Supplemental information:

Supplementary Figure Legends:

Figure S1 (related to Fig. 1): Shank3 levels are reduced in the Homer TKO and the generation of the Shank3(+/ΔC) mouse.

A. Representative immunoblots from total cortical lysates of three independent pairs of WT and Homer TKO aged P35. Actin is used as loading control.

B. Quantification of protein levels in A. Data shown are means ± SEMs; n= 3 independent pairs; **P<0.01; *P<0.05 (Student's *t*-test).

C. Representative confocal images from DIV 14-21 WT and Homer TKO cortical cultures co-labeled with Shank3 (JH3025) and Vglut1. (Scale bar, 10 μm).

D. Quantification of puncta density from C. Data shown are means ± SEMs; 5000-6,000 μm of dendrites was examined from culture sets from 3 independent pairs; **P<0.01 (Student's *t*-test).

E. Quantification of synaptic protein per μm from C. Synaptic protein was defined as an area overlap between Shank3 and the synaptic marker Vglut1. Data shown are means ± SEMs; 5000-6,000 μm of dendrites was examined from culture sets from 3 independent pairs; **P<0.01 (Student's *t*-test).

F. Representative immunoblots from total cortical lysates of WT and Homer1 and Homer2/3 double KO. Actin is used as loading control. Lower graphs represent quantification of protein levels. Data shown are means ± SEMs; n= 3 independent pairs; *P<0.05 (Student's *t*-test).

G. Representative images from HTKO cortical cultures transfected with eGFP, H1a, W24A and G89N constructs. Transfected neurons were identified using GFP or Myc signal (not shown). Vglut1 immunoreactivity was used as a control (not shown). Graph shows rescue of Shank3 levels in the HTKO cultures by transfection of full-length Homer 1c but not Homer1c point mutants W24A and G89N – that do not bind Shank3. Rescue was assessed 24-hrs after transient transfection with these constructs. Data shown are means $\pm$ SEMs; 5000-6,000 µm of dendrites was examined from 3 independent culture sets; **P<0.01 (Student's *t*-test).

H. Quantification of brain Shank3 mRNA levels from real-time PCR analysis. Data is presented as fold difference (obtained by the delta-delta Ct method) $\pm$ SEMs; samples were run in triplicates from mRNA from 3 independent pairs; P>0.05 (Student's *t*-test).

I. Schematic representation of the targeting construct and cre mating used to generate the Shank3(+/ΔC) mouse.

J. DNA agarose gel electrophoresis for genotyping of the Δexon21(+/ΔC) Shank3 mice using primers a, b and c whose position is highlighted in H.

K. Schematic representation of the Shank3 protein with domains and antibody epitope for the three Shank3 antibodies. JH3025 and H160 bind to C-terminal Shank3. ΔC is the truncated N-terminal Shank3. The Homer binding site (PPPEEF) is on the proline-rich domain (indicated by arrow).

Figure S2 (related to Fig. 2): Shank3 polyubiquitination is increased in the Homer TKO mice

A. Co-immunoprecipitation assay from cortical lysates to asses *in vivo* poly-ubiquitination of Shank3 in the Homer TKO mice. Protein samples in the input were immunoprecipitated with non-saturating amounts of the Shank3 antibody (middle blot) and analyzed for polyubiquitination using the FK1 antibody (right blot).

B. Quantification of Shank3 polyubiquitination from A. Y-axis is FK1 polyubiquitin signal (right blot) relative to immunoprecipitated Shank3 (middle blot). Data shown are means ± SEMs; n= 3 independent pairs; *P<0.05 (Student's *t*-test).

C. Representative confocal images from DIV 14-21 cortical cultures from WT and Homer TKO cells co-labeled with Shank3 (JH3025) and the proteasomal marker Rpt6. (Scale bar, 5μm).

D. Quantification of colocalization (area overlap per μm of dendrite) between Shank3 and Rpt6 from C. Data shown are means ± SEMs; 5000- 6000 μm of dendrites was examined from culture sets from 3 independent pairs; **P<0.01 (Student's *t*-test)

E. Representative immunoblots of total Shank3 levels from DIV-14 WT cortical cultures transfected with either GFP-Sindbis or Myc-tagged Homer1a Sindbis virus. Actin is used as loading control.

F. Quantification of protein levels in E. Data shown are means ± SEMs; n= 3 independent sets of cultures; *P<0.05 (Student's *t*-test).

