A synthetic small molecule Isoxazole-9 protects against methamphetamine relapse

Supplementary methods and figures

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Supporting Materials and Methods

**Animals:** Surgical and experimental procedures were carried out in strict adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication number 85–23, revised 1996) and approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute. One hundred-fifty two, male Wistar rats (Charles River), weighing 200-250 g (8 weeks old) at the start of the experiment, were housed two per cage in a temperature-controlled vivarium under a reverse light/dark cycle (lights off 8:00 AM–8:00 PM) for at least one week prior to surgery. The number of animals for each experiment is indicated in the figure legends, and the sample size for each experiment indicated adequate power (>80%) when analyzed with GraphPad StatMate software. All experimental analysis were conducted as a double blinded study where the investigator performing and examining postmortem tissue analysis did not have the code for the animal group or treatment.

**Intravenous Catheter Surgery:** One hundred and nine rats underwent surgery for catheter implantation for intravenous self-administration. Rats were anesthetized with 2–3% of isofluorane mixed in oxygen and implanted with a sterilized silastic catheter (0.64 ID x 1.19 OD mm; Dow Corning Co.) into the right jugular vein under aseptic conditions. The distal end of the catheter was threaded under the skin to the back of the rat and exited the skin via a metal guide cannula (22G, Plastic One, Inc.). Immediately after surgery, Flunixin® (2.5 mg/kg, s.c.; Bimeda – MTC Animal Health Inc) was given as analgesic. The rats were subjected to antibiotic therapy with Cefoxitin or Cefazolin during 10 days after the surgeries. Catheters were flushed daily with antibiotic in
heparinized saline (30USPunits/ml) and tested for patency using methohexital sodium (Brevital®, 10mg/ ml, 2 mg/rat; King Pharmaceutical Inc.).

**Methamphetamine Self-Administration**: Following 4 days of recovery after surgery, ninety-nine animals were trained to lever press for i.v. infusions of methamphetamine (0.05 mg/kg per infusion) in an operant chamber (context A) on an FR1 schedule for 6 hours per session for 17 sessions. Animals were primed for the first hour of the session for the first two sessions. Animals had 17 sessions of methamphetamine self-administration followed by 12 days of abstinence during which animals received one i.p. injection of isoxazole-9 or vehicle (25% HBC) each day, starting on day 1 of abstinence and continued injections for 12 days into abstinence1-3 (treatment was based on an in vivo study1). The day after last isoxazole-9 injection, a subset of animals received one i.p. injection of BrdU (150 mg/kg). Animals remained in their home cages for an additional 13 days followed by extinction and reinstatement sessions.

**Saline self-administration**: Following 4 days of recovery after surgery, ten animals were trained to lever press for i.v. saline (0.9% sterile) in an operant chamber (context A) similar to the paradigm used for methamphetamine self-administration (FR1; 6 hours per session for 17 sessions). Animals had 17 sessions of saline self-administration followed by 12 days of withdrawal. On day thirteen, five animals received one i.p. injection of BrdU (150 mg/kg). Animals remained in their home cages for an additional 13 days followed by extinction and reinstatement sessions.
Extinction: Extinction sessions for methamphetamine and saline animals were performed in a new context (context B) for 1h for six sessions. In this context, animals were not attached to the drug infusion apparatus, white noise was added during the entire session, a house light was turned on for the entire session, and black colored tape was pasted on the operant door. Responses on either the active or inactive lever were recorded and did not result in programmed consequences (i.e., no infusions and no conditioned stimulus presentations). Extinction was defined as reduced (<50%) active lever pressing on days 4-6 vs. day 1 of extinction.

Reinstatement: Twenty-four hours after the final extinction session, animals underwent context-induced reinstatement in which they were placed into the methamphetamine- or saline- paired context (context A) for 1 h, during which they were connected to the infusion apparatus to allow for a similar interaction with the spatial elements of the context as during methamphetamine self-administration training. Lever presses were used as a measure of drug seeking, and responses on either the active or inactive lever were recorded and did not result in an infusion of fluids through the catheter or other programmed consequences (i.e., conditioned stimulus presentations). The next day, animals underwent context-plus-cue-induced reinstatement in which conditions were the same as context-induced reinstatement, and responses on the active lever resulted in the conditioned stimulus light presentation. One hour after the end of the session, animals were euthanized by rapid decapitation or transcardial perfusions.
**Dose-response:** Sixteen animals experienced 14 days of 6h methamphetamine self-administration sessions as described above. The four following days, animals underwent dose-response studies in which the methamphetamine dose administered was changed each day: 0.01, 0.05, 0.1, and 0.2 mg/kg/injection. Dose-response sessions were maintained at 6h and FR1 schedule.

