The Nucleosome Assembly Protein TSPYL2 Regulates the Expression of NMDA Receptor Subunits GluN2A and GluN2B

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TSPYL2 is an X-linked gene encoding a nucleosome assembly protein. TSPYL2 interacts with calmodulin-associated serine/threonine kinase, which is implicated in X-linked mental retardation. As nucleosome assembly and chromatin remodeling are important in transcriptional regulation and neuronal function, we addressed the importance of TSPYL2 through analyzing Tspyl2 loss-of-function mice. We detected down-regulation of N-methyl-D-aspartate receptor subunits 2A and 2B (GluN2A and GluN2B) in the mutant hippocampus. Evidence from luciferase reporter assays and chromatin immunoprecipitation supported that TSPYL2 regulated the expression of Grin2a and Grin2b, the genes encoding GluN2A and GluN2B. We also detected an interaction between TSPYL2 and CBP, indicating that TSPYL2 may activate gene expression through binding CBP. In terms of functional outcome, Tspyl2 loss-of-function impaired long-term potentiation at hippocampal Schaffer collateral-CA1 synapses. Moreover, mutant mice showed a deficit in fear learning and memory. We conclude that TSPYL2 contributes to cognitive variability through regulating the expression of Grin2a and Grin2b.

Neurodevelopmental disorders, such as autism, schizophrenia and idiopathic learning disabilities, are more common or severe in males, where one likely cause is the involvement of X-linked genetic factors1,2. Given the heterogeneous nature of these disorders, functional studies using mutant mouse models are important for understanding the role of individual genes. TSPYL2, an X-linked gene that encodes a nucleosome assembly protein (NAP) in neurons, is a good candidate for neurodevelopmental disorders. TSPYL2 is also called CINAP (CASK-interacting NAP) as it binds calmodulin-associated serine/threonine kinase (CASK)3, which when mutated is clearly associated with X-linked mental retardation1,4.

The NAP domain binds histone for nucleosome remodeling, an important process in the regulation of gene expression5. Furthermore, NAPs have been proposed to regulate gene expression through bridging the transcriptional co-activator complex and chromatin6. The NAP superfamily is divided into NAP1-like (NAP1L), suppressor of variegation-enhancer of zeste-trithorax (SET) and Testis-specific protein, Y-encoded-like (TSPYL) families according to sequence homology of the NAP domain6. NAP1L1, NAP1L4 and SET (previously called NAP1, NAP2 and TAF, respectively) interact with histone acetyltransferase p300, a co-activator of gene expression7, p300 and the closely related CREB binding protein (CBP) cooperate with NAP1 to promote nucleosome eviction at the HTLV-1 promoter and transcriptional activation8. In differentiating neurons, NAP1L2 controls the expression of Cdkn1c by promoting histone H3K9/14 acetylation8. By contrast, SET is a subunit of the inhibitory complex of histone acetyltransferases9. SET negatively regulates the transcription of a subset of neuronal markers in neuroblastoma cells9. These studies demonstrate a role of NAPs in histone acetylation and transcriptional regulation.
Tspyl2 is expressed in neurons in multiple brain regions. Hsueh and colleagues (2004) showed that TSPYL2 forms a complex with CASK and T-box brain gene 1 (Tbr-1), a transcription factor essential for cerebral cortex development. In primary hippocampal neurons, TSPYL2 activates the transcription of the Tbr-1 target gene Grin2b. N-methyl-d-aspartate (NMDA) receptors are involved in memory performance and neurodevelopmental disorders, therefore mutations affecting their expression might be expected to result in defects in these processes. However, Hsueh’s group subsequently reported that mice homozygous for a targeted mutation of Tspyl2 exhibit normal levels of Grin2B in various brain regions; and no learning and memory defects as expected for a reduction in NMDA receptor function was detected. We have generated an independent null mutant allele of Tspyl2 (Tspyl2<sup>tm1.Sich</sup> synonym Tspyl2<sup>−/−</sup>) on a different genetic background. In contrast to the earlier study, here we show that TSPYL2 is an important transcriptional regulator of both Grin2a and Grin2b. We also found that Tspyl2 mutant mice indeed exhibit deficits in both long-term potentiation (LTP) and fear-associative learning.

