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Nonmuscle myosin II inhibition disrupts methamphetamine-associated memory in females and adolescents

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Abstract

Memories associated with drug use can trigger strong motivation for the drug, which increases relapse vulnerability in substance use disorder (SUD). Currently there are no treatments for relapse to abuse of psychostimulants, such as methamphetamine (METH). We previously reported that storage of memories associated with METH, but not those for fear or food reward, and the concomitant spine density increase are disrupted in a retrieval-independent manner by depolymerizing actin in the basolateral amygdala complex (BLC) of adult male rats and mice. Similar results are achieved in males through intra-BLC or systemic inhibition of nonmuscle myosin II (NMII), a molecular motor that directly drives actin polymerization. Given the substantial differences in physiology between genders, we sought to determine if this immediate and selective disruption of METH-associated memory extends to adult females. A single intra-BLC infusion of the NMII inhibitor Blebbistatin (Blebb) produced a long-lasting disruption of context-induced drug seeking for at least 30 days in female rats that mirrored our prior results in males. Furthermore, a single systemic injection of Blebb prior to testing disrupted METH-associated memory and the concomitant increase in BLC spine density in females. Importantly, as in males, the same manipulation had no effect on an auditory fear memory or associated BLC spine density. In addition, we established that the NMII-based disruption of METH-associated memory extends to both male and female adolescents. These findings provide further support that small molecular inhibitors of NMII have strong therapeutic potential for the prevention of relapse to METH abuse triggered by associative memories.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to report.

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Keywords

Actin; memory; synapse; retrieval; addiction; drug seeking; dendritic spine

INTRODUCTION

Recurring bouts of relapse are one of the main defining characteristics of substance use disorder (SUD). At the time of drug use, strong associative memories are formed between surrounding environmental cues and the drug experience, which can later exert motivation for the drug and promote drug seeking [1]. These deeply engrained memories are numerous, highly specific to the individual and often abstract in nature [2]. Currently, there are no available pharmacotherapies for psychostimulant abuse or the prevention of relapse to their use.

Dendritic spines are small, highly dynamic postsynaptic structures that contribute to the physical storage of memory [3]. By enabling input-specific biochemical and electrical isolation of synapses, dendritic spines facilitate signal transduction and information storage during memory formation. Furthermore, dendritic spines undergo volumetric and functional changes at the time of learning that are thought to be critical for stabilizing synapses and long-term memories [4, 5]. The ability of dendritic spines to undergo rapid changes in response to stimulation is governed by dynamic changes to the local actin cytoskeleton [6, 7]. Indeed, the dendritic spine enlargement that occurs during learning is driven by actin polymerization, the process of adding actin monomers (G-actin) to actin filaments (F-actin). Evidence from synaptic plasticity and fear memory studies indicate that actin becomes highly dynamic within minutes, and perhaps seconds, of stimulation, but also rapidly stabilizes, such that long-term potentiation (LTP) and memory quickly become invulnerable to actin depolymerizing agents, such as Latrunculin A (LatA) [8–11].

Evidence from our group suggests that, unlike LTP and memories associated with fear and food reward, the F-actin supporting METH-associated memories remains dynamic long after learning within the BLC, a subregion of the brain's emotional memory center [9, 10, 12, 13]. This renders METH-associated memories selectively vulnerable to disruption by actin depolymerization days to weeks after learning. Indeed, a single intra-BLC infusion of LatA is capable of producing an immediate and long-lasting disruption of METH-associated memories in adult male rats and mice without the need for retrieval. This memory loss is accompanied by a reversal of BLC spine density to pre-METH control levels [12].

While promising, this discovery has limited therapeutic potential because of actin's many roles in the periphery. Indeed, systemic delivery of an actin depolymerizer would likely be fatal because β -actin participates in numerous cellular processes, such as cell polarity, adhesion, and migration [14]. Therefore, we shifted focus upstream of actin polymerization to the molecular motor nonmuscle myosin II (NMII), which we have previously demonstrated to have a critical and temporally restricted role in synaptic actin polymerization and fear memory [9, 10]. As with targeting actin polymerization directly, inhibiting NMII with a single infusion of Blebb, the small molecular inhibitor of NMII, in the BLC immediately disrupted METH-associated memories in adult males, without the

need for retrieval and continued to prevent drug seeking behavior for at least one month [13]. Importantly, we have found that systemic injections of Blebb are tolerated by rodents. Furthering this, we found that Blebb is highly brain penetrant, enabling systemic injections to disrupt METH-associated memories and reverse the associated changes in BLC spine density [13].

