LETTER

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Autistic-like social behaviour in *Shank2*-mutant mice improved by restoring NMDA receptor function

Hyejung Won[1,2]*, Hye-Ryeon Lee[3]*, Heon Yung Gee[4]*, Won Mah[1,2]*, Jae-Ick Kim[3]*, Jiseok Lee[1,2], Seungmin Ha[1,2], Changuk Chung[1,2], Eun Suk Jung[4], Yi Sul Cho[5], Sae-Geun Park[1], Jung-Soo Lee[4], Kyungmin Lee[6], Daesoo Kim[1], Yong Chul Bae[5], Bong-Kiun Kaang[3,7], Min Goo Lee[4] & Eunjoon Kim[1,2,8,9]

Autism spectrum disorder (ASD) is a group of conditions characterized by impaired social interaction and communication, and restricted and repetitive behaviours. ASD is a highly heritable disorder involving various genetic determinants[1]. Shank2 (also known as ProSAP1) is a multi-domain scaffolding protein and signalling adaptor enriched at excitatory neuronal synapses[2–4], and mutations in the human *SHANK2* gene have recently been associated with ASD and intellectual disability[5]. Although ASD-associated genes are being increasingly identified and studied using various approaches, including mouse genetics[6–16], further efforts are required to delineate important causal mechanisms with the potential for therapeutic application. Here we show that *Shank2*-mutant (*Shank2*$^{-/-}$) mice carrying a mutation identical to the ASD-associated microdeletion in the human *SHANK2* gene exhibit ASD-like behaviours including reduced social interaction, reduced social communication by ultrasonic vocalizations, and repetitive jumping. These mice show a marked decrease in NMDA (*N*-methyl-D-aspartate) glutamate receptor (NMDAR) function. Direct stimulation of NMDARs with D-cycloserine, a partial agonist of NMDARs, normalizes NMDAR function and improves social interaction in *Shank2*$^{-/-}$ mice. Furthermore, treatment of *Shank2*$^{-/-}$ mice with a positive allosteric modulator of metabotropic glutamate receptor 5 (mGluR5), which enhances NMDAR function via mGluR5 activation[17], also normalizes NMDAR function and markedly enhances social interaction. These results suggest that reduced NMDAR function may contribute to the development of ASD-like phenotypes in *Shank2*$^{-/-}$ mice, and mGluR modulation of NMDARs offers a potential strategy to treat ASD.

Mutations in the *SHANK2* gene have recently been identified in individuals with ASD and intellectual disability[5,18]. Among these mutations, one *de novo SHANK2* microdeletion found in ASD leads to loss of exons 6 and 7 and a frame shift, with concomitant removal of the PDZ and following domains in SHANK2 proteins. To explore the possibility that this deletion causes ASD in humans, and to study the mechanisms underlying the development of ASD, we generated transgenic mice carrying a mutation identical to the human microdeletion (exons 6 and 7 deletion and a frame shift), which affects both splice variants of *Shank2* (*Shank2a* and *Shank2b*) in mice (Fig. 1a). The deletion was verified by Southern blotting and various PCR methods (Supplementary Fig. 1). Shank2 proteins were undetectable in the brain (Fig. 1b), and there were no compensatory increases in Shank1 or Shank3 (Supplementary Fig. 1). The *Shank2*$^{-/-}$ mice showed normal reproduction and brain structure (Supplementary Fig. 2).

We first examined whether *Shank2*$^{-/-}$ mice displayed autistic-like impairments in social interaction. In a home-cage social interaction assay, *Shank2*$^{-/-}$ mice showed reduced interaction with normal target mice, as compared with wild-type animals (Supplementary Fig. 3). In a three-chamber social interaction assay, wild-type animals preferred to explore the first novel mouse introduced (stranger 1) over an inanimate object relatively more than did *Shank2*$^{-/-}$ mice (Fig. 1c, d and Supplementary Fig. 4). Next, when the object was replaced by another novel mouse (stranger 2), *Shank2*$^{-/-}$ mice preferred to explore stranger 2 over stranger 1, similar to wild-type animals (Fig. 1e), indicative of normal levels of social novelty recognition. Similar results were obtained when we used juvenile *Shank2*$^{-/-}$ mice (Supplementary Fig. 5). *Shank2*$^{-/-}$ mice had normal olfactory function (Supplementary Fig. 6).