**Results**

**TSPYL2 regulates the levels of GluN2A and GluN2B in hippocampus.** We have previously reported that Tspyl2 is expressed in the cortex and hippocampus of adult mice and that both the size and gross morphology of the Tspyl2 mutant brain are normal. Nissl staining on adult forebrain slices showed normal neuroanatomy in the mutant brain (Fig. 1A). To determine whether the expression of specific glutamate receptors is affected by the Tspyl2 mutation, we examined the protein levels of the key glutamate receptor subunits, including NMDA receptor subunits GluN1, GluN2A, GluN2B, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor subunit GluA1 and metabotropic glutamate receptor subtype mGluR5 in the mutant hippocampus. Western blot analysis and densitometry scans revealed that the levels of GluN2A and GluN2B were reduced significantly in the mutant hippocampus (p < 0.05), whereas the levels of the other glutamate receptors were unaffected (Fig. 1B). To test whether this is due to reduced transcript levels, quantitative RT-PCR was performed and the results indicated that the levels of GluN2A (Grin2a) and GluN2B (Grin2b) mRNA were reduced in the mutant hippocampus (p < 0.05). As expected, the mRNA levels of the other glutamate receptors were similar between the wild-type and mutant. Interestingly, the transcript level of Reln, a Tbr-1 target gene, was also unaffected (Fig. 1C). Since the transcript levels of Grin2a and Grin2b were reduced in the mutants, we wondered whether it was due to reduced transcription or reduced mRNA stability. RNA stability assays were performed by adding actinomycin D to block transcription in primary neuron cultures derived from wild-type and mutant hippocampi. From quantitative RT-PCR, the degradation rates of Grin2a and Grin2b transcripts in the mutant hippocampal neurons were similar to that of the wild-type (Fig. 1D). Together, these data suggest that TSPYL2 is important for Grin2a and Grin2b transcription.

**TSPYL2 activates transcription of Grin2a and Grin2b.** TSPYL2 is a NAP, which is expected to function in the nucleus to regulate the expression of multiple genes. To confirm the nuclear localization of TSPYL2, we transfected cells with plasmids expressing TSPYL2 tagged either with HA at the N-terminus or GFP at the C-terminus. The staining patterns of both forms of tagged-TSPYL2 were the same and the result for HA-TSPYL2 is shown in Fig. 2A. Tagged-TSPYL2 was localized in the nucleus in both the neuroblastoma-glioma fusion cell line NG108-15 and primary hippocampal neurons. TSPYL2 could also be observed in the cytoplasm in primary hippocampal neurons, but not NG108-15, upon prolonged exposure. The data show that TSPYL2 is mainly localized in the nucleus.

From luciferase reporter assays, TSPYL2 was found to activate the Grin2b promoter in primary hippocampal neurons, but to inhibit it

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**Figure 1 | Reduced expression of Grin2a and Grin2b in Tspyl2 mutant hippocampus.** (A), Nissl staining of coronal brain sections from 2 month-old mice. Cellular composition of hippocampal substructures and layer development of cortex were indistinguishable between wild-type and mutant. Scale bars: 100 μm. (B), The protein level of key glutamate receptors in 2-month-old hippocampi was detected by western blot and quantitated by densitometry. Cropped gel images are shown and the gels were run under the same experimental conditions. The protein level was normalized to actin in the wild-type and was set as 1. The protein levels of GluN2A and GluN2B were significantly reduced (n = 4 mice per genotype). (C), Transcript levels of the above genes in 2-month-old hippocampi were detected by quantitative RT-PCR. Relative mRNA level was normalized to Hprt and quantitated by densitometry. Cropped gel images are shown and the gels were run under the same experimental conditions. The protein level was normalized to actin and the wild-type level was set as 1. The protein levels of GluN2A and GluN2B were significantly reduced (n = 4 mice per genotype). Expression of Reln was unchanged. (D), RNA stability of Grin2a and Grin2b was determined by adding 10 μg/ml actinomycin D to hippocampal cultures at 7 days in vitro. Quantitative RT-PCR was done on samples collected at 0, 4, 8, 16 and 24 hr after treatment. No difference between wild-type and mutant neurons was observed (n = 3 samples per genotype). Error bars represent SEM. * P < 0.05, Student’s t-test. Abbreviations: +/-Y, wild-type male; m/Y, mutant male; WT, wild-type.
independent samples). Error bars represent SEM. Promoters in primary hippocampal neurons cultured for 14 days (n = 3 independent experiments). (C), Chromatin immunoprecipitation (ChIP) of Reln promoter in NG108-15 but activated it in hippocampal neurons (n = 3 independent experiments). (C), Chromatin immunoprecipitation (ChIP) of Reln promoter in NG108-15 but activated it in hippocampal neurons (n = 3 independent experiments). (D), Co-localization between HA-TSPYL2 and endogenous p300 or CBP in primary hippocampal neurons by confocal microscopy. Scale bar: 10 μm. (E), Interaction between TSPYL2 and CBP in mammalian-two-hybrid assay. Primary hippocampal neurons were transfected with various plasmids against HA. Both promoters were pulled down by anti-HA antibody in HA-TSPYL2 transfected cells. p21 promoter was used as a negative control. HA-SUN2, a nuclear envelope protein, which should not bind any promoter. In this case the Grin2a and Grin2b promoters could not be detected by ChIP using the HA antibody. These data show that the binding of TSPYL2 to the Grin2a and Grin2b promoters is specific.