**Progressive ratio:** The day following the last dose-response session, responding was reinforced on a progressive ratio (PR) schedule of reinforcement. On this schedule, the number of responses required for reinforcement incremented progressively, and each session continued until a breakpoint (defined as the number of infusions obtained before 1h elapsed with no infusions) was reached. Breakpoint was determined for 0.05 mg/kg/infusion methamphetamine.

**Functional observational battery tests:** Animals either received 12 injections of Isx-9 (1 injection per day; 20 mg/kg in 25% HBC, i.p.; n=15) or none (controls, n=12) and were weighed after the last day of injections. Following body weight measures, animals were allowed to freely move in an enclosed chamber fitted with a video tracking system for 3 minutes and locomotor activity was measured during the 3 minute duration. Following activity measures, animals were trained on a trace fear conditioning paradigm to determine any effects of Isx-9 injections on motor and sensory reflexes. Animals received 5 CS-US pairings of a 30 second (s) tone (80 dB, CS) that was followed by 45s trace period which terminated with a 2 s footshock (0.5 mA, US) presented on a 20-90 s
variable ITI. All animals remained in the chamber for 4 minutes following the final footshock. After conditioning, animals were immediately returned to the colony room.

**Brain methamphetamine quantification:** Following the completion of the last self-administration session (progressive ratio schedule), methamphetamine (0.4mg/kg, i.v.) was injected into the rats (n=16) via the indwelling jugular catheter. Within an hour, the rats were deeply anesthetized (isoflurane) and killed by decapitation. The striatum and the hippocampus from the left hemisphere of the brain were rapidly dissected, and weighed in microcentrifuge tubes. Tissue methamphetamine concentrations were measured using a previously published liquid chromatography-tandem mass spectrometry assay with minor modifications. Briefly, striatal and hippocampal tissue samples were homogenized by sonication in 500 μl deionized sterile water, and stored at -80 °C. The entire sample homogenate was used for analysis.

**Preparation of Samples and Standards:** On the day of the assay, the homogenates were equilibrated to room temperature. Deuterated methamphetamine (250 ng; METH-d8) was added as internal standard to 500 μl of homogenate. Samples were vortexed for 5 s and then made strongly basic by adding alkaline (pH > 12) with 100 μl of concentrated ammonium hydroxide. Homogenates were extracted into 6 ml of a 4:1 v/v mixture of n-butyl chloride and chloroform for 30 min with gentle shaking. Samples were centrifuged for 10 min at 1200g. The organic phase containing the analyte of interest was transferred to a 16 x 100-mm silanized glass screw-capped test tube and evaporated to dryness at 20°C. Residues were reconstituted with 100 μl of 95:5 formic acid (0.1%) and 5% acetonitrile (v/v) prior to analysis by liquid chromatography/tandem
mass spectrometry. A multipoint calibration curve ranging from 1 to 1500 ng/ml homogenate was prepared with drug-free rat plasma and extracted as described above. Analytical intra-assay accuracy and the lack of matrix effect were verified by concurrent analysis of quality control (known standard) samples that were prepared in drug-free rat brain tissue homogenate.

**Determination of Methamphetamine Concentration:** Concentrations of methamphetamine were determined with Agilent 6490 triple quadrupole mass spectrometer with an electrospray ionization source operated in MRM/positive ion mode (Agilent Technologies, Santa Clara, CA). Separation by liquid chromatography was carried out using a 2.1x50mm Symmetry ® LC C18 column (Waters, Milford, MA). Water (with 0.1% formic acid) and acetonitrile (with 0.1% formic acid) were used for the gradient mobile phase; gradient used was as follows – 100/0 at T=0 min, 100/0 at T=1 min, 65/35 at T=5 min, 0/100 at T=6, Off at T=9 min. A 4-min re-equilibration step was included between two consecutive sample injections. Transition states monitored were m/z 150.1 -> 91.0 for methamphetamine and m/z 158.2 -> 93.1 for METH-d8. The limit of detection in these experiments was 100 fg/μl. Accuracy was within 10% of the methamphetamine concentrations from brain homogenate quality control (known standard) samples.