All of our work on NMII to date has been performed in adult male rodents. Men account for more than half of the illicit substance use in the United States [15]. However, men and women have an equal likelihood to develop SUD and women are approximately twice as likely to use METH as their primary substance of abuse [16, 17]. In addition to rates of use, gender differences are also apparent in the different phases of substance use, motivation to use and biological effects of abused drugs. For instance, women tend to escalate to addiction faster, and be more susceptible to feelings of craving and relapse, relative to men [18–23]. Moreover, greater bioavailability of psychostimulants has been shown in female animals, as compared to males, and consistent with this, females tend to self-administer lower drug doses [24]. Adolescents are another population that is under-studied and at significant risk for SUD. Many individuals experiment with substance use and develop SUD during adolescence. These factors combine to make adolescence through mid-twenties the age range associated with the highest amount of substance use [25]. Therefore, it is crucial that the efficacy of a potential pharmacotherapy for relapse to METH use be determined in females and adolescents.

MATERIALS AND METHODS

Animals

All procedures were performed in accordance with the Scripps Research Institute Animal Care and Use Committee and national regulations and policies. All animals were handled at least 3 days prior to the start of training. Heterozygous Thy1-GFPm adult female mice (2–3 months old) were bred on site. This line was used because of the Golgi-like GFP expression in a sparse subset of neurons (the Thy1 lineage), enabling dendritic spine density analysis. Adult Sprague-Dawley female rats were obtained from Charles River (300g). To ensure sufficient numbers of mice for adolescent experiments, E16 pregnant C57BL6J females were obtained from Jackson Laboratory. Mice were weaned at postnatal day (PND) 23 and training began on PND 28.

Drugs

Several different doses of methamphetamine hydrochloride (Sigma-Aldrich) were used, depending on age, species and behavioral task. Adult rats undergoing self-administration received METH IV at 0.02 mg in a 0.05 ml infusion. For conditioned place preference (CPP), adult female mice received 2mg/kg of METH IP and adolescent mice received one of three METH doses IP (1, 1.5 or 2 mg/kg). Racemic Blebb (Tocris) was diluted to 1 mg/ml in a vehicle of 0.9% saline and 6.7% DMSO/25% Hydroxypropyl β -Cyclodextrin (HP β CD) and delivered to mice at 10mg/kg (IP). For intracranial infusions, both enantiomers of Blebb (- = active Blebb, += inactive Blebb [Vehicle control]; Calbiochem) were infused into the BLC at a concentration of 90ng/ μ l in 10% DMSO and 0.9% saline. The delivery rate of intra-BLC

infusions was 0.25 μ l/min over 2 min for rats. Injectors were left in place for 1 min following infusion to allow for sufficient diffusion of drug away from the needle tip.

Surgery

Anesthesia, BLC cannulation, intra-jugular catheterization and post-operative care were performed as previously described [12, 13]. Briefly, rats were implanted bilaterally with 26G guide cannulae (Plastics One) 2mm above the BLC (lateral and basolateral nuclei; AP -2.9 mm, ML $+5$ mm relative to bregma; DV -6.7 mm from skull, (Paxino & Watson)). Cannula and needle tip placement was verified, following completion of behavior, by checking 40 μ m Cresyl Violet-stained sections. Animals were excluded from the study if the needle tip was located outside the BLC.