Shank2$^{-/-}$ mice showed impaired spatial learning and memory in the Morris water maze, although novel object recognition memory was normal (Supplementary Fig. 7). These results suggest that *Shank2*$^{-/-}$ mice have partially impaired learning and memory, consistent with the idea that the deletion in exons 6 and 7 in humans causes ASD and mild to moderate intellectual disability[5].

Shank2$^{-/-}$ mice showed impairments in social communication by ultrasonic vocalizations (USVs). When allowed to interact with a novel wild-type female mouse, *Shank2*$^{-/-}$ male mice uttered USVs less frequently than did wild-type animals, and took longer to make the first call (Fig. 1f–h). In a pup retrieval assay, *Shank2*$^{-/-}$ female mice retrieved the pups less efficiently than did wild-type mice (Fig. 1i and Supplementary Fig. 8, Supplementary Movies 1 and 2).

Shank2$^{-/-}$ male animals exhibited other autistic-like abnormalities. When kept alone in stranger-free home cages, *Shank2*$^{-/-}$ mice showed enhanced jumping mostly mixed with upright scrabbling, normal grooming, and decreased digging behaviours (Fig. 1j and Supplementary Movies 3–5). *Shank2*$^{-/-}$ mice also displayed impaired nesting behaviour, hyperactivity in assays including the open field test, anxiety-like behaviour in an elevated plus maze, and increased grooming in a novel object recognition arena (Supplementary Figs 4, 6 and 7). *Shank2*$^{-/-}$ female mice showed similar repetitive jumping, hyperactivity in an open field, and anxiety-like behaviour in an elevated plus maze, although not in a light-dark box (Supplementary Fig. 8). These data collectively suggest that *Shank2*$^{-/-}$ mice show ASD-like behaviours. It should be noted that the hyperactivity and anxiety-like behaviours might contribute to the impaired social interaction in *Shank2*$^{-/-}$ mice by limiting target exploration or evoking anxiety-like responses.

We also characterized heterozygous *Shank2* (*Shank2*$^{+/-}$) mice, because human gene mutations are mostly heterozygotic. *Shank2*$^{+/-}$ mice showed hyperactivity, similar to *Shank2*$^{-/-}$ mice (Supplementary Fig. 9). However, they showed no abnormalities in social interaction,

[1]Department of Biological Sciences, KAIST, Daejeon 305-701, Korea. [2]National Creative Research Initiative Center for Synaptogenesis, KAIST, Daejeon 305-701, Korea. [3]National Creative Research Initiative Center for Memory, Department of Biological Sciences, College of Natural Sciences, Seoul National University, Gwanangno 599, Gwanak-gu, Seoul 151-747, Korea. [4]Department of Pharmacology, Brain Korea 21 Project for Medical Sciences, Severance Biomedical Science Institute, Yonsei University College of Medicine, Seoul 120-752, Korea. [5]Department of Oral Anatomy and Neurobiology, School of Dentistry, Kyungpook National University, Daegu 700-412, Korea. [6]Department of Anatomy, School of Medicine, Brain Science & Engineering Institute, Kyungpook National University, Daegu 700-412, Korea. [7]Department of Brain and Cognitive Sciences, Seoul National University, Seoul 151-747, Korea. [8]Graduate School of Nanoscience and Technology (World Class University), KAIST, Daejeon 305-701, Korea. [9]Center for Synaptic Brain Dysfunctions, Institute for Basic Science, Daejeon 305-811, Korea.
*These authors contributed equally to this work.