NAP1L1, NAP1L4 and SET have been reported to interact with CBP or p300. Therefore we wondered whether TSPYL2 was recruited to the Grin2a and Grin2b promoters through CBP or p300. We found colocalization of immunofluorescence signals for HA-tagged TSPYL2 with endogenous CBP and p300 in primary hippocampal neurons (Fig. 2D). Next, we detected functional interaction between TSPYL2 and CBP in a mammalian two-hybrid assay with primary hippocampal neurons (Fig. 2E). As expected from the histone acetyltransferase activities of p300 and CBP, both GAL4-binding domain (BD)-p300 and BD-CBP activated the reporter activity. Together with the expression plasmid for activation domain (AD)-TSPYL2, the reporter activity doubled for BD-CBP (p < 0.01) but no change was observed for BD-p300 (p = 0.69). Lastly, the importance of TSPYL2 in the assembly of the transcriptional complex in neurons was tested by comparing the binding of p300 and CBP to promoter regions in wild-type and mutant hippocampal neurons. In mutant primary neurons, ChIP analysis revealed significantly reduced binding of p300 (p < 0.05) and CBP (p < 0.001) in the Grin2a and Grin2b promoter, respectively (Fig. 2F). Possibly, TSPYL2 activates transcription by interacting with p300 or CBP, and bridges the transcriptional complex and histones as shown for several other NAPs.

Long-term potentiation is impaired in Tspyl2 mutant neurons. NMDA receptors are important in synaptic function and memory. Using whole-cell patch clamp electrophysiological techniques, the basal excitability of CA1 neurons in both mutant and wild-type mice was examined. The ability of the neurons to fire action potentials in response to current injection as well as the input resistance of the neurons were normal in the mutant (Fig. 3A & B). Furthermore, in hippocampal slices prepared from wild-type and mutant mice, field excitatory postsynaptic potentials (fEPSPs) in the CA1 region were evoked by stimulating the Schaffer collaterals. The basal synaptic transmission, as assessed by the output-input relationship (fEPSP vs stimulation intensity) and the fEPSP to fiber volley, was not affected by the mutation (Fig. 3C). The paired-pulse ratio of the fEPSP also did not change, indicating a lack of effect on short-term synaptic plasticity (Fig. 3D). These
results were consistent with the finding that the expression of the AMPA receptor subunit GluA1 was not affected. To examine potential defects in long term plasticity in Tspyl2 mutant mice, fEPSPs at the hippocampal Schaffer collateral-CA1 synapses were assessed using the conventional high frequency stimulation induction protocol consisting of a 1 second train of 100 Hz stimulation. When compared with wild-type, both male and female mutant mice showed a significant reduction in LTP. The impairment was 18% in male and 30% in female (p < 0.001; Figs. 3E & F). Basal synaptic transmission was compared by the field excitatory post-synaptic potential (fEPSP)-stimulation relationship. No significant differences were observed between the two groups throughout the range of stimulation intensities. There was also no difference between the fEPSP to fiber volley ratio between the two groups (10 slices from 3 +/Y mice, 12 slices from 3 m/Y mice). (D), The paired-pulse ratio of the evoked fEPSP was not affected by the mutation (10 slices from 3 +/Y mice, 12 slices from 3 m/Y mice). (E), LTP of 2-month old male and female mice. FEPSP was measured from the dendritic layer of CA1 neurons in the Schaffer collateral pathway. Period 1 was the baseline fEPSP while period 2 was the fEPSP after induction. Arrow indicated the electrical stimulation (1 train, 100 Hz, 1 s). Representative recorded potentials were shown on the top. (F), Quantitative change in the average amplitude of the fEPSP taken from 50 to 60 min after induction. LTP was impaired in the mutant (n = 12 for male, n = 9 for female). Error bars represent SEM. ***p < 0.001, Student’s t-test. Abbreviations: AP, Action potential; +/Y, wild-type male; m/Y, mutant male; +/+ , wild-type female; +/m, heterozygous female; m/m, mutant female.

