

Supplementary Information for

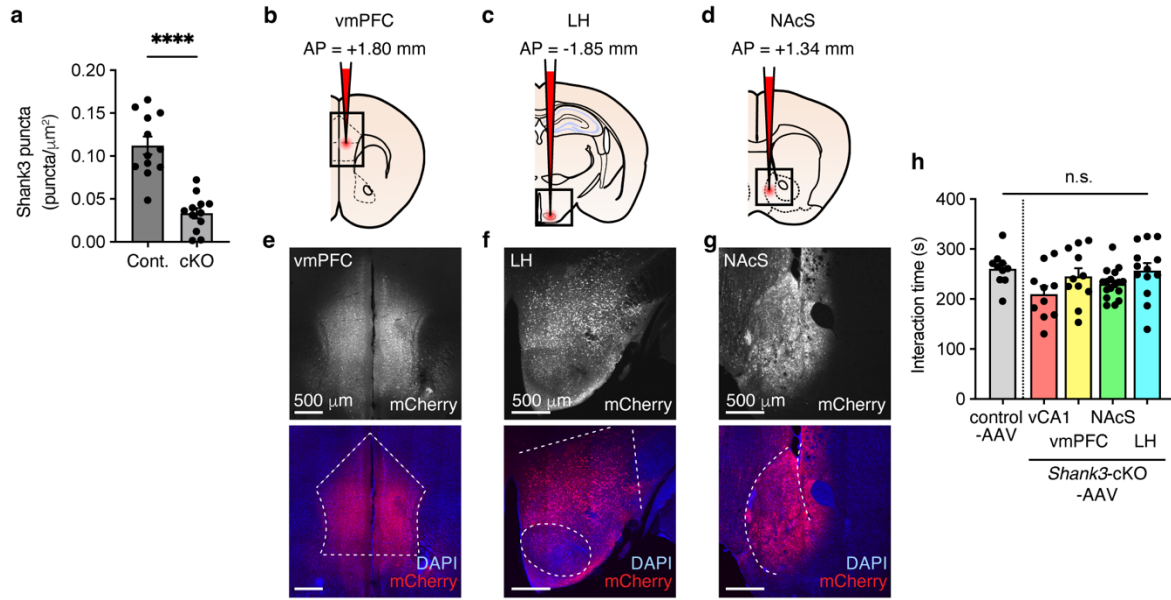
Conditional knockout of *Shank3* in the ventral CA1 by quantitative *in vivo* genome-editing impairs social memory in mice

