

Retrieval-Driven Hippocampal NPTX2 Plasticity Facilitates the Extinction of Cocaine-Associated Context Memory

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ABSTRACT

BACKGROUND: Postretrieval extinction attenuates the pathological memory associated with psychiatric states such as drug addiction in both humans and rodents. The extinction of a learned response requires gene transcription and protein synthesis after memory retrieval in a time-dependent manner, yet the precise physiological basis after retrieval to allow extinction to neutralize a learned behavior is not fully understood.

METHODS: In a cocaine conditioned place preference paradigm, we used a ribosomal tagging strategy to measure the translational state of hippocampal pyramidal neurons after the retrieval of cocaine-associated context memory. Using approaches of electrophysiology, neuronal tracing, and a doxycycline-dependent robust activity marking system, we investigated the cellular and molecular basis of retrieval-induced plasticity that facilitated the extinction.

RESULTS: Bioinformatics analysis discovered the specific translational regulation of signaling pathways by retrieval and revealed *Nptx2* as the hub gene. Manipulating *Nptx2* in dorsal hippocampus bidirectionally regulated the extinction of cocaine-associated context memory as well as the retrieval-driven synaptic remodeling. The pentraxin (PTX) domain of NPTX2 recruited GluA1-AMPA receptors and enhanced the extinction and excitatory synaptic transmission that was prevented by overexpressing carboxyl cytoplasmic tail of GluA1. Furthermore, *Nptx2* in retrieval-activated neurons was required for the extinction.

CONCLUSIONS: The retrieval-driven upregulation of *Nptx2* contributes to the synaptic remodeling in dorsal hippocampus and facilitates the extinction of cocaine-associated context memory, indicating a potential target for the treatment of cue-induced cocaine seeking.

Keywords: Cocaine, Dorsal hippocampus, Extinction, GluA1, NPTX2, Retrieval

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Drug addiction is considered a disorder of aberrant learning and memory (1). The environmental cues, strongly associated with the rewarding effects of the drug, trigger craving and relapse (2). Immediately after retrieval by associative cues, the consolidated memory destabilizes and requires gene expression to be restabilized (3), which is a cascade of events that ultimately lead to changes in neuronal plasticity and mediate memory updating (4). The bidirectional and adaptive nature of reconsolidation provides therapeutic opportunity to update the pathological memories (5). Repeated retrieval by the cue without reward or punish stimulant reduces the behavioral response through a process called extinction (6). Increasing evidence has implicated that neural circuitry and molecular pathways for extinction are distinct from those required for initial learning (7–10). However, the molecular basis of the reconsolidation-based therapies leading to a loss of cue-induced response in the long term remains largely unknown.

Specific synaptic connections within hippocampus are required for acquisition, storing, and retrieving of contextual memories (11). Long-lasting changes in synaptic efficacy induced by gene transcription, protein synthesis, and the

changed strength of hippocampal synapses through AMPA receptor (AMPA) turnover are believed to be critical for learning and memory (12,13). Research studies on memories have identified regulators in experience- and activity-dependent changes of synaptic transmission in CA3–CA1 synapses (14). A recently identified member of the Shisa family, *Shisa7*, is important for hippocampal glutamatergic synaptic plasticity as well as contextual memory (15). *Noelin1*, expressing a secreted glycoprotein at synapses in an activity-dependent manner, reduces lateral mobility of both synaptic and extrasynaptic AMPARs and affects short-term plasticity (16). Synaptotagmin-3, an integral membrane protein of synaptic vesicles that is not required for learning, mediates AMPAR internalization in an activity-induced way to weaken synapse strength and mediate forgetting (17).

Detecting and analyzing the gene translation following memory retrieval opens the possibility to study this process in the context of associative memories. To explore which genes are controlled and how they are regulated in hippocampus during reconsolidation to allow extinction to neutralize learned behavior driven by cocaine conditioning, we used *CamkIIα*-

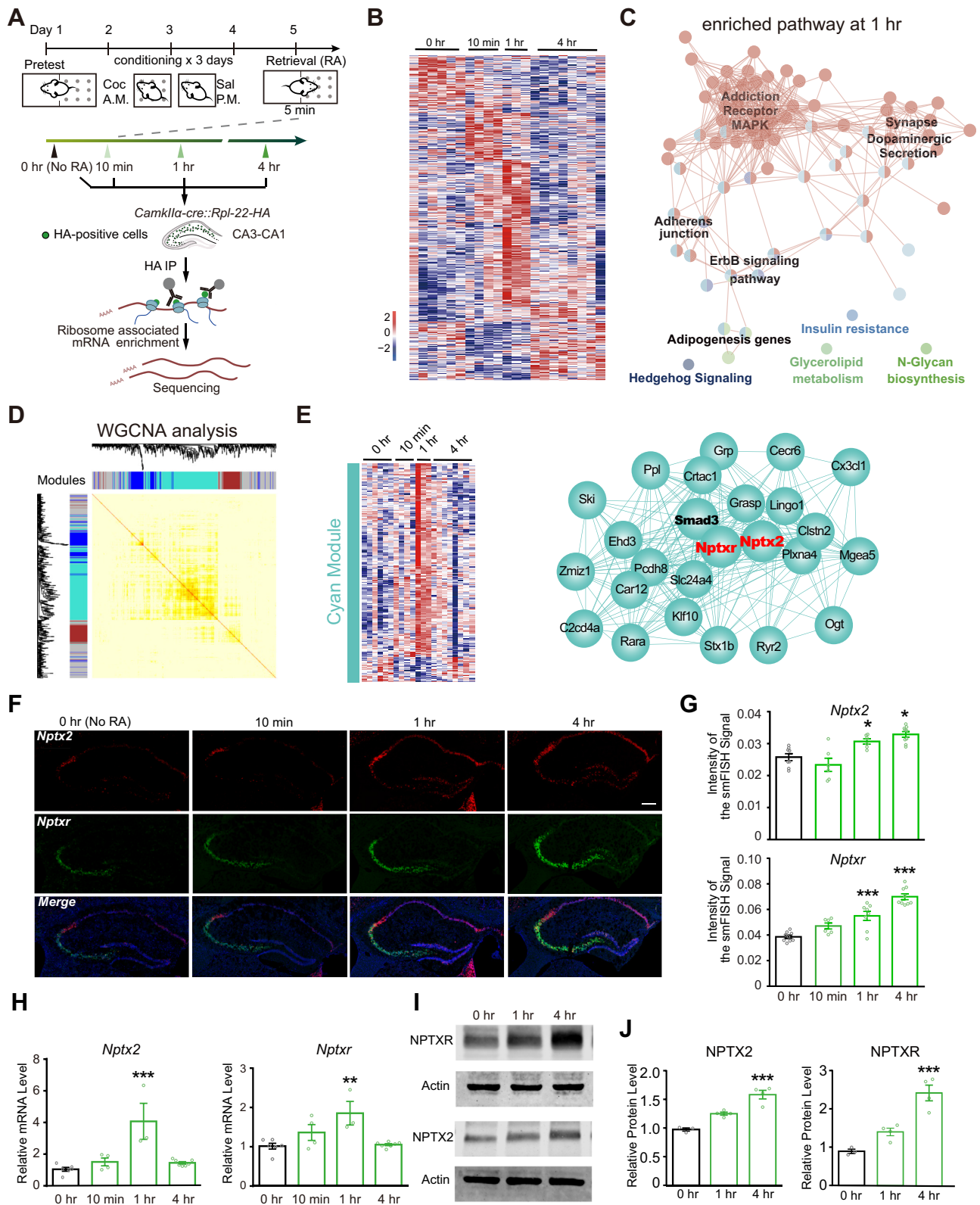


Figure 1. Translational dynamic analysis of CA1–CA3 $\text{CamkII}\alpha^+$ pyramidal neurons following the retrieval of cocaine-associated context memory unveils *Nptx2* as a hub regulator. **(A)** Schematics of RiboTag enrichment of retrieval-induced transcriptome alterations. CA1–CA3 regions of *CamkIIa-Cre::Rpl22-HA* mice were dissected at 0 hours, 10 minutes, 1 hour, or 4 hours after the retrieval of cocaine-associated context memory. Ribosome-associated messenger RNA (mRNA) was enriched and subjected

Cre::Rpl22-HA mice to measure the comprehensive neuronal translational dynamics in hippocampal CA3–CA1 following memory retrieval, and we uncovered *Nptx2* and its anchor partner *Nptxr* as hub genes that are upregulated. *Nptx2* is a neuronal immediate early gene (IEG) belonging to a member of the neuronal pentraxin family, including *Nptx1*, *Nptx2*, and *Nptxr*. NPTXs regulate synaptogenesis and glutamate signaling by clustering AMPARs and contribute to multiple forms of developmental and adult synaptic plasticity (18–22). We found that the upregulation of NPTX2 caused activity-dependent synaptic remodeling by recruiting GluA1-containing AMPAR on the neuronal membrane to accelerate the extinction of cocaine-associated context memory, suggesting that manipulating NPTX2 signaling might be a potential strategy for the treatment of craving triggered by drug-associative cues.