Behavior

Self-administration—Self-administration training procedures and animal care were conducted as previously described [12, 13]. Rats underwent 20 hrs of FR1 food training prior to jugular catheterization and BLC cannulation. All self-administration phases were conducted in Coulbourn Rat Test Cages, which had different contextual and olfactory cues, as previously described [12]. One week after surgery, animals underwent 14 days of FR1 METH training with a 30 sec time-out between infusions in Context A for 2 hours each day. Five females failed to achieve the training criteria of an average of 10 active lever presses per day over the course of training and were excluded from the experiment. The females that successfully completed training were pseudorandomly assigned to either vehicle or Blebb groups, while ensuring equivalent means between the groups, and went on to extinction in Context B. Animals underwent 6–14 days of extinction, depending on when animals met their extinction criteria. Extinction criteria was 3 consecutive days of lever pressing less than 25% of their average lever pressing on the last 3 days of training. Twenty-four hours after meeting their extinction criteria, animals underwent a 1 hr reinstatement session in the training context (Context A).

Conditioned Place Preference—CPP was conducted in mice as previously described [13]. CPP consisted of three different phases: pretesting, training and testing. For the 2 days of pretesting, animals were injected with saline and allowed free access to all three chambers for 30 min. The final 15 min of the second 30 min session was used to assess pre-drug compartment preference and assign either the white or black chamber as an individual animal's METH-paired chamber (conditioned stimulus; CS+). Adult animals demonstrated no initial place preference, so CS+ assignment was balanced between the white and black side. Adolescents consistently demonstrated an initial place preference, though not for one particular chamber, so CS+ was assigned to each animal's least preferred side to avoid biasing the results towards a place preference with drug treatment. Animals were trained twice daily in 30 min training sessions over four consecutive days, such that animals received both saline in the CS- chamber and METH in the CS+ each day. Two days after the final training day, animals were tested by allowing them 15 min of free access to all chambers.

Fear Conditioning—Modified mouse Noldus Phenotypers were used for auditory fear conditioning as previously described [12, 13]. Mice were first habituated to the training context for a total of 12 minutes over three exposures. For training, mice were habituated to the chamber for 3 min before receiving 3 pairings of a 30 sec auditory tone (6kHz, 85dB) that coterminated with a footshock (1 sec, 0.5mA). Inter-trial intervals (ITIs) lasted for 30 sec each. Forty-eight hours after training, mice were allowed to explore the novel testing context for 3 min before experiencing three 30 sec presentations of the tone with 30 sec ITIs. Freezing behavior was determined by averaging percent freezing across all three tones.

Spine Density Analysis

Collection and processing of Thy-GFPm tissue was conducted as previously described [12, 13]. For each animal, a 4x magnification image of the whole BLC was taken to identify regions with low enough dendrite density to provide accurate resolution and analysis of individual dendritic spines. Once a region was selected, a z-stack was taken through the section with a 40x oil immersion lens (N.A. 1.3) with 1.7x scan zoom (7 animals per group; N = 1 stack per animal; 50–80 images per stack; z-distance between two serial images = 0.44 μ m). Morphometric analysis was conducted using Image J software (<http://rsbweb.nih.gov/ij/>). Spine density was determined by analyzing 11 dendrite sections per animal, ranging from 15–30 μ m in length and less than 1 μ m in width (to target tertiary dendrites). Protuberances were counted as a spine if there was a clear connection of the spine head to the dendrite shaft.

Statistical Analysis

Wilcoxon sign rank tests, one-way analysis of variance, two-way analysis of variance and repeated measures ANOVAs were used. Bonferroni corrections were applied when appropriate. Statistical significance was set at $P = 0.05$.

RESULTS

Disruption of context-induced reinstatement of METH seeking in adult females by nonmuscle myosin II inhibition

The potential for NMII inhibition (NMIIi) to disrupt context-induced reinstatement of METH seeking, a gold standard model of memory-induced drug seeking, in females was first tested. Female rats were trained to lever press for METH over 14 daily sessions in Context A, which had a distinct set of contextual and olfactory cues (Fig 1. A–B). The instrumental lever pressing response was then extinguished in Context B, which had a set of visual, tactile and odor cues unique from Context A (Fig. 1B). Twenty-four hours after reaching the extinction criterion, animals received intra-BLC infusions of Vehicle or Blebb, ensuring equivalent self-administration behavior between the two groups (Average active lever presses over the final three days of training: Veh -52.6 ± 13.9 and Blebb -52.1 ± 22.6 ; One Way ANOVA, $F_{(1,13)} < 0.0001$, $P > 0.05$), and were returned to Context A 30 minutes later. Context-induced reinstatement of drug seeking (R1) was assessed by the number of lever presses in the absence of METH reinforcement. Blebb substantially reduced the number of active lever presses upon reexposure to the METH-paired context (Fig. 1C: Repeated Measures ANOVA, Active Lever Veh vs. Blebb: $F_{(1,13)}=9.88$, $P = 0.01$; One Way

