days. *Susd4* KO, n=17; WT, n=15.
Data represent the mean ± SEM. **: $p<0.005$.

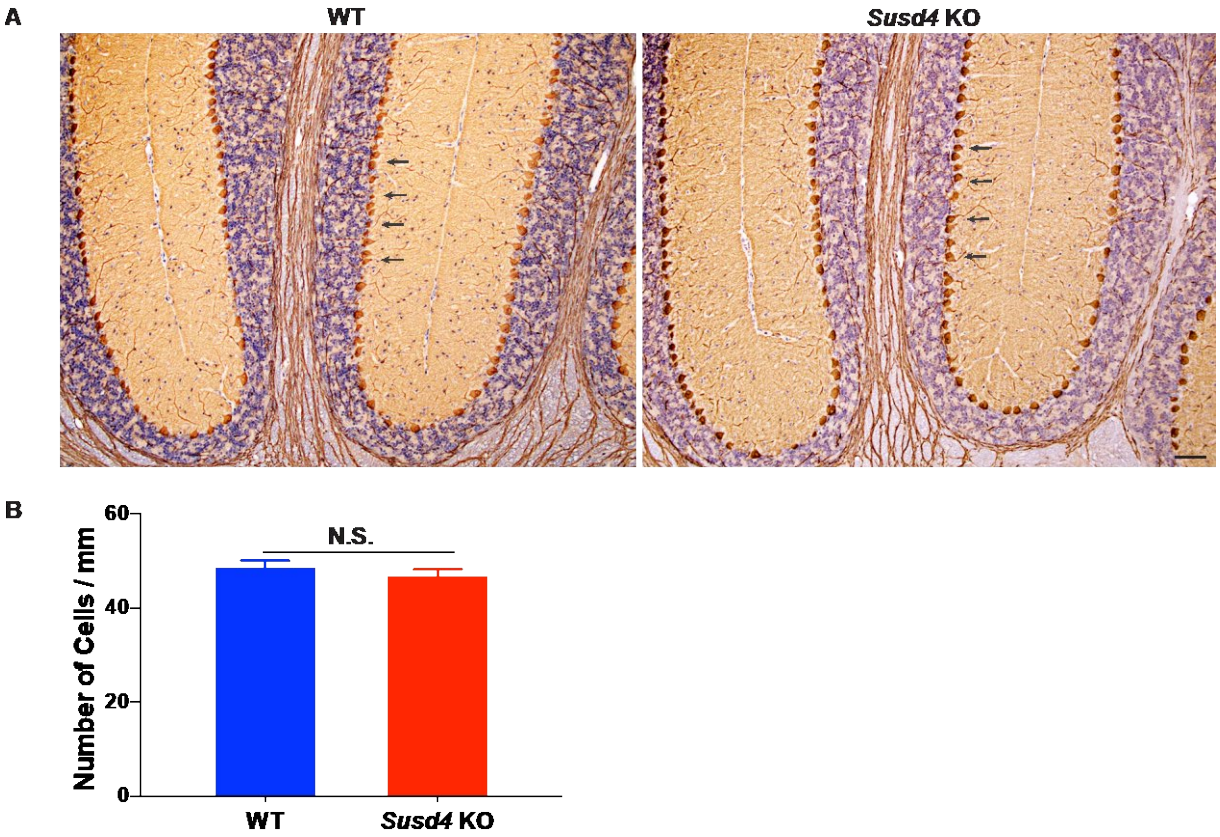

Figure S2. Purkinje Cells Are Not Lost in the Cerebellum of *Susd4* KO Mice, Related to Figure 2.
(A) Representative immunostaining for calbindin in a sagittal view of the cerebellum from a WT and a *Susd4* KO mouse. Arrows indicate calbindin-labeled Purkinje cells. Scale bar, 100 μm. (B) Quantification of the number of Purkinje cells per mm in cerebellar sections of WT and *Susd4* KO mouse brains. Data represent the mean ± SEM. n=3 mice per group. N.S.: not significant.