METHODS AND MATERIALS

Animals

Adult C57BL/6J male mice were obtained from the Shanghai Laboratory Animal Center (CAS, Shanghai, China). *CamkIIα-Cre* mice (No. 005359) and *Rpl22-HA* mice (No. 011029) were purchased from the Jackson Laboratory (Sacramento, CA). *CamkIIα-Cre::Rpl22-HA* mice were generated by crossing *CamkIIα-Cre* mice with *Rpl22-HA* mice. All mice were housed on a 12-hour light/dark cycle (lights on from 8 AM to 8 PM) with access to food and water ad libitum. Male mice at 8 to 10 weeks of age were used for the study. All experiment procedures were strictly in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the animal facility at Fudan University.

Cocaine Conditioned Place Preference

Cocaine conditioned place preference (CPP) was performed in a two-chamber apparatus (15 × 15 × 20 cm) with distinct visual and tactile environments (Med Associates, St. Albans, VT). The procedure consisted of three different sessions: pretest, conditioning, and extinction (23). During the pretest session, mice were placed in the middle of the apparatus and allowed to explore the entire CPP apparatus freely for 15 minutes. The time that the mice spent in each chamber was recorded. Mice showing a strong preference for one of the chambers (>10

minutes) were excluded. During the conditioning sessions, mice were given an intraperitoneal injection of cocaine (15 mg/kg) and confined to one of the chambers (drug paired) for 30 minutes, and 6 hours later they received an intraperitoneal injection of saline (equivalent volume to that of cocaine) and were confined to the other chamber for 30 minutes (conditioning). Mice were placed into the middle of the apparatus and allowed to freely explore both chambers (5 minutes for the retrieval trials and 10 minutes for the extinction trials). The time that the mice spent in each chamber was recorded. The CPP score was defined as the time in the cocaine-paired chamber minus the time in the saline-paired chamber (in seconds per minute).

Ribosome-Associated Messenger RNA Purification/RiboTag

Hippocampi from *CamkIIα-Cre::Rpl22-HA* mice with or without 5-minute retrieval were quickly isolated, and the dentate gyrus (DG) was removed and discarded. The remaining subregions containing CA1–CA3 were used for enrichment of ribosome-associated transcripts as described previously (24). The brain tissue was homogenized in supplemented hybridization buffer containing dithiothreitol, cycloheximide (Cayman, Ann Arbor, MI), heparin (Sigma-Aldrich, St. Louis, MO), protease inhibitors (Roche, Mannheim, Germany), and RNase inhibitor (Promega, Madison, WI). The supernatant was incubated with anti-HA (hemagglutinin) antibody (No. H6908; Sigma-Aldrich) and Dynabeads Protein G (Invitrogen, Carlsbad, CA) for 12 hours. Purified messenger RNA (mRNA) was eluted from the Dynabeads using TRIzol LS (Invitrogen). An Agilent RNA 6000 Pico Kit (No. 5067-1513; Agilent, Santa Clara, CA) and an Agilent 2100 bioanalyzer were used to evaluate the quality of purified mRNA. Purified mRNA samples with RNA integrity number < 7 were discarded.

Virus Constructs and Stereotaxic Surgery

The *scramble* short hairpin RNA (shRNA) (5'-TCGAAG-TATCCGCGTACGTT-3') and shRNA coding sequences targeting mouse *Nptx2* (5'-GAAGACATTGCTGAGCTGTA-3') or *Nptxr* (5'-GACAGCACTGGCACCATTATC-3') were cloned into the *pAAV-CMV-bGlobin-Flex-EGFP-MIR30shRNA* vector (Obio Technology, Shanghai, China). The coding sequences of mouse *Nptx2* and *Nptx2-PTX* were subcloned into *pAAV-CMV-DIO-Mus-2A-EGFP* vector (Obio Technology). The

to RNA sequencing. (B) Heat map of the significantly changed ribosome-associated transcripts of genes in *CamkIIα⁺* neurons at different time points following retrieval of cocaine-associated context memory. Each row in the heat map corresponds to a single gene. The color of the heat map represents the log₂ (fold change) for each gene. (C) Representation of the retrieval-regulated signaling network enrichment analysis corresponding to 1-hour regulated module. The links between terms are calculated using chance-corrected kappa statistics based on shared genes. For each group with more than five pathways, the top three most frequently appearing terms are used as representation of the group. Circle color represents clusters generated by the ClueGO Cytoscape plug-in, and circle size corresponds to ratio of genes enriched in each pathway. (D) WGCNA (weighted gene coexpression network analysis) of differentially expressed ribosome-associated transcripts 1 hour after retrieval. (E) Heat map of genes (left) and hub genes (right) in cyan module, which exhibit the most significant expression alteration at 1 hour after retrieval. (F, G) Representative confocal images (F) and quantification (G) of the single-molecule fluorescence in situ hybridization (smFISH) intensity of *Nptx2* and *Nptxr* in dorsal hippocampus. Red: *Nptx2*; green: *Nptxr*; blue: DAPI. Scale bar: 200 μm. *Nptx2*: $F = 13.172$, $p < .001$; *Nptxr*: $F = 32.878$, $p < .001$; one-way analysis of variance by Tukey's post hoc analysis. (H) Quantitative real-time polymerase chain reaction analysis of ribosome-associated transcripts of *Nptx2* and *Nptxr* in *CamkIIα⁺* neurons. *Nptx2*: $\chi^2 = 11.241$, $p = .01$, Kruskal–Wallis H test with Dunn's post hoc test; *Nptxr*: $F = 7.633$, $p = .002$, one-way analysis of variance by Tukey's post hoc analysis. (I, J) Lysates of dorsal hippocampus were subjected to Western analysis with indicated antibodies. Representative immunoblots (I) and quantification results (J) of the protein level are shown. NPTX2: $F = 32.481$, $p < .001$; NPTXR: $F = 27.875$, $p < .001$; one-way analysis of variance by Tukey's post hoc analysis. Data are presented as mean ± SEM. * $p < .05$, ** $p < .01$, *** $p < .001$ vs. 0 hours. Coc, cocaine; HA, hemagglutinin; IP, immunoprecipitation; MAPK, mitogen-activated protein kinase; No RA, no retrieval; Sal, saline.