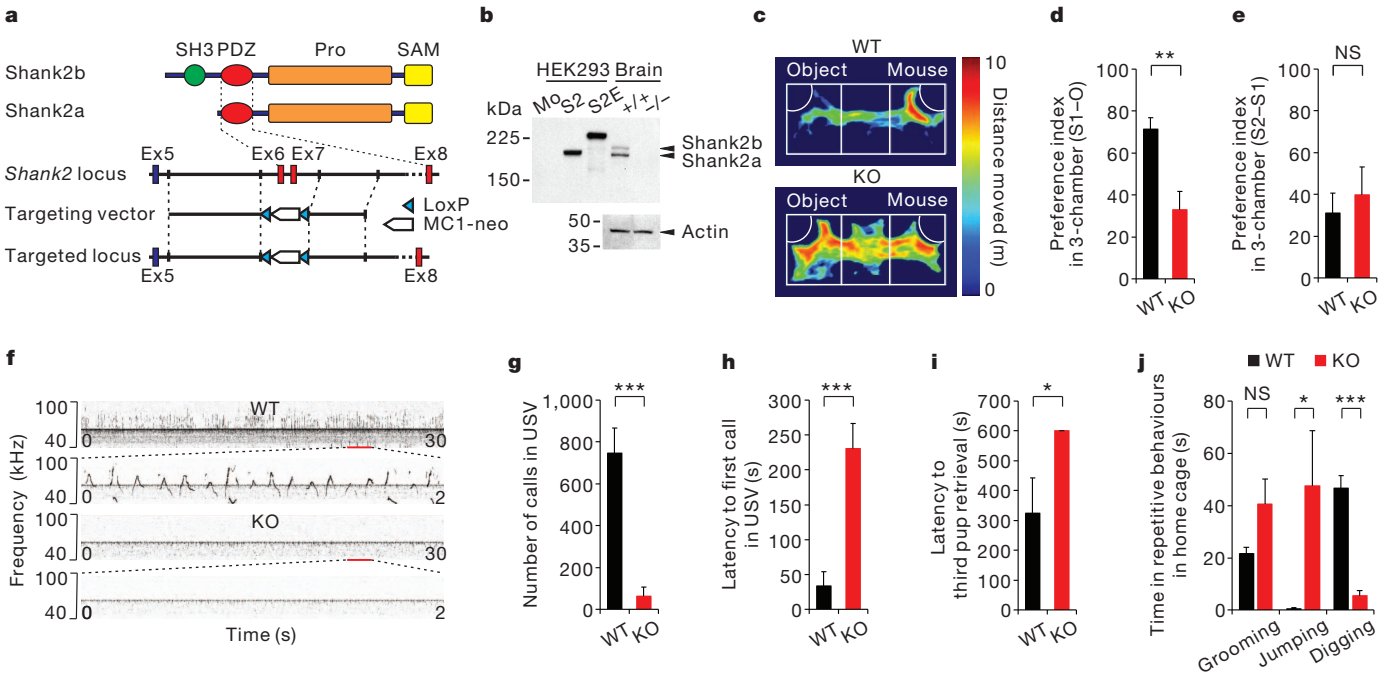

Figure 1 | *Shank2*$^{-/-}$ mice exhibit ASD-like impaired social interaction and social communication, and repetitive jumping. **a**, Targeting of the *Shank2* gene in mice. Ex, exon. **b**, *Shank2*$^{-/-}$ brain lacks expression of both *Shank2a* and *Shank2b* splice variants. Mo, mock; S2, Shank2; S2E, epithelial form of Shank2. **c**–**e**, Impaired social interaction of *Shank2*$^{-/-}$ mice in three-chamber assays. KO, knockout; WT, wild type. **d**, Social preference (object versus stranger 1 (S1–O)). **e**, Social novelty recognition (stranger 1 versus stranger 2 (S2–S1)). $n = 11$ (WT), 10 (KO). **f**–**h**, Impaired social communication by USVs in *Shank2*$^{-/-}$ mice. $n = 10$ (WT), 10 (KO). **i**, Impaired pup retrieval in *Shank2*$^{-/-}$ mice. $n = 5$ (WT), 5 (KO). **j**, Stereotypical behaviours in *Shank2*$^{-/-}$ mice. $n = 11$ (WT), 11 (KO). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, NS, not significant. Data represent mean ± standard error.

repetitive behaviours, or anxiety-like behaviours, reflecting intrinsic differences between humans and mice.