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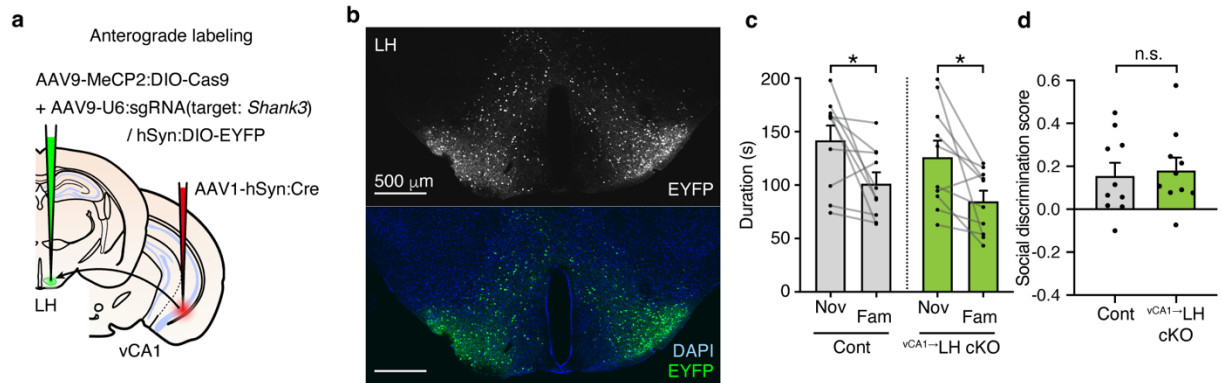
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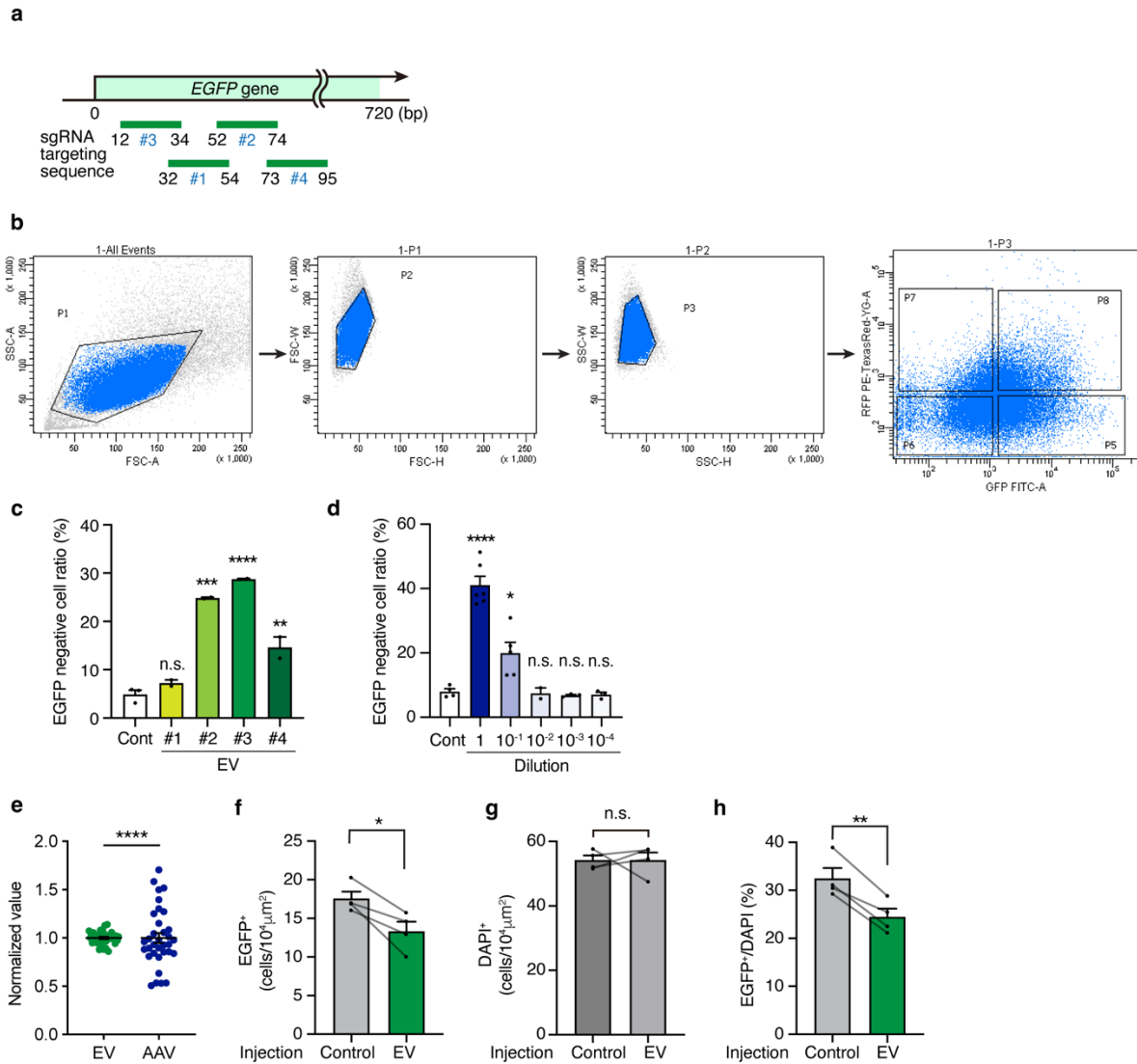
Supplementary Fig. 1. Validation of the AAV-mediated *in vivo* genome editing.