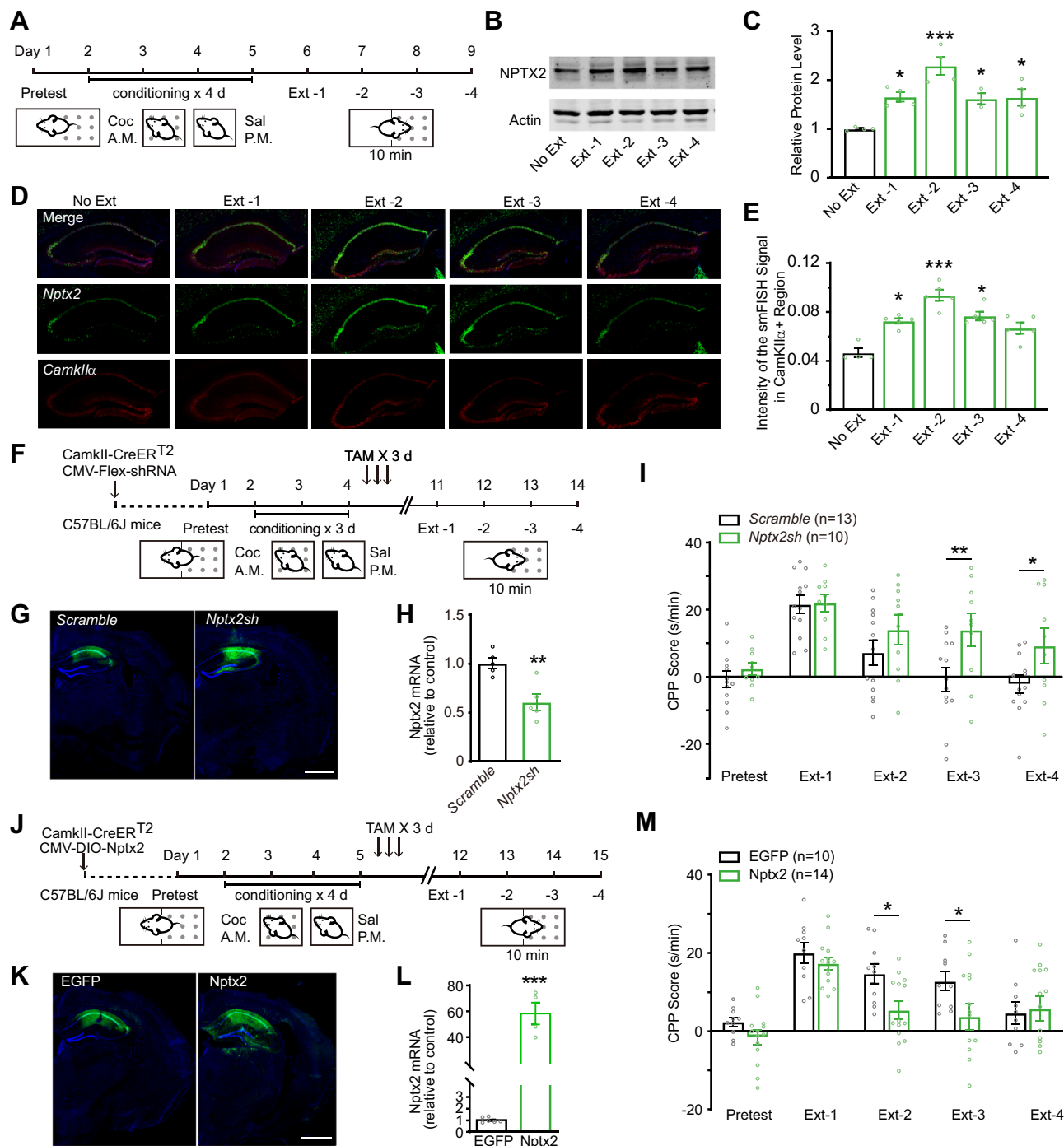


Figure 2. NPTX2 in dorsal hippocampal pyramidal neurons directionally regulates the extinction of cocaine-associated context memory. **(A)** Experimental procedure of the extinction of cocaine-associated context memory. **(B, C)** Representative Western blots **(B)** and quantification **(C)** of NPTX2 protein level across the extinction process. $F = 4.680$, $p < .05$, one-way analysis of variance by Tukey's post hoc analysis. **(D, E)** Representative confocal images **(D)** and quantification **(E)** of the single-molecule fluorescence in situ hybridization (smFISH) intensity of *Nptx2* in *CamkIIα*⁺ neurons across the extinction process. Green: *Nptx2*; red: *CamkIIα*. Scale bar: 200 μ m. $\chi^2 = 11.241$, $p = .002$, Kruskal-Wallis H test with Dunn's post hoc test. **(F-I)** AAV-*CaMKIIα*-*CreER*^{T2} mixed with either AAV-*Flex-scramble*-shRNA-EGFP or AAV-*Flex-Nptx2*-shRNA-EGFP was bilaterally injected into dorsal hippocampus of C57BL/6J mice, and the mice were injected with tamoxifen (TAM) (100 mg/kg) for 3 consecutive days after three pairs of cocaine conditioning. Experimental procedure of the extinction **(F)**. Representative confocal images show short hairpin RNA (shRNA) enhanced green fluorescent protein (EGFP) expression **(G)** and quantitative real-time polymerase chain reaction analysis **(H)** of the relative messenger RNA (mRNA) level of *Nptx2*. $p = .005$, two-tailed unpaired t test. Green: EGFP; blue: DAPI. Scale bar: 1 mm. **(I)** Downregulating *Nptx2* in dorsal hippocampal pyramidal neurons impaired the extinction of the cocaine-associated context memory. $F_{\text{Treatment} \times \text{Ext}(3,63)} = 4.411$, $p = .007$, two-way repeated-measures analysis of variance by Bonferroni post hoc analysis. **(J-M)** Experimental procedure of the

coding sequence of enhanced green fluorescent protein (EGFP) in *pAAV-Ef1 α -DIO-EGFP* (Addgene: No. 27056) was replaced with *GluA1-C-tail-mCherry* (*GluA1CT-mCherry*) (25) to generate *pAAV-Ef1 α -DIO-GluA1-C-tail-mCherry*. The coding sequence of *Cre^{ERT2}* obtained from *p-FosCre^{ERT2}* (Addgene: No. 46388) was subcloned into *pAAV-CaMKII α -hChR2(H134R)-EGFP* (Addgene: No. 26969) (26,27) to generate *pAAV-CaMKII α -Cre^{ERT2}*. The coding sequence of *mCherry-P2A-Cre* obtained from *pLM-CMV-R-Cre* (Addgene: No. 27546) was subcloned into *pAAV-RAM-d2tTA::TRE-EGFP* (Addgene: No. 84469) to generate *pAAV-RAM-d2tTA::TRE-mCherry-P2A-Cre*. Adeno-associated viruses (AAVs) described above were packaged by Obio Technology into serotype 2/9. Mice were anesthetized by 2% isoflurane during the surgery and were bilaterally injected with 0.5 μ L of purified and concentrated AAV (10^{12} IU/mL) with a slow injection rate (0.1 μ L/min) into dorsal hippocampus (coordinates: ± 1.5 mm mediolateral, -1.5 mm anteroposterior, -1.5 mm dorsoventral). All mice were given at least 3 weeks to recover before the behavioral tests, immunostaining, or electrophysiological recordings.

Spine Morphology Analysis

Slices containing dorsal hippocampus were prepared, and the pyramidal neurons expressing EGFP in CA1 were randomly selected and filled with Alexa Fluor 568 hydrazide (No. A10441; Thermo Scientific, Waltham, MA) for 10 minutes of diffusion. Slices were then fixed in 4% paraformaldehyde dissolved in 0.1 M phosphate-buffered saline overnight and mounted. Z-series images with a 0.5- μ m interval were taken using a 60 $\times$ oil lens of a Nikon-A1 confocal microscope (Tokyo, Japan). Reconstruction and analysis of synaptic structures were carried out by the Imaris software package (Bitplane, St. Paul, MN). For spine density measurements, apical dendritic segments of dye-fulfilled CA1 cells were chosen. Here 30- to 50- μ m segments beginning >50 μ m and ending <150 μ m distal to the soma and after the first branch point were quantified. The morphology of the spine was defined as follows: thin (mean width [head] $\geq$ mean width [neck]); mushroom (length < 3 μ m and maximum width [head] $> 2 \times$ mean width [neck]).

Statistics

All data were presented as mean $\pm$ standard error of the mean. Statistical analyses were performed by SPSS 20.0 software (IBM Corp., Armonk, NY). The normality test of the data sets was performed by the Shapiro-Wilk test. The homoscedasticity test was performed by Levene's test. Two-tailed unpaired *t* test was used for comparing two independent groups. One-way analysis of variance (ANOVA) was performed for comparing more than two groups. Bonferroni post hoc analysis was subsequently performed after two-way ANOVA or two-way repeated-measures ANOVA within different factors. Data that were not normally distributed were

analyzed using a nonparametric test (**p* $< .05$, ***p* $< .01$, and ****p* $< .001$).