G. Representative immunoblots from total cortical lysates of two independent pairs of WT and Homer1a KO mice aged P65. Actin is used as loading control.

H. Quantification of protein levels in G. Data shown are means ± SEMs; n= 3 independent pairs of mice; *P<0.05 (Student's *t*-test).

I. Co-immunoprecipitation assay from cortical lysates to asses *in vivo* poly-ubiquitination of Shank3 in the Homer1a mice. Protein samples in the input were immunoprecipitated with the Shank3 antibody (middle blot) and analyzed for polyubiquitination using the FK1 antibody (right blot).

J. Quantification of Shank3 polyubiquitination from I. Y-axis is FK1 polyubiquitin signal (right blot) relative to immunoprecipitated Shank3 (middle blot). Data shown are means ± SEMs; n= 3 independent pairs; *P<0.05 (Student's *t*-test).

Figure S3 (related to Fig. 3): NR1 polyubiquitination is increased in the Homer TKO mice

A. Representative immunoblots from total cortical lysates of two independent pairs of WT and Homer TKO mice aged P24. Actin is used as loading control.

B. Quantification of protein levels in A. Data shown are means ± SEMs; n= 3 independent pairs of mice; *P<0.05 (Student's *t*-test).

C. Co-immunoprecipitation assay from cortical lysates to assess *in vivo* polyubiquitination of NR1 in the HTKO mice. Protein samples in the input were immunoprecipitated with the NR1 antibody (middle blot) and analyzed for polyubiquitination using the FK1 antibody (right blot).

D. Quantification of *in vivo* NR1 polyubiquitination from C. Y-axis is FK1 polyubiquitin signal (right blot) relative to immunoprecipitated NR1 (middle blot). Data shown are means ± SEMs; n= 3 independent littermate pairs; *P<0.05 (Student's *t*-test).

Figure S4 (related to Fig. 4): Reduction of NMDA receptor-mediated synaptic responses in neocortex and hippocampus of Shank3(+/ΔC) mice, without changes in spine number or anatomy.

A. Representative (gray) and averaged (black) traces from a series of 20 consecutive evoked EPSCs in cortical slices (8-to 9-week-old male mice). Peak AMPA currrents were measured at -70 mV, and NMDA currents at +40 mV in the presence of NBQX. The NMDA/AMPA ratio is diminished in Shank3(+/ΔC) neurons (n=7) compared with the WT (n=11). Error bars represent the mean ± SEM. **p<0.005, Student's *t*-test. (Scale bars, 50 pA/50 ms). Values: WT- 0.37 ± 0.05, +/ΔC- 0.16 ± 0.02

B. Raw (gray) and averaged (black) traces from a series of 20 consecutive evoked EPSCs recorded from hippocampal Schaffer collateral-CA1 synapses (3-to 4-week-old male mice). Peak AMPA currrents were measured at -70 mV, and NMDA currents at +40 mV in the presence of NBQX. The NMDA/AMPA ratio is diminished in Shank3(+/ΔC) neurons (n=14) compared with the WT (n=12). Error bars represent the mean ± SEM. **p<0.005, Student's *t*-test. (Scale bars, 50 pA/50 ms). Values: WT- 0.57 ± 0.06, +/ΔC- 0.19 ± 0.01

C. Cresyl Violet staining of sagittal slices from the somatosensory cortex and Hippocampus of WT and Shank3(+/ΔC) mice aged P65 showing normal formation of cell body layers. Scale bar= 100 µm

D. Representative image of DiI-labeled spines from Shank3(ΔC/ΔC) mice. Quantification of spine density (spines/µm) and volume in layer V, II/III and CA1 pyramidal neurons from WT and Shank3(ΔC/ΔC) mice. n=500-1100 spines from 3 independent littermate pairs. Difference is non-significant; Student's *t*-test.

Figure S5 (related to Fig. 5): Normal presynaptic properties and synapse formation in the Shank3(+/ΔC) mice.