**Corticosterone assay:** Plasma corticosterone was measure in rats self-administering methamphetamine and in rats self-administering saline using the DetectX ® Corticosterone Enzyme Immunoassay Kit (Arbor Assays, Ann Arbor, MI) following manufacturer instructions. Briefly, 100-150 ul tail blood was collected from the rats
before and/or after the self-administration session in specialized heparin coated microcentrifuge tubes. Plasma was separated by centrifugation of the blood samples at 3000 rpm for 15 min at 4°C, and stored at –80°C. On the day of the assay, the plasma were allowed to thaw on ice and the reagents of the Enzyme Immunoassay Kit were allowed to warm to room temperature prior to use. Samples were prepared by mixing plasma with the Dissociation Reagent in a 1:1 ratio, and then diluting the mixture with Assay Buffer to get a final dilution of 1:100 for the plasma. The manufacturer provided corticosterone standard (100 ng/ml) was serially diluted to generate an 8-point standard curve ranging from 78.125 pg/ml to 10,000 pg/ml. Standards and the diluted samples were added to microtiter plate coated with secondary antibody against sheep. A sheep polyclonal antibody against corticosterone and corticosterone-peroxidase conjugate were added to sample and standard wells. Following an hour of incubation, binding of the corticosterone and corticosterone-peroxidase conjugate to the plate was stopped by washing the wells. Then a peroxidase substrate was added to the wells that produced a colorimetric reaction with the bound corticosterone-peroxidase conjugate. This reaction was stopped and the intensity of the generated color (or optical density) was measured at 450nm using a microtiter plate reader. The concentration of corticosterone in the samples was calculated from the 4-parameter logistic non-linear regression obtained from concentration-optical density plot generated by the 8 known standard dilutions using Graphpad Prism.

Sucrose Self-administration: Twelve rats were trained to orally self-administer sucrose solution (10% sucrose on an FR1 schedule; context A) for seventeen sessions. The first
four sessions served as training for the animal to learn to 1) press the correct lever for a fluid reward and 2) drink the administered fluid from the delivery cup. The first two training sessions were conducted overnight in the operant chambers and active lever presses were rewarded with tap water while the following four sessions were two hours in duration and 10% sucrose was delivered following an active lever press. Subsequent sessions were 30 minutes in duration, and each active lever press activated delivery of the 10% sucrose solution into the delivery cup. Operant sessions were conducted for 5 days followed by 2 days of abstinence for a total of 17 30-minute sessions. Following the last operant session, animals were injected i.p. with either 20mg/kg body weight Isoxazole-9 or vehicle (25% HBC) once per day for 12 days. Subsequently, animals received one i.p. injection of BrdU (150 mg/kg). 13 days following BrdU administration, animals were trained to extinguish their previous operant training. For this, the session duration was the same (30 minutes) but the extinction sessions occurred in a novel context B and active lever presses were not rewarded. Following six extinction sessions, animals were then returned to their original operant context for a single reinstatement session (30 min); however, responses on the active lever did not result in programmed consequences. One hour after the end of the session, animals were euthanized by transcardial perfusions.

Synthesis and MS of Isoxazole-9: Isx-9 was prepared according to cyclodehydration route outlined by Schneider et al 2007 2. Supplementary Figure 2 demonstrates the 1H NMR and HPLC of Isx-9 to confirm identity and purity. The concentration of Isx-9 in plasma or hippocampus tissue was measured via MS using a previously reported
Rats were injected with Isx-9 (20 mg/kg, i.p.) and were decapitated 10, 30 or 60 minutes after the injection (n = 3 each time point) and trunk blood was collected in heparin coated microfuge tubes and hippocampal tissue was dissected, weighed and snap frozen on dry ice. Plasma was isolated and 500 μl of plasma was used for MS. Brain tissue was homogenized in 500 μl of acetonitrile and used for MS. Samples were run on Agilent 6490 triple quadrupole mass spectrometer by the core facility at Scripps Research Institute to estimate amount of Isx-9 (ng) per weight unit (g tissue or ml plasma) (supplementary figure 2).

Retroviral Constructs and Virus Preparations. The mCherry plasmid and retrovirus was generated as previously reported in Magill et al 2010.

Intracranial Surgery: Intracranial surgery was performed the day following the last isoxazole-9 injection or 13 days after the last methamphetamine self-administration session. Animals were anesthetized with isoflurane (2-3%) mixed with oxygen and received stereotaxic bilateral infusions of mCherry retrovirus in the dorsal hippocampus (AP, -4.3mm from bregma; ML, +/- 2.6mm from bregma ; DV, -3.4, -3.25, and -3.0 from dura; 9) with a stainless steel cannula attached to a syringe pump connected by plastic tubing. Animals were infused at a flow rate of 1 μl/min with a total volume of 4.5 μl (1.5 μl infused at -3.4mm, 2 μl infused at -3.25mm, and 1 μl infused at -3.0mm). Immediately after surgery, Flunixin® (2.5 mg/kg, s.c.; Bimeda – MTC Animal Health Inc) was given as analgesic, and Cefoxitin was administered as antibiotic.
Perfusions and brain tissue collection: One hour after behavioral experiments animals were fully anaesthetized using chloral hydrate (240 mg/kg, i.p.). Rats were then transcardially perfused with phosphate-buffered saline (2 min at 15 ml/min and 4 % paraformaldehyde (20 min at 15 ml/min). The brains were dissected out and postfixated in 4 % paraformaldehyde at 4ºC for 16–20 h and sectioned in the coronal plane at a thickness of 40 µm on a freezing microtome. The sections through the brain were collected in nine vials [containing 0.1 % NaN3 in 1X phosphate-buffered saline (PBS)] and stored at 4 ºC. One ninth of the brain region through the hippocampus and the prefrontal cortex was used for immunohistochemical analysis.