RESULTS

Translational Dynamic Analysis of CA1–CA3 Pyramidal Neurons Following the Retrieval of Cocaine-Associated Context Memory Unveils *Nptx2* as Hub Regulator

To address how the hippocampal CA1–CA3 regions process reward extinction, we used cocaine CPP procedures to investigate the dynamics of the translational profiles in CA1–CA3 pyramidal neurons following memory retrieval. *CamkII α -Cre::Rpl22-HA* mice, which express HA-tagged ribosomal protein specifically expressed in pyramidal neurons (Supplemental Figures S1 and S2A), were trained daily for 3 days and reexposed to the CPP apparatus to retrieve cocaine CPP memory. The ribosome-associated transcripts from hippocampal CA1–CA3 region were isolated at 0 hours (no retrieval), 10 minutes, 1 hour, and 4 hours after retrieval (Figure 1A). Sequence analysis of ribosome-associated mRNA using the Shannon entropy-based method and ANOVA revealed significant changes among different time points after retrieval, and notably $\sim 45\%$ of genes exhibited translational activation/suppression 1 hour after retrieval (Figure 1B). Given that IEGs respond to memory retrieval–extinction incidents, we first overlapped our data with previous screenings on IEGs (28–30) for data validation (Supplemental Figure S2B). Consistently, all the overlapped IEGs exhibited significant upregulation after memory extinction. *Dusp1* and *Btg2* were most upregulated at 10 minutes, while the other conventional IEGs such as *Arc*, *Fos*, and *Egr1* exhibited most significant enrichment 1 hour after retrieval. Among all the genes significantly changed after retrieval, insulin signaling and apoptosis pathways exhibited sustained downregulation following retrieval (Supplemental Figure S2C, upper left) (31–34). Proteins involved in gap junction (35,36) and fatty acid biosynthesis (37) were actively translated 10 minutes after retrieval (Figure S2C, lower left). Genes involved in regulating addiction, dopaminergic, synapse, and secretion (38) and adherence junction (39) were actively translated 1 hour after retrieval (Figure 1C), while the genes related to protein processing in endoplasmic reticulum and *N*-glycan biosynthesis were transiently suppressed and recovered at 4 hours after retrieval (40) (Supplemental Figure S2C, right).

To discover the regulators underlying memory extinction, WGCNA (weighted gene coexpression network analysis) (41) data mining of genes with changed expression after retrieval identified four modules of interest (Figure 1D). The gray and brown modules represent early- and late-responding genes (10 minutes and 4 hours, respectively), while the blue and cyan modules represent intermediate phase-activated modules (Figure 1E and Supplemental Figure S2D). Hub-gene-network

extinction (J). AAV-CaMKII α -Cre^{ERT2} mixed with either AAV-EF1 α -DIO-Nptx2-EGFP or AAV-EF1 α -DIO-EGFP was bilaterally injected into dorsal hippocampus of C57BL/6J mice, and TAM was injected for 3 consecutive days after 4 days of cocaine conditioning. Representative images (K) and quantification (L) of the mRNA level of *Nptx2* after TAM injection are shown. *p* = .011, Mann-Whitney *U* test. (M) Overexpression of *Nptx2* in dorsal hippocampal pyramidal neurons promoted cocaine-associated memory extinction. $F_{\text{Treatment} \times \text{Ext}(3,66)} = 3.734$, *p* = .015, two-way repeated-measures analysis of variance by Bonferroni post hoc analysis. Data are presented as mean $\pm$ SEM. **p* $< .05$, ***p* $< .01$, ****p* $< .001$ vs. *scramble* group. Coc, cocaine; CPP, conditioned place preference; Ext, extinction; Sal, saline.

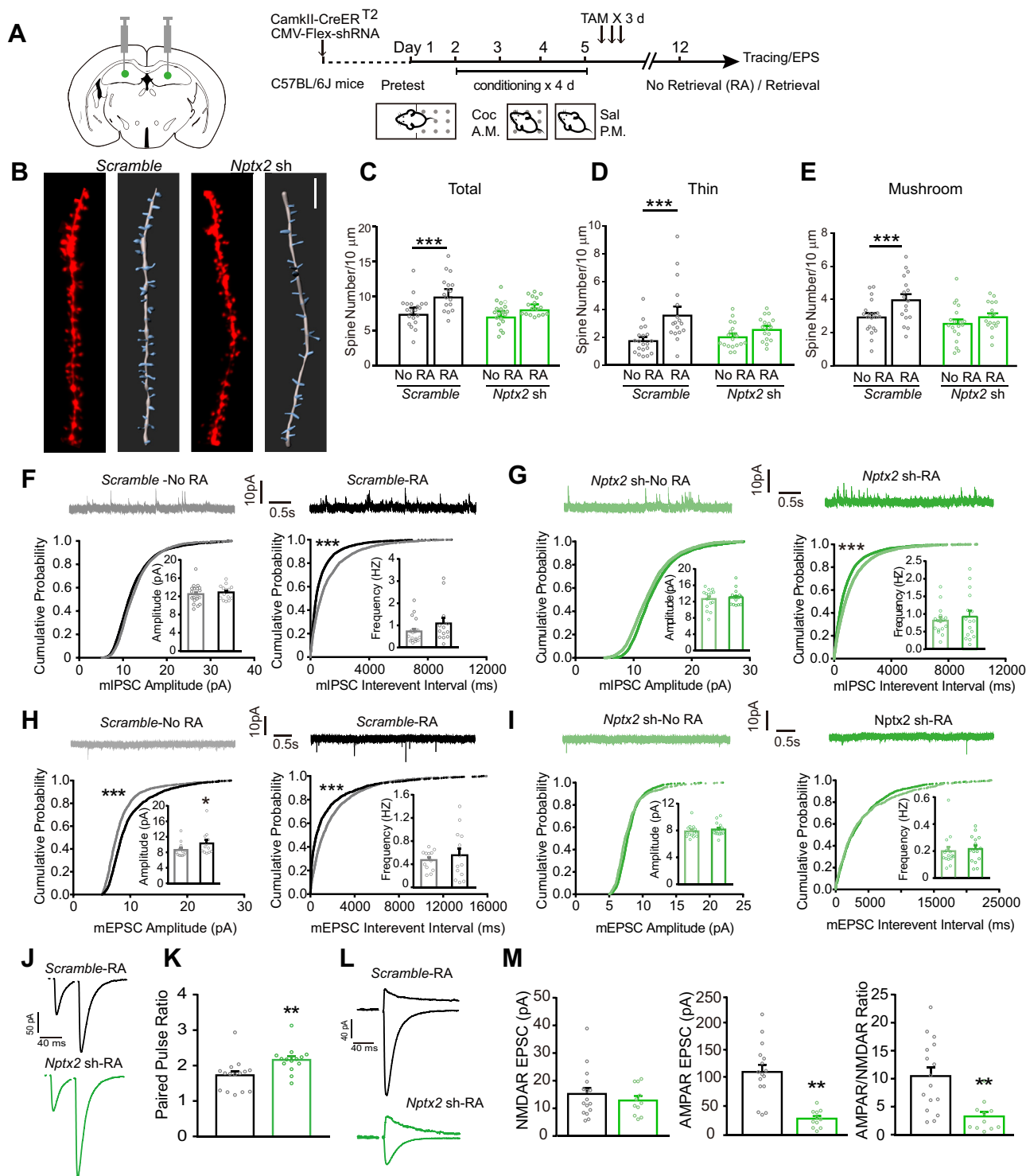


Figure 3. NPTX2 is required for retrieval-driven synaptic plasticity in dorsal CA1. **(A)** Experimental procedure to assess retrieval-driven synaptic changes in dorsal CA1. AAV-CamKII α -CreER^{T2} mixed with either AAV-Flex-scramble-shRNA-EGFP or AAV-Flex-Nptx2-shRNA-EGFP was bilaterally injected into dorsal hippocampus of C57BL/6J mice. After cocaine conditioning, mice were injected with tamoxifen (TAM) to downregulate NPTX2, and the slice containing dorsal hippocampus was prepared from mice with or without retrieval (RA). **(B-E)** The pyramidal neurons expressing enhanced green fluorescent protein (EGFP) in dorsal CA1 were randomly selected and filled with Alexa Fluor 568 hydrazide. Representative confocal images of the dendrite segments **(B)** and the quantification results **(C-E)** of the spine density are shown. Scale bar: 5 μ m. Total: $F_{\text{Treatment} \times \text{Ext}(1,69)} = 2.638$, $p = .109$; thin: $F_{\text{Treatment} \times \text{Ext}(1,69)} = 5.82$, $p = .018$; mushroom: $F_{\text{Treatment} \times \text{Ext}(1,69)} = 1.494$, $p = .226$; two-way analysis of variance with Bonferroni post hoc analysis. **(F)** Representative traces, the cumulative

analysis showed that the blue module includes a majority of IEGs encoding transcription factors, as well as *Arc*, which is involved in the regulation of synaptic plasticity (Supplemental Figure S2D). Two neuronal pentraxins, *Nptx2* and its anchor partner *Nptxr* (Figure 1E, highlighted in red), constitute the hub genes of the cyan module.