A. Current-voltage (I-V) relationship in pyramidal neurons derived from WT and Shank3 (+/ΔC) cortical slices. Raw traces show evoked EPSCs obtained while holding postsynaptic cell at membrane potentials ranging from -70 mV to +60 mV in 10 mV steps recorded in the presence of D-AP5 (50 µM) to isolate AMPAR-mediated EPSCs. Graph shows normalized I-V relationship of the peakEPSC amplitude. The data were fitted by a straight line giving an estimate for the reversal potential of -2.5 mV (WT in red) and -1.9 mV (+/ΔC in black), respectively (corrected for liquid junction potential). Note the lack of rectification in both WT and Shank3(+/ΔC) pyramidal cells. (Scale bars, 50 pA/10 ms)

B. Representative confocal images of Synapsin labeling of DIV14-21 cortical cultures from WT and Shank3(+/ΔC) mice. (Scale bar, 10µm)

C. Quantification of Synapsin puncta/μm from B. Data shown are means ± SEMs; 7000-8,000 μm of dendrites was examined from culture sets from 3 independent littermate pairs; difference is non-significant; Student's *t*-test.

D. Representative traces in response to paired pulses in hippocampal slices derived from WT and Shank3(+/ΔC) mice. (Scale bars, 0.5 mV/50 ms). fEPSPs were evoked with inter-stimulus intervals of 30, 60, 90, 120 and 150 ms (mean ± SEM). The quantification shows the relationship between paired-pulse interval and paired-pulse ratio (PPR) of the Schaffer collateral-CA1 synapses of WT and Shank3 (+/ΔC) mice.

E. Relationship between fiber volley (FV) amplitude and fEPSP slope of the Schaffer collateral-CA1 synapses of WT and Shank3 (+/ΔC) mice (Scale bars, 1 mV/20 ms). Each point represents the mean ± SEM for a narrow range of fiber volley amplitudes and fEPSP slopes.

F. Raw (gray) and averaged (black) traces from a series of 30 consecutive population spikes in hippocampal slices. The quantification shows the relationship between presynaptic fiber volley (FV) amplitude and population spike (PS) amplitude of the Schaffer collateral-CA1 synapses of WT and Shank3(+/ΔC) mice. Each point represents the mean ± SEM for a narrow range of fiber volley amplitudes. (Scale bars, 1 mV/5 ms)

Figure S6 (related to Fig. 6): Additional measures of cognitive function and social interactions in Shank(3+/ΔC) mice.

(A) Latency to reach the hidden platform in the water maze in consecutive days of training. Genotype x Sex x Day ANOVA yielded significant effect of Day ($F(8,320)=59.82$, $P<0.000001$) but no genotype or sex-related differences or interactions. Shank3(+/ΔC) (n=22) and control mice (n=22).

(B) Spatial memory for the platform tested in the probe trial 24 hr after the end of Morris water maze training. Time spent in different quadrant of the water maze is shown. Both WT and Shank3(+/ΔC) spent significantly more time in the target quadrant than all other quadrants. Genotype x Sex x Quadrant ANOVA yielded significant effect of Quadrant ($F(3,120)=71.32$, $P<0.000001$) but no genotype- or sex-related differences or interactions. Numbers of mice are the same as in L.

(C) Freezing behavior during pre-training and testing for context- and cue-related fear memory. Both, Shank3(+/ΔC) (n=22) and control mice (n=22) spent equal percent of time freezing in context and cued fear conditioning tests (ANOVA; no significant effects). Control tests showed no differences in sensitivity to shock (Table S2).

(D) Extinction of memory to context shown as percentage of time spent freezing during repeated presentations of context without presentation of unconditioned stimulus. Genotype x Sex x Day ANOVA revealed significant effect of Day ($F(4,160)=40.15$, $P<0.0000001$) but no genotype- or sex-related differences or interactions.

(E) Time to fall from the accelerating rotarod, a test for motor learning and coordination. Genotype x Sex x Trial ANOVA showed significant effect of Trial

(F(7,287)=22.91, P<0.000001) but no effect of Sex, Genotype or their interaction (Shank3(+/ΔC) n=22 and WT control n=22).

(F) Total number of aggressive episodes is shown for the groups of mice that engaged in aggressive behaviors during habituation-dishabituation paradigm (see Fig. 6C-D). Shank3(+/ΔC) mice showed increased number of aggression episodes in the last trial of the testing as compared to wild type controls (Genotype x Trial ANOVA: significant effect of Trial (F=3.30, P<0.025) and Genotype x Trial interaction (F=3.53, P<0.021). Asterisks indicate significant between-genotype differences (P<0.01, Fisher LSD post-hoc test).

(G) Duration of social investigation in the habituation-dishabituation test with free-roaming stimulus mice of the 129 strain background. Left and right panels show “non-aggressive” and “aggressive” mice, respectively. Similar to results shown in Fig. 6C (Swiss Webster stimulus mice) and Fig. S6H (C57Bl/6J stimulus mice), the presence of aggression in Shank3(+/ΔC) mice disrupted normal habituation-dishabituation curves of social investigation when tested with stimulus mice of the 129 strain background. See Fig. S6I for the percentages of mice involved in aggression.