Immunohistochemistry: The following primary antibodies were used for immunohistochemistry (IHC): Ki-67 (1 : 1000), BrdU (1 : 400), activated caspase-3 (AC-3, 1:500) and c-Fos (1 : 1000). For Ki-67, BrdU and AC-3 IHC, left and right hemispheres of every ninth section through the hippocampus and mPFC (including the anterior cingulate, prelimbic, and infralimbic cortices) were slide-mounted, coded, and dried overnight before IHC. For Fos IHC, two bilateral sections that contained the dentate gyrus, two bilateral sections that contained the mPFC were used for cell quantification from each rat from each group. The sections were pretreated, blocked, and incubated with the primary antibodies (BrdU, Ki-67, AC-3, Fos) followed by biotin-tagged secondary antibodies. Immunoreactive cells in the SGZ (ie, cells that touched and were within three cell widths inside and outside the hippocampal granule cell-hilus border for BrdU, Ki-67 and AC-3) or granule cell layer (GCL; Fos) were quantified with a Zeiss AxioImagerA2 (×400 magnification) using the optical fractionator method, in which
sections through the dentate gyrus (−1.4 to −6.7 mm from bregma;⁹) were examined. Cells in the SGZ and GCL were summed and divided by the area of the granule cell layer to give the total number of cells per mm². BrdU and Fos cells in the mPFC (3.7–2.2 mm from bregma) were examined, and cells from the left and right hemispheres that were localized in the counting frame (mPFC, 0.09 mm²) were visually quantified. The total number of immunoreactive cells both in the left and right hemispheres was counted and are expressed as the total number of immunoreactive cells per mm² in each rat averaged across rats within a group.