TSPYL2 is important in fear-associative learning. As the molecular defect in the Tspyl2 mutant neuron leads to impaired LTP, we searched for learning deficits in the mutant mouse. Our mutant mice are on a 129/SvEv background, which are good learners for learning and memory tests. We therefore performed Morris water maze tests to assess spatial memory, and contextual fear conditioning tests to assess fear-associative memory. Our analysis demonstrated that Tspyl2 mutant mice have normal vision and swim speed during visual tests (Fig. 4A), and behaved similarly to wild-type in our Morris water maze settings for reference memory and probe tests (Fig. 4B, C). Both genotypes learned with the escape latency decreased significantly across trials in the reference memory test (Days: F2,24 = 38.46, p < 0.001; genotype: F1,24 = 1.06, p = 0.323 by repeated measures ANOVA) and increased

Figure 3 | Tspyl2 mutation impairs long term potentiation (LTP). (A), Membrane excitability of hippocampal CA1 neurons was measured by whole-cell patch-clamp recording in 2-month old mice. Series of current steps between −160 pA and +160 pA (−55 mV) in 20 pA increments were applied. No difference was observed between wild-type and mutant in action potential (n = 4 each). Three representative recorded action potentials (40, 100, 160 pA) were shown on the right. (B), Input resistance of hippocampal CA1 neurons was measured by whole-cell patch-clamp recording in 2-month old mice at a current of −60 mV. No significant difference was observed between wild-type and mutant (n = 43 for +/Y, n = 34 for m/Y). (C), Basal synaptic transmission was compared by the field excitatory post-synaptic potential (fEPSP)-stimulation relationship. No significant differences were observed between the two groups throughout the range of stimulation intensities. There was also no difference between the fEPSP to fiber volley ratio between the two groups (10 slices from 3 +/Y mice, 12 slices from 3 m/Y mice). (D), The paired-pulse ratio of the evoked fEPSP was not affected by the mutation (10 slices from 3 +/Y mice, 12 slices from 3 m/Y mice). (E), LTP of 2-month old male and female mice. FEPSP was measured from the dendritic layer of CA1 neurons in the Schaffer collateral pathway. Period 1 was the baseline fEPSP while period 2 was the fEPSP after induction. Arrow indicated the electrical stimulation (1 train, 100 Hz, 1 s). Representative recorded potentials were shown on the top. (F), Quantitative change in the average amplitude of the fEPSP taken from 50 to 60 min after induction. LTP was impaired in the mutant (n = 12 for male, n = 9 for female). Error bars represent SEM. ***p < 0.001, Student’s t-test. Abbreviations: AP, Action potential; +/Y, wild-type male; m/Y, mutant male; +/+ , wild-type female; +/m, heterozygous female; m/m, mutant female.
time spent in the target quadrant in the probe test. To be eligible for fear conditioning tests, mutant mice were first examined and shown to have normal pain sensation (Fig. 4D). In addition, exploratory activity during habituation was not significantly different between genotypes (Genotype: F(1, 242) = 1.95, p = 0.176; time: F(11, 242) = 3.76, p < 0.0001; interaction: F(11, 242) = 2.42, p < 0.05 by repeated measures ANOVA, Fig. 4E, left). Noticeably, Tspy2 mutant mice exhibited significantly impaired contextual fear conditioning (p < 0.05, Student’s t-test, Fig. 4E, right), indicating a deficit in fear-associative memory.

