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A synthetic small molecule Isoxazole-9 protects against methamphetamine relapse

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Abstract

Adult neurogenesis in the dentate gyrus (DG) is strongly influenced by drug taking behavior and may have a role in the etiology of drug seeking behavior. However, mechanistic studies on the relationship of neurogenesis on drug seeking are limited. Outbred Wistar rats experienced extended access methamphetamine self-administration and individual differences in drug taking defined animals with higher preferred and lower preferred levels of drug intake. Forced abstinence from higher preferred levels of drug taking enhanced neurogenesis and neuronal activation of granule cell neurons (GCNs) in the DG and produced compulsive-like drug reinstatement. Systemic treatment with the drug Isoxazole-9 (a synthetic small molecule known to modulate neurogenesis in adult rodent brain) during abstinence blocked compulsive-like context-driven methamphetamine reinstatement. Isoxazole-9 modulated neurogenesis, neuronal activation and structural plasticity of GCNs, and expression of synaptic proteins associated with learning and memory in the DG. These findings identify a subset of newly born GCNs within the DG that could directly contribute to drug-seeking behavior. Taken together, these results support a direct role for the importance of adult neurogenesis during abstinence in compulsive-like drug reinstatement.

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Conflict of Interest

The authors declare no competing financial interests in relation to the work described.

Keywords

Self-administration; psychostimulants; hippocampus; neurogenesis; BrdU; CaMKII

Introduction

Methamphetamine addiction is a serious public health problem and the rate of recovery from methamphetamine addiction is extremely low. One aspect of addiction, compulsive drug seeking, can be modeled in rodents with extended access to methamphetamine [1]. One distinct advantage of these models is that the neuroplasticity in neurobiological function can be delineated in rodents based on individual differences in drug seeking animals [2–4]. These findings demonstrate that animals with preferentially higher levels of drug intake are powerful models for identifying neurobiological factors involved in the acquisition, maintenance and risk of relapse and provide a means to increase our understanding of addiction-like behaviors using rodent models.

Functional granule cell neurons (GCNs) are generated in the granule cell layer (GCL) of the dentate gyrus (DG) of the hippocampus throughout life by a multistep process called neurogenesis [5, 6]. The process of neurogenesis involves stem-like precursor cells that proliferate into preneuronal progenitors, which in turn differentiate into immature neurons and eventually mature into GCNs [7]. GCNs generated during adulthood assist with neuronal turnover [8]. Computational and behavioral models combined with electrophysiological findings indicate that the DG participates in an array of behaviors to assist with hippocampal dependent spatial memory [9]. For example, GCNs in the DG communicate with CA3 neurons, mossy cells and hilar interneurons to modulate interference between similar spatial inputs via cognitive discrimination. Furthermore, newly born GCNs modulate sparseness of activity of pre-existing GCNs through recruitment of feedback inhibition, and via adaptive changes to DG network excitability affect and strengthen cognitive discrimination [10, 11]. Neurogenesis may also enable animals to distinguish related stimuli and events rapidly and support contextual discrimination. In addition to their role in discrimination, new evidence supports the functional significance of neurogenesis in hippocampal memory clearance [12, 13], suggesting that endogenous alterations in neurogenesis and DG excitability could contribute to memory-related disorders [14].

Neuroanatomical studies in the hippocampus support segregation of neuronal outputs along the dorso-ventral axis whose connectivity may influence the expression of neurogenesis in the DG and behavior dependent on the hippocampus [15, 16]. For example, the dorsal hippocampus has higher levels of neurogenesis and is vital for spatial learning, and is particularly critical in mediating contextual discrimination [17]. In contrast, the ventral hippocampus has lower levels of neurogenesis and is strongly associated with negative affective symptoms that promote propensity for reinstatement of drug seeking [18, 19]. Similar functional differences have been noted along the septo-temporal axis of the hippocampus in humans, with ventral hippocampus demonstrating greater activity in response to negative affective symptoms [20]. Given the functional distinction between the dorsal and ventral

hippocampal regions, the role of neurogenesis and hippocampal synaptic events along the dorsal-ventral gradient in regulating contextual discrimination should be investigated.

In the context of substance and alcohol use disorders, it is predicted that hippocampal neurogenesis protects the neural and behavioral plasticity suppressed by drugs of abuse and alcohol. For example, reinforcing doses of stimulants, opiates and alcohol during drug taking suppress proliferation, differentiation and survival of neural progenitors. Forced abstinence from stimulants and alcohol stimulate proliferation and enhance survival of neural progenitors, suggesting a rebound effect [21]. These findings demonstrate that suppression of, and stimulation of neurogenesis are being observed at various stages of substance abuse disorders. Therefore, it has been hypothesized that spontaneous neurogenesis during forced abstinence may block memories associated with the contextual reinstatement of drug seeking or promote extinction learning [22]. However, the reduction in spontaneous neurogenesis during self-administration and robust rebound in neural progenitors and neurogenesis during abstinence are associated with enhanced propensity for reinstatement in methamphetamine experienced animals [23], suggesting reinforcement of drug memories by enhanced neurogenesis during abstinence. This led us to hypothesize that spontaneous neurogenesis during abstinence may produce productive effects on reinstatement of drug seeking by strengthening drug associated memories (enhance contextual discrimination between drug-paired context and drug-unpaired context and prevent memory clearance). Conversely, we hypothesized that inhibiting or preventing neurogenesis during abstinence would clear drug associated memories and reduce the efficacy of context-driven reinstatement. To test this hypothesis, a synthetic small molecule isoxazole-9 (Isx-9; [N-cyclopropyl-5-(thiophen-2-yl)isoxazole-3-carboxamide]; [24–26]) was administered during forced abstinence to evaluate the efficacy of the molecule in modulating the neurogenesis response during abstinence, and in reducing context-driven reinstatement of drug seeking.

Materials and Methods

Detailed methods are provided for all behaviors and procedures conducted in the supplementary methods section. One hundred-fifty two, adult male Wistar rats (Charles River), weighing 200–250 g (8 weeks old) at the start of the experiment were used for the study. All procedures were approved by the IACUC at The Scripps Research Institute. One hundred and nine rats underwent surgery for catheter implantation for intravenous self-administration (Supplementary methods). 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) and ten animals were trained to lever press for i.v. infusions of saline (0.9%) in an operant chamber (context A) on an FR1 schedule for 6 hours per session for 17 sessions. Some animals were trained to self-administer sucrose (context A, oral, 10%w/v). After 17 sessions of methamphetamine or sucrose self-administration animals experienced forced abstinence for 24 days. During abstinence animals received one i.p. injection of Isx-9 or vehicle (25% HBC; Supplementary methods) each day, starting on day 1 of abstinence and continued injections for 12 days into abstinence [26–28] (treatment was based on an *in vivo* study [27]). The day after last isoxazole-9 injection, a subset of animals received one i.p. injection of BrdU (150 mg/kg) or i.c. injection of mCherry retrovirus (Supplementary

methods). Animals then experienced extinction (context B) and reinstatement (context A) sessions (Supplementary methods). One hour after reinstatement session animals were euthanized and brain tissue was processed for histology, Western blotting analysis, and cellular quantification (Supplementary methods). Statistical analysis were conducted using one-way, two-way ANOVAs followed by Student-Newman-Keuls *post hoc* test (Supplementary methods).