a. The number of Shank3 puncta in vCA1 of control-AAV injected and *Shank3*-cKO-AAV injected mice (Control AAV: $n = 12$ sections/4 mice; vCA1-*Shank3*-cKO: $n = 12$ sections/4 mice, unpaired t-test, two-tailed, $t(22) = 6.505$, **** $p < 0.0001$). **b-d.** Schematic illustration of AAV microinjection into vmPFC (**b**), LH (**c**), and NAcS (**d**). **e-g** Representative confocal microscopy images of AAV-injected vmPFC (**e**), LH (**f**), and NAcS (**g**) stained with anti-RFP (red, for mCherry) and DAPI (blue). Scale bar = 500 μm . **h** Comparison of the total social interaction time (Novel + Familiar) between the AAV-injected groups (Control AAV, $n = 10$; vCA1- *Shank3*-cKO, $n = 10$; vmPFC-*Shank3*-cKO, $n = 11$; NAcS-*Shank3*-cKO, $n = 17$; LH-*Shank3*-cKO, $n = 13$. One-way ANOVA, $F[4,56] = 1.862$, $p = 0.0670$). Data presented as the mean $\pm$ SEM. Source data are provided as a Source Data file.



Supplementary Fig. 2. *Shank3*-cKO in the $vCA1 \rightarrow LH$ neurons does not alter social memory.

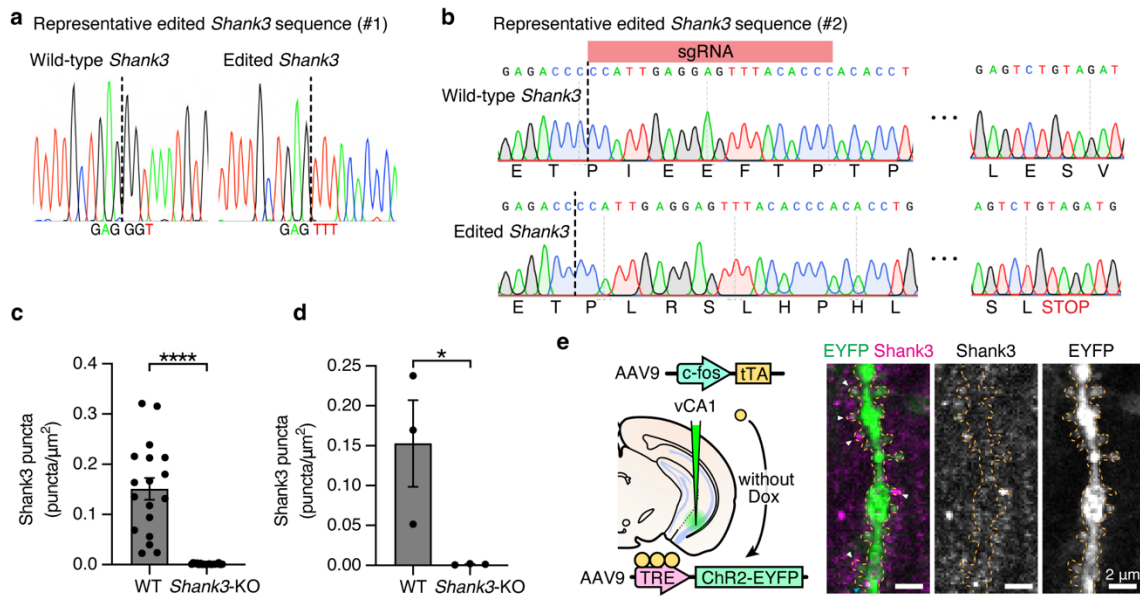
a. Schematic illustration of viral injection strategy of *Shank3*-cKO in the $vCA1 \rightarrow LH$ neurons. Cre is derived anterogradely from vCA1 to LH, and Cas9 is expressed in a Cre-dependent manner in LH. **b.** Representative microscopic image of LH stained with anti-GFP (green, EYFP) and DAPI (blue). **c.** Social discrimination test of the *Shank3* $vCA1 \rightarrow LH$ cKO mice (Control: n = 10 mice, $vCA1 \rightarrow LH$ cKO: n = 10 mice. Two-way mixed-model ANOVA, Familiarity $\times$ Group, $F[1,18] < 0.0001$, $p = 0.9784$; Familiarity, $F[1,18] = 15.99$; *** $p = 0.0008$; Group, $F[1,18] = 1.289$, $p = 0.2712$; Control, $p = 0.0237$; cKO, $p = 0.0162$). **d.** Social discrimination score (Control: n = 10 mice, $vCA1 \rightarrow LH$ cKO: n = 10 mice, unpaired t-test, two-tailed, $t(18) = 0.3006$, $p = 0.7672$). Data presented as the mean $\pm$ SEM. Source data are provided as a Source Data file.



