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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 factors.

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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 factors. 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), which when mutated is clearly associated with X-linked mental retardation.

The NAP domain binds histone for nucleosome remodeling, an important process in the regulation of gene expression. Furthermore, NAPs have been proposed to regulate gene expression through bridging the transcriptional co-activator complex and chromatin. 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 domain. NAP1L1, NAP1L4 and SET (previously called NAP1, NAP2 and TAF, respectively) interact with histone acetyltransferase p300, a co-activator of gene expression. p300 and the closely related CREB binding protein (CBP) cooperate with NAP1 to promote nucleosome eviction at the HTLV-1 promoter and transcriptional activation. In differentiating neurons, NAP1L2 controls the expression of Cdkn1c by promoting histone H3K9/14 acetylation. By contrast, SET is a subunit of the inhibitory complex of histone acetyltransferases. SET negatively regulates the transcription of a subset of neuronal markers in neuroblastoma cells. These studies demonstrate a role of NAPs in histone acetylation and transcriptional regulation.
Tsyl2 is expressed in neurons in multiple brain regions13. 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 performance13-15 and neurodevelopmental disorders16, 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 *Tsyl2* exhibit normal levels of GluN2B in various brain regions; and no learning and memory defects as expected for a reduction in NMDA receptor function was detected17. We have generated an independent null mutant allele of *Tsyl2* (Tsyl2<sub>mut, rich</sub>, synonym *Tsyl2*<sub>mut</sub>) on a different genetic background18. 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 *Tsyl2* 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 *Tsyl2* is expressed in the cortex and hippocampus of adult mice and that both the size and gross morphology of the *Tsyl2* mutant brain are normal18. 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 *Tsyl2* 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<sub>4</sub>, 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.
in Neuro-2A neuroblastoma cells. To investigate whether TSPYL2 also regulates the expression of Grin2a, we performed luciferase reporter assays using the Grin2a promoter. In NG-108-15, the activity of the Grin2a promoter was 10 fold that of the pGL3-basic vector. Co-transfection with an HA-TSPYL2 expression plasmid inhibited the activity of the Grin2a promoter by about 0.3 fold compared to the control with co-transfection of pcDNA3 (p < 0.05). In contrast, with primary neurons, the activity of the Grin2a promoter was 3 fold that of the pGL3-basic vector, while co-transfection with the HA-TSPYL2 expression plasmid enhanced the promoter activity of Grin2a by about 8.6 fold when compared to the control with co-transfection of pcDNA3 (p < 0.05). As a negative control, the activity of the Reln promoter was 200 and 5 fold that of pGL3-basic vector in NG108-15 and primary hippocampal neurons, respectively. Transfection of HA-TSPYL2 had no significant effect on the activity of the Reln promoter (Fig. 2B).

Next, we tested whether TSPYL2 was tethered to the native Grin2a and Grin2b promoters by using chromatin immunoprecipitation (ChIP) with cross-linking. We transfected NG108-15 cells with HA-tagged TSPYL2, and performed ChIP using IP-grade antibodies against HA. Both Grin2a and Grin2b promoters, but not the negative control p21 promoter, were pulled down by the HA antibody (Fig. 2C). As a negative control, cells were transfected with HA-tagged 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.

NAPIL1, NAPIL4 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. 3 A & 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). The normal membrane excitability and basal synaptic transmission in the mutant hippocampus implicated that the impairment in LTP was not due to defects in transmitter release machinery or fast AMPA receptors, but were in line with postsynaptic problems such as NMDA receptor malfunction.