Results

Extended access methamphetamine self-administration in 99 outbred adult Wistar rats demonstrates high and low preferred intake in methamphetamine

Animals were separated into compulsive-like responders (high responders, HR) and noncompulsive-like responders (low responders, LR) based on escalation criteria (defined as greater than 150 % change in active lever (reinforced) responses during sessions 13–17 compared with sessions 1–5 after a median split analysis on their reinforced lever responses during sessions 13–17. HR have higher responding on reinforced (active) levers indicated by a significant methamphetamine group × active lever responses interaction $F(16,1581)=8.2$, main effect of methamphetamine group $F(1,1581)=608.4$ and number of active lever presses $F(16,1581)=10.2$ by two-way ANOVA, $P<0.01$ (Figure 1A–B). HR have higher responding on the non-reinforced (inactive) levers compare to LR (no interaction, no effect of days, significant effect of methamphetamine group $F(1,1585)=88.3$, $P<0.01$; Figure 1C; Supplementary Figure 1A–B). HR have higher responding on the active levers during the 1st hour of the 6 hour session, indicated by a significant methamphetamine group × active lever responses interaction $F(16,1456)=3.3$, main effect of methamphetamine group $F(1,1456)=289.9$ and number of active lever presses $F(16,1456)=4.296$ $P<0.01$ by two-way ANOVA (Figure 1D). HR exhibit an upward shift in peak self-administration rates, and a rightward shift in the descending limb of the self-administration dose–response curve compared to LR indicated by a significant methamphetamine group × dose interaction $F(3,42)=4.018$, main effect of methamphetamine group $F(1,14)=11.62$ and number of active lever presses $F(3,42)=12.96$ $P<0.01$ by two-way ANOVA (Figure 1E). Methamphetamine self-administration data converted to dose-intake curves shows that HR take greater daily amounts of methamphetamine at doses on the descending limb of the dose–response curve compared to LR, $P<0.01$ (Figure 1F). HR demonstrate higher escalation in drug self-administration ($t=5.46$, $df=61.08$, $P<0.0001$ by unpaired t test; Figure 1G). HR demonstrate uncontrolled responding during time-out indicated by main effect of methamphetamine group $F(1,1536)=250.4$, $P<0.0001$ by two-way ANOVA, indicating inability to suppress unrewarded behavior, while maintaining the ability to discriminate reinforced active lever responses from non-reinforced inactive lever responses (Supplementary Figure 1C–D). HR demonstrate higher motivation to seek methamphetamine as measured by infusions earned during self-administration on a progressive ratio schedule ($t=2.4$, $df=13$, $P<0.05$ by unpaired t test; Figure 1H). Amount of meth measured by mass spectroscopy in the hippocampus following meth challenge (0.4 mg/kg, i.v.) showed no significant differences between HR and LR (Figure 1I). Plasma corticosterone levels collected at two time points during animals' dark cycle before initiation of self-administration and after completion of the session on the same day shows no difference between HR and LR, however, demonstrate a

significant increase in basal corticosterone levels in HR and LR compared to saline self-administering animals (F=13.1, $P<0.01$; Figure 1J).

Synthetic small molecule Isoxazole-9 reduces drug seeking during context- and cue-induced reinstatement in abstinent HR

Isx-9 was synthesized according to [26], and pharmacokinetic studies indicate that systemic injections of the compound crosses the blood-brain barrier and into the hippocampus with a half-life of 29.2 minutes (Supplementary Figure 2A–C). To determine whether Isx-9 itself produced any confounding behavioral responses we treated a separate set of drug and behavior naïve rats with Isx-9 (Supplementary Figure 3A) and investigated the potential effects of Isx-9 in functional observational battery tests. Isx-9 treatment did not alter body weight, locomotor activity and sensory/motor reflex responses (Supplementary Figure 3B–D; all Ps>0.05).

Prior to initiation of Isx-9 administration (vehicle [HBC; (2-hydroxypropyl)-β-cyclodextrin] and Isx-9 rats were matched for self-administration behavior (Figure 2B). HBC-HR responded higher than HBC-LR during days 1–3 of extinction (significant methamphetamine group × extinction days interaction F(5,210)=3.027, main effect of extinction days F(5,210)=29.61 and methamphetamine group F(1,42)=7.339, $P<0.001$ by two-way ANOVA; Figure 2E,F). Isx-9-HR responded higher than Isx-9-LR during days 1–3 of extinction (no significant interaction, main effect of extinction days F(5,175)=24.05 and methamphetamine group F(1,35)=7.165, $P<0.001$ by two-way ANOVA). Lever responses on the previously paired active levers were higher than inactive levers in HBC-HR, HBC-LR and Isx-9-LR animals ($P<0.05$). HBC-HR responded higher than HBC-LR and Isx-9-HR during context-driven reinstatement (significant methamphetamine group × lever responses interaction F(6,154)=6.215, P<0.001; main effect of reinstatement F(2,154)=30.24, P<0.01; and methamphetamine group F(3,77)=4.710, $P<0.001$ by repeated measures two-way ANOVA; Figure 2G). Lever responses on the previously paired active levers were higher than inactive levers in HBC-HR ($P<0.05$). HBC-HR responded higher than HBC-LR and Isx-9-HR during contextual cued reinstatement (significant meth group × lever responses interaction F(6,154)=4.911, P<0.001; main effect of reinstatement F(2,154)=20.88, P<0.01; and meth group F(3,77)=7.798, $P<0.001$ by repeated measures two-way ANOVA; Figure 2H).

Isoxazole-9 produced distinct alterations in neurogenesis and neuronal activation of GCNs in the dorsal and ventral DG in abstinent HR

17-day-old 5-bromo-2'-deoxyuridine (BrdU) cells in HBC and Isx-9 HR and LR were examined in the GCL immediately after the reinstatement session to quantify the number of newly born GCNs (BrdU colabeled with neuronal marker neuronal nuclease, NeuN), and activation of newly born GCNs (BrdU colabeled with NeuN and Fos). Brain tissue was also processed for Ki-67 to quantify proliferation of newly born progenitors and Fos to quantify activation of GCNs that were preexisting relative to newly born (BrdU) GCNs. Isx-9 controls had higher number of BrdU and Ki-67 cells compared to HBC controls; Isx-9-HR had reduced number of BrdU and Ki-67 cells compared with HBC-HR and similar number of cells compared to HBC controls. Two-way ANOVA demonstrated a significant

methamphetamine group × Isx-9 interaction F(3,35)=4.111, main effect of methamphetamine F(3,35)=3.437 and main effect of Isx-9 F(1,35)=5.694 on BrdU (Figure 3C; *P*s<0.01), and a significant methamphetamine group × Isx-9 interaction F(2,31)=6.092 on Ki-67 cells (Figure 3D; *P*<0.01). Isx-9-HR and -LR had similar number of activated caspase-3 cells compared with HBC-HR and -LR and controls (Supplementary Figure 4A). Isx-9 controls had similar ratio of the phenotype of BrdU cells compared to HBC controls. Isx-9-HR and -LR had similar ratio of the phenotype of BrdU cells compared to HBC-HR and -LR and controls. Two-way ANOVA demonstrated a main effect of methamphetamine group F(5,72)=5.69 and main effect of phenotype F(2,72)=62.9 in dorsal BrdU cells (Figure 3G; *P*<0.001), and a significant methamphetamine group × Isx-9 interaction F(10,69)=2.1, main effect of methamphetamine group F(5,69)=5.4 and main effect of phenotype F(2,69)=52.04 in ventral BrdU cells (Figure 3H; *P*<0.001). Quantitative analysis showed higher number of nonneuronal BrdU cells in dorsal GCL and higher number of neuronal BrdU cells in dorsal and ventral GCL in Isx-9 controls compared to HBC controls (Figure 3G, H). HBC-HR had higher number of neuronal BrdU cells compared to HBC controls in dorsal and ventral GCL (*P*s<0.05). Isx-9 controls did not have higher number of activated BrdU cells in dorsal and ventral GCL compared to HBC controls. Isx-9-HR had reduced number of activated BrdU cells in dorsal GCL compared to HBC-HR (*P*<0.05; Figure 3G).

Quantitative analysis of Fos expressing GCNs demonstrated an increase in the number of activated cells in the dorsal and ventral GCL in HBC-HR and -LR compared to controls and a reduction in the number of activated cells in Isx-9-HR in the ventral GCL compared to HBC-HR (main effect of methamphetamine in the dorsal hippocampus F(2,24)=10.96, *P*<0.01; a significant interaction F(2,22)=3.863 and main effect of methamphetamine in the ventral hippocampus F(2,22)=7.759, *P*<0.01 by two-way ANOVA; Figure 3K).

The number of BrdU cells were quantified in the prefrontal cortex, a brain region where newly born progenitors mostly differentiate into oligodendrocyte progenitors [29]. We demonstrate that Isx-9's effects were attributable to specific effects in the GCNs as no alterations were found in gliogenic progenitors in the medial prefrontal cortex (Supplementary Figure 7).