ANOVA, R1: $F_{(1,13)}=6.45$, P 0.05; Repeated Measures ANOVA, Inactive Lever Veh vs. Blebb: $F_{(1,13)}=0.002$, $P>0.05$). As previously demonstrated in males [13], this single NMIIi infusion prior to R1 disrupted METH seeking behavior in females during subsequent test sessions, performed 24 hours (R2) and 30 days (R30) later (Fig. 1C: One Way ANOVA, Active Lever Veh vs Blebb R2: $F_{(1,13)}=4.48$, P 0.05; R30: $F_{(1,13)}=7.37$, P 0.05). The continued suppression of METH seeking one month after a single NMIIi infusion indicates that the effect is long-lasting, without spontaneous recovery of the memory. Cannula and needle placements were subsequently determined using Cresyl Violet (Fig. 1D).

METH-associated memories are uniquely vulnerable to disruption by nonmuscle myosin II inhibition in adult females

The effect of systemic (IP) Blebb on METH-associated memory was next assessed in adult female mice with CPP, using the same behavioral training, dose and route of administration we previously found to disrupt METH-associated memory in adult males. Two days after the final METH CPP training session, females received a single IP injection of Blebb 30 minutes prior to assessment of METH-associated memory in the absence of METH reinforcement (Fig. 2A). Vehicle-treated animals spent significantly more time in the METH-paired (CS+) chamber, as compared to the Saline-paired (CS-) chamber (Fig. 2B: Wilcoxon Signed Rank, Veh: $z=-2.55$, P 0.05), indicating a strong METH-associated memory. Blebb treatment, on the other hand, disrupted the memory (Fig. 2B: Wilcoxon Signed Rank, IP Blebb: $z=-0.89$, $P>0.05$).

METH-associated memory is accompanied by an increase in BLC spine density in males [12] that returns to baseline levels following intra-BLC LatA or systemic Blebb treatment [12, 13]. Therefore, we next determined if the memory disrupting effects of systemic Blebb in females was accompanied by a similar decrease in BLC spine density. The behavioral data depicted in Figure 2A–B was collected in Thy1-GFP(m) mice, allowing for the imaging and quantification of BLC spine density (Fig. 2C). Dendrites chosen for spine analysis had similar dendritic width across groups (One Way ANOVA, $F_{(1,12)}=0.015$, $P>0.05$). As with males, the disruption of METH-associated memory by systemic Blebb was accompanied by a decrease in BLC spine density (Fig. 2C–D: One Way ANOVA, $F_{(1,12)}=6.28$, P 0.05).

We have also previously shown that the disruption of METH-associated memory in adult males is highly selective, as pre-test infusions of Blebb have no effect on BLC-dependent memories associated with fear or food reward [12]. Here we confirmed a similar specificity of Blebb's memory disrupting effects. Unlike the effect on METH-associated memory (Figures 1–2), pre-test Blebb injections had no effect on auditory fear memory in female mice (Fig. 2E–F: Repeated Measures ANOVA, $F_{(1,13)}=0.04$, $P>0.05$). Furthermore, systemic Blebb had no effect on BLC spine density in these animals (Fig 2G–H: One Way ANOVAs, Spine Density: $F_{(1,13)}=0.39$, $P>0.05$ and Dendritic Width: $F_{(1,13)}=0.18$, $P>0.05$).