Supplementary Fig. 3. *In vitro* and *in vivo* application of EGFP gene targeting EV and AAV.

a. Schematic illustration of the positions of the EGFP gene-targeting sgRNA on the EGFP gene. sgRNAs #1–#4 were used to produce EV-#1–#4. **b.** Gating strategy for flow cytometry. FSC/SSC gates were used to exclude potential doublet cells. **c.** Quantification of the EGFP-negative cell ratio in the negative control (control-EV) and EGFP-targeting EV (EV-#1–#4)-transduced HEK293-EGFP cells. (Control: $n = 3$, EV-#1–#4: $n = 2$. One-way ANOVA followed by Dunnett's multiple comparison test, two-sided. $F(4, 6) = 66.82$, **** $p < 0.0001$; Control vs. EV-#1, $p = 0.8145$; Control vs. EV-#2, *** $p = 0.0001$; Control vs. EV-#3, **** $p < 0.0001$; Control vs. EV-#4, ** $p = 0.0076$). **d.** Quantification of the EGFP negative cell ratio of the negative control (control-AAV), undiluted (1), and serially diluted (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4}) EGFP-targeting AAV transduced HEK293-EGFP cells (Control: $n = 4$, 1: dilution 1: $n = 6$, Dilution 10^{-1} : $n = 5$, Dilution 10^{-2} : $n = 2$, Dilution 10^{-3} : $n = 3$, Dilution 10^{-4} : $n = 3$, One-way ANOVA followed by Dunnett's multiple comparison test, two-sided. $F(5, 17) = 34.02$, **** $p < 0.0001$; Control vs. 1, **** $p < 0.0001$; Control vs. 10^{-1} , * $p = 0.0130$; Control vs. 10^{-2} , $p > 0.9999$; Control vs. 10^{-3} , $p = 0.9982$; Control vs. 10^{-4} , $p = 0.9994$). **e.** Mean normalized values of the EGFP-negative cell ratio in EGFP-targeting EV- and AAV-treated

HEK293-EGFP cells (Fig. 2e, g). EV: n = 36 samples; AAV: n = 34 samples. F-test. $p < 0.0001$. **f-h**. Comparison of the number of EGFP-expressing cells (**f**, n = 4, paired t-test, two-sided, $*p = 0.023$), DAPI-positive cells (**g**, n = 4, paired t-test, two-sided, $p > 0.99$), and the EGFP+/DAPI+ cell ratio (**h**, n = 4, paired t-test, two-sided, $**p = 0.0050$) in the control and *EGFP*-targeting EV-injected NAc of *Drd1*-EGFP mice. $*p < 0.05$, $**p < 0.01$, $**p < 0.001$, $***p < 0.0001$. Data presented as the mean $\pm$ SEM. Source data are provided as a Source Data file.



Supplementary Fig. 4. Confirmation of *Shank3*-cKO by EV-mediated methods.

a. Representative wild-type (unedited) and edited *Shank3* sequences (#1). **b.** Representative wild-type (unedited) and edited *Shank3* sequences near the sgRNA sequence (#2). Edited *Shank3* contains a premature termination codon. **c-d.** Shank3 puncta analysis of WT and *Shank3*-KO mice by section (**c**, $n = 18$ sections each, unpaired t-test, two-sided, **** $p < 0.0001$) and individual (**d**, $n = 3$ mice each, unpaired t-test, two-sided, * $p = 0.049$). **e.** Shank3 puncta in the spine-like structure of the social memory neuron in vCA1. Left, Schematic illustration of the strategy of social memory neuron labeling; Right, Representative microscopic image of a social memory engram neuron stained with anti-GFP (green, EYFP) and anti-Shank3 (magenta, Shank3). White arrowhead, Shank3 puncta at the spine-like structure; Blue arrowhead, puncta in the dendrite. Bar = 2 μm . * $p < 0.05$, **** $p < 0.0001$. Data presented as the mean $\pm$ SEM. Source data are provided as a Source Data file.