Isoxazole-9 produced alterations in structural plasticity of pre-existing and newly born GCNs in the dorsal DG

17-day-old retrovirus expressing mCherry (mCherry) cells in HBC and Isx-9 HR and LR were examined in the GCL immediately after the reinstatement session to quantify the dendritic structure of newly born GCNs. Brain tissue was also processed for Golgi-Cox analysis to examine structural alterations in GCNs that were preexisting relative to newly born (mCherry) GCNs. Isx-9-HR showed enhanced structural plasticity of mCherry and Golgi-Cox GCNs compared to HBC-HR, indicated by enhanced spine density and dendritic extent (main effect of Isx-9 by two-way ANOVA F(1,103)=7.543, *P*<0.01, Figure 4G), dendritic extent of Golgi-Cox GCNs (main effect of Isx-9 by two-way ANOVA F(1,127)=7.249; *P*<0.01, Figure 1H) and mCherry GCNs (main effect of methamphetamine by two-way ANOVA F(2,125)=7.138, *P*<0.01, Figure 1I). 3D Sholl analyses of Golgi-Cox and mCherry GCNs demonstrated a significant neuron type × distance from soma

interaction (F(15,690)=7.166, $P<0.01$, Figure 4J). 3D Sholl analyses of Golgi-Cox GCNs demonstrated main effect of methamphetamine (F(5,386)=6.506, P<0.05) and distance from soma (F(15,386)=43.40, $P<0.01$, Figure 4K). 3D sholl analyses of mCherry GCNs demonstrated main effect of methamphetamine (F(5,559)=10.97, P<0.01) and distance from soma (F(15,559)=34.83, $P<0.01$, Figure 4L).

Isoxazole-9 alters the expression of synaptic plasticity proteins in the dorsal and ventral DG

Brain tissue was snap frozen one hour after the reinstatement session and micropunches enriched in the dorsal and ventral DG GCNs were separated and homogenized and cytoplasm enriched fractions were processed for immunoblotting. Isx-9-HR showed reduced density of total N-methyl-D-aspartate (NMDA) glutamate receptor 2B (GluN2B [30]), and enhanced expression of phosphorylated GluN2B in dorsal DG without producing any changes in the ventral DG (Dorsal dentate gyrus: tGluN2B-main effect of Isx-9: F(1,41)=4.741, $P<0.05$; pGluN2B- main effect of methamphetamine F(2,41)=4.218, $P<0.05$; main effect of Isx-9 F(1,41)=9.845, $P<0.01$, Figure 5E). Isx-9 controls showed higher levels of phosphorylated Ca^{2+}/calmodulin (CaM)-dependent protein kinase (CaMK), pCaMKII, in the dorsal and ventral DG compared to HBC controls. Isx-9-HR and -LR showed higher levels of pCaMKII in dorsal DG compared to HBC-HR and -LR. Isx-9-HR showed lower levels of pCaMKII in ventral DG compared to HBC-HR (Dorsal DG: pCaMKII- main effect of Isx-9 F(1,43)=13.10, $P<0.001$, Figure 5G; Ventral DG: pCaMKII- methamphetamine group × Isx-9 treatment interaction F(2,38)=6.561, $P<0.01$; main effect of methamphetamine F(2,38)=6.273, $P<0.01$, Figure 5H). Isx-9-HR showed higher levels of phosphorylated class IIa histone deacetylase (HDAC5) in dorsal DG compared to HBC-HR (pHDAC5-methamphetamine group × Isx-9 treatment interaction F(2,40)=3.820, $P<0.05$, Figure 5I).

Discussion

Compulsive-like Methamphetamine Intake Predicts Higher Reinstatement of Methamphetamine Seeking

We demonstrate compulsive-like behavior in HR self-administering intravenous infusions of methamphetamine in an extended access schedule of reinforcement. This was evident as increased responding during self-administration sessions and during time-out periods, increased peak self-administration rates and responding during progressive-ratio schedules reflecting enhanced motivation for methamphetamine [2]. These behavioral differences between HR and LR cannot be explained by differential metabolism or bioavailability of methamphetamine, or differences in circadian dependent corticosterone release by methamphetamine. These findings demonstrate that HR are powerful models for identifying neurobiological factors involved in determining risk for relapse and will improve our understanding of addiction-like behavior with regard to its translational value to human addiction.

To determine differences in propensity for relapse, HR and LR were withdrawn from methamphetamine and after a period of protracted abstinence (22 days; a timeframe required for preneuronal progenitor cells to become GCNs), all animals were tested for reinstatement

of drug seeking in an A-B-A self-administration-extinction-reinstatement paradigm [31]. HR demonstrated significantly greater drug seeking behavior (as defined by lever pressing) during extinction compared to LR. However, responding was significantly reduced in both HR and LR rats, reaching equivalent levels of performance after 6 days of extinction training. Following extinction, reinstatement of drug-seeking behavior was tested by re-exposing the animal to the training drug context (context A) without cues (context only) or with cues (context A + cues). HR exhibited greater methamphetamine-seeking behavior on the previously associated drug-paired lever compared to LR despite the continued absence of the drug reinforcer. These findings provide further validity to the enhanced compulsive-like drug seeking observed in HR, as they demonstrated enhanced propensity for reinstatement after protracted abstinence via enhanced contextual discrimination.

Isoxazole-9 Reduces Reinstatement of Methamphetamine Seeking in HR

Robust modulation of neurogenesis is achieved by pharmacological agents (e.g., antidepressants, anticonvulsants, synthetic small molecules [32]). *In vitro* studies demonstrate that the synthetic small molecule Isx-9 triggers release of intracellular calcium (Ca^{2+}), specifically in neuronal progenitor cells via high-voltage Ca^{2+} channels and GluN2B, suggesting neurotransmitter-like properties selective to this cellular population [24, 26, 28]. Increases in intracellular Ca^{2+} by Isx-9 produce cellular excitation in progenitor cells that drives expression of genes and epigenetic factors such as HDAC5 via CaMK activity to direct the phenotype of progenitors into neurons [26, 33]. Isx-9 also increases neurogenesis *in vivo* [25, 27]. These findings suggest that Isx-9 can modulate excitatory neurotransmission and synaptic plasticity, particularly in the hippocampus via GluN2B [26], and mediate synaptic events in the hippocampus and long-term memory storage dependent on the hippocampus via CaMKII [34]. Notably, Isx-9 produces growth arrest by inhibiting cell differentiation in cells selectively sensitive to disturbances in Ca^{2+} homeostasis (e.g., cells born during hyperglutamatergic state or cells expressing altered GluN2B), providing a potential target mechanism for reducing cells of an immature progenitor status with compromised function [28]. Therefore, Isx-9 could be used to modulate neurogenesis and thereby influence contextual discrimination by either increasing or reducing the available pool of adult generated neurons to alter their capacity for information processing.

To investigate the role of Isx-9 in context-driven reinstatement, we injected HR and LR with Isx-9. Following extinction, Isx-9 treated HR failed to reinstate drug-seeking responding compared to vehicle treated HR. These findings demonstrate that Isx-9 reduces propensity for reinstatement, possibly by promoting methamphetamine context-specific extinction learning mechanisms.

Isx-9 reduces the neuronal activation of newly born GCNs in HR

We studied Isx-9-induced alterations in the number of newly born GCNs and activation of GCNs that could be associated with reduced methamphetamine seeking. Quantification of BrdU cells revealed that Isx-9 increased the number of cells in methamphetamine naïve and sucrose self-administering animals. In methamphetamine treated animals, forced abstinence increased the number of BrdU cells by 70% and 52% in vehicle treated HR, relative to both methamphetamine naïve controls and LR. Isx-9 treatment in methamphetamine treated

animals did not increase the number of BrdU cells during forced abstinence compared to vehicle treated controls, and this effect could be due to a hostile cellular environment in the progenitor pool created during methamphetamine experience [35]. For example, methamphetamine experience reduces net proliferation of progenitors and immature neurons by reducing the number of proliferating preneuronal neuroblasts and increasing the number of proliferating preneuronal progenitor cells [35], suggesting that a decrease in the number of progenitors and immature neurons, to a large degree, is attributable to the decrease in the ability of neuroblasts to divide and produce stable progenitor cells that survive as immature neurons [35, 36]. However, abstinence from methamphetamine experience increases net proliferation of progenitors and survival of newly born GCNs, suggesting that cell intrinsic signals that maintain cell proliferation are differentially regulated during abstinence from the drug [23]. The increases in net proliferation and survival during abstinence observed in vehicle treated HR were not evident in Isx-9 treated HR when compared to drug naïve controls. Taken together, while the effects of Isx-9 on proliferation and survival in methamphetamine naïve and methamphetamine treated animals are quantitatively different, further investigation is required to determine the cellular mechanism underlying the difference. Labeling for activated caspase-3 and analyses of pro- and anti-cell death factors indicated that the higher number of BrdU cells in vehicle treated HR and similar number of BrdU cells in Isx-9 treated HR compared to drug naïve controls was not correlated with alterations in apoptosis.