The pentraxins family, which is composed of NPTX1, NPTX2, and NPTXR (42), has been proposed to play an important part in synapse organization and memory loss (21,43). We performed single-molecule fluorescence in situ hybridization of *Nptx1*, *Nptx2*, and *Nptxr* to determine their distribution in dorsal hippocampus. *Nptx2* was distributed around CA1–CA3, *Nptxr* was enriched in CA3, and *Nptx1* was enriched in both DG and CA3 (Figure 1F and Supplemental Figure S3). The mRNA of *Nptx2* and *Nptxr* in dorsal hippocampus were increased 1 hour after the retrieval of cocaine CPP memory (Figure 1G), while the mRNA level of *Nptx1* was downregulated following retrieval (Supplemental Figure S3). Quantitative real-time polymerase chain reaction confirmed that the ribosome-associated transcripts of *Nptx2* and *Nptxr* in dorsal hippocampus were upregulated 1 hour after retrieval (Figure 1H). Consistently, the protein levels of NPTX2 and NPTXR following retrieval were also increased (Figure 1I, J). These data indicate that the retrieval of cocaine-associated context memory increases the transcription and translation of *Nptx2* and *Nptxr*.

NPTX2 Within Dorsal Hippocampal *CamkIIα*⁺ Neurons Was Required for the Extinction of Cocaine-Associated Context Memory

To assess the role of NPTX2 in dorsal hippocampal pyramidal neuron, an inducible Cre-dependent shRNA expression vector *AAV-Flex-Nptx2-shRNA-EGFP* was made to downregulate *Nptx2*. Knockdown of *Nptx2* in dorsal hippocampus did not affect the locomotor activity and novel object recognition, but it impaired the acquisition of cocaine CPP (Supplemental Figure S4A–D). Knockdown of *Nptx2* after CPP conditioning did not affect the memory retrieval in a 5-minute exploration test (Supplemental Figure S4E, F). We then tested the expression of *Nptx2* after each of four 10-minute extinction trials. The protein level and the mRNA level of *Nptx2* in dorsal hippocampus were remarkably increased 1 hour after each extinction trial (Figure 2A–E). To temporally regulate *Nptx2* expression, the *AAV-CamKIIα-CreER^{T2}* was used for tamoxifen (TAM)-inducible activation of *CreER^{T2}* (26,44). Mice were coinjected with *AAV-CamKIIα-CreER^{T2}* and *AAV-Flex-shRNA-EGFP* in dorsal hippocampus. Very few EGFP-labeled cells were in dorsal hippocampus of the mice without TAM injection (Supplemental Figure S4G), but in mice treated with TAM for 3

consecutive days (100 mg/kg intraperitoneal) there was a majority of EGFP⁺ cells in dorsal hippocampus (Figure 2G). The mRNA level of *Nptx2* in dorsal hippocampus was significantly downregulated by *Nptx2-shRNA* (Figure 2H), indicating TAM-dependent knockdown efficiency of *Nptx2-shRNA*.

Mice were injected with TAM to temporally knock down the *Nptx2* in hippocampal pyramidal neurons after three pairs of cocaine conditioning. As shown in Figure 2I, these mice spent significantly more time in the cocaine-paired chamber compared with the *scramble-shRNA* group (Figure 2I) at the extinction trials, indicating the impaired extinction of cocaine CPP memory. *AAV-CamKIIα-CreER^{T2}* with either *AAV-EF1α-DIO-Nptx2-EGFP* or *AAV-EF1α-DIO-EGFP* was injected into dorsal hippocampus of mice to overexpress *Nptx2* under the control of TAM. The mice were treated with TAM after extended conditioning (4 days) to induce the expression of *Nptx2* (Figure 2J–L). The mice overexpressing NPTX2-EGFP spent less time in the cocaine-paired chamber in the second and third extinction trials (Figure 2M), indicating the enhanced extinction of cocaine-associated context memory. Downregulating *Nptxr* in dorsal hippocampus did not affect the acquisition and extinction of cocaine-associated context memory (Supplemental Figure S5A–F). These data indicate that NPTX2 in dorsal hippocampus bidirectionally regulates the extinction of cocaine CPP memory.

NPTX2 Was Required for Retrieval-Driven Synaptic Plasticity in Dorsal CA1

Given that extinction derived from the cellular consequences of adaptive changes of synaptic plasticity after memory retrieval (45), we then investigated whether NPTX2 mediates the retrieval-driven synaptic remodeling. Mice were injected with TAM to induce the expression of *Nptx2-shRNA* or *scramble-shRNA* after cocaine conditioning, and the brain slices containing dorsal hippocampus were isolated from the mice that experienced retrieval or no retrieval. The EGFP⁺ neurons in dorsal CA1 underwent fluorescent dye tracing or electrophysiological recording (Figure 3A). The total and mature spine density was increased after retrieval in neurons expressing *scramble-shRNA* but not in neurons expressing *Nptx2-shRNA* (Figure 3C–E). The frequency of miniature inhibitory postsynaptic currents and the amplitudes and frequency of miniature excitatory postsynaptic currents (mEPSCs) were increased by retrieval. However, in neurons expressing *Nptx2-shRNA*, the amplitudes and frequency of mEPSCs were not changed by retrieval, while the frequency of miniature inhibitory postsynaptic currents was not affected (Figure 3F–I). The Schaffer collaterals were stimulated to activate the glutamatergic inputs to CA1 pyramidal neurons.

probability distribution, and the average amplitude and frequency of miniature inhibitory postsynaptic currents (mIPSCs) of *scramble* groups with or without retrieval. Amplitude: $p = .551$, two-tailed unpaired t test; frequency: $p = .151$, Mann-Whitney U test. (G) Representative traces, the cumulative probability distribution, and the average amplitude and frequency of mIPSCs of *Nptx2sh* group. Amplitude: $p = .970$; frequency: $p = .940$; Mann-Whitney U test. (H) Representative traces, the cumulative probability distribution, and the average amplitude and frequency of miniature excitatory postsynaptic currents (mEPSCs) in *scramble* groups with or without retrieval. Amplitude: $p = .043$; frequency: $p = .773$; Mann-Whitney U test. (I) Representative traces, the cumulative probability distribution, and the average amplitude and frequency of mEPSCs in the *Nptx2sh* groups with or without retrieval. Amplitude: $p = .330$; frequency: $p = .430$; Mann-Whitney U test. (J, K) Paired-pulse ratio of evoked EPSCs in neurons expressing *Nptx2sh* or *scramble* after retrieval. $p = .002$, Mann-Whitney U test. (L, M) The amplitude of AMPA current, NMDA current, and AMPA receptor (AMPA)/NMDA receptor (NMDAR) ratio in neurons expressing *Nptx2sh* or *scramble* assessed 1 hour after memory retrieval NMDAR: $p = .753$; AMPAR: $p < .001$; AMPAR/NMDAR ratio: $p = .001$; Mann-Whitney U test. Data are presented as mean $\pm$ SEM. * $p < .05$, ** $p < .01$, *** $p < .001$ vs. *scramble*. Coc, cocaine; EPS, electrophysiological study; Sal, saline.