METH-associated memories are similarly disrupted in adolescent males and females by nonmuscle myosin II inhibition

Substance use is the highest in late teens through the mid-twenties [25]. Therefore, we also determined the impact of NMIIi on METH-associated memory in adolescent males and

females. There was no significant difference between male and female adolescent behavior so data was collapsed across sex ($P > 0.05$ for all gender comparisons, One Way ANOVAs with Bonferroni Correction). Prior to testing the effect of Blebb treatment, we determined the optimal dose for METH CPP in adolescents. CPP training began when animals were postnatal day (PND) 28 and testing occurred on PND 35 (Fig 3A). As mentioned in the Methods, unlike adults, adolescents consistently displayed a bias for one chamber during the pretest, which was paired with saline (CS-) during training (Fig 3B: Wilcoxon Signed Rank, $z = -5.83$, $P = 0.0001$). However, the bias was not for one specific chamber (One Way ANOVA, $F_{(1,10)} = 3.67$, $P > 0.05$). Given the differences between adolescent and adult pharmacodynamics [26], we first examined the effect of different doses on the ability for adolescent mice to successfully form METH-associated CPP. Interestingly, adolescent animals require half of the adult training dose (1mg/kg) to successfully form CPP (Fig 3C: Two Way ANOVA, Day x dose comparison: $F_{(3,60)} = 3.44$, $P = 0.05$; Bonferroni Correction Post hoc comparisons (P set at < 0.008 for significance): Saline vs 1mg/kg $P = 0.0001$, Saline vs 1.5mg/kg $P = 0.035$, Saline vs 2mg/kg $P > 0.05$). Difference scores (Pretest CS+ time subtracted from Test CS+ time) were also examined to directly compare the degree of preference induced by each training dose. Indeed, 1mg/kg resulted in the greatest increase in time spent in the CS+ from the pre- to post-test (Fig. 3D: One Way ANOVA, $F_{(3,56)} = 4.46$, $P = 0.01$; Post hoc comparisons with Bonferroni Correction (P set at < 0.008 for significance): Saline vs 1mg/kg $P = 0.008$ and 1mg/kg vs 2mg/kg $P = 0.008$; Saline vs 1.5mg/kg $P = 0.041$ and 1mg/kg vs 2mg/kg $P = 0.016$). To test the effect of Blebb on adolescent METH-associated memory, 1mg/kg of METH was used to train the animals and systemic Blebb was administered 30 minutes prior testing (Fig. 3E). Consistent with the previous groups of adolescent mice, an initial chamber bias was present during the pretest (Fig. 3F: Wilcoxon Signed Rank, $z = -5.97$, $P = 0.0001$). Again, the preferred chamber was paired with saline (CS-). As expected, vehicle-treated animals demonstrated a strong preference for the METH-paired chamber (Fig. 3G: Two Way ANOVA Day x Group, $F_{(1,44)} = 16.7$, $P = 0.001$; One Way ANOVA, Veh: $F_{(1,21)} = 147.0$, $P = 0.0001$). Animals that received Blebb demonstrated a slight increase in time spent in the CS+ chamber from the pre- to post-test (One Way ANOVA, IP Blebb: $F_{(1,23)} = 14.4$, $P = 0.001$) that was similar to the shift that occurred in saline-treated mice (Fig. 3C–D). Directly comparing the difference scores between vehicle- and Blebb-treated mice indicates a significant attenuation of the METH-associated memory (Fig. 3H: One Way ANOVA, $F_{(1,44)} = 16.5$, $P = 0.001$).

DISCUSSION

Our previous work with actin depolymerization and NMII inhibition has been conducted in adult male animals [12, 13]. Given the potential therapeutic implications of NMII for targeting relapse-inducing memories, as well as gender and age differences associated with SUD, it was crucial that NMII be tested in adult females and adolescents of both sexes. The results of the experiments presented here establish that NMII is equally efficacious between sexes and at different ages. Importantly, as previously demonstrated in males, a single intra-BLC infusion of Blebb prior to testing immediately disrupted memory-induced METH seeking for at least 30 days and prevented spontaneous recovery in females. This is important in light of the “incubation of craving” that has been found to occur in both humans

and rodents, such that motivation for the drug increases with longer periods of abstinence [27–29]. Incubation, as measured by greater lever pressing at 30 days, is likely not seen here because of the extinction effect of three reinstatement tests. We also established that peripherally administered Blebb crosses the blood brain barrier to rapidly disrupt METH-associated memories in adult females and adolescents of both sexes. As in males, the same manipulation had no effect on fear-associated memories or spine density, pointing to selectivity of the effect for METH-associated memories.