(H) Duration of social investigation in the habituation-dishabituation test with free-roaming stimulus mice of the C57Bl/6J strain background. Left and right panels show “non-aggressive” and “aggressive” mice, respectively. Note that presence of aggression in Shank3(+/ΔC) mice disrupted normal habituation-dishabituation curves of social investigation. See Fig. S6I for the percentages of mice involved

in aggression. A Control test for olfactory discrimination of socially-relevant stimulus (urine) was similar between genotypes (Fig. S6K-L).

(I) Proportions of Shank3(+/ΔC) and wild type mice that demonstrated aggressive behaviors during habituation-dishabituation testing with stimulus mice of different strain backgrounds. In concert with previously published data showing that males from the same strain display different scores of aggression with opponents belonging to different strains (François et al., 1990), in our hands, the strain background of stimulus mice significantly modulated aggressive behaviors in the test mice. However, proportions of Shank3(+/ΔC) mice engaged in aggression were not significantly different from that in the control mice. Pounds indicate significant effects of strain background (*t*-test for proportions, $P<0.05$) observed in each of the genotypes. The same Shank3(+/ΔC) (n=11) and wild type mice (n=13) were tested with stimulus mice of 129 and C57Bl/J backgrounds and separate group of mice was tested with SW stimulus mice (n=20 per genotype).

(J) Number of aggression episodes observed during a 10-min test in a standard clean mouse cage (neutral arena) or in a test mouse's home cage. There were no significant genotype-related differences. See Fig. 6F for the latency to the first aggression episodes in the same mice.

(K) A control test for olfactory perception conducted as an olfactory discrimination between a socially-relevant stimulus (fresh urine from an adult C57Bl/6J male mouse) and water. Duration of the first investigation of a perforated plastic cup containing a drop of urine (3μl) or an equivalent volume of water is shown. Both Shank3(+/ΔC) (n=12) and wild type mice (n=14) investigated a cup with urine

significantly longer than a cup with water. Genotype x Stimulus ANOVA revealed significant effect of Stimulus ($F(1,24)=40.89$, $P<0.0001$) but not Genotype. Total duration of olfactory investigation during a 3-min test revealed similar findings (Genotype x Stimulus ANOVA; effect of Stimulus ($F(1,24)=18.78$, $P<0.0002$ but not Genotype; data not shown). These data indicate that mice of both genotypes successfully discriminated a cup with socially-relevant stimulus.

(L) A control test for olfactory perception conducted as an olfactory habituation-dishabituation paradigm (Silverman et al, Brain Research 2010). Duration of olfactory investigation of cotton swabs dipped in distilled water, almond or cinnamon extracts (both McCormick, Hunt Valley, MD; 1:100 dilution) or wiped across the bottom of a cage housing sex-matched mice of a C57BL6/J or 129s6 strain is shown. Each odor was presented in three consecutive trials (2 min each). No genotype-related differences were detected (three-way Genotype x Odor x Trial ANOVA; effect of Genotype $F(1,14)=0.01$, $P>0.90$). Both genotypes investigated social odors more than non-social ones (effect of Odor ($F(4,56)=24.02$, $P<0.0001$) and decreased investigation with repeated presentation of the same odor (effect of Trial $F(2,28)=50.73$, $P<0.0001$; n=8 per genotype)

Figure S7 (related to Fig. 7): Measures of startle reactivity, novelty-induced exploration and other behavioral tasks in Shank3(+/ΔC) mice.

(A) Omission of startle reactions. Probability of startle responses was calculated as a percent of reactions with the amplitude at least 5 STDEV above the

amplitudes observed in “no stimulus” trials. Probability of startle responses was significantly lower in Shank3(+/ΔC) mice (n=12) than in WT (n=14) (Genotype x Stimulus Intensity ANOVA, effect of Genotype $F(1,24)=6.45$, $P<0.018$).

(B) Amplitude of startle reactions in ‘no-omission’ trials. To assess whether low levels of the average startle amplitude observed in Shank3(+/ΔC) mice (Fig. X2A) were due to omission of startle reactions in ~20% of trials (Fig. SX2A), we analyzed the amplitude of startle reactions in “no omission” trials. In these trials, Shank3(+/ΔC) mice showed lower amplitudes as well (Genotype x Stimulus Intensity ANOVA, effect of Genotype $F(1,24)=4.72$, $P<0.039$) indicating that lower reactivity of Shank3(+/ΔC) mice to startle stimuli was due to both the omission of startle reactions and the low amplitude of the reaction when it occurs.