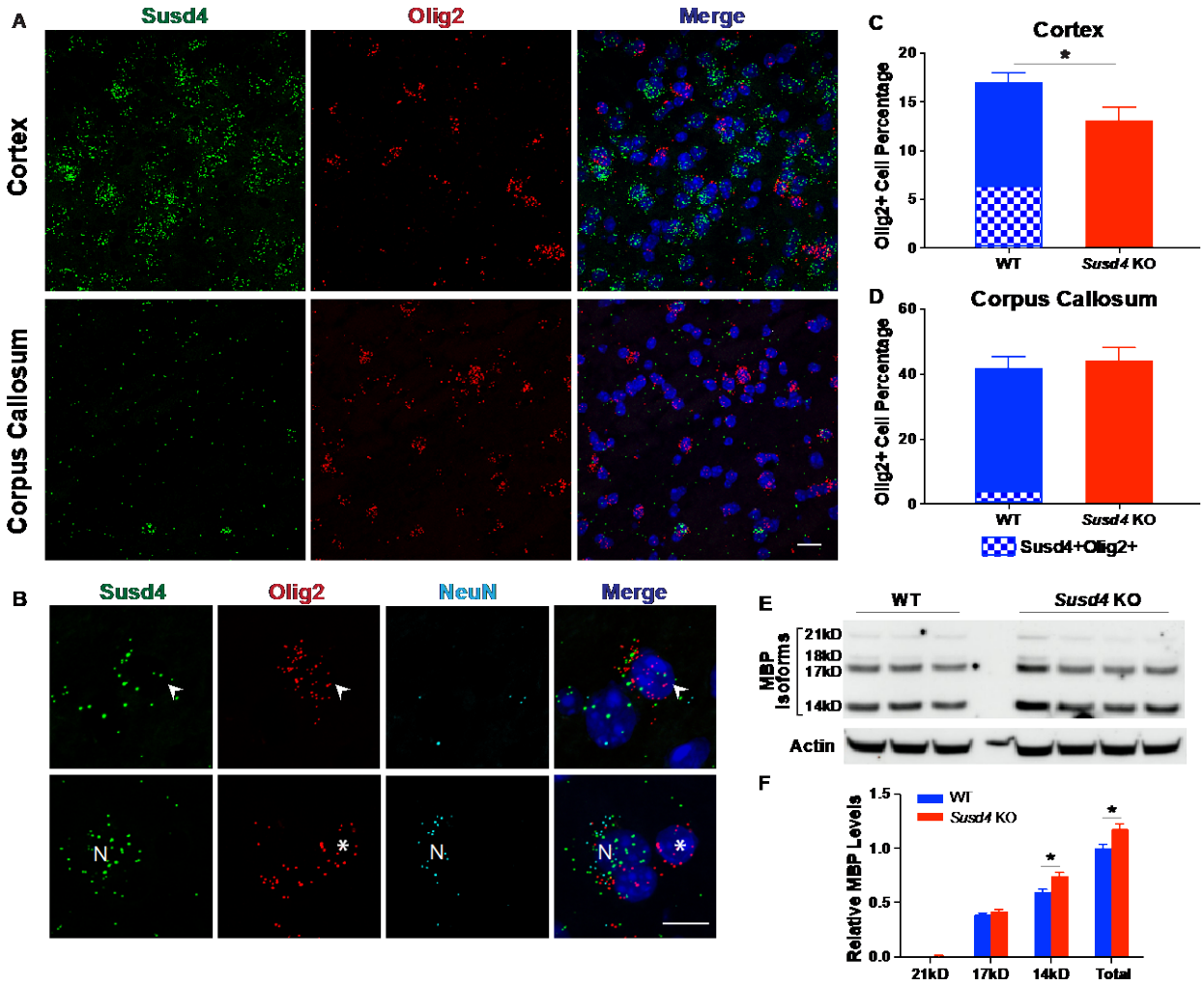

Figure S3. *Susd4* mRNA Is Expressed in Oligodendrocyte Lineage Cells, Related to Figure 3.