Discussion

TSPYL2 is within the chromosome region linked to neurodevelopmental syndromes\(^1\). In this study, we investigated whether TSPYL2 plays a role in cognitive function through transcriptional regulation of neuron-specific genes. We examined the expression of glutamate receptors in the hippocampus, a key brain area involved in learning and memory\(^1\), in Tspy2 mutant mice and found a reduction in the expression of genes encoding the GluN2A and GluN2B subunits in the mutant hippocampus. Furthermore, ChIP analysis indicated that Grin2a and Grin2b promoters are indeed targets of TSPYL2. Our data illustrate the importance of TSPYL2 in the transcriptional regulation of both GluN2A and GluN2B subunits, and suggest a role for TSPYL2 in learning and memory.

NMDDA receptors play a critical role in some forms of synaptic plasticity and learning\(^1\). Mice with loss of GluN2A\(^2\) or GluN2B\(^3\) show impaired hippocampal LTP, and impaired spatial memory in the Morris water maze. In our Tspy2 mutant mice, which show reduced expression of both GluN2A and GluN2B instead of total loss of either one receptor subunit, we did not identify spatial memory defects in a conventional water maze paradigm, but we did observe disrupted contextual fear conditioning. Other tests of spatial learning with different sensorimotor and motivational demands, such as appetitive motivated maze tasks, may reveal deficits in mutant mice having normal water maze performance\(^4,5\). For the fear conditioning task, it is dependent on amygdala-hippocampal function\(^6,7\). We attribute the deficits in our mice at least partly to disrupted NMDA signaling in the hippocampus because this is important in the process of fear conditioning\(^8,9\). Recently, de novo mutations in GRIN2A and GRIN2B affecting protein functions were identified in individuals with mental retardation\(^10\) and autism spectrum disorders\(^11\). Reduction or loss of function of GluN2A and GluN2B, together with other genetic factors, is likely to cause variable neurodevelopmental phenotypes.

How does TSPYL2 activate the transcription of both Grin2a and Grin2b in the hippocampus? Previously it has been shown that TSPYL2 forms a complex with Tbr-1 through interaction with CASK, and activates the Grin2b promoter\(^1\). Our finding of TSPYL2 regulating the transcription of Grin2a is novel. As there are multiple reports of direct interactions between NAPs and CBP or p300\(^12,13\), recruitment of TSPYL2 to the various promoters by transcription regulators such as CASK, p300 and CBP may be a general mechanism. In return, TSPYL2 will help to anchor the transcriptional complex to chromatin through its binding to histone. In future, the...
availability of antibodies for specific immunoprecipitation of TSPYL2 will allow us to further confirm the binding of TSPYL2 to the promoters of Grin2a and Grin2b in specific brain regions, as well as to identify other in vivo gene targets by ChIP-sequencing. In addition, insights into how TSPYL2 regulates the transcription of the Grin2a promoter can be gained by identifying other interacting proteins of TSPYL2, including transcription factors and transcription regulators.

CBP interacts with over 400 transcription factors and its importance for memory formation is well established. A recent study shows that p300 is required for recognition memory and contextual fear memory. Interestingly, we found co-localization of HAACTTTCCCAGGGCTCTCT; GGTTTGGACTCAT TGGGAGCT; Grin2a; CTCACGACAGGGGCTCTCA; GGGTTGGAACCTTATCA; CAGTCTGAATGGC TDYPACT; Grial1; ACGTCTTCCACAGGGCTCTCTG; ACACCGCTAAG TTCAGGACCTCTCT; Grm5; GAGACCTTGACAGTTGTGTA; GATCTTCTCTTGC TGCCAC; Rehn; GAAAGCTTCACAGGGCTCTCTG; ATGCTTATAGGA GACACC; Hprt; AATCTGGAAAGAAGTCTTGTAGTG; TCAATATCCAA AAGTCCTGGC; Primer for promoters: Grin2a; CCGAGAGGCTGTGTTTACGCA; GGAAAAAGGCC GACCTAGG; Grin2b; CACACACGTGCTCTTTGTTTGC; GTCCTGCTGTGG ATTGCGGCTT; p21: AGTCCCTGGTGGGAGGACTAGC; CCACCTGGACAGTA GCAGCC.