Shank2 is an important regulator of excitatory synaptic structure and function[2–4,19]. *Shank2* deletion, however, had minimal effects on excitatory or inhibitory synapses (Supplementary Fig. 10). In addition, electron microscopy revealed that excitatory synapse number and postsynaptic density morphology were unaltered (Supplementary Fig. 11).

We next measured synaptic transmission at hippocampal Schaffer-collateral–CA1-pyramidal (SC–CA1) synapses. Basal excitatory transmission such as input–output and paired-pulse ratio was unchanged in *Shank2*$^{-/-}$ mice (Fig. 2a, b). In addition, spontaneous transmission and membrane excitability were normal in mutant animals (Supplementary Fig. 12). When synaptic plasticity was tested, long-term potentiation (LTP) induced by high-frequency stimulation or theta-burst stimulation was severely impaired in *Shank2*$^{-/-}$ mice (Fig. 2c and Supplementary Fig. 13). Long-term depression (LTD) induced by low-frequency stimulation was completely abolished also in *Shank2*$^{-/-}$ mice (Fig. 2d). Because LTD induced by low-frequency stimulation activates both NMDARs and mGluRs[20], we isolated mGluR LTD by bath-applying (*RS*)-3,5-dihydroxyphenylglycine (DHPG), an agonist of mGluR5, but found no difference between genotypes (Supplementary Fig. 13). This suggests that the observed reductions in LTP and LTD may be due to NMDAR hypofunction.

We thus measured the NMDA/AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) ratio at *Shank2*$^{-/-}$ SC–CA1 synapses. Indeed, the NMDA/AMPA ratio was reduced relative to wild-type synapses (Fig. 2e). Meanwhile, both the decay kinetics of NMDAR excitatory post-synaptic currents (EPSCs) and GluN2B-mediated EPSCs (GluN2B also known as NR2B or Grin2b) were indistinguishable between genotypes, suggesting that GluN2A- (also known as NR2A or Grin2a) and GluN2B-containing NMDARs were equally affected (Supplementary Fig. 14). Given that AMPA receptor (AMPAR)-mediated transmission is normal (Fig. 2a), these results suggest that NMDAR-mediated transmission is selectively decreased. The NMDA/AMPA ratio in the medial prefrontal cortex, however, was unaltered in *Shank2*$^{-/-}$ mice (Supplementary Fig. 14), suggesting that the reduced NMDA/AMPA ratio is not a change uniformly occurring in all brain regions.

Shank2 deletion may also affect NMDAR-associated signalling that critically regulates various synaptic events including LTP and LTD[21,22]. In immunoblot analyses, phosphorylation but not total levels of CaMKII-α/β (T286), ERK1/2 (p42/44) and p38 were significantly reduced in the *Shank2*$^{-/-}$ brain (Supplementary Fig. 15). A similar decrease was observed in phosphorylation of the AMPAR subunit GluA1 (S831 and S845). There were no changes in phosphorylation of PAK1/3 and mTOR, total levels of glutamate receptors (GluN2A, GluA2 (also known as GluR2 or Gria2) and mGluR1/5 (also known as Grm1/5)), or total levels of synaptic scaffolds and signalling adaptors/proteins directly or indirectly associated with Shank2 including PSD-95 (also known as Dlg4), SAP97 (also known as Dlg1), GKAP (also known as Dlgap1), SynGAP1, Homer1, Arhgef6/7 (also known as α/βPIX), GIT1 and PLC-β3. The increase in GluN1 expression may reflect a compensatory increase. These results suggest that Shank2 deficiency leads to impairments in NMDAR-associated signalling.