**Western Blotting:** Procedures optimized for measuring both phosphoproteins and total proteins were employed.¹¹ Animals were euthanized via rapid decapitation under light isoflurane anesthesia 1 hour after end of reinstatement session. Brains were quickly removed and flash-frozen. Brain tissue was cut at the mid-sagittal axis and the right hemisphere was processed for Western blotting. Tissue punches from dorsal and ventral hippocampal formation from 500 μm thick sections were homogenized on ice by sonication in buffer (320 mM sucrose, 5 mM HEPES, 1 mM EGTA, 1mMEDTA, 1% SDS, with Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktails II and III diluted 1:100; Sigma), heated at 100 °C for five minutes, and stored at−80 °C until determination of protein concentration by a detergent-compatible Lowry method (Bio-Rad, Hercules, CA). Samples were mixed (1:1) with a Laemmli sample buffer containing β-mercaptoethanol. Each sample containing protein from one animal was run (20 μg per lane) on 8–12% SDS-PAGE gels (Bio-Rad) and transferred to polyvinylidene fluoride membranes (PVDF pore size 0.2 μm). Blots were blocked with 2.5% bovine serum
albumin (for phosphoproteins) or 5% milk (w/v) in TBST (25 mM Tris–HCl (pH 7.4), 150 mM NaCl and 0.1% Tween 20 (v/v)) for 16–20 h at 4 °C and were incubated with the primary antibody for 16–20 h at 4 °C: antibody to NR1 (1:200, Santa Cruz Biotechnology cat. no. sc-1467, predicted molecular weight 115 kDa, observed band ~115 kDa), antibody to NR2A (1:200, Santa Cruz Biotechnology cat. no. sc-9056, predicted molecular weight 177 kDa, observed band ~170 kDa), antibody to pNR2A Tyr-1325 (1:200, PhosphoSolutions cat. no. p1514-1325, predicted molecular weight 180 kDa, observed band ~180 kDa), antibody to NR2B (1:200, Santa Cruz cat. no. sc-9057, predicted molecular weight 178 kDa, observed band ~180 kDa), antibody to pNR2B Tyr-1472 (1:200, Cell Signaling cat. no. 4208S, predicted molecular weight 190 kDa, observed band ~180 kDa), antibody to pNR2B Ser-1480 (1:200, Thermo Scientific cat. no. PA14733, predicted molecular weight 190 kDa, observed band ~180 kDa), antibody to CamKII (1:200, Abcam cat. no. ab52476, predicted molecular weight 47 and 60 kDa, observed band ~47 and 60 kDa), antibody to pCamKII Tyr-286 (1:200, Abcam cat. no. ab5683, predicted molecular weight 50 kDa, observed band ~50 kDa), antibody to HDAC5 (1:200, Abcam cat. no. ab50001, predicted molecular weight 122 kDa, observed band ~130 kDa), antibody to pHDAC5 Ser-259 (1:200, Abcam cat. no. ab192339, predicted molecular weight 122 kDa, observed band ~130 kDa), antibody to pHDAC5 Ser-498 (1:200, Abcam cat. no. ab47283, predicted molecular weight 122 kDa, observed band ~130 kDa), Bcl-2 (1:500, R&D Systems cat. no. MAB8272, predicted molecular weight 24 kDa, observed band ~25 kDa), Bax (1:500, Santa Cruz cat. no. sc-493, predicted molecular weight 23 kDa,
observed band ~20 kDa), and antibody to PSD-95 (1:500, Millipore, cat. no. 04-1066, predicted band size 95 kDa, observed band ~95 kDa). Blots were then washed three times for 15 min in TBST, and then incubated for 1 h at room temperature (24 °C), appropriately with horseradish peroxide-conjugated goat antibody to rabbit or horseradish peroxide–conjugated goat antibody to mouse (1:10,000, BioRad) in TBST. After another three washes for 15 min with TBST, immunoreactivity was detected using SuperSignal West Dura chemiluminescence detection reagent (Thermo Scientific) and collected using HyBlot CL Autoradiography film (Denville Scientific) and a Kodak film processor. Following chemiluminescence detection, blots were stripped for 20 minutes at room temperature (Restore, Thermo Scientific) and reprobed for total protein levels of β-Tubulin (1:4000, Santa Cruz cat. no.sc-53140, predicted molecular weight 50 kDa, observed band ~50 kDa), for normalization purposes. Densitometry was performed using ImageStudio software (Li-Cor Biosciences). X-ray films were digitally scanned at 600 dpi resolution, then bands of interest were selected in identically sized selection boxes within the imaging program which included a 3 pixel extended rectangle for assessment of the background signal. The average signal of the pixels in the ‘background’ region (between the exterior border of the region of interest selection box and the additional 3 pixel border) was then subtracted from the signal value calculated for the band of interest. This was repeated for β-Tubulin, and the signal value of the band of interest following subtraction of the background calculation was then expressed as a ratio of the corresponding β-Tubulin signal (following background subtraction). This ratio of expression for each band was then expressed as a percent of the drug naïve control animals included on the same blot.
Golgi-cox staining and neuron morphology analysis: For determination of the effect of methamphetamine on hippocampal neuronal architecture, the left hemisphere was processed for Golgi–Cox staining. The other half (right hemisphere) was used for Western blotting analysis (See above). For Golgi–Cox staining, the brain was submerged in Golgi–Cox solution A+B (FD Neurotechnologies Inc.) for 8 days at room temperature, followed by solution C for 4 days at room temperature and stored at −80 °C until processed for staining. Frozen brain tissue was coronally cut on a cryostat at 100 μm-thick sections and stained with solution D + E and dehydrated according to manufacturer’s instructions. Brains were coded before sectioning to ensure that experimenters were blind to treatments. To evaluate hippocampal neuron morphology, a Zeiss Axiophot microscope and a computer-based system (Neurolucida; Micro Bright Field) was used to generate three-dimensional neuron tracings that were subsequently visualized and analyzed using NeuroExplorer (Micro Bright Field). In order for a neuron to be selected the following four criteria were met: (1) the neuron was in the region of interest (DG [outer granule cell layer of the superior or inferior blade] of the hippocampus, −2.56 to −3.8mm to bregma), (2) the neuron was distinct from other pyramidal and interneurons to allow for identification of dendrites, (3) the neuron was not truncated or broken, and (4) the neuron exhibited dark, well filled staining throughout including spines. For each animal, 4 granule cell neurons in the DG were traced at 40× magnification. No more than one neuron per region per section was used in the structural analysis. Both the apical and basal trees were traced, and morphological measurements were analyzed separately. For each reconstructed
neuron, an estimate of dendritic complexity was obtained using the Sholl ring method. A 3D Sholl analysis was performed in which concentric spheres of increasing radius (starting sphere 10 μm and increasing in 20 μm increments) were layered around the cell body until dendrites were completely encompassed. The number of dendritic intersections at each increment was counted, and results were expressed as total intersections and the number of intersections per radial distance from the soma. Additionally, total dendritic length and longest soma-to-tip length were calculated for each tree of each reconstructed neuron. For all neurons, spine density was measured at 60×–100× magnification with an oil immersion lens (equipped with a 10× eye piece) on the same dendritic branches used for Sholl analysis. Dendritic spine density was measured on three dendritic segments from both the apical tree. For the apical tree, spines were counted along 10 μm segments of primary apical branches located at least 50 μm away from the cell body. All measurements of spine density were taken from 1–2-μm-thick dendritic segments to minimize the number of spines hidden by the dendritic shaft and ensure that the number of hidden spines was proportional across all segments counted and among treatment groups. The total number of dendritic spines visible along both sides of the segment were counted and expressed as number of spines per total length of dendrite.