Supplementary Table 1. Statistical information for Figures			
Figure	Sample Size	Analysis	Statistical Test Values (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)
Figure 1g	n = 61 mice Control: 10 mice vCA1-cKO: 10 mice vmPFC-cKO: 11 mice NAcS-cKO: 17 mice LH-cKO: 13 mice	Two-way mixed model ANOVA	(Familiarity×Group) F(4,56) = 6.270; ***p = 0.0003 (Familiarity) F(1,56) = 21.65; ****p < 0.0001 (Group) F(4,56) = 2.331; p = 0.0670 (Control) F(1,9) = 23.63; ***p = 0.0009 (vCA1-cKO) F(1,9) = 0.103; p = 0.756 (vmPFC) F(1,10) = 9.158; *p = 0.013 (NAcS-cKO) F(1,16) = 4.649; *p = 0.047 (LH) F(1,12) = 0.012; p = 0.913
Figure 1h	n = 61 mice Control: 10 mice vCA1-cKO: 10 mice vmPFC-cKO: 11 mice NAcS-cKO: 17 mice LH-cKO: 13 mice	One-way ANOVA Scheffe's multiple comparison	F(4,54) = 5.377; **p = 0.0010 Control vs. vCA1-cKO: **p = 0.0032 Control vs. vmPFC-cKO: p = 0.2968 Control vs. NAcS-cKO: p = 0.0817 Control vs. LH-cKO: **p = 0.0037 vCA1-cKO vs. vmPFC-cKO: p = 0.3709 vCA1-cKO vs. NAcS-cKO: p = 0.5304 vCA1-cKO vs. LH-cKO: p = 0.9989 vmPFC-cKO vs. NAcS-cKO: p = 0.9917 vmPFC-cKO vs. LH-cKO: p = 0.4637 NAcS-cKO vs. LH-cKO: p = 0.6457
Figure 2e	n = 36 samples Control: 6 samples 1/1: 6 samples 1/2: 6 samples 1/4: 6 samples 1/8: 6 samples 1/16: 6 samples	One-way ANOVA Dunnett's multiple comparisons test	F(5,30) = 343.4, ****p < 0.0001 Control vs. 1/1: ****p < 0.0001 Control vs. 1/2: ****p < 0.0001 Control vs. 1/4: ****p < 0.0001 Control vs. 1/8: **p = 0.0028 Control vs. 1/16: p = 0.8521
Figure 2g	n = 34 samples Control: 4 samples 1/1: 6 samples 1/2: 5 samples 1/4: 6 samples 1/8: 7 samples 1/16: 6 samples	One-way ANOVA Dunnett's multiple comparisons test	F(5,28) = 13.19, ****p < 0.0001 Control vs. 1/1: ****p < 0.0001 Control vs. 1/2: ***p = 0.0001 Control vs. 1/4: **p = 0.0017 Control vs. 1/8: p = 0.0273 Control vs. 1/16: p = 0.3276
Figure 3d	n = 4 brain sections	Paired t-test (two-tailed)	t(3) = 5.535, *p = 0.0116

Figure 3e	n = 16 brain sections 1/1: 4 samples 3/5: 4 samples 2/5: 4 samples	One-way ANOVA Tukey's multiple comparisons test	$F(2,9) = 6.164$, * $p = 0.021$ 1/1 vs. 3/5: $p = 0.1747$ 1/1 vs 2/5: * $p = 0.0166$ 3/5 vs. 2/5: $p = 0.3233$
Figure 4d	n = 26 brain samples Control-EV: 13 samples Shank3-cKO-EV: 13 samples	Unpaired t-test (two-tailed)	$t(24) = 2.336$, * $p = 0.0206$
Figure 4f	n = 69 brain sections 1/64: 30 sections 1/128: 25 sections Control: 14 sections	One-way ANOVA Dunnett's multiple comparisons test	$F(2,66) = 7.073$, ** $p = 0.0016$ Control vs. 1/64: *** $p = 0.0007$ Control vs. 1/128: $p = 0.0554$
Figure 4g	n = 110 mice 1/1: 9 mice 1/2: 10 mice 1/4: 12 mice 1/8: 11 mice 1/16: 5 mice 1/32: 6 mice 1/64: 5 mice 1/128: 15 mice 1/256: 11 mice 1/512: 12 mice Control: 14 mice	Two-way mixed model ANOVA	(Familiarity×Group) $F(10,99) = 1.937$; * $p = 0.0488$ (Familiarity) $F(1,99) = 7.530$; ** $p = 0.0072$ (Group) $F(10,99) = 0.8769$; $p = 0.5573$ 1/1: $F(1,8) = 0.034$, $p = 0.857$ 1/2: $F(1,9) = 0.563$, $p = 0.472$ 1/4: $F(1,11) = 0.052$, $p = 0.823$ 1/8: $F(1,10) = 0.260$, $p = 0.621$ 1/16: $F(1,4) = 0.033$, $p = 0.865$ 1/32: $F(1,5) = 0.144$, $p = 0.720$ 1/64: $F(1,4) = 0.057$, $p = 0.824$ 1/128: $F(1,14) = 4.595$, $p = 0.050$ 1/256: $F(1,10) = 4.698$, $p = 0.050$ 1/512: $F(1,11) = 15.52$, ** $p = 0.002$ Control: $F(1,13) = 7.759$, * $p = 0.015$
Figure 4h	n = 110 mice 1/1: 9 mice 1/2: 10 mice 1/4: 12 mice 1/8: 11 mice 1/16: 5 mice 1/32: 6 mice 1/64: 5 mice 1/128: 15 mice	One-way ANOVA	$F(10,99) = 2.009$, * $p = 0.0401$