Antibodies. The sources of antibodies were: actin (A5060), GluN1 (G9813), GluN2A (M264), HA (H3663 for immunocytochemistry) from Sigma; GluLa1(L8441), mGluR5 (K651) from bioworld; GluN2B (606-609) from Upstate; CBP (sc-369), HA (sc-805 for ChIP) and p300 (sc-585) from Santa Cruz. Plasmids. A cDNA clone containing full length human TSPYL2 was obtained by library screening using a partial fragment isolated previously. The entire coding region of TSPYL2 (493 amino acids) was PCR-amplified using forward and reverse primers, 5′-ACGGAAATTTCTTGGCCACCCGGCATAGG-3′ and 5′-AACGCT GGAAATCTGGTACCGGTCGAGGC-3′. The purified PCR product was cloned into pT-Adv (Clontech), then subcloned into EcoRI/Sall sites of pEGFP-N1 (Clontech) and a modified pcDNA3 vector (Invitrogen) with the HA tag inserted. All plasmids were verified by sequencing. Plasmid of HA-SUN2 was cloned as described previously. Firefly reporter plasmids Grin2a-1253 containing 1253 bp upstream of translation start was kindly provided by Dr. Andres Buonanno; and Reln-514 containing 514 bp upstream of transcription start site by Dr. Dennis R. Grayson. Plasmids for mammalian two-hybrid assay (pG4-p300, pG4-CBP) were gifts from Dr. Neil D. Perkins; and Reln-514 containing 514 bp upstream of transcription start site by Dr. Dennis R. Grayson.

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Quantitative RT-PCR. RNA was extracted using Trizol solution (Invitrogen). Two μg of total RNA was reverse transcribed in 20 μl with Oligo(dT). Quantitative PCR was done with 0.5 μl of cDNA by using Quantifast SYBR green PCR kit (Qiagen) in 9700 HT Fast Real-Time PCR System (Applied Biosystems). For RNA stability assay, hippocampal neurons collected from littermate embryos were cultured for 7 days. Neurons were then incubated with 10 μg/ml actinomycin D (Sigma) to inhibit transcription. RNA samples were collected at 0, 4, 8, 16 and 24 hr after treatment. Immunocytochemistry. Cells grown on coverslips coated with gelatin or poly-L-lysine were washed with PBS and fixed in 4% paraformaldehyde for 15 min. Cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min. Standard staining procedures were used. Images were acquired with epifluorescence microscope (Carl Zeiss, Axiosplan 2 imaging) or LSM710 Meta laser scanning confocal microscope (Carl Zeiss). The setting of the confocal microscope was 40X oil objective lens; numerical aperture 1.4.
Luciferase assay and mammalian two-hybrid assay. Expression plasmids were co-transfected with the firefly reporter plasmid (Grin2a-1253 or Ren-514) and TK-Renilla reporter pRL-TK into NG108-15 cells (in 24-well plates) or hippocampal neurons at 6 days in culture (in 12-well plates) using Lipofectamine 2000 (Invitrogen). Lysates were prepared 24–48 hr after transfection and measured with Dual-Glo Luciferase assay system (Promega). Firefly luciferase activities were standardized to the corresponding Renilla luciferase activities. For the mammalian two-hybrid assay, plasmid concentrations were optimized to give ~1.1 molar ratio of AD-TSPYL2 and interacting proteins to be tested. Primary hippocampal neurons were cultured for 6 days in 12-well plates and transfected by calcium phosphate precipitation with a total of 2 μg of plasmids containing 200 ng pRL-TK, 800 ng pG5-luc, 300 ng of AD-TSPYL2 together with pGBK-T7-CBP. Assays were carried out 24 hours after transfection using Dual Luciferase assay system (Promega). Experiments were performed in triplicate and n = the number of independent experiments.

Chromatin immunoprecipitation. Cells were grown on 10 cm dishes. For primary neurons, they were collected from the whole litter of wild-type or mutant embryos obtained on the same day and cultured for 14 days in parallel. Cells were cross-linked by 1% formaldehyde for 10 min and harvested in lysis buffers with protease inhibitors and sonicated (Soniprep 150, MSE). An aliquot of chromatin from 1 million cells was used for each IP, washed five times with RIPA buffer and once with TE buffer. Standard protocols were then followed for DNA extraction and qPCR®. Promoter regions −1002 to −917 of Grin2a, and −291 to −132 of Grin2b were amplified.