**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: $F_{2,24} = 38.46$, $p < 0.001$; genotype: $F_{1,24} = 1.06$, $p = 0.323$ by repeated measures ANOVA) and increased...
Figure 4 | Normal spatial memory but impaired fear-associative memory in Tspyl2 mutant mice. (A–C), Tspyl2 mutant mice perform normally in Morris water maze (n = 7 per genotype). (A), Visual test was performed in clear water. There was no significant difference between genotypes in swim speed (p = 0.160) and latency (p = 0.613) to find the platform. (B), Spatial reference memory was tested with a fixed, hidden platform. Mutant mice learned normally. (C), Probe test was done by removing the platform after the spatial reference memory test. Wild-type and mutant mice spend similar time in the target quadrant. (D), Normal pain sensation in hot plate test (n = +/Y = 15, m/Y = 5) as one of the prerequisites for fear conditioning test. (E), Results of fear conditioning tests. Mutant and wild-type mice did not differ significantly in exploratory activities during the 6 min habituation on day 1 (left). The freezing rate during habituation was shown as basal. After conditioned stimulus-unconditioned stimulus training (shock) on day 1, the mice were tested in a novel chamber with the same tone presented (cue) on day 2 and in the training chamber without tone stimulus (context) on day 3. As reflected by freezing behavior, mutant mice showed significantly impaired contextual fear conditioning (Error bars represent SEM, *p < 0.05, Student’s t-test, n = 12 per group). Abbreviations: +/Y, wild-type male; m/Y, mutant male.

Discussion

TSPYL2 is within the chromosome region linked to neurodevelopmental syndromes21–23. 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 memory24, in Tspyl2 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.

NMDA receptors play a critical role in some forms of synaptic plasticity and learning.4 Mice with loss of GluN2A25 or GluN2B26 show impaired hippocampal LTP, and impaired spatial memory in the Morris water maze. In our Tspyl2 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 performance27,28. For the fear conditioning task, it is dependent on amygdala-hippocampal function29,30. We attribute the defects in our mice at least partly to disrupted NMDA signaling in the hippocampus because this is important in the process of fear conditioning.31 Recently, de novo mutations in GRIN2A and GRIN2B affecting protein functions were identified in individuals with mental retardation32 and autism spectrum disorders33. 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. Our finding of TSPYL2 regulating the transcription of Grin2a is novel. As there are multiple reports of direct interactions between NAPs and CBP or p300–4,5,33, 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-seqencing. 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 established1,12. A recent study shows that p300 is required for recognition memory and contextual fear memory13. Interestingly, we found co-localization of HA-TSPYL2 with CBP and p300 in primary hippocampal neurons and we have confirmed this result in various cell lines. Our data from mammalian-two-hybrid assays further suggested functional interaction between TSPYL2 and CBP. However, we cannot find evidence for an interaction between p300 and TSPYL2 even though they co-localized. One possibility is the transient or weak nature of their interaction. To address this we investigated the datasets from a proteomic study designed to detect weak protein interactions by utilizing high levels of reciprocal in 3290 immunoprecipitations with transcriptional coregulators in cell lines. TSPYL1 is found to form a stable complex with TSPYL2, and this complex interacts with p300. On the other hand, multiple immunoprecipitations with CBP or p300 antibodies did not reveal a steady-state stoichiometric partner40. Taken together, we propose that TSPYL2 interacts with CBP and p300 to regulate transcription in neurons.

The phenotypic outcome of a mutation can be modified by other genes and varies between genetic backgrounds11,12. With another Tspyl2 targeted mutation in mice on a C57/BL6 background, both the level of GluN2B in various brain regions and fear-conditioning were reported to be normal while locomotor activities are increased15. We only observed a marginal increase in locomotion in terms of mean speed (p = 0.160) and exploration (p = 0.176) in our mutant mice. The targeted allele in both studies is likely to be a null allele. The full-length TSPYL2 protein was eliminated and there was no abnormal protein being detected in theirs17 or in our mutant mouse brain (Supplementary Fig. 1). Differences in phenotype among inbred strains are not unexpected due to the polymorphisms in promoter sequences. In this case, the polymorphism in the promoters of TSPYL2 will allow us to further confirm the binding of TSPYL2 to a steady-state stoichiometric partner40. Taken together, we propose that TSPYL2 interacts with CBP and p300 to regulate transcription in neurons.