Isx-9 increased neurogenesis in the dorsal and ventral GCL, and enhanced generation of nonneuronal cells in the dorsal GCL compared to vehicle treated controls. Colabeling analysis of BrdU with Fos demonstrated neuronal activation of BrdU GCNs, however, context-driven reinstatement did not alter the activity of BrdU GCNs in vehicle treated HR and LR compared to controls. Notably, Isx-9 treatment reduced the activity of dorsal BrdU GCNs in HR compared to vehicle treated HR. These findings demonstrate that the behavioral effects of reduced context-driven reinstatement produced by Isx-9 in HR is associated with reduced activation of newly born GCNs in the dorsal GCL. Examination of Fos activation of preexisting GCNs demonstrated that significant number of GCNs were activated during context-driven reinstatement in dorsal and ventral GCL in HR (450%) and LR (300%) relative to methamphetamine naïve controls. Isx-9 treatment did not alter reinstatement-induced Fos activation in the dorsal GCL. In contrast, Isx-9 abolished Fos activation in the ventral GCL in HR compared to vehicle treated HR. Therefore, the findings with Fos demonstrate that dorsal and ventral GCNs respond similarly to context-driven reinstatement-elicited brain activity, and reduced reinstatement is associated with reduced activation of newly born GCNs in the dorsal GCL and preexisting GCNs in the ventral GCL in Isx-9 treated HR. Taken together, Isx-9 treatment may have reduced the incentive motivational effects of the drug context to some degree, resulting in the reduced Fos expression in GCNs.

To examine the specificity of these findings, separate groups of animals were trained to self-administer sucrose or saline and tested using an identical extinction-reinstatement procedure (Supplementary Figure 5–6). The results of these studies show that neurogenesis and Fos responses were not altered in sucrose-trained or saline-trained animals suggesting that Isx-9's effects on alterations in neurogenesis, neuronal activation and the behavioral

responses are attributable to drug specific effects and not to general nondrug reward seeking or other procedural influences. Therefore, while these experimental groups served to demonstrate specificity of Isx-9 on drug reward, additional studies are needed to determine the mechanisms underlying Isx-9's effects on neurogenesis and neuronal activation of GCNs and reduced methamphetamine seeking in HR.

The neuronal phenotype of newly born GCNs was further confirmed by retroviral labeling studies, where 17-day-old mCherry-labeled cells exhibited neuron-like morphology with apical dendrites arborizing in the molecular layer of the DG. Because Isx-9 reduced activation of BrdU GCNs in the dorsal GCL, we examined alterations in structural plasticity in newly born and preexsisting Golgi-Cox labeled dorsal GCNs. Three dimensional Sholl analysis demonstrated that newly born GCNs have distinct arborization profiles relative to older preexisting GCNs. Further analysis revealed that HR exhibited reduced arborization of newly born and preexisting GCNs, and reduced dendritic extent in distal dendrites and reduced spine density relative to methamphetamine naïve controls, and this effect was inhibited by Isx-9 treatment. The effects in structural changes were not evident in LR. These findings demonstrate that forced abstinence from methamphetamine increases neurogenesis with compromised structural arborization of newly born GCNs in the dorsal GCL and the effect was specifically seen in animals that demonstrated compulsive-like behavior and higher propensity for reinstatement. Isx-9 treatment reduced context-driven reinstatement of drug seeking in HR and reduced activation of, and modified the structure of newly born GCNs. Taken together, these results demonstrate a novel relationship between abstinence-induced alterations in newly born GCNs and enhanced propensity for reinstatement.

We also studied possible synaptic and epigenetic mechanisms underlying the changes in neuronal activation and structural plasticity, using standard immunoblotting techniques. Density of total and phosphorylated proteins was evaluated for GluN2B, CaMKII, and HDAC5 (Figure 5; Supplementary Table 1). Density of total GluN2B were reduced and phosphorylated GluN2B at Tyr1472 and phosphorylated CaMKII at Thr286 were enhanced by Isx-9 treatment in the dorsal DG, indicating enhanced activity of synaptic plasticity proteins [34, 37, 38]; whereas an opposite effect of Isx-9 treatment on these phosphorylated subunits were observed in ventral DG (GluN2B no change; CaMKII reduced phosphorylation). Enhanced CaMKII activity in the dorsal DG correlated with enhanced phosphorylation of HDAC5 at Ser259, supporting epigenetic alterations and derepression of target gene expression [26]. The opposing effects of Isx-9 on the activity of GluN2B, CaMKII and HDAC5 in the dorsal and ventral DG support the functional dissociation that exists along the dorsal-ventral gradient in the rat hippocampus [15, 16]. In summary, the most parsimonious interpretation of our results is that Isx-9 treatment during abstinence protects against context-driven reinstatement and these behavioral benefits were associated with reduced activation and enhanced structural plasticity of newly born GCNs in the dorsal GCL and reduced activation of preexisting GCNs in the ventral GCL. Mechanisms underlying this protection include enhanced expression of synaptic proteins that are known to promote long-term memory storage of extinguished memory in the dorsal DG. Further studies are now needed to explore the circuit-level consequences of the small yet significant number of newly born GCNs during abstinence and how they influence other cognitive behaviors that are context-driven, and pathologies associated with other types of drugs of abuse.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Kitamura O, Wee S, Specio SE, Koob GF, Pulvirenti L. Escalation of methamphetamine self-administration in rats: a dose-effect function. Psychopharmacology (Berl). 2006; 186(1):48–53. [PubMed: 16552556]
2. Piazza PV, Deroche-Gamonent V, Rouge-Pont F, Le Moal M. Vertical shifts in self-administration dose-response functions predict a drug-vulnerable phenotype predisposed to addiction. J Neurosci. 2000; 20(11):4226–4232. [PubMed: 10818158]
3. Deroche-Gamonet V, Belin D, Piazza PV. Evidence for addiction-like behavior in the rat. Science. 2004; 305(5686):1014–1017. [PubMed: 15310906]
4. Sutton MA, Karanian DA, Self DW. Factors that determine a propensity for cocaine-seeking behavior during abstinence in rats. Neuropsychopharmacology. 2000; 22(6):626–641. [PubMed: 10788762]
5. Spalding KL, Bergmann O, Alkass K, Bernard S, Salehpour M, Huttner HB, et al. Dynamics of hippocampal neurogenesis in adult humans. Cell. 2013; 153(6):1219–1227. [PubMed: 23746839]
6. Garcia AD, Doan NB, Imura T, Bush TG, Sofroniew MV. GFAP-expressing progenitors are the principal source of constitutive neurogenesis in adult mouse forebrain. Nat Neurosci. 2004; 7(11): 1233–1241. [PubMed: 15494728]
7. Abrous DN, Koehl M, Le Moal M. Adult neurogenesis: from precursors to network and physiology. Physiol Rev. 2005; 85(2):523–569. [PubMed: 15788705]
8. Ming GL, Song H. Adult neurogenesis in the mammalian brain: significant answers and significant questions. Neuron. 2011; 70(4):687–702. [PubMed: 21609825]
9. Sahay A, Scobie KN, Hill AS, O'Carroll CM, Kheirbek MA, Burghardt NS, et al. Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. Nature. 2011; 472(7344):466–470. [PubMed: 21460835]
10. Niibori Y, Yu TS, Epp JR, Akers KG, Josselyn SA, Frankland PW. Suppression of adult neurogenesis impairs population coding of similar contexts in hippocampal CA3 region. Nat Commun. 2012; 3:1253. [PubMed: 23212382]
11. Park EH, Burghardt NS, Dvorak D, Hen R, Fenton AA. Experience-Dependent Regulation of Dentate Gyrus Excitability by Adult-Born Granule Cells. J Neurosci. 2015; 35(33):11656–11666. [PubMed: 26290242]
12. Akers KG, Martinez-Canabal A, Restivo L, Yiu AP, De Cristofaro A, Hsiang HL, et al. Hippocampal neurogenesis regulates forgetting during adulthood and infancy. Science. 2014; 344(6184):598–602. [PubMed: 24812394]
13. Epp JR, Silva Mera R, Kohler S, Josselyn SA, Frankland PW. Neurogenesis-mediated forgetting minimizes proactive interference. Nat Commun. 2016; 7:10838. [PubMed: 26917323]
14. Frankland PW, Josselyn SA. Hippocampal Neurogenesis and Memory Clearance. Neuropsychopharmacology. 2016; 41(1):382–383. [PubMed: 26657960]
15. Amaral DG, Witter MP. The three-dimensional organization of the hippocampal formation: a review of anatomical data. Neuroscience. 1989; 31(3):571–591. [PubMed: 2687721]
16. Snyder JS, Radik R, Wojtowicz JM, Cameron HA. Anatomical gradients of adult neurogenesis and activity: young neurons in the ventral dentate gyrus are activated by water maze training. Hippocampus. 2009; 19(4):360–370. [PubMed: 19004012]