(C) Latencies of startle reactions in “no omission” trials. Average latencies of startle reaction were significantly longer in Shank3(+/ΔC) mice than in WT littermates ($F(1,24)=4.51$, $P<0.045$) .

(D) Cumulative distributions of startle latencies. Shank3(+/ΔC) and WT littermates display similar medians of startle latencies (shown as a horizontal line). The differences in means between Shank3+/ΔC and WT mice were due the presence of significantly higher proportion of long latencies in Shank3(+/ΔC) mice (Kolmogorov-Smirnov two-sample test, MaxDif=0.24, $P<0.001$, skewness 3.88 and 7.41 for WT and Shank3(+/ΔC) mice, respectively).

(E) Startle amplitude as a function of startle latency. The averages were calculated from 283 trials with a 120dB startle stimulus. In both, Shank3(+/ΔC) and WT control mice, the startle reactions with longer latencies had smaller

amplitudes. The negative relations observed in both genotypes between amplitude and latency of the startle reaction indicate that changes in average latencies in Shank3(+/ΔC) mice may be secondary to their deficits in the amplitude of startle reaction.

(F) Novelty-induced motor activation in a novel open field. No differences were detected between Shank3(+/ΔC) (n=11) and control mice (n=15). To assess locomotor response to novelty in Shank3(+/ΔC) mice, we used additonal tasks that consist of a short (5-10 min) exposure to novel Y maze (Fig. S7H) or plus maze (Table S2). Novelty-induced activation in Shank3(+/ΔC) mice was not different from controls in any of these new environments. Anxiety levels assessed in some of these new environments (the open field, plus maze and light-dark apparatus; Table S2) were not different between genotypes.

(G) Total motor activity accumulated over a 2-hour testing in the open field. Asterisks indicates a significant between-genotype difference (paired *t*-test, $p<0.05$; n=22 per genotype). These data indicate that when the duration of exposure to the new environment was increased to ~2 hrs, Shank3(+/ΔC) mice displayed significantly lower levels of motor activity implicating a faster habituation to the environment than in control mice. No differences between Shank3(+/ΔC) and WT control mice were observed in a light-dark transition or marble burying tasks (Table S2), which has been described as a task relevant to anxiety and to obsessive-compulsive / repetitive behaviors (Andersen et al., 2010).

(H) Novelty-induced motor activation in a novel Y maze. Similar to the open field (Fig. S7F), there were no genotype differences in novelty-induced activation in Y maze. Number of mice is the same as in Fig. S7F.

(I) Nest building skills were measured in Shank3(+/ΔC) (n=22) and control mice (n=22) by increases in width of nesting material during a 90-min observation period. Genotype x Sex x Time ANOVA yielded a significant effect of Time ($F(2,80)=17.73$, $P<0.000001$) but no genotype- or sex-related differences or interactions.

(J) Body weights were similar at the range of ages used in behavioral studies. Data for male mice are shown. Shank3(+/ΔC) n=13,19 and WT mice n=16,13 for 3 and 5 months of age, respectively.

Table S1 (related to Fig. 4): Kinetic analysis of mEPSCs in neocortical pyramidal neurons

Table S2 (related to Fig. 6): Measures of memory, anxiety levels and pain sensitivity in Shank3(+/ΔC) mice and their WT littermates. NS indicates “non-significant”.

Table S3 (related to Fig. 7): Pearson correlations between the amplitudes of startle reaction and prepulse inhibition in WT control mice (shown below the diagonal marked in grey; n=14) and Shank3(+/ΔC) mice (shown below the diagonal; n=12). Correlations marked in bold are significant at $P<0.05$. P levels

(p) are shown below each correlation coefficient. Variables S120dB and S110dB indicate the startle amplitudes in trials with 120 and 110 dB, respectively. Other variables represent prepulse inhibition in four types of trials in which a prepulse (PP) of 4 or 8 dB above background was followed by a startle stimulus of 110 or 120 dB. Note the amplitudes of startle reactions to 120 and 110 dB were strongly correlated ($R=0.971$, $P<0.000$) allowing using just one of these variables (S120dB) as a covariate in the ANCOVA in prepulse inhibition (Fig. 7E).