	1/256: 11 mice 1/512: 12 mice Control: 14 mice		
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Supplementary Table 1. Statistical information for Figures.

Supplementary Table 2		
Name	Primer	Sequence (5'-3')
Genotyping		
Drd1-EGFP	Forward	5'-CCT ACG GCG TGC AGT GCT TCA GC-3'
	Reverse	5'-CGG CGA GCT GCA CGC TGC GTC CTC-3'
Shank3-KO	Forward	5'-GAG CTC TAC TCC CTT AGG ACT T-3'
	Reverse-1	5'-TCC CCC TTT CAC TGG ACA CCC-3'
	Reverse-2	5'-TCA GGG TTA TTG TCT CAT GAG C-3'
AAV-mediated CRISPR/Cas9 delivery		
Shank3-sgRNA-1	Forward	5'-CAC CGG CAG GGG CGT GTC CAG GTT AG-3'
	Reverse	5'-AAA CCT AAC CTG GAC ACG CCC CTG CC-3'
Shank3-sgRNA-2	Forward	5'-ACC AGG GGC GTG TCC AGG TTA GG-3'
	Reverse	5'-AAC CCT AAC CTG GAC ACG CCC CT-3'
EGFP-sgRNA-3	Forward	5'-ACC GGG CGA GGA GCT GTT CAC CG-3'
	Reverse	5'-AAC CGG TGA ACA GCT CCT CGC CC-3'
EV-mediated CRISPR/Cas9 delivery		
EGFP-EV#1	Forward	5'-CAC CGA CCA GGA TGG GCA CCA CCC-3'
	Reverse	5'-AAA CGG GTG GTG CCC ATC CTG GTC-3'
EGFP-EV#2	Forward	5'-CAC CGA GCT GGA CGG CGA CGT AAA-3'
	Reverse	5'-AAA CTT TAC GTC GCC GTC CAG CTC-3'
EGFP-EV#3	Forward	5'-CAC CGG GCG AGG AGC TGT TCA CCG-3'
	Reverse	5'-AAA CCG GTG AAC AGC TCC TCG CCC-3'
EGFP-EV#4	Forward	5'-CAC CGG CCA CAA GTT CAG CGT GTC-3'
	Reverse	5'-AAA CGA CAC GCT GAA CTT GTG GCC-3'
Shank3- cKO-EV	Forward	5'-CAC CGG GTG TAA ACT CCT CAA TGG-3'
	Reverse	5'-AAA CCC CAC ATT TGA GGA GTT ACC-3'
Quantitative RT-PCR (qRT-PCR)		
Actb	Forward	5'-GAC CCA GAT CAT GTT TGA GAC C-3'
	Reverse	5'-GCT GTG GTG GTG AAG CTG TA-3'
Shank3	Forward	5'-AAG CAG AGA CCC CCA TTA G-3'
	Reverse	5'-CAG TTC GAA GTC CAG CCC TC-3'

Supplementary Table 2. List of primer sequences used in the study.