**mCherry staining and neuron morphology analysis:** For detecting retrovirus labeled newly born neurons, animals were perfused as previous indicated and brain tissue was sectioned on a cryostat at 100um thickness. Sections were stained with primary antibody against mCherry (1:500, Clontech Laboratories Inc., cat. No. 632543) and
stained neurons were traced to evaluate hippocampal neuron morphology as described for Golgi-Cox analysis.

**Statistical analyses:** The methamphetamine self-administration data is expressed as the mean mg/kg per session of methamphetamine self-administration. The effect of session duration on methamphetamine self-administration during the 6h session and during the first hour of the 6h session was examined over the 17 escalation sessions using a two-way repeated-measures analysis of variance (ANOVA; session duration × daily session) followed by the Student-Newman-Keuls *post hoc* test. The pattern of responding for methamphetamine is expressed as the mean mg/kg per hour over 6 h sessions and active and inactive lever responses in HR and LR rats and were compared between the first and >10th escalation sessions. Differences in the rate of responding between the first and other escalation sessions were evaluated using the paired *t*-test. The effect of methamphetamine withdrawal on extinction and reinstatement in vehicle treated and Isx-9 treated animals was examined using a two-way repeated-measures (ANOVA; meth group x session) followed by the Student-Newman-Keuls *post hoc* test. Similar analyses were performed for sucrose and saline self-administering animals. For the Ki-67, BrdU, AC-3, Fos Golgi-Cox, mCherry and Western blotting analyses, two-way ANOVA was used. The data are expressed as mean ± SEM in all graphs.
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Supplementary figure 1: Extended access methamphetamine self-administration in 99 outbred adult Wistar rats demonstrates enhanced compulsive-like responses in HR animals. (A-B) Active and inactive lever responses in LR (A) and HR (B). LR animals have higher responding on reinforced (active) levers indicated by a significant number of sessions x lever responses interaction F (16, 1498) = 3.247, main effect of number of sessions F (16, 1498) = 2.258 and lever presses F (1, 1498) = 148.5 by repeated measures two-way ANOVA, P<0.01. HR animals have higher responding on reinforced (active) levers indicated by a significant effect of lever presses F (1, 1585) = 88.31 by repeated measures two-way ANOVA, P<0.01. (C) HR animals have higher responding on the active levers during time-out, no interaction, no effect of days, significant effect of session length F (1, 1536) = 250.4, P<0.01. (D) HR animals have higher responding on the inactive levers during time-out, significant session length x inactive lever responses interaction F (16, 1480) = 2.028, main effect of meth group F (1, 1480) = 118.9 and number of sessions F (16, 1480) = 3.249, P<0.01 by two-way ANOVA. (E) Active lever presses during the first hour of the six hour session during self-administration sessions, followed by lever presses during extinction sessions and reinstatement sessions. *P<0.05 vs. LR; #P<0.05 vs. day 17 compared to days 43 and 44; #P<0.05 vs. day 48 compared to days 49 and 50.

Supplementary figure 2: Synthesis of Isx-9 and pharmacokinetics of Isx-9 in adult Wistar rats. HPLC (A) and MS (B) data for Isx-9. Isx-9 was synthesized at the Scripps Research Institute according to 2. (c) MS analysis of Isx-9 in plasma and hippocampus tissue. Isx-9 crosses the BBB, as shown by the concentration of Isx-9 (ng/g) in hippocampus tissue or plasma (ng/ml) 10, 30, and 60 min after 1 i.p. injection of Isx-9 (n=3 per time point). Two-way ANOVA demonstrated a significant Isx-9 amount x time after injection interaction F (2, 9) = 17.35, significant increases in brain Isx-9 vs. plasma F (1, 9) = 32.31, and significant effect of time after injection F (2, 9) = 47.30, P<0.01. Posthoc analysis revealed higher levels of Isx-9 10 min after injection compared with other time points in the adult rat hippocampus tissue, supporting previously reported pharmacokinetic data in the adult mouse brain tissue 1.