Reduced NMDAR function and associated signalling may contribute to ASD-like behaviours in *Shank2*$^{-/-}$ mice. To test this hypothesis directly and restore NMDAR function, we used D-cycloserine, a partial agonist at the glycine-binding site of NMDARs, which has been shown to rescue repetitive grooming in neuroligin-1-deficient mice associated with a reduced NMDA/AMPA ratio[23]. We found that D-cycloserine fully recovered the NMDA/AMPA ratio (Fig. 3a). In addition, D-cycloserine-treated *Shank2*$^{-/-}$ mice showed improved social interaction in three-chamber social interaction assays (Fig. 3b–d and Supplementary Fig. 16).

To explore further the association between reduced NMDAR function and ASD-like behaviours in *Shank2*$^{-/-}$ mice, we used 3-cyano-*N*-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB), a membrane-permeable positive allosteric modulator of mGluR5, which increases the responsiveness of mGluR5 to glutamate and enhances

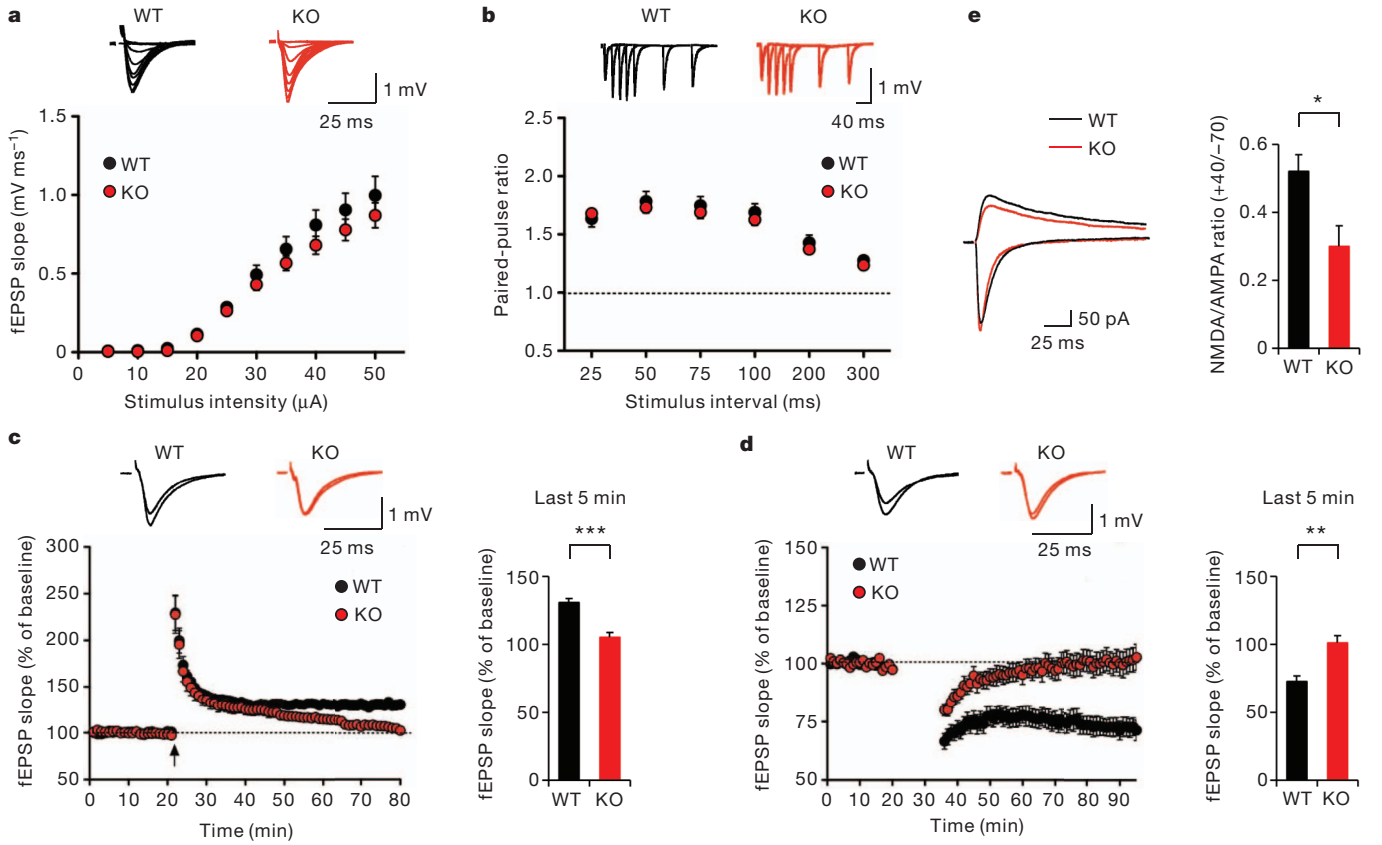

Figure 2 | Impaired NMDAR-dependent synaptic plasticity in *Shank2*$^{-/-}$ mice. **a**, Normal input–output curve at hippocampal SC–CA1 synapses in *Shank2*$^{-/-}$ mice. $n = 7$ (WT), 8 (KO). fEPSP, field excitatory postsynaptic potential. **b**, Normal paired-pulse ratio. $n = 10$ (WT), 8 (KO). **c**, Impaired LTP. $n = 6$ (WT), 8 (KO). **d**, Impaired LTD. $n = 10$ (WT), 9 (KO). **e**, Reduced NMDA/AMPA ratio. $n = 8$ (WT), 8 (KO). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Data represent mean ± standard error.