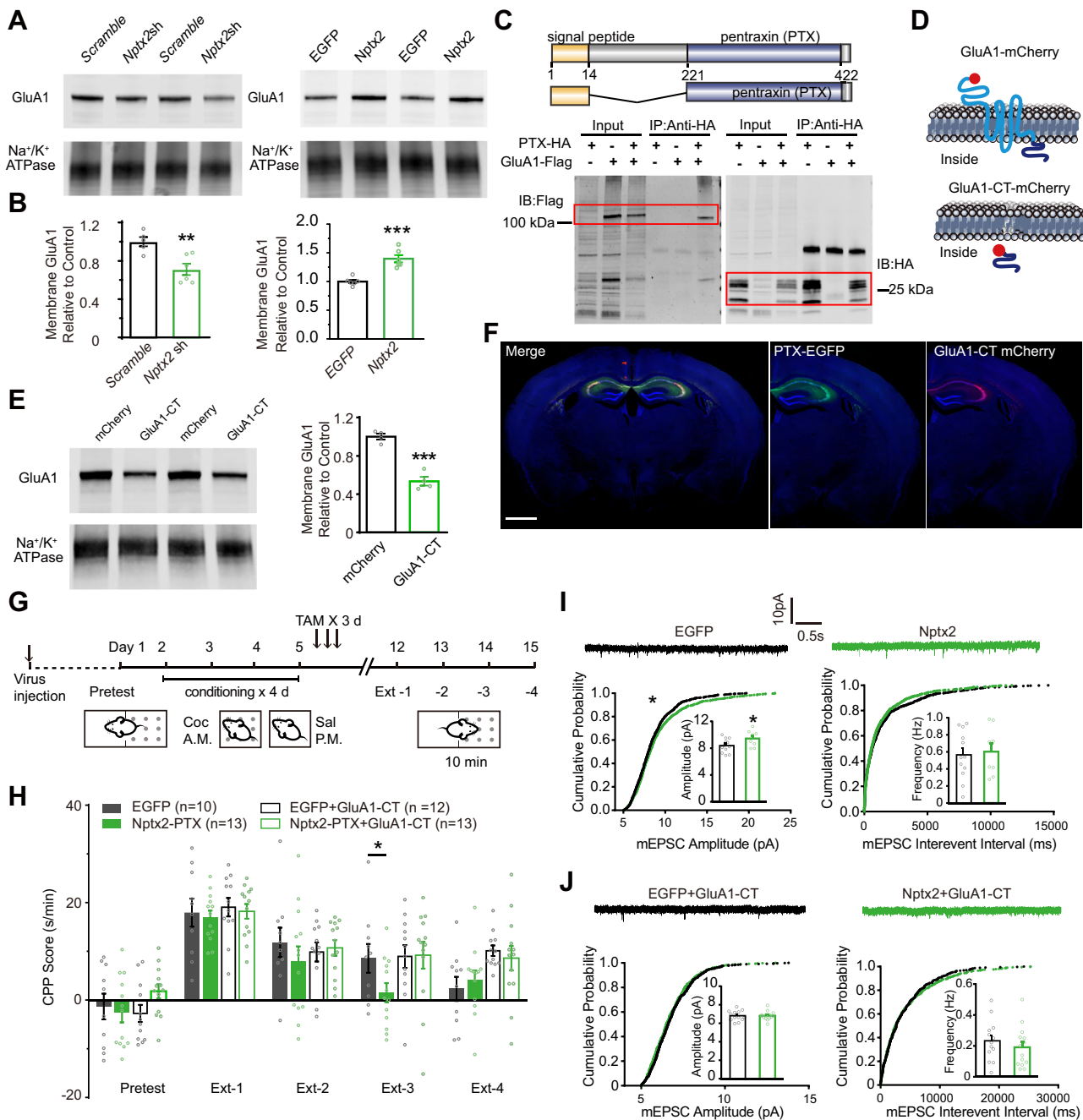


Figure 4. NPTX2 promotes the extinction of cocaine-associated context memory via recruiting GluA1. (**A, B**) Dorsal hippocampus of the *CamkIIα-Cre* mice was infected with viruses to knock down or overexpress Nptx2. Lysates of the membrane protein extracted from dorsal hippocampus were subjected to Western analysis with indicated antibodies. Representative immunoblots (**A**) and quantification of the GluA1 protein level (**B**) are shown. *Scramble* vs. *Nptx2sh*: $p = .005$; enhanced green fluorescent protein (EGFP) vs. *Nptx2*: $p < .001$; two-tailed unpaired t test. (**C**) Pentraxin (PTX) domain of NPTX2 mediates the recruitment of GluA1. Upper panel: Schematic diagram of full-length and truncated NPTX2, including the signal peptide and PTX domain. Lower panel: Recombinant PTX-HA and GluA1-Flag in HEK293 cells as pulled down with anti-HA (hemagglutinin) beads; the precipitates were analyzed by immunoblotting with anti-Flag and anti-HA antibodies. (**D**) Schematic diagram of a fusion protein of mCherry and the last C-terminal 81 amino acids of the GluA1 subunit (GluA1-CT-mCherry). (**E**) Dorsal hippocampus of the *CamkIIα-Cre* mice were infected with AAV-DIO-GluA1CT-mCherry or AAV-DIO-mCherry virus, and lysates of the membrane protein extracted from dorsal hippocampus were subjected to Western analysis with indicated antibodies. $p < .001$ vs. *DIO-mCherry*, two-tailed unpaired t test. (**F-H**) AAV-*CamkIIα-CreER*^{T2} and AAV-DIO-PTX-EGFP mixed with either AAV-DIO-mCherry or AAV-DIO-GluA1CT-mCherry were bilaterally injected into dorsal hippocampus of wild-type mice. After cocaine conditioning, mice were injected with tamoxifen (TAM) to induce the expression of PTX or GluA1-CT. (**F**) Representative confocal images verified the expression of the PTX-EGFP and GluA1CT-mCherry in dorsal hippocampus after TAM injection. Green: PTX-EGFP; red: GluA1CT-mCherry; blue: DAPI. Scale bar: 1 mm. (**G, H**) Experimental procedure (**G**) and the conditioned place preference (CPP) score (**H**) during the extinction of cocaine-associated context memory are shown. EGFP vs. NPTX2-PTX: $F_{\text{Treatment} \times \text{Ext}(3,63)} = 2.936$, $p = .040$; EGFP + GluA1CT vs.

Dramatically decreased amplitude of AMPAR EPSCs and minor increased paired-pulse ratio (PPR) were observed in neurons expressing *Nptx2-shRNA* 1 hour after memory retrieval (Figure 3J–M), suggesting that NPTX2 regulates retrieval-induced glutamatergic synaptic transmission via both pre- and postsynaptic mechanisms.

NPTX2 Promotes the Extinction of Cocaine-Associated Context Memory Via Recruiting GluA1

Accumulating evidence has shown that the membrane trafficking of AMPARs is essential for memory extinction (46). We extracted the membrane proteins of dorsal hippocampus from the mice infected with viruses to conditionally knock down or overexpress *Nptx2*. Membrane GluA1 was decreased by *Nptx2-shRNA*, while it was increased notably by overexpression of *Nptx2* (Figure 4A, B). The pentraxin (PTX) domain of NPTXR binds to AMPARs and mediates its synapse induction activity (21). NPTX2 contains a large C-terminal PTX domain homologous to NPTXR, and this PTX domain of NPTX2 recruited GluA1 (Figure 4C). We constructed an AAV vector encoding the carboxyl cytoplasmic tail (81 amino acids) of GluA1 (*GluA1-CT*) fused with mCherry, which acts as a dominant-negative mutant to prevent synaptic incorporation of endogenous GluA1 receptors (12,25). Overexpressing *GluA1-CT* in the dorsal hippocampus decreased the membrane recruitment of GluA1 (Figure 4D, E). Expressing recombinant PTX decreased the time spent in the cocaine-paired chamber during the extinction trials, while the expression of *GluA1-CT* prevented the PTX-promoted extinction of cocaine-associated context memory (Figure 4F–H). In addition, expression of *GluA1-CT* blocked the NPTX2-enhanced amplitude of mEPSCs 1 hour after memory retrieval (Figure 4I, J). These findings revealed that NPTX2 promotes the extinction of cocaine-associated context memory via recruiting GluA1 to the membrane.