The ability of actin depolymerizing agents, such as LatA or Blebb, to influence memory requires that actin be actively cycling. This allows one to infer the state of actin dynamics based on the ability of the inhibitor to disrupt memory. Previous LTP and fear conditioning studies indicate that actin dynamics are rapidly triggered to facilitate dendritic spine dynamics and support plasticity and memory formation at the time of learning, but then stabilize within minutes. Indeed, we and others have demonstrated that pre-training, but not pre-testing, actin depolymerization in the BLC disrupts auditory and contextual fear memory [9, 13]. METH-associated memories, on the other hand, remain susceptible to LatA and Blebb long after learning, indicating that METH-associated memories are uniquely dependent on continuously cycling actin [12, 13].

However, assessing memory stability over time is not without its challenges. Parsons and Davis report that infusion of ZIP into the amygdala only disrupts memory when testing occurs fairly close to the time of infusion (2 hours or 2 days, but not 10 or more days). Because ZIP is still effective at short infusion-test intervals, even when there is a long training-infusion interval (e.g. 20 days), the authors conclude that ZIP likely has a performance effect, rather than there being a change in reliance of the memory trace on the amygdala [30] (see also [31, 32]). Indeed, others have shown that testing can impact performance in subsequent tests [33], which could explain the lasting effect observed in the Parson and Davis study when the first test was given close to ZIP delivery. Because we have not assessed a window greater than one day between Blebb treatment and testing in the current study, there is the possibility of an initial performance effect, followed by a lasting disruption somehow produced by a performance deficit during an active attempt to retrieve. While longer delays between treatment and retrieval are less therapeutically relevant because individuals with SUD constantly encounter triggers, the possibility of a lasting performance deficit produced by Blebb should be considered. Three points argue against this possibility. First, Blebb is fully cleared from the brain within two hours [13]. This indicates that it could not be directly disrupting memory in the experiments where testing is performed 24 hours after Blebb treatment [13]. However, the possibility of some residual, network effect capable of disrupting performance remains. To address this, we point to our previous experiments demonstrating a similar memory disruption with intra-amygdala Latrunculin A, an actin depolmerizing agent [12], and genetic knockdown of the nonmuscle myosin IIB heavy chain, Myh10 [13]. The likelihood of all three interventions creating similar, lasting performance effects does not seem high. Third, the effect of Blebb on METH-associated memory is far more selective than we initially hypothesized. Indeed, pre-test Blebb similarly disrupts amphetamine-associated memory [34], but has no effect on the expression of an auditory fear memory, or a conditioned place preference for food reward, cocaine or

morphine [12, 13, 34]. Nevertheless, future studies will directly assess longer intervals between training and treatment and between treatment and testing.

While the ability of NMII to disrupt METH-associated memories and associated METH seeking is similar between females and males, we did notice subtle, qualitative gender differences in the METH self-administration behavior of males [12, 13] and females. Females pressed the METH-associated active lever less and received fewer METH infusions during self-administration training, when compared to our previous experiments with males. While we have not made direct, statistical comparisons because the experiments were not performed side-by-side, this is consistent with previously published studies demonstrating a greater sensitivity to psychostimulants in females, likely due to greater bioavailability [24]. Another sex difference noted when comparing the current data in females to our prior data in males is related to BLC spine density. The average dendritic width and spine density were lower in control females, as qualitatively compared to our previous measures in males. Because the goal of the current study was to determine the therapeutic potential of NMIIi for future use in women, females were left reproductively intact. Spine density in some brain regions, such as the CA1 region of the hippocampus, have been shown to fluctuate in response to an animal's estrous cycle [35]. However, it is unlikely that the apparent difference in spine density between males and females here is due to a specific phase in estrus, as the data we report in Figure 2 represents two cohorts of females run at different times. Direct comparison of spine density between males and females will be required to firmly conclude sex differences in the BLC and to determine if this is a specific interaction with METH. But regardless, the magnitude of Blebb-induced reduction of spine density was comparable in females to what we have previously reported in males, indicating that METH-associated memories are likely to be similarly supported in the BLC of males and females by dynamic actin.