(A-D) Using RNAscope Multiplex Fluorescent Assay, mRNA of *Susd4* (green), Olig2 (red), and NeuN (cyan) was detected *in situ* on cerebral cortex sections from WT and *Susd4* KO mice. Nuclei were counterstained with DAPI (blue). (A) Representative images show the partially co-localized Olig2 and *Susd4* mRNA expression in WT cortex (upper panels) and corpus collosum (lower panels). Scale bar, 20 μm. (B) Representative higher magnification view shows a cell expressing both *Susd4* and Olig2 mRNA (arrow head) in upper panels, while a cell expressing Olig2 without *Susd4* (star) in lower panels. N, neuron. Scale bar, 10 μm. (C-D) Percentage of cells positive for Olig2, both positive and negative for *Susd4*, in the cerebral cortex region (C) and the corpus collosum region (D) of WT and *Susd4* KO mouse brains. Total cells were quantified by counting DAPI stained nuclei. Data represent the mean ± SEM. n=3 mice per group. (E) Western blot analysis of myelin basic protein (MBP) isoforms (21, 18, 17, and 14 kD) in cerebral cortex lysates obtained from 3 WT and 4 *Susd4* KO mice. β-actin was detected on the same blot as a loading control. (F) Quantification of MBP isoforms expression from Western blots. Expression was normalized to β-actin expression. Data represent the mean ± SEM. WT values of total MBP were set to 1.0. *: $p<0.05$