NPTX2 in Neurons Activated by Cocaine-Paired Context Is Required for Mediating the Extinction of Cocaine-Associated Context Memory

A fraction of neurons can be activated by memory retrieval and facilitate memory extinction (47). We took advantage of a recently developed robust activity marking system, which is a modified doxycycline (Dox)-dependent Tet-Off system, to temporally label neurons activated by a specific experience (48). AAV-RAM-EGFP was infected into dorsal hippocampus of the mice to label the homecage-activated neurons in the absence of Dox, whereas c-Fos staining was used to label the neurons activated by cocaine-paired context (Figure 5A). There was rare colocalization of EGFP⁺ neurons with the c-Fos⁺ neurons (Figure 5B), indicating that the homecage and cocaine-paired context-labeled neurons were two distinct cell populations. We then assessed whether NPTX2-mediated

plasticity in the neurons activated by the cocaine-paired context was required for memory extinction. We used the robust activity marking system to drive the expression of Cre recombinase, allowing temporal control of gene manipulation in mice (Figure 5D). AAV-RAM-mCherry-P2A-Cre was infected with either AAV-Flex-*Nptx2-shRNA-EGFP* or AAV-Flex-scramble-*shRNA-EGFP* into dorsal hippocampus of mice. The mice experienced cocaine conditioning when on Dox and then were taken off Dox to induce Cre-induced recombination in neurons activated in homecage or cocaine-paired context (Figure 5F). The densities of mCherry⁺ neurons in dorsal hippocampus were dramatically increased in the absence of Dox, and the densities of the cells activated by homecage or cocaine-paired context exposure were similar between the *Nptx2-shRNA* and scramble groups (Figure 5G). Downregulation of *Nptx2* in the homecage-activated neurons caused no effect on extinction learning (Figure 5I), while with downregulation of *Nptx2* in neurons activated by cocaine-associated context the mice showed impaired extinction (Figure 5J). These data show that NPTX2-mediated plasticity in retrieval-activated neurons, which might encode a cue formerly associated with drug reward, is required for extinction.

DISCUSSION

The potential therapeutic targets based on reconsolidation that might adapt the content of addiction memories, leading to a loss of cue-induced relapse and craving, need to be investigated. By analyzing the translational profiling following the retrieval by cocaine-paired context, we found that *Nptx2* was a hub regulator orchestrating cellular alterations in dorsal hippocampal pyramidal neurons. Our results showed that NPTX2 recruited GluA1-AMPA to the neuronal membrane and enhanced the synaptic remodeling to accelerate the extinction of cocaine-associated context memory. NPTX2-mediated synaptic plasticity in the neurons activated by the context cue is essential for the extinction of the drug-associated memory.

Neuronal Pentraxins in Pathological Diseases

Neuronal pentraxins have been implicated in pathological states. NPTX1 contributes to the neuronal damage evoked by amyloid- β and is present around plaques in postmortem Alzheimer's disease brain (49). The expression of NPTX2 is reduced in human Alzheimer's disease and Down syndrome brain (43) and is correlated with cognitive decline (50,51). NPTX2 in hippocampus is increased in mice treated with an antidepressant and regulates anxiety (52). Results of single-molecule fluorescence in situ hybridization of *Nptx1*, *Nptx2*, and *Nptxr* in mice after cocaine CPP memory showed that *Nptx2* was distributed predominantly in CA1–CA3, *Nptxr* was enriched in CA3, and *Nptx1* was enriched in both DG and CA3 (Figure 1F and Supplemental Figure S3). The expression of

Nptx2-PTX + GluA1CT: $F_{\text{Treatment} \times \text{Ext}(3,69)} = 0.259$, $p = .855$; two-way repeated-measures analysis of variance by Bonferroni post hoc analysis. (I, J) The slices containing dorsal hippocampus were prepared from the mice 1 hour after retrieval, and the neurons expressing indicated proteins were recorded. Representative traces, the cumulative probability distribution, and the average amplitude and frequency of miniature excitatory postsynaptic currents (mEPSCs) were analyzed. (I) EGFP vs. NPTX2: $p = .048$ for amplitude, $p = .774$ for frequency. (J) EGFP + GluA1CT vs. NPTX2 + GluA1CT: $p = .969$ for amplitude, $p = .419$ for frequency; two-tailed unpaired t test. Data are presented as mean $\pm$ SEM. * $p < .05$, ** $p < .01$, *** $p < .001$. Coc, cocaine; Ext, extinction; IB, immunoblot; IP, immunoprecipitation; Sal, saline.