17. Wells AM, Lasseter HC, Xie X, Cowhey KE, Reittinger AM, Fuchs RA. Interaction between the basolateral amygdala and dorsal hippocampus is critical for cocaine memory reconsolidation and subsequent drug context-induced cocaine-seeking behavior in rats. Learn Mem. 2011; 18(11):693–702. [PubMed: 22005750]
18. Rogers JL, See RE. Selective inactivation of the ventral hippocampus attenuates cue-induced and cocaine-primed reinstatement of drug-seeking in rats. Neurobiol Learn Mem. 2007; 87(4):688–692. [PubMed: 17337218]
19. Lasseter HC, Xie X, Ramirez DR, Fuchs RA. Sub-region specific contribution of the ventral hippocampus to drug context-induced reinstatement of cocaine-seeking behavior in rats. Neuroscience. 2010; 171(3):830–839. [PubMed: 20870011]
20. Lau JY, Goldman D, Buzas B, Hodgkinson C, Leibenluft E, Nelson E, et al. BDNF gene polymorphism (Val66Met) predicts amygdala and anterior hippocampus responses to emotional faces in anxious and depressed adolescents. Neuroimage. 2010; 53(3):952–961. [PubMed: 19931400]
21. Mandyam CD, Koob GF. The addicted brain craves new neurons: putative role for adult-born progenitors in promoting recovery. Trends Neurosci. 2012; 35(4):250–260. [PubMed: 22265158]
22. Canales JJ. Comparative neuroscience of stimulant-induced memory dysfunction: role for neurogenesis in the adult hippocampus. Behav Pharmacol. 2010; 21(5–6):379–393. [PubMed: 20700045]
23. Recinto P, Samant AR, Chavez G, Kim A, Yuan CJ, Soleiman M, et al. Levels of neural progenitors in the hippocampus predict memory impairment and relapse to drug seeking as a function of excessive methamphetamine self-administration. Neuropsychopharmacology. 2012; 37(5):1275–1287. [PubMed: 22205547]
24. Koh SH, Liang AC, Takahashi Y, Maki T, Shindo A, Osumi N, et al. Differential Effects of Isoxazole-9 on Neural Stem/Progenitor Cells, Oligodendrocyte Precursor Cells, and Endothelial Progenitor Cells. PLoS One. 2015; 10(9):e0138724. [PubMed: 26407349]
25. Bettio LE, Patten AR, Gil-Mohapel J, O'Rourke NF, Hanley RP, Kennedy S, et al. ISX-9 can potentiate cell proliferation and neuronal commitment in the rat dentate gyrus. Neuroscience. 2016; 332:212–222. [PubMed: 27373772]
26. Schneider JW, Gao Z, Li S, Farooqi M, Tang TS, Bezprozvanny I, et al. Small-molecule activation of neuronal cell fate. Nat Chem Biol. 2008; 4(7):408–410. [PubMed: 18552832]
27. Petrik D, Jiang Y, Birnbaum SG, Powell CM, Kim MS, Hsieh J, et al. Functional and mechanistic exploration of an adult neurogenesis-promoting small molecule. FASEB J. 2012
28. Zhang L, Li P, Hsu T, Aguilar HR, Frantz DE, Schneider JW, et al. Small-molecule blocks malignant astrocyte proliferation and induces neuronal gene expression. Differentiation; research in biological diversity. 2011; 81(4):233–242. [PubMed: 21419563]
29. Somkuwar SS, Fannon MJ, Staples MC, Zamora-Martinez ER, Navarro AI, Kim A, et al. Alcohol dependence-induced regulation of the proliferation and survival of adult brain progenitors is associated with altered BDNF-TrkB signaling. Brain Struct Funct. 2015
30. Collingridge GL, Olsen RW, Peters J, Spedding M. A nomenclature for ligand-gated ion channels. Neuropharmacology. 2009; 56(1):2–5. [PubMed: 18655795]
31. Shaham Y, Shalev U, Lu L, De Wit H, Stewart J. The reinstatement model of drug relapse: history, methodology and major findings. Psychopharmacology (Berl). 2003; 168(1–2):3–20. [PubMed: 12402102]
32. Kheirbek MA, Klemenhagen KC, Sahay A, Hen R. Neurogenesis and generalization: a new approach to stratify and treat anxiety disorders. Nat Neurosci. 2012; 15(12):1613–1620. [PubMed: 23187693]
33. Linseman DA, Bartley CM, Le SS, Laessig TA, Bouchard RJ, Meintzer MK, et al. Inactivation of the myocyte enhancer factor-2 repressor histone deacetylase-5 by endogenous Ca(2+) // calmodulin-dependent kinase II promotes depolarization-mediated cerebellar granule neuron survival. J Biol Chem. 2003; 278(42):41472–41481. [PubMed: 12896970]
34. Irvine EE, von Hertzen LS, Plattner F, Giese KP. alphaCaMKII autophosphorylation: a fast track to memory. Trends Neurosci. 2006; 29(8):459–465. [PubMed: 16806507]

35. Yuan CJ, Quiocho JM, Kim A, Wee S, Mandyam CD. Extended access methamphetamine decreases immature neurons in the hippocampus which results from loss and altered development of neural progenitors without altered dynamics of the S-phase of the cell cycle. Pharmacol Biochem Behav. 2011; 100(1):98–108. [PubMed: 21855565]

36. Mandyam CD, Wee S, Crawford EF, Eisch AJ, Richardson HN, Koob GF. Varied access to intravenous methamphetamine self-administration differentially alters adult hippocampal neurogenesis. Bi ol Psychiatry. 2008; 64(11):958–965.

37. Prybylowski K, Chang K, Sans N, Kan L, Vicini S, Wenthold RJ. The synaptic localization of NR2B–containing NMDA receptors is controlled by interactions with PDZ proteins and AP-2. Neuron. 2005; 47(6):845–857. [PubMed: 16157279]

38. Lavezzari G, McCallum J, Lee R, Roche KW. Differential binding of the AP-2 adaptor complex and PSD-95 to the C-terminus of the NMDA receptor subunit NR2B regulates surface expression. Neuropharmacology. 2003; 45(6):729–737. [PubMed: 14529712]