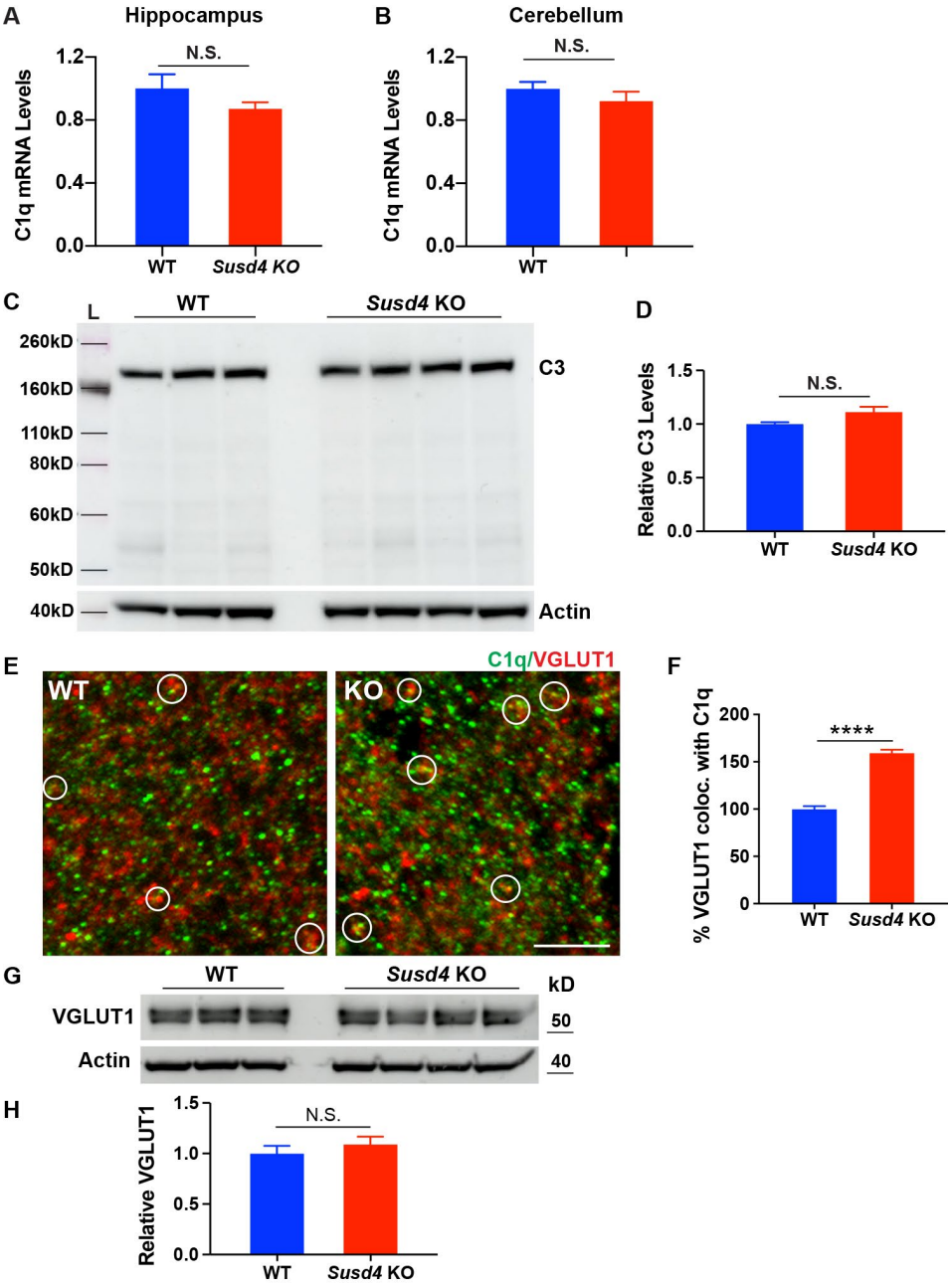

Figure S4. Determination of C1qa mRNA levels and C3 protein levels, Related to Figure 4.

(A-B) mRNA expression of C1q determined by qPCR from WT and *Susd4* KO hippocampal (A) and cerebellar (B) tissues. Expression was normalized to GAPDH expression. (C) Western blot analysis of complement C3 in hippocampal lysates obtained from 3 WT and 4 *Susd4* KO mice. β-actin was detected on the same blot as a loading control. L, molecular weight ladder sample. (D) Quantification of C3 expression (~180,000 MW band) from Western blots. Expression was normalized to β-actin expression. For A, B, and D, data represent the mean ± SEM. WT values were set to 1.0. N.S.: not significant. (E) Representative immunostaining for C1q (green) and VGLUT1 (red) in the WT and *Susd4* KO hippocampus sections. Circles indicate the C1q and VGAT colocalized puncta in the sections. Scale bar, 5 µm. (F) Quantification using ImageJ shows that % VGLUT1 found colocalized with C1q is significantly higher in *Susd4* KO. Data represent the mean ± SEM. n=3 mice per group. ****: $p<0.0001$. (G) Western-blot analysis of VGLUT1 in hippocampal lysates obtained from 3 WT and 4 *Susd4* KO mice. β-actin was detected on the same blot as a loading control. (H) Quantification of VGLUT1 protein levels from Western blots. Expression was normalized to β-actin expression. WT values were set to 1.0. N.S., not significant.

Table S1. Measures of General Health and Reflexes, Related to Figure 1.

General Health	WT (15)	*Susd4* KO (17)
Body weight (g)	26.73 ± 1.19[1]	26.41 ± 0.79
Body length: nose-tail base (cm)	9.27 ± 0.12	9.40 ± 0.04
Poor fur/skin condition (%)	0	0
Bald patches (%)	20[2]	0
Missing whiskers (%)	0	0
Piloerection (%)	0	0
Head shape (% of normal)	100	100
Nose (% of good)	100	100
Ears (% of good)	100	100
Eyes morphology (% of normal)	100	100
Teeth (% of good)	100	100
Limb (% of normal)	100	100
Tail (% of normal)	100	100
Body lumps (%)	0	0
Physical abnormality (%)	0	0
Body Temperature (°C)	35.69 ± 0.12	35.42 ± 0.07
Mucous membrane (% of normal)	100	100
Drooling (%)	0	0
Respiratory Pattern (% of normal)	100	100
Reactivity		
Eye blink (%)	100	100
Pupillary Reflex (%)	100	100
Vocalizations (%)	100	100
Biting (%)	0	0
Handling hypersensitivity (%)	0	0
Support hind end (%)	100	100
Seizures (%)	0	0
Motoric abilities		
Catalepsy test	< 1 sec	< 1 sec
Akinesia test	3.53 ± 0.69	3.80 ± 0.53
Inverted grid test[3]	90.0 ± 0.0	89.4 ± 0.6
Ladder climb test	4.52 ± 0.29	4.91 ± 0.28

1: Data represent the mean ± SEM.
2: Bald spots of 3 WT mice were caused by littermate barbering in one cage.
3: The maximum hanging time for the test is 90 sec.

TRANSPARENT METHODS

Animals

All mouse experiments were performed according to protocols approved by the Animal Care and Use Committee of the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health. In the mouse facility, a 12 dark/12 light cycle was used, and mice were housed under standard conditions with *ad libitum* access to water and food.