NMDAR function[17,24,25]. CDPPB has antipsychotic and pro-cognitive activities[17,24,26–28], and facilitates behavioural flexibility[29]. In addition, CDPPB restores reduced excitatory transmission and ERK phosphorylation caused by *Shank3* knockdown[25], and CDPPB and its derivative (VU-29) enhance both LTP and LTD, and spatial learning[27].

Consistent with previous findings, CDPPB also normalized the NMDA/AMPA ratio in *Shank2*$^{-/-}$ brain slices (Fig. 4a). Moreover, CDPPB restored the impaired LTP and LTD at SC–CA1 synapses (Fig. 4b, c), without affecting basal synaptic transmission (Supplementary Fig. 17). Biochemically, CDPPB treatment of *Shank2*$^{-/-}$ mice fully normalized NMDAR signalling in *Shank2*$^{-/-}$ whole brains and also in *Shank2*$^{-/-}$ synaptosomes (Supplementary Figs 18). The smaller extent of signalling deficits in older mice (8 weeks) relative to younger mice (3–4 weeks) may reflect age-dependent reductions in NMDAR-mediated currents and/or compensatory changes in NMDAR signalling.

Behaviourally, *Shank2*$^{-/-}$ mice treated with CDPPB showed substantial recoveries in social interaction to a greater extent than those treated with D-cycloserine, while having no effect on social novelty recognition (Fig. 4d–f and Supplementary Fig. 19). A lower dose of CDPPB did not rescue impaired social interaction (Supplementary Fig. 20), indicative of a dose-dependent action. Notably, CDPPB did not rescue impaired pup retrieval, repeated jumping, anxiety-like behaviours and hyperactivity (Supplementary Fig. 21), suggesting that CDPPB selectively rescues social interaction, but not other behaviours.