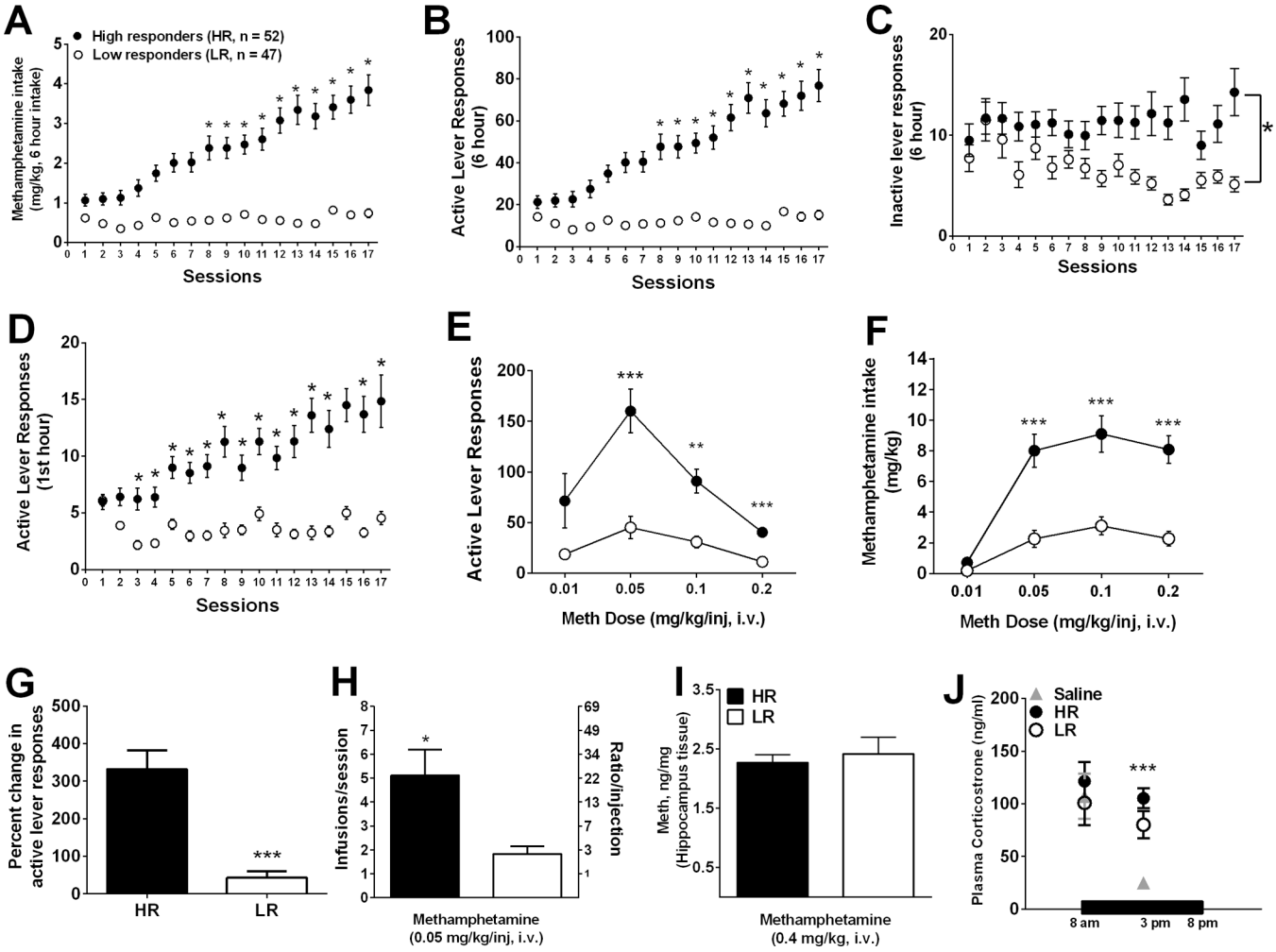

Figure 1. Extended access methamphetamine self-administration in 99 outbred adult Wistar rats demonstrates high and low preferred intake in methamphetamine

(A) Methamphetamine intake during six hour sessions, (B) active lever responses during six hour sessions (C) inactive lever responses during six hour sessions and (D) active lever responses during the first hour of the six hour session in High Responders (HR, n=52) and Low Responders (LR, n=47). HR and LR phenotype during methamphetamine self-administration were determined by the median of average daily methamphetamine self-administration (0.05 mg/kg/injection, FR1 schedule of reinforcement) during the 14 of 17 six hour self-administration sessions. (E) HR animals (n=10) exhibit an upward shift in peak self-administration rates, and a rightward shift in the descending limb of the self-administration dose–response curve compared to LR animals (n=6). (F) Methamphetamine self-administration lever responses shown in (E) converted to dose-intake curves shows that HR animals take greater daily amounts of methamphetamine compared to LR animals at doses on the descending limb of the dose–response curve shown in panel (E). (G) Percent changes in active lever responses in HR (n=52) and LR (n=47) animals when lever responses during sessions 13 to 17 were compared with sessions 1 to 5. (H) Methamphetamine-maintained breakpoints in HR (n=10) and LR (n=6) rats when tested in on a progressive ratio schedule of reinforcement. Left axis in (H) depicts the number of infusions obtained;

right axis in (H) depicts the final ratio value completed. (I) Brain methamphetamine levels are similar in HR (n=10) and LR (n=6) animals. Brain tissue was collected 45 min after a 0.4 mg/kg intravenous methamphetamine injection and methamphetamine levels were analyzed by gas chromatography/mass spectrophotometry methods. (J) Plasma corticosterone (CORT) levels evaluated by ELISA demonstrates that HR (n=10) and LR (n=6) have similar peak CORT levels compared to animals that self-administered saline (n=8). However, nadir CORT was higher in HR and LR rats compared to saline rats. Saline, n=8; HR, n=10–52; LR, n=6–47 each group). Data shown are represented as mean +/− SEM. * $P < 0.05$, compared to session 1 (A); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs.LR (C–E), or saline (G) by repeated measures or standard two way analysis of variance (ANOVA) and Student-Newman-Keuls posttests.

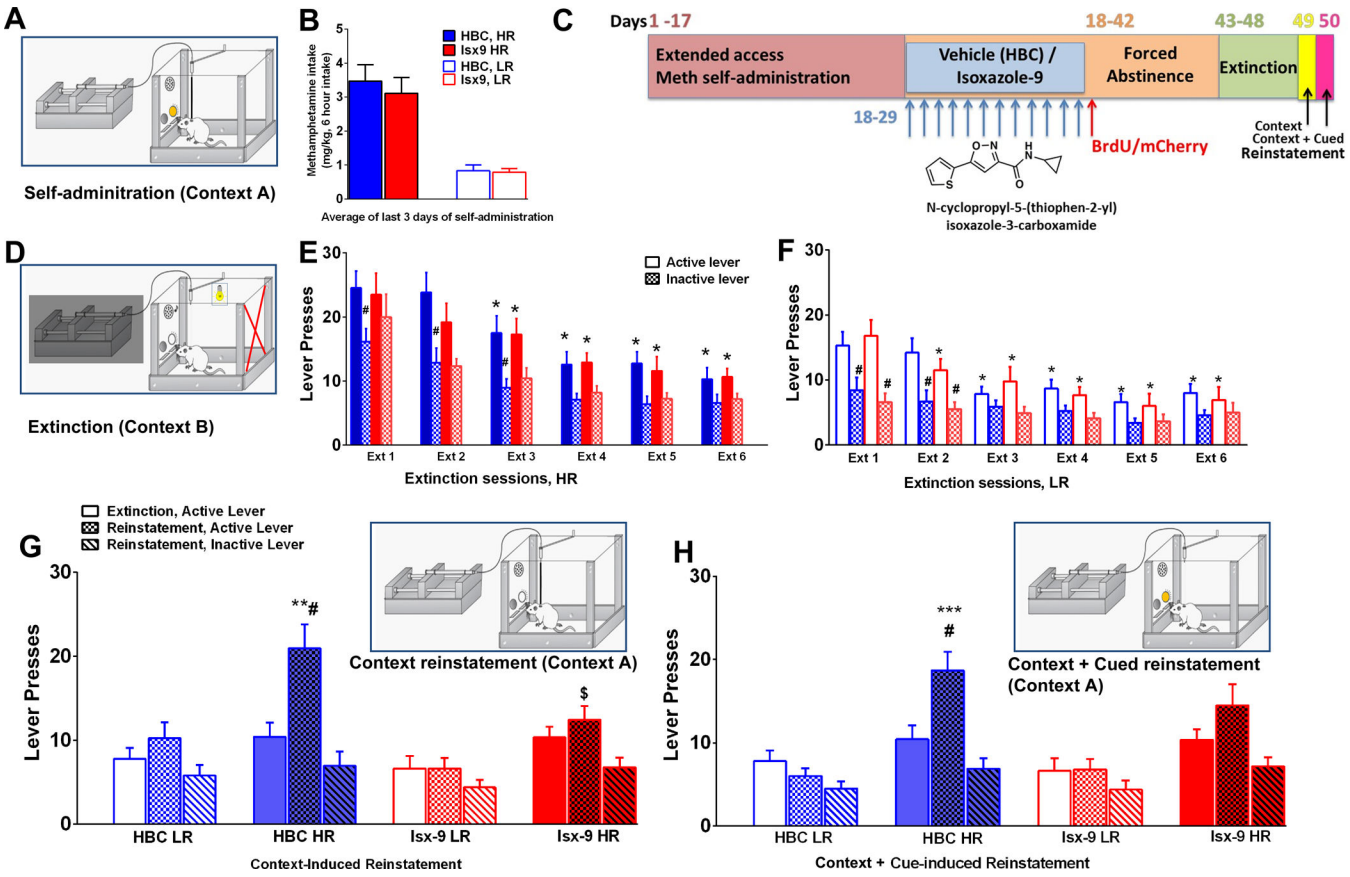

Figure 2. Neurogenic small molecule Isoxazole-9 reduces drug seeking during context- and cue-induced reinstatement in methamphetamine addicted animals

(A) Cartoon representation of operant box ‘context A’ for methamphetamine (meth) self-administration modified from Watterson and Olive 2014. (B) Average meth intake during last three sessions of self-administration in HR and LR animals injected with HBC control or Isx-9 during abstinence. (C) Schematic of experimental design indicating self-administration, Isx-9 injections (20 mg/kg, i.p.), BrdU injection (150 mg/kb, i.p.), abstinent days, extinction days and reinstatement days. (D–F) Cartoon representation of operant box ‘context B’ for extinction sessions (D), and active and inactive lever responses during extinction sessions in HR (E, control and Isx-9) and LR (F, control and Isx-9) animals. (G–H) Cartoon representation of operant box for contexual and cued reinstatement (insets in G-H), and active and inactive lever responses during contextual (G) and contextual-cued (H) reinstatement sessions in HR and LR, control and Isx-9 animals. HBC-LR, n=20; HBC-HR, n=24; Isx9 LR, n=19; Isx9-HR, n=18. Data shown are represented as mean +/− SEM. # P<0.05, vs. active lever presses and * P <0.05 vs. day 1 active lever presses in E-F by repeated measures two way ANOVA and Bonferroni posttests. ** P <0.01, *** P <0.001 vs. extinction session 6, # P<0.05, vs. inactive lever presses, $ P<0.01 vs. HBC-HR active lever presses in G-H by repeated measures two way ANOVA and Student-Newman-Keuls posttests.