Susd4^{tmLex} mice were obtained from Mutant Mouse Resource & Research Centers, USA (stock #: 032614-UCD, Davis, CA). The *Susd4* coding exon 1 and preceding sequence was targeted by homologous recombination to knock out the gene . For genotyping, the knockout allele was identified by PCR using 5'-GAA CAA GAT GGA TTG CAC GCA G-3' (forward primer) and 5'-CTT GAG CCT GGC GAA CAG TTC-3' (reverse primer), which generated a product of 520 bp, and the WT allele was identified using 5'-CTG TGG TTT CAA CTG GCG CTG TG-3' (forward primer) and 5'-CCG GTG GGT GTG CGA ACC TA-3' (reverse primer), which generated a product of 259 bp. The mice were backcrossed at least 7 generations into a C57BL6/J (Stock# 000664, The Jackson Laboratory, Bar Harbor, ME) genetic background for this study.

qPCR

Total RNA from mouse tissue was extracted using the RNeasy Plus Mini Kit (Qiagen, Germantown, MD). The quality and quantity of RNA was measured using the Agilent RNA 6000 Nano Kit (Agilent, Santa Clara, CA), then transcribed into cDNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). TaqMan Gene Expression Assay predesigned qPCR primers and probes for mouse *Susd4* (Mm01312134_m1, Mm00460598_m1), *Gapdh* (Mm99999915_g1), and *C1qa* (Mm00432142_m1) were obtained from Applied Biosystems. qPCR was performed with a reaction volume of 20 µl and under following thermocycle conditions: 2 min of initial incubation at 50°C; 10 min initial activation and denaturation at 95°C; 40 cycles of 15 sec denaturation at 95°C, 1 min annealing and extension at 60°C. mRNA expression levels were calculated using the $\Delta\Delta C_t$ analysis method . The Susd4 tissue expression pattern was profiled using the Mouse Multiple Tissue cDNA Panel I (Takara Bio USA, Mountain View, CA), which contains a set of first-strand cDNA from 12 different tissues: heart (whole);

brain (whole); spleen; lung; liver (whole); skeletal muscle; kidney (whole); testis, 7-day embryo; 11-day embryo; 15-day embryo; and 17-day embryo.

RNAscope

The RNAscope Multiplex Fluorescent Assay (Advanced Cell Diagnostics, Newark, CA) was performed with fresh frozen 10-micron brain sections, strictly following the manufacturer's protocols. In brief, sections were first fixed in 4% paraformaldehyde for 15 min, dehydrated through serial ethanol, and permeabilized with Pretreat 4 for 30 min at room temperature. Sections were then hybridized with predesigned assay-on-demand probes of Mm-Susd4-C1, Mm-Gfap-C2 or Mm-Olig2-C2, and Mm-Rbfox3 (NeuN)-C3 (Advanced Cell Diagnostics, Newark, CA) in a HybEZ Oven (Advanced Cell Diagnostics) for 2 h at 40°C. Sections were washed twice with 1X Wash Buffer and hybridized sequentially with Amp 1-FL, Amp2-FL, Amp 3-FL, and Am 4-FL (with 2 washes in-between each hybridization). At the end, sections were counterstained with DAPI (Thermo Fisher Scientific, Rockford, IL) and mounted with ProLong Diamond Antifade Mountant (Thermo Fisher Scientific). Sections were examined and imaged on a Zeiss Axio Observer Z1 confocal microscope (Thornwood, NY). Olig2/*Susd4* labeled nuclei were counted using 'cell counter' mode of ImageJ/Fiji program (National Institutes of Health, Bethesda, MD; http://imagej.nih.gov/ij/).

Behavioral studies

All behavioral analysis was performed in the Murine Phenotyping Core Facility of the National Heart, Lung, and Blood Institute, National Institutes of Health. All tests were performed during the day light cycle on male *Susd4* KO (n=17) and WT (n=15), starting at the age of 3-5 months. Mice underwent each test one time, with a minimum of one week rest between the tests.

DigiGait analysis: Gait dynamics is a sensitive indication for locomotor deficits in diseases or drug treatments (Hampton et al., 2004). Mice were placed on a transparent treadmill positioned above a high-speed digital camera (DigiGait Imaging System, Mouse Specifics Inc., Boston MA), and run at a speed of 18 cm/sec for approximately 5 min until 12 consecutive strides were videotaped for analysis of braking, swing, and propulsion components of each stride.