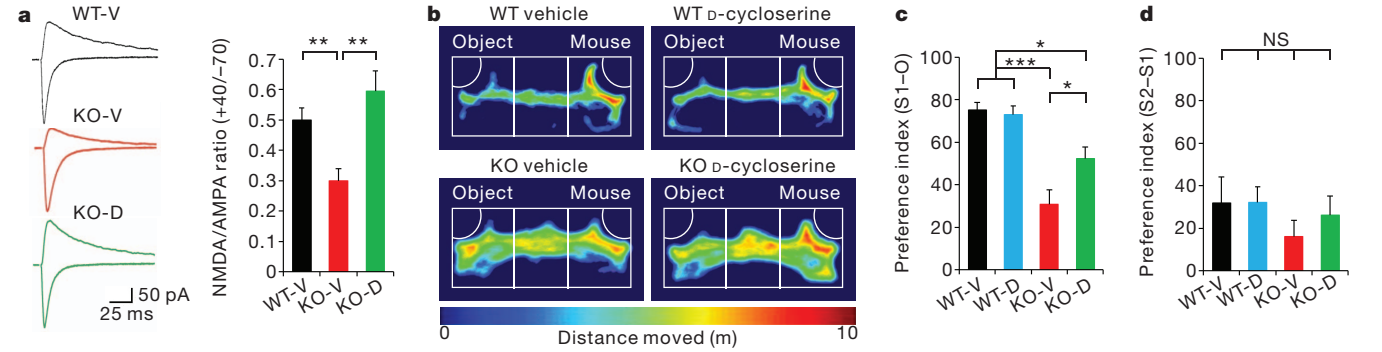

Figure 3 | D-cycloserine normalizes NMDAR function and improves social interaction in *Shank2*$^{-/-}$ mice. **a**, D-cycloserine (20 μM) recovers the reduced NMDA/AMPA ratio. $n = 13$ (wild type, vehicle (WT-V)), 9 (knockout, vehicle (KO-V)), 9 (knockout, D-cycloserine (KO-D)). **b–d**, D-cycloserine (20 mg kg^{-1}) partially normalizes the impaired three-chamber social interaction in *Shank2*$^{-/-}$ mice. $n = 9$ (WT-V), 10 (WT-D), 11 (KO-V), 10 (KO-D). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, NS, not significant. Data represent mean ± standard error.