Currently Blebb is the only small molecular inhibitor of the nonmuscle class of myosin II's and is an excellent starting point for medicinal chemistry. While Blebb is efficacious in SUD animal models, is highly brain penetrant, and has low molecular weight, it needs improvement from a neurotherapeutic perspective to address its phototoxicity when exposed to blue light [36, 37] selectivity [38] and solubility. Blebb's sensitivity has recently been overcome with the development of the C15 nitro derivative [39]. Further efforts using medicinal chemistry to improve Blebb's selectivity and solubility would be beneficial for understanding NMII's role in spine dynamics and as a potential SUD neurotherapeutic.

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HIGHLIGHTS

- NMIIi disrupted memory-induced METH seeking for at least 30 days in females
- NMIIi disrupted METH, but not fear, memories and related spine density in females
- Adolescent mice required a lower training dose to form CPP, relative to adults
- As in adults, NMIIi disrupted METH-associated memory in adolescents

Figure 1. Long-lasting disruption of context-induced METH seeking in females by NMII inhibition in the BLC

(A) A schematic of the experimental design for context-induced reinstatement of instrumental METH seeking. (B) In Context A, animals learned to associate the active lever with METH reinforcement. After 14 days of training animals underwent extinction of the instrumental response, lever pressing, in novel Context B. (C) The effect of a single intra-BLC infusion of Blebb ($n = 7$) prior to testing on context-induced METH seeking immediately (R1) and 30 days later (R30). Vehicle ($n = 8$) was the inactive enantiomer of Blebb. Error bars represent SEM and * $P < 0.05$ for Veh vs Blebb active lever presses.

Figure 2. Systemic NMII inhibition disrupts METH-, but not fear-, associated memories and related dendritic spine density changes in female mice

(A) Schematic of the experimental design for testing the effect of systemic Blebb treatment (IP) on METH-associated memory. (B) Effect of IP Blebb ($n = 9$) on a consolidated METH-associated memory, * $P < 0.05$ for CS+ vs CS-. Vehicle ($n = 9$) was the inactive enantiomer of Blebb. (C) Representative images of Thy1-GFP(m) BLC expression and dendritic spine density from animals depicted in (B). Scale bar is equal to $2\mu\text{m}$. (D) Dendritic spine density in animals treated with systemic Blebb prior to testing, error bars represent SEM and * $P < 0.05$ for Veh vs IP Blebb. (E) Schematic of experimental design for testing fear-associated memory. (F) Effect of systemic Blebb delivery on the expression of consolidated fear-associated memory (Vehicle $n = 7$; IP Blebb $n = 8$). (G) Representative images of Thy1-GFP(m) BLC expression and dendritic spine density from groups presented in (B). Scale bar is equal to $2\mu\text{m}$. (H) Effect of systemic Blebb on BLC spine density. Error bars represent SEM.

Figure 3. Systemic NMII inhibition disrupts METH-associated memory in male and female adolescent mice

(A) Schematic for experimental design for adolescent CPP (Saline $n = 15$ [7 female, 8 male]; 1mg/kg $n = 17$ [8 female, 9 male]; 1.5mg/kg $n = 16$ [8 female, 8 male]; 2mg/kg $n = 16$ [8 female, 8 male]). (B) Unlike adult mice, adolescent animals display an initial, pre-training preference for one chamber (assigned as saline-paired, CS- for training). (C) Determination of optimal METH dose for CPP in adolescent mice, as assessed by the change in CS+ time from pre- to post-training and (D) by a difference score (Test – PreTest CS+ time). (E) Experimental design for testing the effect of systemic Blebb treatment on METH-associated memory in adolescent (Veh $n = 22$ [11 female, 11 male]; IP Blebb $n = 24$ [12 female, 12 male]). (F) Pretraining preference, with the preferred side assigned as the saline-paired CS-. (G) Determination of the effect of Blebb on METH-associated memory in adolescent mice, as assessed by the change in CS+ time from pre- to post-training and (H) by a difference score (Test – PreTest CS+ time). Error bars represent SEM and t (trend) represent comparisons that reached statistical significance with Fisher's prior to Bonferroni correction.