Electrophysiology. Two-month old littermate animals were employed and the detailed procedures were described previously54. Briefly, 250 μm thick parasagittal sections were maintained at 34°C on whole-cell patch-clamp recordings. Pipettes with low KCl internal solution was used to record the electrophysiological properties of hippocampal CA1 neurons. The holding current was adjusted until the membrane potential was held at −70 mV or −50 mV. For characterization of the membrane excitability and properties of the action potentials, a series of current steps between −160 pA and +160 pA in 20 pA increments were applied. To study the basal synaptic transmission and E-LTP, 300 μm hippocampal slices were prepared. To increase the efficiency and to minimize variations in the results arising from differences in incubation times, a maximum of four slices were studied simultaneously. The slices were placed on probes fabricated with 8 × 8 electrode arrays. EPSPs were recorded from the dentritic layer of CA1 neurons by choosing an electrode in the Schaffer collateral pathway as the stimulating electrode. Since there was no difference in the size of fiber volley evoked by various stimulation currents between the wild-type and the mutant, the basal synaptic transmission was compared by assessing the fEPSP-stimulus current relationship and also the fEPSP to fiber volley ratio. The ratio with an fEPSP of 50 ms was compared. For the LTP, based on the stimulus-response curve, we chose a stimulation intensity that evoked fEPSP with a magnitude of 30–40% of the maximum response (around 1 mV in most cases). After allowing a stable baseline of 30 min, 1 train of 100 Hz stimulus that lasted for 1 s was applied and the field potential response for 1 hr after the tetanus was recorded. LTP was quantified as % change in the average amplitude of fEPSPs taken from 50 to 60 min interval after the induction, compared with that of baseline.

Morris water maze. Two to three months old male littermate mice were subject to a reverse dark cycle and dark prior to tests which were performed in the dark cycle. Mutant mice were confirmed to be of normal vision and swimming ability in the visual test, where a platform was put in clear water in standard water maze settings. Two-month old littermate animals were employed and the detailed procedures were described previously54. Two- to three-month-old male C57BL6J mice weighing 25–28 g were used. Food and water were available ad libitum. The water was changed daily to remove any sediments. The mice were acclimated for 1 week in the test environment before commencing the behavioral testing. The water was at 20°C. A systematic, large-scale resequencing screen of X-chromosome regions 291 to 132 of Grin2b were amplified. A linkage study of schizophrenia to markers within Xp11 near the MAOB gene. Psychol Med 29, 406–419 (1996). A within-subjects, within-task demonstration of intact spatial reference memory and placebo effect in humans. J. Neurosci., 217–222 (2003). A systemic, large-scale resequencing screen of X-chromosome regions 291 to 132 of Grin2b were amplified. A linkage study of schizophrenia to markers within Xp11 near the MAOB gene. Psychol Med 29, 406–419 (1996). A within-subjects, within-task demonstration of intact spatial reference memory and placebo effect in humans. J. Neurosci., 217–222 (2003). A within-subjects, within-task demonstration of intact spatial reference memory and placebo effect in humans. J. Neurosci., 217–222 (2003). A within-subjects, within-task demonstration of intact spatial reference memory and placebo effect in humans. J. Neurosci., 217–222 (2003). A within-subjects, within-task demonstration of intact spatial reference memory and placebo effect in humans. J. Neurosci., 217–222 (2003). A within-subjects, within-task demonstration of intact spatial reference memory and placebo effect in humans. J. Neurosci., 217–222 (2003). A within-subjects, within-task demonstration of intact spatial reference memory and placebo effect in humans. J. Neurosci., 217–222 (2003).
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Author contributions
S.Y.C., W.H.Y., G.M., Y.S.C. designed the experiments and provided essential reagents and materials, K.H.T., S.K.L., Q.L., H.L., P.H.S.M., C.C.P.N. prepared samples, performed the experiments and prepared figures, S.Y.C., K.H.T., W.H.Y., G.M. wrote the main manuscript text. All authors reviewed the manuscript.

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