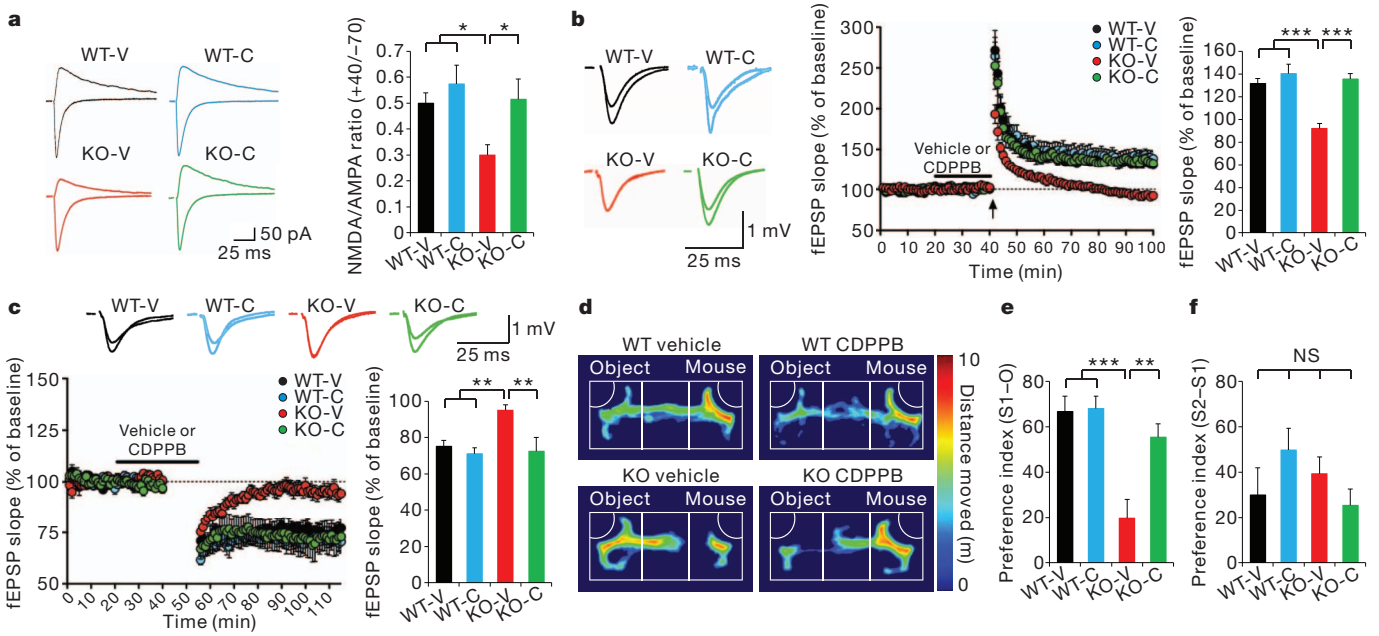

Figure 4 | CDPPB normalizes NMDAR function and substantially improves social interaction in *Shank2*$^{-/-}$ mice. **a**, CDPPB (10 μM) restores the NMDA/AMPA ratio at *Shank2*$^{-/-}$ SC–CA1 synapses. $n = 13$ (wild type, vehicle (WT-V)), 8 (wild type, CDPPB (WT-C)), 9 (knockout, vehicle (KO-V)), 9 (knockout, CDPPB (KO-C)). **b**, **c**, CDPPB (10 μM) recovers impaired LTP and LTD. $n = 5$ (WT-V), 5 (WT-C), 5 (KO-V), 6 (KO-C) for high-frequency stimulation LTP, and $n = 5$ (WT-V), 6 (WT-C), 7 (KO-V), 6 (KO-C) for low-frequency stimulation LTD. **d**–**f**, *Shank2*$^{-/-}$ mice treated with CDPPB (10 mg kg^{-1}) show substantially improved social interaction in three-chamber assays. **e**, **f**, Quantification of the results in **d**. $n = 8$ (WT-V), 8 (WT-C), 9 (KO-V), 9 (KO-C). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, NS, not significant. Data represent mean ± standard error.

These results, together with the D-cycloserine results, suggest that reduced NMDAR function and signalling lead to impaired social interaction in *Shank2*$^{-/-}$ mice, although NMDAR-independent mechanisms may also have a role.

Recently, another line of *Shank2*$^{-/-}$ mice produced by deleting exon 7 has been reported to display multiple phenotypes, including reduced spine number, reduced basal transmission, elevated NMDAR currents and ASD-like behavioural changes[30]. Given that our *Shank2*$^{-/-}$ mice lack both exons 6 and 7, the observed differences in mouse phenotype might reflect the differences in genetic deletions and are in line with the different ASD symptoms observed in humans[5]. In addition, the fact that both reduced and enhanced NMDAR functions lead to the same ASD-like phenotypes in mice suggest that maintaining normal levels of NMDAR function is important.

We have demonstrated that NMDAR function is an important mechanism underlying the development and rescue of ASD-like phenotypes in *Shank2*$^{-/-}$ mice, and that mGluR5 may be a novel target for the treatment of ASD involving altered NMDAR function.

METHODS SUMMARY

Animals and statistical analysis. Numbers, genders and ages of mice used for behavioural and other assays are summarized in Supplementary Table 1. All behavioural and electrophysiological assays were performed and analysed in a blind manner. Statistical analyses were performed using SPSS 12.0 (SPSS) and OriginPro (OriginLab), and details of the results are described in Supplementary Table 2.

Experimental details of mouse generation and characterization by behavioural, electrophysiological, biochemical and immunohistochemical analyses are described in Supplementary Methods.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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