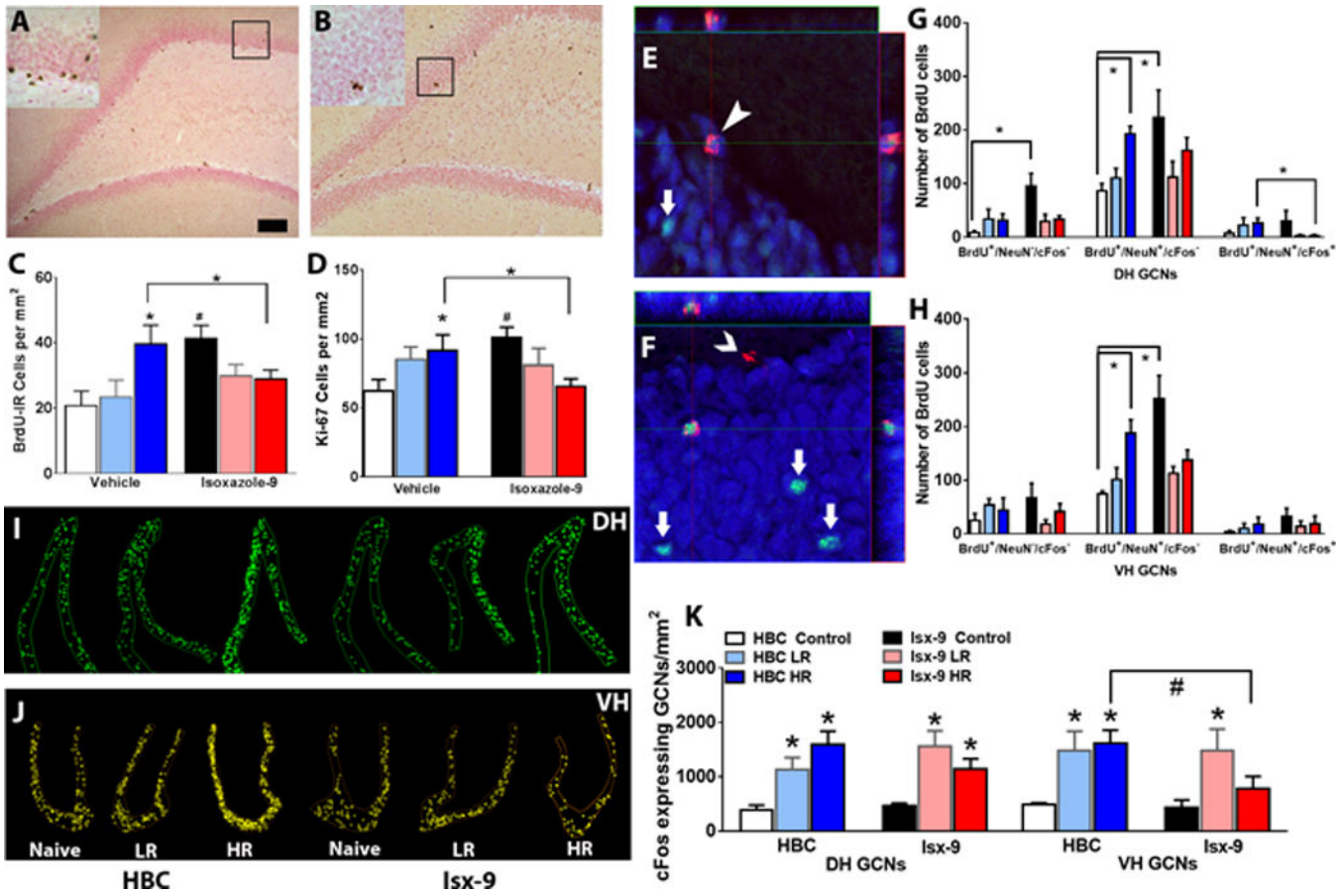

Figure 3. Isoxazole-9 prevented methamphetamine abstinence-induced alterations in neurogenesis and neuronal activation of GCNs in the dentate gyrus

(A–B) Photomicrographs of 17-day-old BrdU (A) and Ki-67 (B) labelled cells in the dorsal dentate gyrus granule cell layer. (C–D) Quantitative analysis of BrdU (C) and Ki-67 (D) labelled cells. (E–F) Orthogonal z stack confocal images of BrdU (red, CY3) cells expressing NeuN (blue, CY5), and/or cFos (green, FITC) in dorsal (E) and ventral (F) GCNs. Open arrowhead, BrdU alone; filled arrowhead, BrdU/NeuN; thin arrow, BrdU/NeuN/cFos; thick arrows, cFos/NeuN. (G–H) Phenotypic analysis of BrdU labelled cells in dorsal (G) and ventral (H) GCNs. (I–J) Schematic representation of expression of cFos in dorsal (I) and ventral (J) GCNs as depicted in StereoInvestigator that was used for cell quantification. (K) Quantitative analysis of cFos expressing GCNs. HBC control, n=4; HBC-LR, n=5; HBC-HR, n=7; Isx-9 control, n=6; Isx-9 LR, n=5; Isx-9 HR, n=7. Scale bar in A, main panel is 100 um (applies A-B), is 15 um (applies E-F). Data shown are represented as mean +/− SEM. *P<0.05 vs. vehicle control, #P<0.05 vs. Isx-HR in C-D, *P<0.05 vs. vehicle control in (K) by two way ANOVA and Student-Newman-Keuls posttests.