Supplementary figure 3: Functional observational battery tests were performed on animals treated with Isx-9 to determine the effects of the small molecule on general behavioral endpoints. (A) Schematic of experimental design and behavioral tests. (B) Body weight measured in grams. (C) Locomotor activity indicated as distance travelled in cm in seconds. (D) Freezing behavior in response to CS-US pairings indicated as percent time freezing normalized to baseline freezing. No significant differences were observed between controls and Isx-9 animals in any of measures indicated.

Supplementary figure 4: Cell death measured by activate caspase-3 is not altered by Isx-9 treatment in the granule cell layer of the hippocampus. (A) Quantitative analysis of the number of activated caspase-3 cells in the granule cell layer of the hippocampus does not demonstrate an effect of methamphetamine or Isx-9. (B) Density of cell death factors in the dorsal and ventral dentate gyrus enriched regions demonstrates no significant effect in any groups.
**Supplementary figure 5: Synthetic small molecule Isx-9 does not alter reinstatement of sucrose seeking behaviors.** (A) Sucrose consumption increases over days of self-administration. Repeated measures one-way ANOVA demonstrates significant increase in sucrose consumption ($F(16, 176) = 4.03, P=0.005$). Post hoc analysis indicated sustained increase in consumption during days 9-17 when compared to the first day of sucrose experience ($Ps <0.05$ by Fishers LSD). (B) Animals injected with Isx-9 did not differ in self-administration behavior compared with animals injected with vehicle control. (c) Vehicle and Isx-9 injected animals do not differ in their latency to extinguish sucrose-seeking behaviors, all animals extinguished before reinstatement session. (D) Vehicle and Isx-9 animals do not differ in their reinstatement to sucrose seeking triggered by sucrose context. (E) Withdrawal and protracted abstinence from sucrose self-administration does not enhance survival of BrdU cells in the DG. Isx-9 increased survival of BrdU cells in controls and sucrose experienced animals, two-way ANOVA demonstrated a significant main effect of Isx-9, $F(1, 16) = 9.598, P<0.05$. (F) Reinstatement of sucrose seeking increased neuronal activation of GCNs in the dorsal and ventral DG and Isx-9 treatment did not affect these increases, main effect of sucrose $F(3, 32) = 10.40, P <0.01$.

**Supplementary figure 6: Neurogenesis is not altered by drug seeking behaviors in saline self-administering animals.** (A) Animals trained to self-administer saline do not increase lever responses over sessions and do not demonstrate lever discrimination between active (reinforcing) and inactive lever responses. (B) Animals trained to self-administer saline do not seek saline and do not demonstrate extinction behavior. (C) Animals trained to self-administer saline do not reinstate saline seeking triggered by context. (D) Withdrawal and protracted abstinence from saline self-administration does not alter survival of BrdU cells in the DG. (E) Neuronal activation is enhanced in the GCNs in saline seeking animals that did not demonstrate reinstatement triggered by saline context. Two-way ANOVA demonstrated significant effect of saline context on the number of cFos cells $F(1, 14) = 21.55, P<0.05$.

**Supplementary figure 7: Isx-9 does not alter the number of glial progenitors and gliogenesis in the medial prefrontal cortex of methamphetamine seeking rats.** (A) Withdrawal and protracted abstinence from methamphetamine self-administration does not alter the number of glial progenitors in the medial prefrontal cortex. (B) Isx-9 treatment did not alter the number of glial progenitors in the prefrontal cortex. (C) Reinstatement of methamphetamine seeking triggered by drug context and cues enhanced neuronal activation in the medial prefrontal cortex, and Isx-9 did not modify these effects. Main effect of treatment, $F(5, 22) = 4.015, P<0.05$.

**Supplementary table 1: Isx-9 does not alter GluN1, GluN2A receptor and PSD-95 expression in the dentate gyrus.** Withdrawal and protracted abstinence does not alter the expression of other proteins implicated in synaptic activity.
Galinato et al Supplementary Figure 1

A

B

C

D

E

Galinato et al
Galinato et al Supplementary figure 2
Supplementary figure 3

(A) Days 1-12, 13, 14

- Vehicle (HBC) / Isoxazole-9
- Body weight
- Locomotor activity
- Fear conditioning

(B) Body weight (gms)
- Control
- Isoxazole-9

(C) Locomotor activity (cm per second)

(D) % Time Freezing Normalized to Baseline

- Tone 1, 2, 3, 4, 5
Supplementary figure 4

A

![Bar chart showing the number of AC3-IR cells in the Granule Cell Layer for different groups.]