Methods

Animals. Tspo2 mutant mice were maintained in a pure 129SvEv background14,15. Nissl staining was performed on coronal brain sections collected from perfusion-fixed animals. Mouse experiments were approved by the Committee on the Use of Live Animals in Teaching and Research at the University of Hong Kong (Approval no. 1643-08 and 2612-11). All experiments were performed in accordance with the relevant guidelines and regulations of the Laboratory Animal Unit at the University of Hong Kong, which has full accreditation with Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Cell culture. Primary hippocampal neurons were isolated from 16.5 days embryos and seeded in Neurobasal Medium (Invitrogen) supplemented with B27 (Invitrogen), 1% FBS and penicillin/streptomycin on poly-1-lysine coated culture dishes. The seeding cell density was 600 cells/mm2 for Grin2a and 50 cells/mm2 for luciferase assay. NG108-15 cells were cultured in DEMEM supplemented with 10% FBS.

Primers. The sequences of forward and reverse primers (5’ to 3’) were as follows:

Grin1: 5’-CGGAAACCTGCGAGGTAGA; GGCTGTCTTTGGAGACCCT-3’
Grin2a: 5’-CATCAGCAGGCGGCTACCA; GGGTGGAGACTCAT-3’
Grin2b: 5’-CTACATCGAGAGTGTACTAC; CAGTCGTGAATGGC-3’
Grin3: 5’-ACATTTCCCCAGGGCTTGCTTG; AACCGTCTAGGTTAAGGACCTCT-3’
Gccag: 5’-GAGGACTTGAAGGGTGTA; GATCTCTTTCTGC-3’

Antibodies. The sources of antibodies were: actin (A5060), GluN1 (G8913), GluN2A (M268), HA (H3663 for immunocytochemistry) from Sigma; GluA1(L844), nGluR5 (K560), nGluR6 (from both worlds); GluN2B (60-600) 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 previously46. The purified coding region of TSPYL2 (893 amino acids) was PCR-amplified using forward and reverse primers 5’-ACGGAATTTGTAGCACCCTCAGTGGG-3’ and 5’-ACGGTCGAAATCCTGGTTTTCCCCCTCC-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 previously46. Firefly reporter plasmids Grin2a-1253 containing 1253 bp upstream of transcription start site was kindly provided by Dr. Andres Buonanno49; and Reln-514 containing 514 bp upstream of transcription start site was kindly provided by Dr. Dennis R. Grayson50. Primer for promoters:

Grin2a: 5’-CGGAGGAGCGGTCATCAGCA; GGCAACAGGCC-3’
Grin2b: 5’-CACACCTCGGCTCTTGTTCTGTTGCGTCTTGCCTTGTC-3’

Antibodies: actin (A5060), GluN1 (G8913), GluN2A (M268), HA (H3663 for immunocytochemistry) from Sigma; GluA1(L844), nGluR5 (K560), nGluR6 (from both worlds); GluN2B (60-600) from Upstate; CBP (sc-369), HA (sc-805 for ChIP) and p300 (sc-585) from Santa Cruz.

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Immunoblotting. Hippocampi were collected from 2-month old male littermate mice in ice-cold HBSS (Sigma). Tissue was lysed with a dounce homogenizer in RIPA buffer supplemented with complete protease inhibitors (Roche) and 100 µM MG132 (Sigma). Proteins (50 µg) were resolved and detected with standard immunoblotting procedures and ECL reagents (Millipore). Quantitation of the protein bands was done with software from GeneTools (Syngene).

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 QuantFast 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. Confocal images were collected on a 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-2325 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 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 pc5- luc, 300 ng of AD-TSPYL2 and 500 ng of pCDNA3 +4-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 previously [1]. Briefly, 250 μm thick parasagittal sections of the brain were maintained at 34 °C in whole cell patch-clamp recordings. Pipette medium 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. FEPSPs were recorded from the dendritic 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. FEPSP amplitude ratio with the stimulus strength set at 100 Hz stimulation was 128–141 (2011). For the LTP, based on the stimulus-response curve, we chose a stimulation protocol of 100 Hz stimulus that lasted for 1 s was applied and the field potential compared. For the LTP, based on the stimulus-response curve, we chose a stimulation protocol of 100 Hz stimulus that lasted for 1 s was applied and the field potential compared.

Data analysis. Data were analyzed by repeated-measure ANOVA or two-sided paired Student’s t-test. P < 0.05 was considered statistically significant.


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