Horizontal balance-beam test: This test assesses balance and coordination in mice (Luong et al., 2011). It uses a series of 80-cm-long horizontal square beams of different widths that are elevated 50 cm off the floor, with an

opaque shelter placed at the end of each beam. For acclimation, each mouse was first trained to cross a 32-mm-wide beam several times for 2 days. On the third day, each mouse was tested sequentially with beams with a width of 24 mm, 12 mm, and 9.5 mm, and the time to cross was recorded. For each mouse, the test was performed with 3 trials per beam, with a brief rest between trials and beams. One cage of 4 WT mice with barbering behavior were excluded from this test.

Accelerating-rotarod test: This test assesses motor coordination, balance, and equilibrium in mice. Each mouse was brought to the Rotamex 5 rotarod apparatus (Columbus Instruments, Columbus, OH) and first acclimated for 3 days before the test, at a steady speed of 4 rpm, 6 rpm, and up to 8 rpm (maximum 3 min/trial, 3 trials/training, with 10 min resting time between each trial). For the test, the rotarod was set at an accelerating mode (4–40 rpm, 5 min), and latency time for the mouse to fall from the rotarod was recorded. For each mouse, the test was repeated 3 times with 1 h rests between each trial.

Elevated zero-maze test: This test measures anxiety in mice (Bell et al., 2014; Kulkarni et al., 2007). The maze is a 66-cm circular runway 84 cm above the floor; part of the runway is enclosed by walls (closed arms) and other parts are open (open arms). Each mouse was placed on the runway to explore the maze for 5 min. The numbers of entries into, as well as the time and the distance the mouse traveled in, the open arms and the closed arms were recorded.

Open-field test: This test measures locomotor and anxiety-like behavior in mice (Gould et al., 2009). Each mouse was placed in a 16" × 16" × 16" Perspex chamber, and Any-maze software (version 4.99, Stoeling Co.) was used to analyze patterns of mouse movement into the center of the chamber, remaining around the edges, and the distance traveled. Each mouse was observed for 30 min and then returned to its home cage.

Cylinder test: This test assesses early sensorimotor function changes. Each mouse was placed in a small transparent cylinder (15.5-cm height; 12.7-cm diameter) and the number of spontaneous rears, which is defined as the mouse with both forelimbs off the floor and standing only on its hindlimbs, was recorded for 90 s.

Hole-board test: This test assesses anxiety and exploratory drive in mice (File and Wardill, 1975). The test uses an apparatus that is a 40 cm x 40 cm x 35 cm (height) horizontal board with multiple 3-cm holes for mice to explore. For the test, each mouse was placed in the center of the board and allowed explore the apparatus freely for 5 min. Total distance traveled and the number of head-pokings were recorded.

Morris water maze test: This test is designed for assessing spatial memory in rodents (Vorhees and Williams, 2006). It consists of a circular pool (122 cm diameter, 76 cm height) filled with room temperature water (24-30°C). Non-toxic tempura paint was added to the water to make it opaque. A 10-cm square platform was placed in northwest quadrant of the tank 1 cm beneath the water surface. Visual cues were placed around the pool for mice to locate the hidden platform. The mice were trained for consecutive 6 days with four 60-sec trials per day. Distance swam, latency to find platform, swim speed were measured by Any-maze software. On the last day, the 90-sec probe trial was performed in which the platform was removed.

Shirpa screening test battery: The modified Shirpa protocol (Rogers et al., 1997) was used as an initial non-invasive screening to observe any physical and functional abnormalities. The screening includes a gross visual inspection of fur coat, eyes, ears, teeth, nares, limbs and tail; measurement of body weight, length and temperature; a general postural assessment for body position, tremor, aggression, transfer arousal in an arena cage, grooming, freezing, rearing or jumping; sensorimotor detection of an approaching object, eye blink, ear twitch, touch escape & auditory cue; and a sequence of neuromuscular phenotyping screens including an inverted grip test to assess a mouse limb stretch by hanging it on the cage lid for a 90 sec session, a catalepsy test to place the mouse hind limbs on a raised 1.5-inch block to assess its ability to correct an abnormally imposed body position, the akinesia test to detect mobility deficit by the latency for a mouse to move all four limbs after placing it onto a flat surface, and the ladder climb test by record the time for a mouse to cross a 30 cm long metal ladder with steps placed 1.25 cm apart and positioned at a 50° angle vertically.