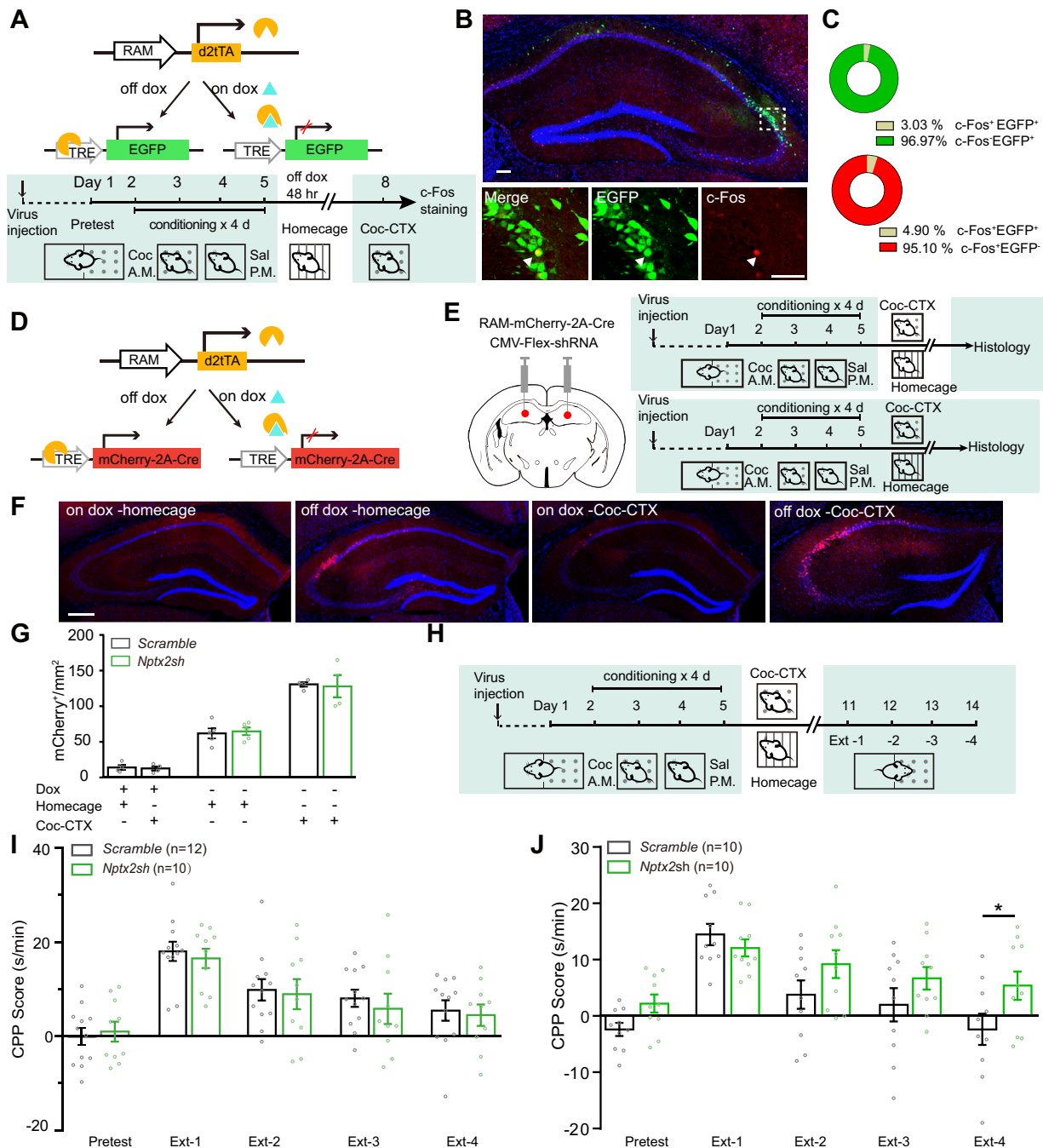


Figure 5. NPTX2 in dorsal hippocampal neurons activated by cocaine context mediated the extinction of cocaine-associated context memory. **(A)** The design of robust activity marking (RAM) system and experimental procedure. The mice were taken off doxycycline (dox) for 48 hours to label the neurons activated at homecage and then put on dox again. Then, 24 hours later, the mice were exposed to the cocaine-associated context (Coc-CTX) and the brains were collected 2 hours later for c-Fos staining. **(B, C)** Representative confocal image and the quantification of EGFP⁺/c-Fos⁺ cells. Green: enhanced green fluorescent protein (EGFP); red: c-Fos; blue: DAPI. Scale bar: top, 300 μ m; bottom, 20 μ m. **(D–G)** AAV-RAM-mCherry-2A-Cre mixed with either AAV-Flex-scramble-shRNA-EGFP or AAV-Flex-Nptx2-shRNA-EGFP was bilaterally injected into dorsal hippocampus of wild-type mice. **(D, E)** The design of the RAM-mCherry-2A-Cre system and the experimental procedure to assess the density of mCherry⁺ neurons with or without dox at homecage or cocaine context. **(F, G)** Representative confocal images **(F)** and quantification **(G)** of mCherry⁺ neurons at homecage or cocaine context. Scale bar: 300 μ m. Off dox/homecage: $p = .762$, two-tailed unpaired t test; off dox/Coc-CTX, $p = 1.00$, Mann-Whitney U test. **(H)** Experimental procedure to investigate the effect of knockdown of NPTX2 in neurons activated by homecage or cocaine-associated context in the extinction of cocaine-associated context memory. **(I)** Homecage: $F_{\text{Treatment} \times \text{Ext}(3,60)} = 0.0654$, $p = .978$. **(J)** Cocaine-associated context: $F_{\text{Treatment} \times \text{Ext}(3,54)} = 3.788$, $p = .015$; two-way repeated-measures analysis of variance with Bonferroni post hoc test. Data are presented as mean $\pm$ SEM. * $p < .05$ vs. scramble. Coc, cocaine; CPP, conditioned place preference; Ext, extinction; Sal, saline.

Hippocampal NPTX2 Plasticity in Drug Memory Extinction

Nptx2 and *Nptxr* was upregulated (Figure 1G), while the expression of *Nptx1* was downregulated, in dorsal hippocampus by retrieval (Supplemental Figure S3). Previous studies have assessed the potential role of neuronal pentraxins in the behavioral effects of addictive drugs. *Nptx2* knockout mice show impaired extinction of morphine CPP (53), which can be rescued by overexpressing *Nptx2* into the medial prefrontal cortex (54). *Nptx2* and *Nptx1* depletion enhances the acquisition of cocaine CPP and blunts AMPAR response, indicating that NPTX2 is required for cocaine-induced neuronal adaption (55,56). Here we found that specific knockdown of *Nptx2* in dorsal hippocampal pyramidal neurons impaired the acquisition and extinction of cocaine CPP without affecting the locomotor activity and the novel object recognition (Supplemental Figure S4), while knockdown of *Nptxr* had no effect on cocaine CPP (Supplemental Figure S5). These data indicate that neuronal pentraxins differentially contribute to addictive drugs or cue-induced behavior changes, and NPTX2 might be required for the plasticity during the consolidation and adaptive reconsolidation of drug-associative memory.

Neuronal Pentraxins in Glutamatergic Transmission

Accumulating evidence reveals a bifunctional role of neuronal pentraxins in strengthening and weakening glutamatergic transmission. NPTX1 aggregates postsynaptic GluA1-containing AMPARs at developing synapses, facilitating functional synapses to form (57). NPTXR is a potent inducer of both excitatory and inhibitory heterologous synapses (21). Cleaved NPTXR recruits AMPAR for endocytosis and is required for mGluR1/5-dependent long-term depression in hippocampal and cerebellar synapses (19). NPTX2 secreted by axon terminals of the pyramidal neurons binds and stabilizes postsynaptic GluA4 in parvalbumin interneurons to mediate activity-dependent strengthening of the pyramidal neuron-parvalbumin interneuron synapses (18,22). Chronic exposure to NPTX1, NPTX2, or NPTXR decreases PPR at mouse CA3–CA1 hippocampal synapses, indicating an increase in glutamate release (58). In this study, knockdown of *Nptx2* after cocaine conditioning substantially decreased the frequency of mEPSCs in dorsal CA1 neurons, which indicates an impaired presynaptic glutamatergic transmission in CA1 or a weakened strength of synaptic connectivity between the hippocampal ensembles recruited during the cocaine conditioning. Knockdown of *Nptx2* abolished retrieval-enhanced glutamatergic transmission (Figure 3H, I), while it had no effect on the inhibitory transmission (Figure 3F, G), indicating that NPTX2 is necessary for the adaptive strengthening of the glutamatergic synapses in dorsal CA1 after exposing to cocaine or cocaine-associative cue. Knockdown of *Nptx2* increased the PPR and decreased AMPAR EPSCs in dorsal CA1 neurons after retrieval (Figure 3J–M), indicating impaired presynaptic releasing and postsynaptic AMPAR functions in CA3–CA1 synapses.

Hippocampal NPTX2 Recruits GluA1-AMPArs to Facilitate Synaptic Remodeling and Enhance the Extinction of Cocaine-Associated Context Memory

Retrieval effectively returns a memory from long-term storage to an unstable state, where the new information may become

incorporated into old memories. The reconsolidation update-based postretrieval extinction therapy has been shown to be effective for the associative learning-based psychiatric diseases (59,60). However, the boundary conditions that constrain this form of therapy lead us to speculate on the molecular pathways orchestrating neuronal plasticity within a fixed time window. Translational analysis in hippocampal pyramidal neurons showed that NPTX2 is the hub regulator following memory retrieval. NPTX2 serves as a regulator in neurite outgrowth and synapse maturation in dorsal hippocampus (22,61) and is enriched at excitatory synapses colocalizing tightly with the GluA1-AMPAr (62). Here we found that the protein and mRNA level of *Nptx2* went up 1 hour after exposure to the cocaine CPP apparatus, and NPTX2 bidirectionally regulated the membrane-associated GluA1 level and the extinction of cocaine-associated context memory. Knockdown of *Nptx2* decreased the spine number and impaired the AMPAR EPSC current 1 hour after retrieval (Figure 3J–M). The expression of NPTX2 went up 1 hour after each extinction trial until the animal no longer exhibited a preference for the cocaine-paired chamber (Figure 2A–E). The upregulation of NPTX2 mirrors the GluA1-AMPAr trafficking, which facilitates synaptic remodeling and memory instability, allowing selective manipulation of cocaine cue memory during a fixed time window to produce the extinction effect.

Conclusions

Retrieval of cocaine-paired context memory upregulates the expression of *Nptx2* in hippocampal pyramidal neurons, leading to recruit GluA1-AMPAr during the synaptic remodeling, and allows manipulation of cocaine-associated memory during a fixed time window to produce the extinction effect. Based on our findings, the dynamic expression of *Nptx2*, which mirrors the synaptic remodeling after memory retrieval, is provided as a potentially therapeutic strategy for addiction.

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FW and LM designed the research. ZW, TJ, QL, CL, and XW performed the research. ZW, TJ, and QL analyzed data and drafted the manuscript. LM and FW revised the manuscript.

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ARTICLE INFORMATION

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Hippocampal NPTX2 Plasticity in Drug Memory Extinction

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