- **HBC, Control**
- **HBC, LR**
- **HBC, HR**
- **Isx-9, Control**
- **Isx-9, LR**
- **Isx-9, HR**

B

| Protein Markers | Density of protein (% of control) |
|-----------------|-----------------------------------|
| **DORSAL Dentate gyrus** | LR, HBC | HR, HBC | drug naive, Isx-9 | LR, Isx-9 | HR, Isx-9 |
| Bax             | 81.90 ± 6.90  | 94.30 ± 15.39 | 101.10 ± 12.73 | 88.99 ± 6.89 | 88.12 ± 12.12 |
| Bcl-2           | 108.30 ± 7.40 | 117.40 ± 8.17 | 116.05 ± 18.34 | 118.80 ± 9.97 | 130.30 ± 17.94 |

| Protein Markers | Density of protein (% of control) |
|-----------------|-----------------------------------|
| **VENTRAL Dentate gyrus** | LR, HBC | HR, HBC | drug naive, Isx-9 | LR, Isx-9 | HR, Isx-9 |
| Bax             | 103.20 ± 9.06  | 103.10 ± 5.80 | 101.40 ± 7.31 | 100.60 ± 6.84 | 96.80 ± 0.42 |
| Bcl-2           | 130.60 ± 33.10 | 139.7 ± 32.15 | 107.70 ± 12.09 | 122.80 ± 30.04 | 136.60 ± 45.07 |
Galinato et al Supplementary figure 5

A

B

C

D

E

F

Galinato et al Supplementary figure 5

A

B

C

D

E

F
Galinato et al Supplementary figure 6

A

Active Lever Responses
Inactive Lever Responses

Sessions

Saline Self-administration, 6h

B

Active Lever Responses
Inactive Lever Responses

Extinction Sessions

Ext 1  Ext 2  Ext 3  Ext 4  Ext 5  Ext 6

C

Extinction, Active Lever
Reinstatement, Active Lever
Reinstatement, Inactive Lever

Lever Presses

Context  Contextual-Cued

D

BrdU-IR Cells per mm^2

Control  Saline

E

Control, n = 4
Saline, n = 10

Dorsal GCNs  Ventral GCNs
Supplementary figure 7

A

B

Medial prefrontal cortex

BrdU-IR Cells per mm^2

HBC Control
HBC LR
HBC HR
Isx-9 Control
Isx-9 LR
Isx-9 HR
HBC Sucrose
Isx-9 Sucrose

Infralimbic Cortex
Prelimbic Cortex
Cingulate Cortex
mPFC Cortex

cFos expressing cells/mm^2

HBC
Isx-9
HBC
Isx-9
HBC
Isx-9
HBC
Isx-9
HBC
Isx-9

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### Galinato et al Supplementary Table 1

#### DORSAL Dentate gyrus

| Protein markers | LR, HBC | HR, HBC | drug naive, lsx-9 | LR, lsx-9 | HR, lsx-9 |
|-----------------|---------|---------|-------------------|-----------|-----------|
| tGluN1          | 112.02 ± 9.69 | 114.8 ± 13.32 | 95.99 ± 6.40 | 109.80 ± 8.97 | 104.40 ± 12.44 |
| tGluN2A         | 113.39 ± 16.40 | 101.30 ± 9.87 | 90.05 ± 12.34 | 108.40 ± 12.89 | 87.57 ± 8.88 |
| pGluN2A Tyr 1246| 107.2 ± 9.59 | 121.6 ± 17.39 | 121.3 ± 20.10 | 119.30 ± 11.27 | 122.60 ± 17.44 |
| pGluN2B Ser1480 | 108.3 ± 7.4 | 117.4 ± 8.1 | 116.0 ± 16.2 | 118.6 ± 9.9 | 130.3 ± 17.9 |
| pHDAC5 Ser498   | 88.1 ± 16.2 | 93.4 ± 10.4 | 101.5 ± 8.1 | 98.1 ± 21.9 | 90.2 ± 15.8 |
| PSD-95          | 80.84 ± 16.18 | 99.43 ± 9.17 | 100.00 ± 13.14 | 122.10 ± 20.54 | 73.39 ± 7.32 |

#### VENTRAL Dentate gyrus

| Protein Markers | LR, HBC | HR, HBC | drug naive, lsx-9 | LR, lsx-9 | HR, lsx-9 |
|-----------------|---------|---------|-------------------|-----------|-----------|
| PSD-95          | 120.05 ± 12.71 | 122.89 ± 13.65 | 96.83 ± 4.57 | 108.00 ± 7.89 | 130.20 ± 15.69 |