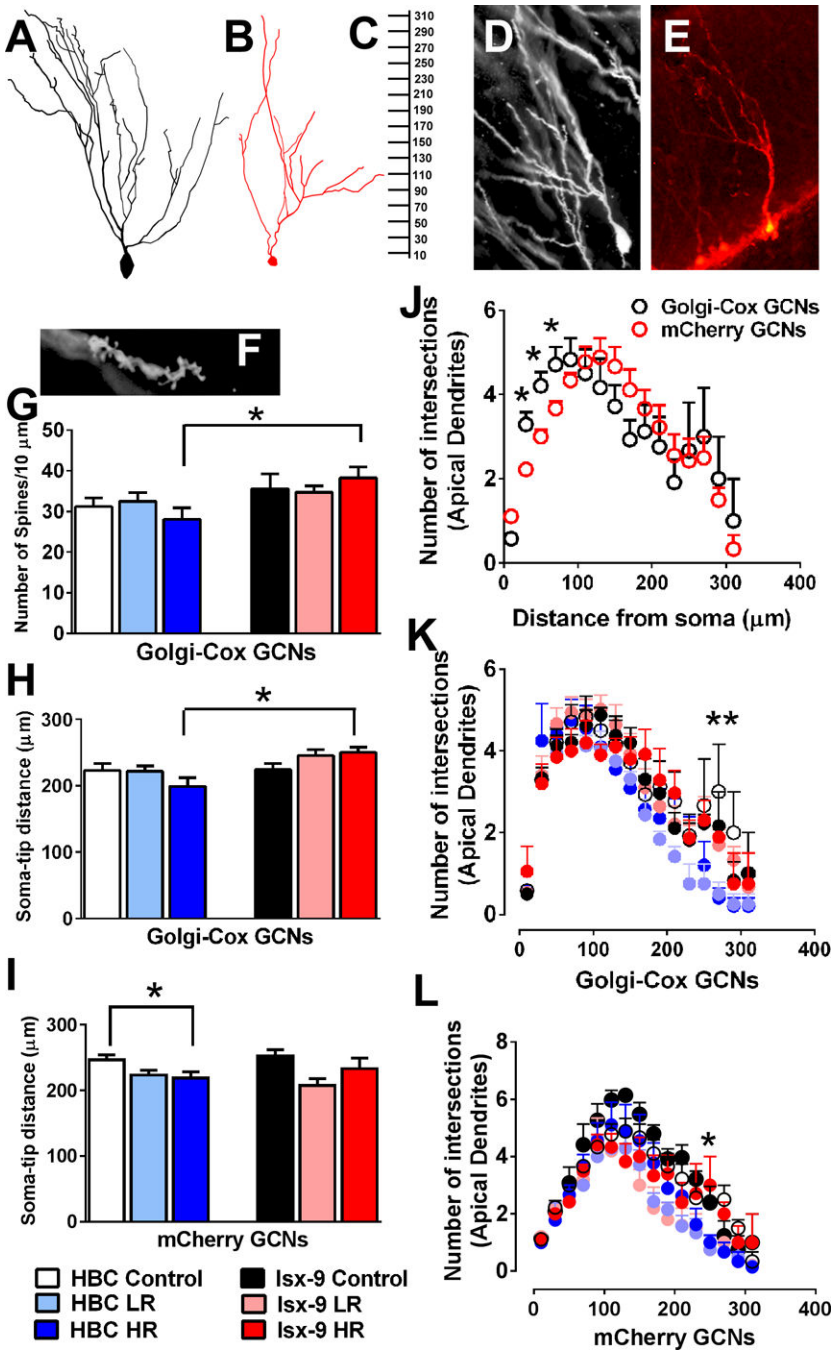

Figure 4. Isoxazole-9 prevented methamphetamine abstinence-induced altered structural plasticity of pre-existing and newly born granule cell neurons (GCNs)

(A–F) 3D tracings of preexisting (A) and newly born (B) GCNs with Sholl ring distance indicated in (C), and images of Golgi-Cox (D, inverted image of black cells over white background) and 17-day-old mCherry (E, red cells over black background) labeled GCNs used for Sholl analysis. (F) Illustration of dendritic spines on Golgi-Cox labeled cells. (G–I) Structural plasticity of Golgi-Cox-labeled and mCherry labeled neurons shown as alterations in the number of dendritic spines on apical dendrites of Golgi-Cox-labeled cells (G), soma-to-tip distance of Golgi-Cox-labeled cells (H) and mCherry-labelled cells (I). (J–L) 3D Sholl

analyses of preexisting and newly born GCNs (J), and newly born GCNs (L). HBC controls (n=6–9), Isx-9 controls (n=6–9), HBC-LR (n=8–9), HBC-HR (n=9–12), Isx-9 LR (n=5–8), Isx-9 HR (n=9–12). Data shown are represented as mean +/− SEM. *P<0.05 in G-I; * P<0.05 vs mCherry GCNs in J; **P<0.01 and *P<0.05 vs. HBC control in K-L compared to groups indicated by lines or to vehicle control by two way repeated measures ANOVA and Student-Newman-Keuls posttests.

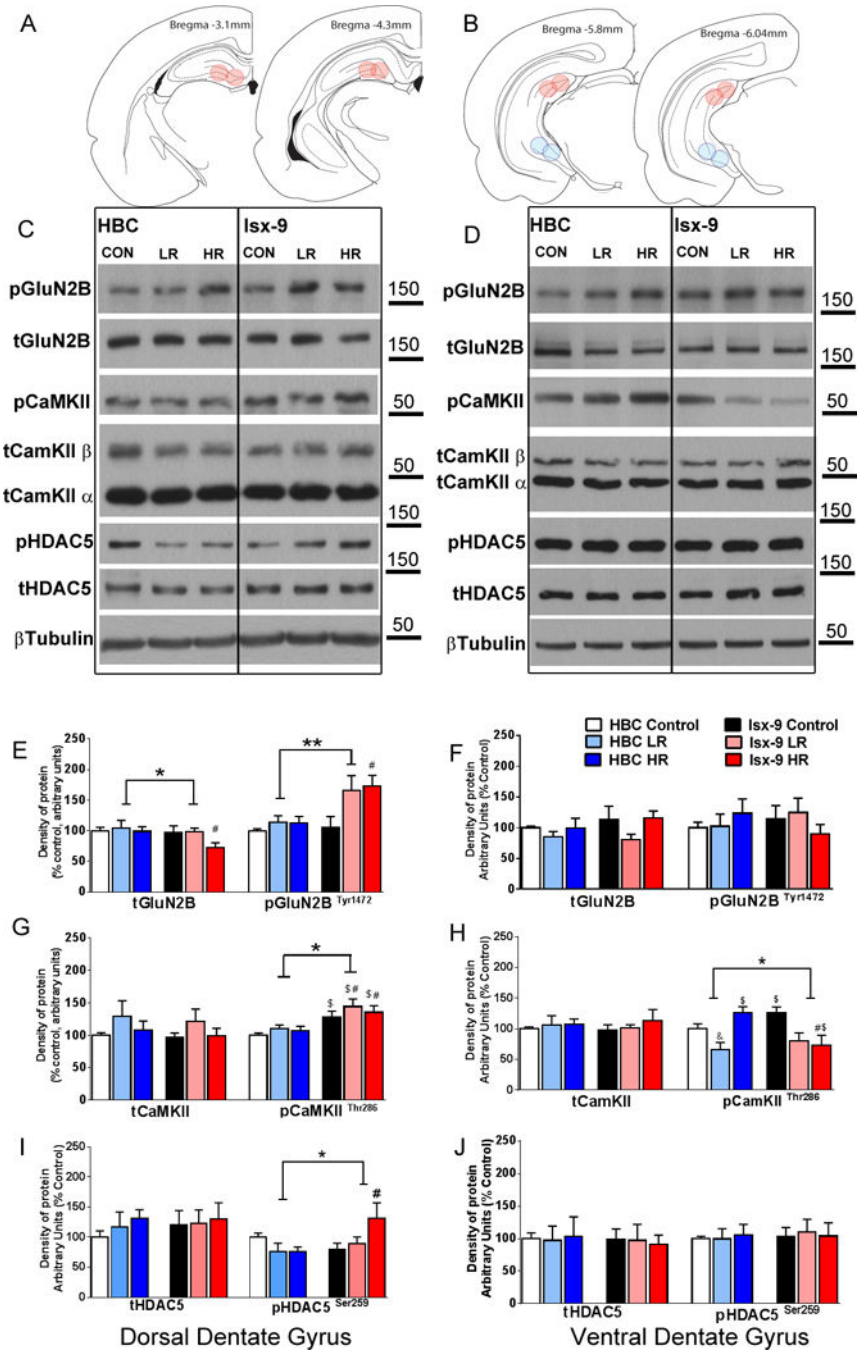

Figure 5. Isoxazole-9 alters the expression of synaptic plasticity proteins in the dorsal and ventral dentate gyrus

(A–B) Schematic showing location of tissue punches taken in the dorsal (A) and ventral (B) dentate gyrus of the hippocampus. (C–D) Representative Western blots for protein expression in dorsal (C) and ventral (D) dentate gyrus enriched tissue of total and phosphorylated GluN2B, CaMKII and HDAC5. (E–F) Density of protein expression for total and phosphorylated GluN2B in dorsal (E) and ventral (F) dentate gyrus (Dorsal dentate gyrus: tGluN2B- main effect of Isx-9: $F(1,41) = 4.741$, $P<0.05$; pGluN2B- main effect of meth $F(2,41) = 4.218$, $P<0.05$; main effect of Isx-9 $F(1,41) = 9.845$, $P<0.01$). (G–H) Density

of protein expression for total and phosphorylated CaMKII in dorsal (G) and ventral (H) dentate gyrus (Dorsal dentate gyrus: pCaMKII- main effect of Isx-9 $F(1,43) = 13.10$, $P<0.001$; Ventral dentate gyrus: pCaMKII- meth group × Isx-9 treatment interaction $F(2,38) = 6.561$, $P<0.01$; main effect of meth $F(2,38) = 6.273$, $P<0.01$). (I–J) Density of protein expression for total and phosphorylated HDAC5 in dorsal (I) and ventral (J) dentate gyrus (Dorsal dentate gyrus: pHDAC5- meth group × Isx-9 treatment interaction $F(2,40) = 3.820$, $P<0.05$). HBC control, n=8; HBC-LR, n=8; HBC-HR, n=8; Isx-9 control, n=8; Isx-9 LR, n=9; Isx-9 HR, n=8. *P<0.05, **P<0.01 by two way ANOVA and Student-Newman-Keuls posttests.