Histochemistry

Bielschowsky silver staining was performed by Histoserv, Inc. (Germantown, MD). For sample preparation, mice were transcardially perfused with PBS followed by 4% paraformaldehyde (PFA), then the brains were dissected and post-fixed for 48 h in 4% PFA. Brain samples were maintained in 70% ethanol and then processed into 5-micron paraffin sections for staining.

Golgi-Cox impregnation was performed with the FD Rapid Golgi Stain™ Kit by FD Neurotechnologies (Columbia, MD). Brain tissues were prepared according to the kit manual. Sagittal brain sections were cut at 100-micron thickness and examined with a Leica DMLB microscope and bright field images under a 40x objective. Neuron dendritic spine density analysis was performed with the ImageJ (Orlowski and Bjarkam, 2012). Five images of

hippocampal CA region per mouse and 3 mice per group were analyzed and spines from secondary or tertiary dendrite segments of pyramidal cells were counted using the ImageJ. Spine density was calculated using obtained spine number divided by the length of the dendrite segment.

Western blot

Dissected mouse brain cerebral, hippocampal, or cerebellar tissues were homogenized in RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with protease inhibitor cocktail (Roche, Mannheim, Germany). Lysed protein samples and molecular weight ladder (Li-Cor Biotechnology, Lincoln, NE) were heated for 10 min at 70 degrees in NuPAGE® LDS Sample Buffer containing lithium dodecyl sulfate and 50 mM dithiothreitol. Samples were separated on precast NuPAGE 4–12% Bis-Tris gels (Thermo Fisher Scientific) under denaturing conditions, and then transferred onto nitrocellulose membranes.

C1q was detected with Odyssey fluorescent western blotting (Li-Cor Biotechnology). In brief, membranes were blocked with Odyssey blocking buffer in PBS for 1 hour at room temperature, then incubated over night at 4°C with primary antibodies anti-C1q (1:50, mouse monoclonal [JL-1, cat #: MA1-40311], Thermo Fisher Scientific) and anti-β-actin (1:1000, goat polyclonal [I-19, cat #: sc-1616], Santa Cruz Biotechnology, Dallas, TX), diluted in antibody dilution buffer (Odyssey blocking buffer in PBS with 0.1% Tween 20). Membranes were washed 3 times in PBS with 0.05% Tween-20 and then incubated for 1 hour at room temperature with secondary antibodies IRDye 680LT donkey anti-mouse (1:5000, cat #: 926-68022, Li-Cor Biotechnology) and IRDye 800CW donkey anti-goat secondary antibodies (1:5000, cat #: 926-32214, Li-Cor Biotechnology). Membranes were imaged with Odyssey CLx (Li-Cor Biotechnology) and protein bands were quantified with ImageJ.

For chemiluminescent Western blotting, membranes were blocked with 5% milk for 1 hour at room temperature, then incubated over night at 4°C with primary antibodies anti-VGAT (1:10000, rabbit polyclonal [cat #: 131 002], Synaptic Systems, Goettingen, Germany), anti-VGLUT1 (1:2000, rabbit polyclonal [cat #: 12331], Cell Signaling Technology, Danvers, MA), anti-MBP(1:10000, rabbit polyclonal [cat#: PA5-78397], Thermo Fisher Scientific), anti-C3 (1:200, mouse monoclonal [cat#: sc-28294], Santa Cruz Biotechnology), Thermo Fisher Scientific), diluted in 5% milk. Membranes were washed 3 times and then incubated for 1 hour at room temperature with peroxidase conjugated secondary antibodies goat anti rabbit IgG (1:3000, cat #: AP132P, Millipore, Burlington, MA), goat anti-mouse IgG (1:2500, cat #: AP124P, Millipore), diluted in 5% milk. Membranes were detected with

ECL prime Western blotting system (Sigma-Aldrich, St. Louis, MO) and imaged with an Amersham Imager 680 (GE Healthcare Life Sciences, Pittsburgh, PA). The same membrane was then incubated for 45 min at room temperature with primary antibody anti-β-actin (1:40000, HRP conjugated mouse monoclonal [cat#: ab49900], Abcam, Cambridge, MA), diluted in 5% milk and detected with ECL.

Immunohistology

Immunofluorescent staining was done with fixed-frozen sections. Anesthetized mice were transcardially perfused with 50 ml PBS and then 100 ml 4% PFA. Brains were extracted and post-fixed in 4% PFA for 24 h at 4°C, then placed in 20% sucrose overnight or until tissues sank. Brain tissues were embedded with O.C.T. (Sakura Finetek, Torrance, CA), and 14-micron sagittal sections were cut with a cryostat (Leica, Buffalo Grove, IL) and stored at -80 °C until usage. For staining, sections were washed in PBS to remove O.C.T. and then blocked with 10% normal goat serum (Thermo Fisher Scientific) and 0.3% Triton X-100 for 1 hour at room temperature. Then sections were incubated overnight at 4°C with primary antibodies anti-C1q (1:100, mouse monoclonal [JL-1, cat #: MA1-40311], Thermo Fisher Scientific), anti-NeuN (1:1000, rabbit polyclonal [cat #: ab128886], Abcam), anti-neurofilament H, phosphorylated (1:1000, mouse monoclonal [SMI 31, cat #: 801601], BioLegend, San Diego, CA), anti-calbindin-D-28K (1:2000, rabbit polyclonal [cat #: CB-38], Swant, Marly, Switzerland), anti-Iba1(1:500, rabbit polyclonal [cat #: 019-19741], Wako, Richmond, VA), anti VGAT(1:1000, rabbit polyclonal [cat #: 131 002], Synaptic Systems) anti-VGLUT1 (1:1000, rabbit polyclonal [cat #: 135 302], Synaptic Systems), diluted in 2% normal goat serum. Sections were washed 3 times in PBS and then incubated for 1 hour at room temperature with secondary antibodies Dylight 488 goat anti-mouse (1:800, cat #:35503, Thermo Fisher Scientific), Dylight 594 goat anti-rabbit (1:800, cat #:35561, Thermo Fisher Scientific). Sections were counterstained with DAPI (Thermo Fisher Scientific) and mounted with ProLong Gold Antifade Mountant DAPI (Thermo Fisher Scientific). Sections were examined and imaged on a Zeiss Axio Observer Z1 confocal microscope (Thornwood, NY). Fluorescence intensity was quantified by ImageJ. In brief, 5-6 regions of interest per section were selected from the hippocampus of *Susd4* KO and WT mice. Measurements were made on 3-4 sections per mouse and 3 mice per group. Iba1 labeled microglia were counted using 'analyze particles' mode of image J. For the final result, 4 images per mouse and 3 mice per group were counted. Colocalization analysis of C1q and VGAT/VGLUT1 colocalization were carried out using 'colocalization threshold' mode of ImageJ.

For the analysis, high-resolution images (5-6 images per section) were taken from the same areas of hippocampi (3-4 sections per mouse) in *Susd4* KO and WT mice (3 mice per group).

For Purkinje cell quantification, sagittal sections of paraffin-embedded mouse brains were immunostained with calbindin antibody to identify Purkinje cells. Deparaffinized and rehydrated sections (5-micron) were subjected to heat-induced antigen retrieval and then stained with anti-calbindin-D-28K antibody (1:500, mouse monoclonal [cat#: CB-955], Sigma-Aldrich) overnight at 4°C. Immunohistochemical detection was performed using the Mouse on Mouse ImmPRESS™ Peroxidase Polymer Kit (cat# MP-2400, Vector Laboratories, Burlingame, CA) and counterstained with hematoxylin (cat# H-3401, Vector Laboratories) following manufacturer's instructions. Cerebellum regions were imaged with a Leica DMLB microscope (Buffalo Grove, IL) and 5 regions per mouse and 3 mice per group were analyzed using the ImageJ to determine the linear cell density (number of Purkinje cells divided by the Purkinje cell layer length).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

All data were analyzed with Prism 7 software package (GraphPad Software, La Jolla, CA) and presented as mean ± standard error of the mean (SEM). Unpaired Student's *t* tests were performed to compare results between *Susd4* KO and WT groups. p-values < 0.05 were considered statistically significant. The number of biological replicates for each type of experiment is outlined in the figure legends.

Supplemental References

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